TRANSCRIPTIONAL CONTROL OF PHOTORECEPTOR AXON GROWTH AND TARGETING IN *DROSOPHILA MELANOGASTER*

by

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A DISSERTATION

Presented to the Department of Biology
and the Graduate School of the University of Oregon
in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy

March 2012

DISSERTATION APPROVAL PAGE

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Title: Transcriptional Control of Photoreceptor Axon Growth and Targeting in *Drosophila melanogaster*

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Degree awarded March 2012

DISSERTATION ABSTRACT

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The nervous system is required for human cognition, motor function, and sensory interaction. A complex network of neuronal connections, or synapses, carries out these behaviors, and defects in neural connectivity can result in developmental and degenerative diseases. In vertebrate nervous systems, synapses most commonly occur at axon terminals. Upon reaching their synaptic targets, growth cones lose their motility and become boutons specialized for neurotransmitter release. I am studying this process in R7 photoreceptors in the *Drosophila* visual system.

In a forward genetic screen, we identified the transcriptional repressor Tramtrack69 (Ttk69) as being required to prevent R7 axon terminals from expanding and overlapping with adjacent R7 targets. Loss of Ttk69 results in R7 axons that grow into neighboring terminals, while premature expression of Ttk69 in R7s prevents their axons from reaching their final target. From this I conclude that Ttk69 is both necessary and sufficient to inhibit R7 axon growth and that the timing of Ttk69 expression is critical to R7 target selection. Rather than forming mature synaptic boutons, *ttk69* mutant R7s develop oversized terminals with ectopic filopodia-like protrusions, suggesting a failure to transition from growth to synapse formation. However, Ttk69 appears to regulate axon growth

independently of synaptogenesis, as I have found that the overgrowth of *ttk69* mutant R7 axons does not depend on the ability of R7s to form synapses nor does it disrupt the initial steps of synapse formation.

To identify the genetic program downstream of Ttk69, I have taken both a genetic and a candidate gene approach. It was previously shown that loss of Activin signaling causes mild overgrowth of R7 terminals. I have subsequently found that Ttk69 is required for normal accumulation of the Activin effector dSmad2 in R7 nuclei, suggesting that Ttk69 promotes Activin signaling to regulate R7 axon growth.

Ttk69 appears to act through additional Activin-independent pathways as well, and research is ongoing to determine these pathways as well as the molecular mechanisms that govern axon growth and the transition to synapse formation.

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ACKNOWLEDGMENTS

First and foremost, I would like to thank my advisor, Tory, for her insight, guidance and patience, as well as for introducing me to the fine programming of Chicago Public Radio. I must also thank all members of the Herman lab for contributions scientific and otherwise, in particular Scott Holbrook for beginning the Tramtrack project and passing it on to me. I am also thankful for the support and helpful comments provided by my committee. Finally, I am grateful for the love and friendship of my family and friends that carried me through the highs and lows of my graduate career.

I dedicate this work to Heather.

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INTRODUCTION TO THE *DROSOPHILA* VISUAL SYSTEM AS A MODEL FOR NEURAL CIRCUIT ASSEMBLY

Nervous System Development and the *Drosophila* Visual System

The nervous system is responsible for controlling and maintaining human cognition, motor function and how we interact with our surroundings. Nervous systems accomplish these tasks by receiving and processing all the internal and external stimuli we encounter. Specialized cells called neurons transmit this information across great distances and through complicated networks by extending processes (axons and dendrites) and forming synaptic connections with other neurons or tissues. Failure to form or maintain these connections impairs the nervous system's ability to carry out its critical functions. Therefore, understanding the structure and function of neurons and their synaptic connections is of great medical importance.

In vertebrate nervous systems, synapses most commonly occur at axon terminals. Upon reaching their synaptic targets, growth cones lose their motility and become boutons specialized for neurotransmitter release. In addition, the organization of these neuronal connections into non-overlapping topographic maps within specific layers is characteristic of both invertebrate and vertebrate brains and is functionally important for resolution of sensory information. However, with approximately 90 billion neurons that each makes approximately 10,000 synaptic connections, the human brain is staggeringly complex (Azevedo et al., 2009). Fortunately, the visual system of *Drosophila melanogaster*, the common fruit fly, is a relatively simple, yet powerful system in which

to analyze the molecular mechanisms that underlie terminal synapse formation and organization. In addition, the genes and genetic mechanisms uncovered through studies in *Drosophila* have proven to be highly conserved through evolution.

The *Drosophila* retina is made up of approximately 750 units (ommatidia) that each contain 8 photoreceptor neurons, or retinula (R) cells, that can be divided into three classes of photoreceptor neurons, R1-R6, R7 and R8. The R1-R6 neurons are required for motion detection and image formation, and project axons to the first optic lobe of the brain, the lamina. R7 and R8 neurons sense UV and blue light, respectively, and project axons to the second optic lobe, the medulla. As each ommatidium perceives light from a slightly different point in space than its neighbor, the R cell axons are transmitting highly specific visual stimuli to the optic lobe. This spatial resolution is preserved by retinotopy, with the axons from a single ommatidium bundled together into fascicles as they enter the brain (Meinertzhagen and Hanson 1993; Ting and Lee, 2007). Upon entering the lamina, R1-R6 axons de-fasciculate and make connections with lamina interneurons. Meanwhile, R7 and R8 axons continue to the medulla where they are organized into individual columns, with R7 axons terminating at synaptic targets slightly deeper than R8 axons. Because R7 neurons are particularly amenable to genetic manipulation, I have chosen to study them as a means to understand axon growth and targeting.

R7 Axon Extension and Synaptic Targeting

R7 neurons are the last of the R cells to differentiate in the developing retina, and therefore also the last to elaborate axons into the brain. Upon differentiation, R7 neurons

extend axons that fasciculate with and follow R8 axons from the same ommatidium into the medulla, which is divided into ten layers, M1-M10 (Ting et al., 2005). At approximately 17 hours after puparium formation (hr APF), R7 axons begin entering the medulla and pause at a temporary layer. R8 axons, on the other hand, are already paused at approximately the M1 layer. Beginning around 40 hr APF, paused R7 axons regain motility and extend to their final synaptic target layer in the M6 layer by 60 hr APF. R8 axons also resume motility, but terminate in the M3 layer. Thus, after arriving in the medulla, R7 axons are no longer following a path laid by R8 axons, but rather extending and ultimately forming synapses independently.

Fruit flies innately phototax towards UV light, a behavior that requires R7 connectivity. *Drosophila* researchers have exploited this behavior to screen for mutations that disrupt both R7 fate and axon targeting (Lee et al., 2001; Clandinin et al., 2001). Visual behavior screens identified the cell adhesion molecule N-cadherin (N-cad), the receptor tyrosine phosphatases PTP69D and LAR, the scaffolding protein Liprin, the guanine nucleotide exchange factor Trio, and the putative GTPase activating protein dSyd-1 as being required for proper R7 target selection (Lee et al., 2001; Newsome et al., 2000; Maurel-Zaffran et al., 2001; Hofmeyer et al., 2006; Hofmeyer and Treisman, 2009; Holbrook et al., 2012). Mutations in any of these genes result in R7 axons that target the M3 instead of the M6 layer. Subsequent studies showed that N-Cad is required on R7 axons and their target neurons in order for R7s to both reach their temporary layer as well as to extend to the M6 layer, with the transcription factor Sequoia regulating temporal expression of Ncad during these steps (Ting et al., 2005; Nern et al., 2005; Petrovic and Hummel, 2008). LAR, Liprin, Trio, and dSyd-1, on the other hand, appear to be required

for synapse formation at R7 terminals, and presumably retract from the M6 to the M3 layer upon failure to form a synapse.

While the above genes affect the layer-specific targeting of R7 axons, mutations have also been found that disrupt the restriction of R7 axons to individual columns within the medulla. R7s that lack components of the Activin signaling pathway extend axons into neighboring columns (Ting et al., 2007). A separate study found that the cell surface protein Turtle is also required to prevent R7 axons from extending laterally and fusing with neighboring axon terminals (Ferguson et al., 2009). R7 axons that lack both Activin signaling and Turtle extend into neighboring columns at an increased frequency over loss of either one alone, suggesting that R7 axon extension is regulated by two partially redundant mechanisms. The focus of this study is to identify additional genes that are required for R7 axon extension and targeting.

A Genetic Screen for Defects in R7 Axon Growth and Targeting

An advantage of studying R7 axons is that we can create mosaic animals in which we have removed gene function only from individual, positively marked R7s. We can do so by inducing mitotic recombination, with FLP recombinase under control of the GMR promoter, in the final cell division that generates R1, R6, and R7 photoreceptor neurons. We use the standard MARCM (mosaic analysis with a repressible cell marker) system to label the resulting homozygous mutant cells with GFP. All cells contain a promoter driving expression of Gal4 as well as a Gal4-responsive UAS element driving expression of a reporter. However, cells that are not homozygous mutant also contain a transgene

that ubiquitously expresses Gal80, which blocks Gal4-mediated activation. Therefore only homozygous mutant cells are able to express the reporter (GFP).

To identify genes required for R7 axon growth and targeting, the lab conducted a behavioral screen for mutations that disrupt R7-dependent phototaxis to UV light. Mutagenized flies were placed in a T-maze and given a choice between UV and white light. Wild-type flies phototax toward white light, while flies with non-functional R7 neurons show no preference for either light source. Mutants that failed to phototax toward UV light were analyzed with the GMR-FLP/MARCM method described above.
This screen yielded a set of three mutations in the transcription factor Ttk69 that disrupt R7 axon growth. In the next chapter I will analyze the role of Ttk69 in restricting R7 axons to individual columns.

CHAPTER II

THE TRANSCRIPTION FACTOR TTK69 IS REQUIRED AND SUFFICIENT TO RESTRICT R7 AXON GROWTH

Introduction

During development, axons navigate to their targets by means of a specialized, motile structure at their tip, the growth cone. Growth cones make decisions on when to advance, pause, turn, or terminate by both responding to extrinsically derived attractive and repulsive cues (Tessier-Lavigne and Goodman, 1996) and coordinating intrinsic changes in gene expression (Goldberg, 2004). These instructions ultimately define growth cone behaviors by regulating actin assembly at the growth cone's leading edge (Pollard and Borisy, 2003). Microtubules within the shaft of the axon also extend into the growth cone and can influence growth cone dynamics, for example, by stabilizing membrane protrusions initiated by actin polymerization (Kalil and Dent, 2005). Furthermore, changes in microtubule organization are associated with the transition from axon outgrowth to synapse formation. Studies at *Drosophila* neuromuscular junction have identified microtubule loops at sites of synaptic bouton formation (Packard et al., 2002), and these loops are also a characteristic of paused growth cones, with splaying of microtubule loops accompanying resumed axon growth (Dent et al., 1999).

Nonetheless, the details of how axons control growth cone activity, in particular starting and stopping, are incomplete. The identification of several transcription factors that regulate the expression of guidance receptors and signals provides evidence that

neurons control axon growth via changes in gene expression (Butler and Tear, 2007). For example, studies in *Drosophila* have shown that the BTB-zinc-finger transcription factor Lola specifies axon growth by regulating expression of guidance signals and receptors (Seeger et al., 1993; Giniger et al., 1994; Goeke et al., 2003). The zinc-finger transcription factor Sequoia controls R7 and R8 photoreceptor axon termination by temporally regulating the competence of cell adhesion molecules to interact with synaptic targets (Petrovic and Hummel, 2008).

We used the *Drosophila* visual system to screen for molecules required for axon targeting and growth cone regulation. The fly eye is composed of approximately 750 repeating units (the ommatidia) that each contain 8 photoreceptor (R) neurons and several non-neuronal accessory cells. The R7 and R8 neurons, required for color vision, project axons retinotopically to two different layers within the optic lobe in the brain. R8 axons project first and pause at a temporary layer in the medulla. R7 axons project later, following the path of R8s, and pause at a slightly deeper temporary layer in the medulla. Both R7 and R8 axons then resume axon extension with R7s terminating in a deeper layer of the medulla than R8s.

The intrinsic growth of R7 axons is, at least in part, regulated by canonical Activin signaling. Loss of the type I Activin receptor Baboon (Babo) or the nuclear effector dSmad2 causes R7 axons to abnormally extend into neighboring axon terminals (Ting et al., 2007). This defect is enhanced by removal of neighboring R7 terminals, suggesting that Activin signaling regulates an intrinsic growth mechanism distinct from the extrinsic repulsive interactions that restrict R7 axons to individual columns in the medulla (Ashley and Katz, 1994; Ferguson et al., 2009).

In this study, I identified the BTB/POZ zinc-finger transcription factor Tramtrack69 (Ttk69), as being required to restrict growth of R7 axons. I show that Ttk69 expression is required in the nuclei of R7 neurons during the final stage of axon extension and premature Ttk69 expression is sufficient to arrest R7 axon extension before it has reached its synaptic target layer. I propose that Ttk69 functions via both canonical Activin signaling and an Activin-independent pathway to regulate the intrinsic growth of R7 axons as they transition from growth cone to synaptic bouton.

Results

ttk69 mutant R7 growth cones display abnormal morphology

To identify genes required for R7 axon growth and targeting, the lab performed a behavior screen for mutations that disrupt R7-dependent phototaxis toward UV light (Lee et al., 2001; Ting et al., 2007). Randomly mutagenized chromosomes were screened and three mutations were identified on 3R that failed to complement each other for lethality. I used MARCM (mosaic analysis with a repressible cell marker) to analyze individual R7 axons homozygous for these mutations. I generated homozygous mutant R7s by expressing FLP recombinase under control of the GMR promoter and positively labeled mutant R7 axons with *mCD8-GFP*. Each mutation resulted in an R7 growth cone morphology defect evocative of loss of Activin signaling (see below; Ting et al., 2007). These mutations were mapped to a region of the distal tip of 3R that contained *tramtrack* (*ttk*) and all three failed to complement known *ttk* alleles for lethality. I obtained the *ttk* allele ttk^{1e11} and analyzed individual mutant R7s at 60 hours after puparium formation (hr

APF) with MARCM. In wild-type animals, R7 axons terminate in the M6 layer of the medulla, where they form spherical synaptic boutons in distinct columns by 60 hr APF (Figure 1A, arrowhead). ttk^{1e11} mutant R7 axons correctly reached the M6 layer, but 21% extended laterally into adjacent columns (Figure 1B). In addition, the ttk^{1e11} R7 axons that remained in their own column often displayed filopodial projections rather than a rounded bouton morphology and their terminals were approximately one and a half time larger than wild-type R7 boutons (Figures 1C and 1H).

Previous studies indicated that R7 axon terminals are mutually repellent (Ashley and Katz, 1994; Ferguson et al., 2009). To determine whether ttk^{1e11} is required for repulsion between adjacent R7s, I genetically removed R7s with the temperature-sensitive *sevenless* allele *V1*. When grown at the restrictive temperature, 29°C, males hemizygous for sev^{V1} lack the majority of their R7s. Despite removal of neighboring R7 axons, wild-type R7 axons correctly target the M6 layer and remain restricted to a single column (Figure 1E). In contrast, the frequency in which ttk^{1e11} mutant R7 axons extended beyond their own column was enhanced from 21% to 50% in the absence of neighboring R7s (Figures 1F and 1G). This result suggests ttk^{1e11} R7 axons are responsive to adjacent R7 terminals and supports a role for ttk^{1e11} in the intrinsic growth of R7 axons.

Ttk encodes two isoforms, Ttk69 and Ttk88, which share a common BTB/POZ domain, but have distinct zinc finger DNA-binding specificities (Read and Manley, 1992). The ttk^{1e11} allele is a putative null for Ttk69, though it may also slightly disrupt Ttk88 (Lai and Li, 1999). To determine which Ttk isoform is required for proper R7 axon growth, I expressed Ttk69 or Ttk88 in ttk^{1e11} mutant R7s. I found that Ttk69 expression fully rescued the axon defects of ttk^{1e11} mutant R7s, while Ttk88 expression did not have

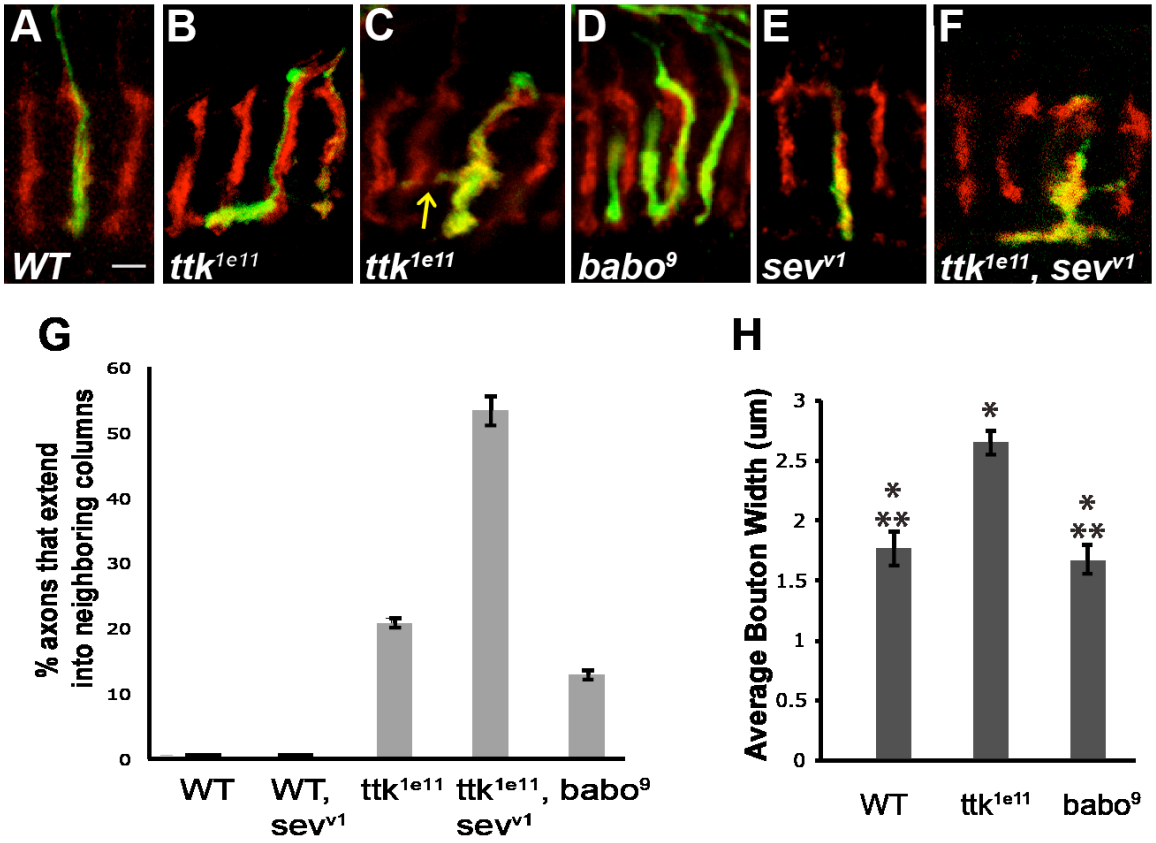

Figure 1. *ttk69* mutant R7 axon terminals invade neighboring columns.
Individual homozygous mutant R7s were generated with GMR-FLP labeled with *mCD8-GFP* (green). Pupal medullas were dissected at 60 hr APF and R7 and R8 axons were visualized with mab24B10 (red). (A) Wild-type R7s terminate in distinct columns within the M6 layer of the medulla. (B) ttk^{1e11} mutant R7s terminate in the M6 layer, but often extend into adjacent columns and overlap with neighboring R7 axon terminals. (C) Excluding ttk^{1e11} mutant R7 axons that had extended into neighboring columns, the remaining ttk^{1e11} R7s often extended aberrant filopodia (arrow) and the tips of the axon terminals were larger than wild type when measured across their width. (D) *babo* mutant R7 axons invade neighboring columns, but the remaining non-invasive *babo* axons are normal with respect to bouton morphology. (E,F) Individual mutant R7s were generated with *GMR-FLP* and labeled with *mCD8-GFP* with MARCM in a sev^{v1} mutant background in which most R7 neurons are absent. In the absence of neighboring R7s, wild-type (E) R7 axons correctly terminate in distinct columns in the M6 layer. (F) The absence of neighboring R7s strongly enhances the frequency of invasion in ttk^{1e11} mutant R7s. (G) Quantitation of mutant R7 axon extension into neighboring columns. (H) Quantitation of mutant R7 bouton width. The difference between ttk^{1e11} and either wild type or babo mutants is statistically significant (single asterisk, $p<0.00001$). Wild type and babo mutants are not significantly different (double asterisk, $p>0.5$).

any rescuing activity (see below). Therefore, I conclude that the Ttk69 isoform has a specific role in regulating R7 axon growth and I will refer to ttk^{1e11} as *ttk69*.

Loss of *ttk69* does not alter R7 neuron fate

To verify that the *ttk69* phenotype is a defect in R7 axon growth and not a byproduct of a failure to properly differentiate, I examined whether loss of *ttk69* altered other aspects of R7 neuron morphology or fate. At 60 hr APF, wild type and *ttk69* mutant R7 cell bodies appear morphologically normal, as assessed by the membrane marker mCD8-GFP, and express the neuronal marker Elav and the R7 fate marker Prospero (Figures 2A-2B"). As R7 neurons are the only cell type in the retina that express this combination of markers, I conclude that loss of *ttk69* is not affecting R7 neuron fate.

However, previous studies have shown that loss of *ttk69* from non-neuronal cells in the retina does alter their fate (Li et al., 1997). Cone cells that lack Ttk69 are transformed into R7 neurons, which I observed as extra R7 cell bodies in some ommatidia (arrows, Figure 2B). These extra R7s extended axons to the medulla, resulting in crowded columns with more than one R7 axon. However, there is strong evidence that the *ttk69* axon overgrowth defect is not due to crowding of R7 axons within a single column. Constitutive activation of Ras1 transforms cone cells into R7s (Fontini et al., 1992), but extra R7s generated in this manner remained restricted to a single column (data not shown; Ashley and Katz, 1994). Additionally, I removed *ttk69* from R7s, but not cone cells, by expressing a *ttk-RNAi* transgene under control of the R neuron-specific driver, Chaoptin-Gal4. I did not observe transformed R7s in these animals, but R7s that expressed *ttk-RNAi* extended into neighboring terminals (Figure 2D). I therefore conclude

that the overgrowth defect in *ttk69* mutant R7 axons is neither a side effect of hyperinnervation of the medulla nor an artifact of transforming cone cells into R7s.

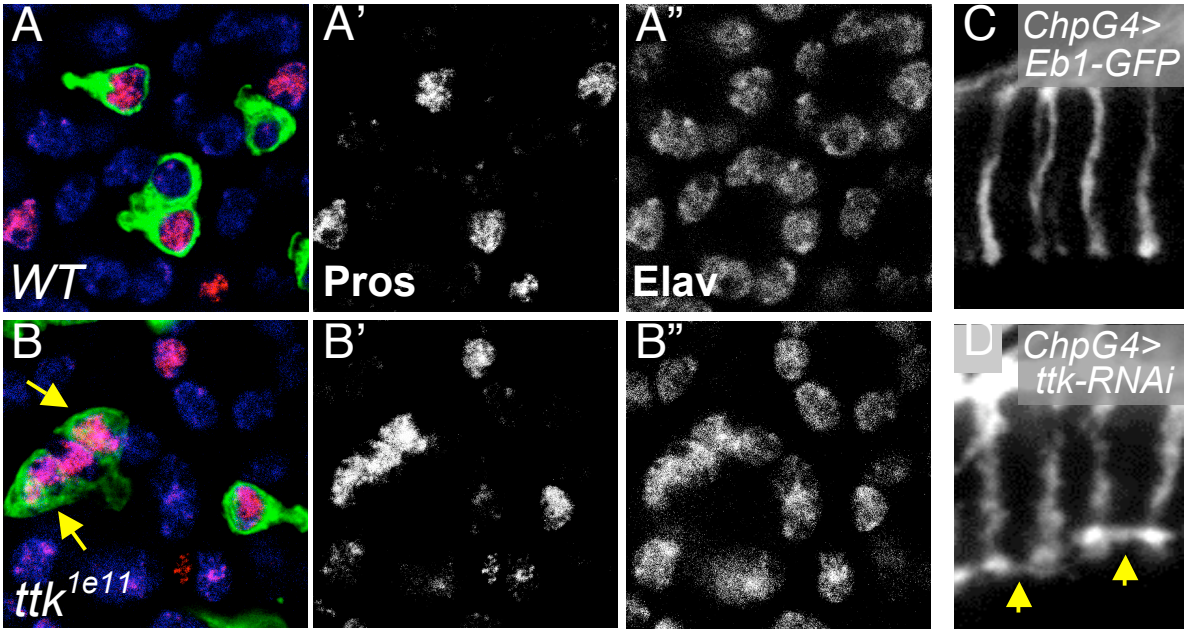

Figure 2. Loss of *ttk69* does not alter R7 neuron fate.
Wild type (A) and *ttk69* (B) mutant R7 neurons are morphologically normal and express Elav (A', B') and Prospero (A", B") at 60 hr APF. Cone cells mutant for *ttk69* are transformed into R7s (arrows). (C) Wild-type R7s that express GFP under control of Chaoptin-Gal4 do not extend into neighboring columns. (D) R7s that express GFP and *UAS-ttk-RNAi* under control of Chaoptin-Gal4 extend into neighboring columns (arrowheads) and exhibit larger terminal boutons.

Ttk69 is both expressed and required in R7 nuclei during R7 target selection

Studies of Ttk69 in the developing retina have primarily focused on its role in non-neuronal cells as a repressor of neural fate (Read and Manley, 1992; Giesen et al., 1997, Badenhorst et al., 2002). To this end, Ttk69 is expressed in all cells in the retina, but is eliminated from cells that will become R neurons (Li et al., 1997). However, work

by Lai and Li identified a positive role for Ttk69 in maintaining differentiated R neurons and showed that Ttk69 is expressed in all R neurons during pupal development (Lai and Li, 1999). In order to determine at precisely which stage of R7 axon extension Ttk69 is acting, I used a Ttk69 antibody to examine Ttk69 expression throughout R7 axon development. I found that Ttk69 is absent from all R neurons at 24 hr APF (Figures 3A and 3A'). However, Ttk69 protein becomes detectable in the nuclei of all R neurons by 30 hr APF (Figures 3B and 3B') and continues through 60 hr APF (Figures 3C-3E'). I did not observe Ttk69 staining in R7 axons, suggesting Ttk69 functions exclusively in the nucleus of R7 neurons. In support of this assertion, I found that loss of Ttk69 from the nucleus phenocopies *ttk69* mutant R7 axons. R7s mutant for the nuclear importer Importin-α3 (*imp-α3*) exclude Ttk69 from the nucleus and *imp-α3* growth cones extend into neighboring columns (Figures 3F and 3G, respectively). *imp-α3* was previously described as a component of Activin pathway signaling in R7 axons (Ting et al., 2007).

To correlate the expression of Ttk69 with the defect in *ttk69* mutant R7 growth cones, I examined the developmental time course of *ttk69* mutant R7 axons. R7 neurons project axons from the retina to their final target layer in the medulla in two steps. First, R7 axons enter the medulla approximately 17 hr APF and pause at a temporary layer. Then, starting between 40 and 50 hr APF, the growth cone transitions to a synaptic

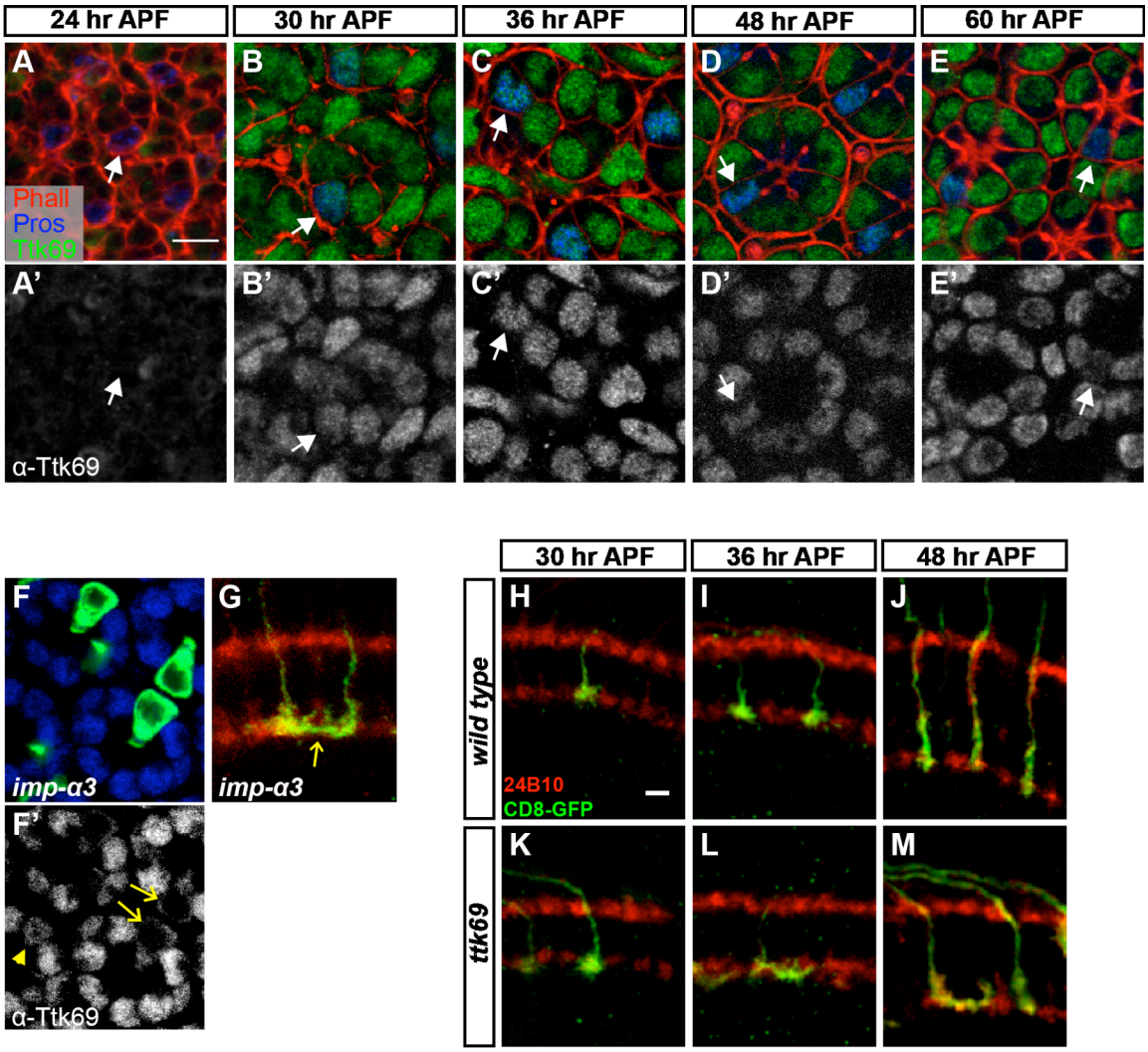

Figure 3. Ttk69 is expressed and required in R7 nuclei as R7 axons complete target selection. (A-E) Pupal retinas in which photoreceptor neurons are outlined by Phalloidin (red). R7 nuclei were visualized with anti-Pros antibodies (blue, arrows). Ttk69 (green) is absent from all R neurons at 24 hr APF (A, A'), but strongly expressed beginning at 30 hr APF (B, B') and continuing to 60 hr APF (C-E). (F) In *imp-α3* mutant R7s generated with GMR-FLP and labeled with *mCD8-GFP*, Ttk69 is mislocalized outside the nucleus at 48 hr APF (arrows indicate *imp-α3* mutant). (G) *imp-α3* mutant R7 axons phenocopy the axon extension phenotype of *ttk69* mutant R7s (arrow). (H-M) Individual wild-type and *ttk69* mutant R7s were generated with GMR-FLP and labeled with *mCD8-GFP*. At 30 hr APF, wild type (H) and *ttk69* (K) R7 axons have extended to their temporary target layer in the medulla. At 36 hr APF, wild-type axons (I) remain paused at their temporary target layer but 10% of *ttk69* mutant axon terminals (L) extend laterally into neighboring columns. At 48 hr APF, wild-type axons (J) have extended to their final target layer and terminate within distinct columns. (M) By 48 hr APF, 22% of *ttk69* mutant R7 axon terminals extend laterally into neighboring columns. Scale bar in A and H represents 3 μm.

bouton at the M6 layer (Ting et al., 2005). Accordingly, at 30 and 36 hr APF individual wild-type R7 axons are paused at their temporary layer (Figures 3H and 3I). By 48 hr APF, wild-type R7s have reached the M6 layer but their terminals have not yet matured into synaptic boutons (Figure 3J). *ttk69* mutant R7 axons resembled wild-type axons at 30 hr APF (Figure 3K), however at 36 hr APF (Figure 3L) 10% of *ttk69* R7s had extended into neighboring columns and by 48 hr APF 22% of *ttk69* axons had invaded neighboring columns (Figure 3M). These results show that the onset of Ttk69 expression coincides with the first signs of *ttk69* mutant R7 axon overgrowth, and suggest that Ttk69 is specifically required during the R7 axon transition from paused growth cone to synapse formation.

Premature Ttk69 expression is sufficient to restrict R7 axon extension

The coincident onset of Ttk69 expression and the *ttk69* mutant axon defect suggests that strict temporal regulation of Ttk69 is necessary to restrict R7 axon growth. Therefore, I hypothesized that premature expression of Ttk69 would disrupt R7 axon extension. I expressed a UAS-Ttk69 transgene in R7s generated with GMR-FLP/MARCM. GMR-FLP induced recombination occurs during third instar larval stage just as R7 neurons are recruited and specified. However, because of Gal80 protein perdurance, labeling of R7s and expression of Ttk69 protein did not occur until approximately 10-15 hours after R7 recruitment. As such, neuronal precursors correctly differentiated into R neurons, but Ttk69 was expressed in labeled R7s much sooner and at much higher levels than normal (Figure 4A-A"). While premature expression of Ttk69 fully rescued the axon defects in *ttk69* mutant R7s, it also prevented 14.3% (n=10) of R7

axons from reaching the M6 layer of the medulla (Figure 4C). To test whether this effect was specific to *ttk69* mutant R7s, I expressed Ttk69 in wild-type R7s. Premature Ttk69 expression resulted in 17.6% (n=9) of wild-type R7 axons failing to reach the M6 target layer (Figure 4D). Quantifying both wild type and *ttk69* mutant R7s revealed that misexpression of Ttk69 slightly favored axon termination at the M1 layer as opposed to intermediate positions between the M1 and M6 layers (9.7% vs. 6.2%, n=19, $p<0.004$). In contrast, misexpressing the Tramtrack isoform Ttk88 failed to rescue *ttk69* mutant R7s and had no effect on R7 axon growth (Figure 4B). To clarify that premature expression of Ttk69 was sufficient for premature R7 axon termination, I also analyzed pupal brains at 24 hr APF, when R7s do not normally express Ttk69. If R7 axons are competent to respond to Ttk69 expression even before it is normally expressed, I predicted early termination of R7 axons at 24 hr APF. Alternatively, if R7 axons are not able to respond to Ttk69 until approximately 30 hr APF, when expression begins in R7s, misexpressing Ttk69 would have little effect on R7 axon growth at 24 hr APF. At 24 hr APF, wild-type R7 axons are paused at a temporary layer in the medulla (Figure 4E; Ting et al., 2005). When I misexpressed Ttk69 in wild-type R7s, I observed R7 axons that failed to reach the temporary layer by 24 hr APF, instead terminating upon entry to the medulla (Figure 4F). These results show that Ttk69 is sufficient to arrest R7 axon growth as soon as R7s express Ttk69. Furthermore, this suggests the precise timing of Ttk69 expression is essential to properly restrict R7 axon growth.

What is controlling the onset of Ttk69 expression at this stage of R7 development? One possibility is that a signal originating at the growth cone is transduced to the nucleus via retrograde axonal transport. To test this, I disrupted

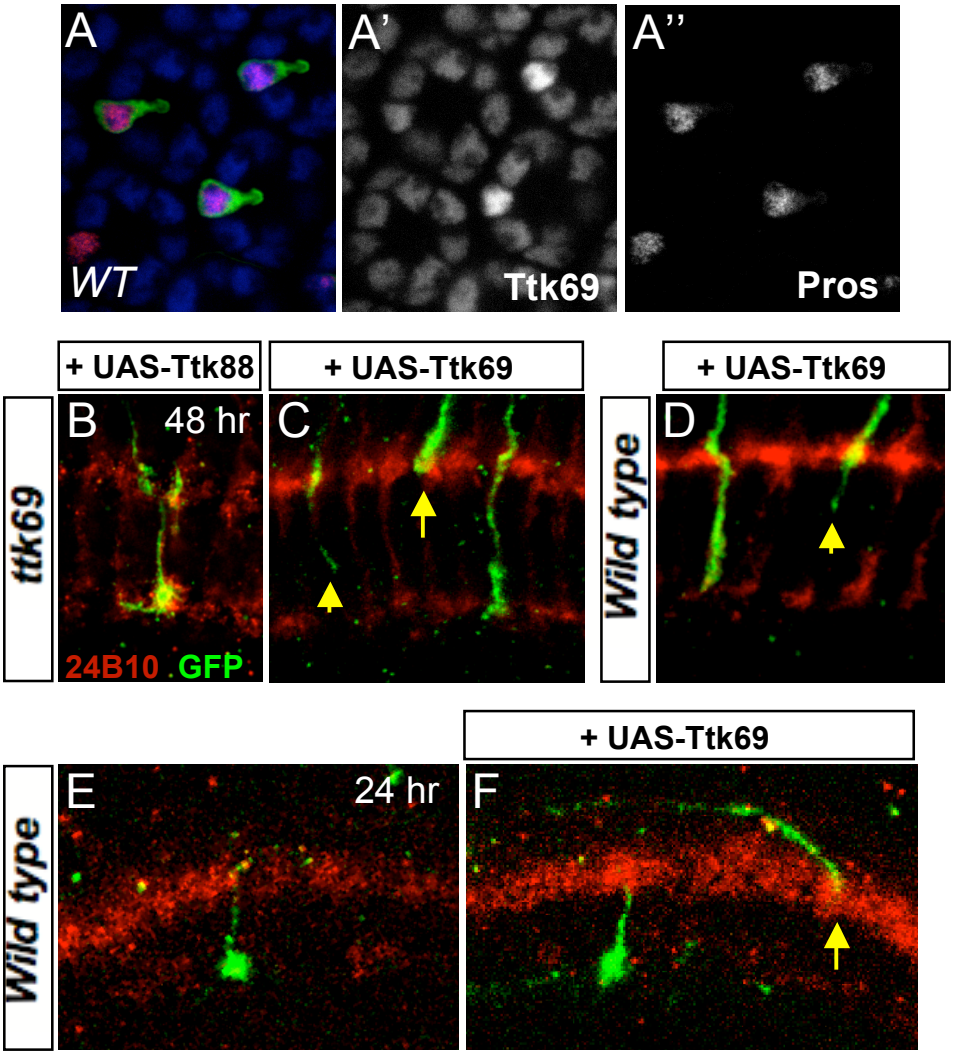

Figure 4. Premature Ttk69 expression is sufficient to restrict R7 axon extension. (A-F) Individual homozygous R7s created with GMR-FLP were labeled with *mCD8-GFP* (green). (A) Expression of UAS-Ttk69 in wild-type R7s does not alter cell body morphology or the R7 cell fate marker prospero (A''). (B) Expression of *UAS-Ttk88* does not rescue *ttk69* mutant R7 axons. (C) Expression of *UAS-Ttk69* rescues the invasion phenotype of *ttk69* mutant R7 axons, but also results in premature termination of R7 axons at the M1 layer (arrow, 8.2%) or an intermediate location between M1 and M6 layers (arrowhead, 6.0%). (D) Wild-type R7s that express *UAS-Ttk69* often fail to reach the M6, stopping at the M1 (11.3%) or an intermediate location between M1 and M6 (arrowhead, 6.3%). (E) At 24 hr APF, R7s normally pause at a temporary layer just beyond the M1 layer. (F) R7s that express *UAS-Ttk69* fail to reach the temporary layer, instead terminating in the M1 layer (arrow).

Dynein/Dynactin-dependent retrograde transport by expressing a dominant negative form of the Dynactin subunit Glued (Gl^{DN}) in individual R7s with GMR-FLP/MARCM (Allen et al., 1999). However, I found that in R7s expressing Gl^{DN}, Ttk69 was expressed normally at 48hr APF (data not shown). This result suggests Ttk69 expression is not dependent on receipt of a growth-cone derived signal, but instead may be controlled intrinsically.

Earlier in development, a Sina/Phyl degradation complex regulates Ttk69 expression (Tang et al., 1997; Li et al., 1997; Li et al., 2002). I hypothesized that relief of this regulation is required for Ttk69 expression in pupal R7 neurons. Phyl is an adaptor protein that links Ttk69 and the E3 ubiquitin ligase Sina, thereby promoting proteosome-dependent degradation of Ttk69. To verify the sufficiency of Phyl to degrade Ttk69 protein, I over-expressed Phyl (*UAS-FLAG-Phyl*) in GMR-FLP/MARCM generated R7s. Phyl over-expression reduced Ttk69 expression and phenocopied the axon defect of *ttk69* mutant R7s at 48 hr APF. Consequently, I predicted that removal of the Sina/Phyl complex would result in premature Ttk69 expression in R7 neurons and premature axon termination. However, when I expressed a RNAi transgene targeting Phyl or Sina under control of longGMR-Gal4 or Chp-Gal4 I did not observe premature Ttk69 expression at 24 hr APF (data not shown). I also expressed a RNAi transgene targeting the putative E3 ligase SinaH (Cooper et al., 2008), but I did not observe premature Ttk69 expression (data not shown). Therefore, while Phyl is sufficient to block Ttk69 expression in R7s, I cannot conclude that downregulation of Phyl-mediated degradation is required for the return of Ttk69 expression in R7s during axon extension.

The *ttk69* mutant axon defect neither prevents nor requires the localization of presynaptic components

We originally identified *ttk69* as a mutation that rendered R7s unable to drive phototaxis to UV light. This raises the possibility that despite reaching the correct synaptic target layer, *ttk69* mutant R7 axons fail to form functional synapses. In mature R7 axon terminals, over 70% of presynaptic sites are located between the M4 and M6 layers of the medulla (Takemura et al., 2008). Accordingly, in wild-type R7 axons, the synaptic vesicle marker Synaptotagmin (visualized with *Syt-GFP*) is predominantly localized between the M4 and M6 layers (Figures 5A and 5A'). To test whether Ttk69 is required to properly localize synaptic vesicles, I expressed *Syt-GFP* in *ttk69* mutant R7s. In *ttk69* mutant R7s, *Syt-GFP* is correctly enriched between M4 and M6, however, there appears to be an increase in the amount of *Syt-GFP* along this region of the axon, consistent with the increased size of *ttk69* R7 terminals (Figures 5B and 5B'). In contrast, R7s mutant for the presynaptic assembly protein dSyd-1 mislocalize *Syt-GFP* along the full length of the axon and have smaller boutons or terminals that fail to form boutons at the M6 layer (Figures 5C and 5C'; Holbrook et al., manuscript in preparation). This result suggests that synaptic components are localizing properly in the absence of Ttk69, but does not resolve whether *ttk69* mutant R7 axons are completing synaptogenesis.

To determine if the ability to form synapses is necessary for the overgrowth of *ttk69* mutant R7 axons, I sought to examine R7s that lack both dSyd-1 and Ttk69. Because *ttk69* and *dsyd-1* are only separated by approximately 100kbp on 3R, we were unable to construct a double mutant chromosome (McQuilton et al., 2011). However, loss of *imp-α3* phenocopies all aspects of *ttk69* mutant R7 axon overgrowth and *Syt-GFP*

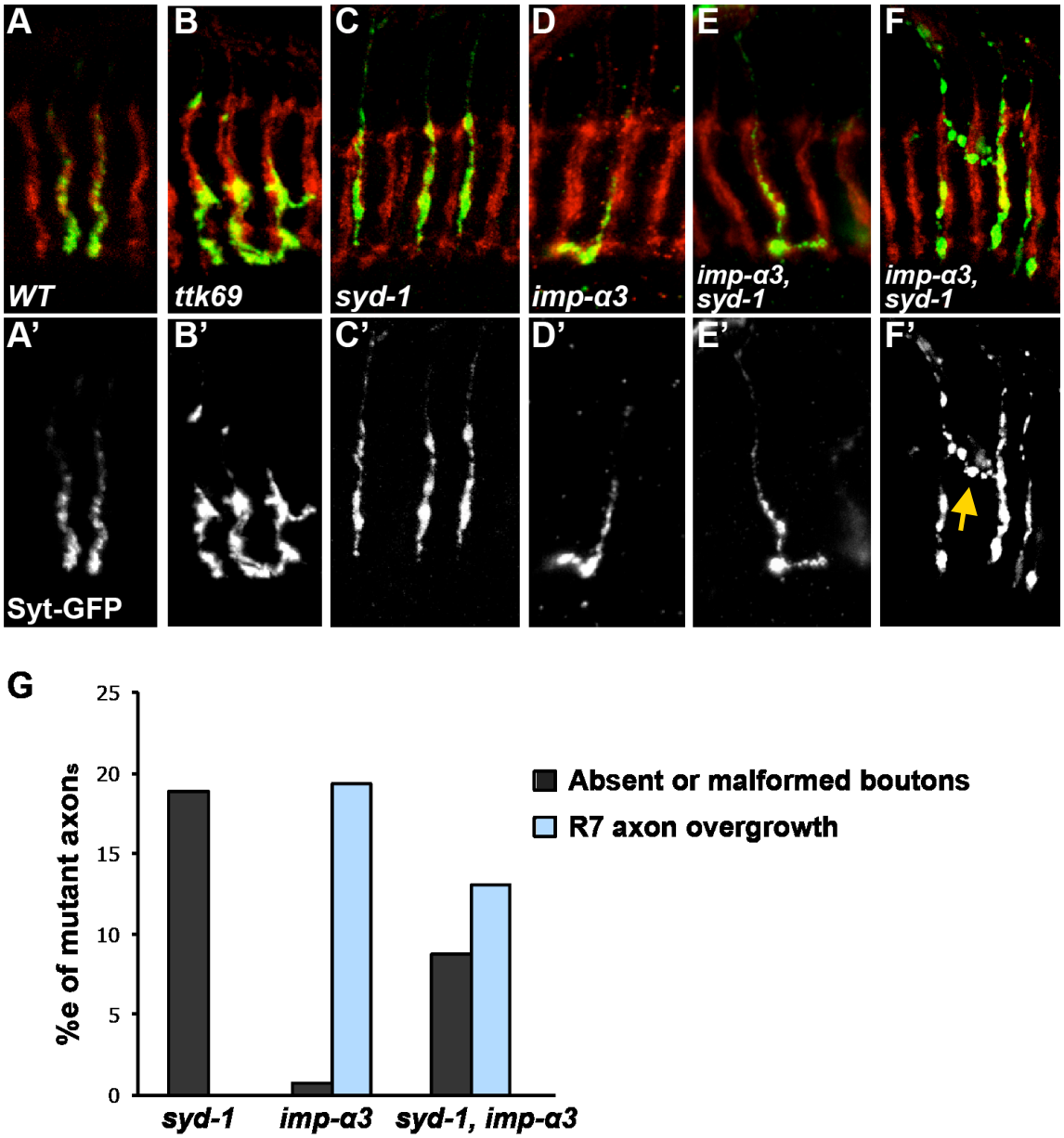

Figure 5. The *ttk69* mutant axon defect neither prevents nor requires the localization of presynaptic components.

Individual homozygous mutant R7s were generated with GMR-FLP and labeled with *Syt-GFP* (green) and medullas were dissected at 60hr APF. (A, A') In wild-type R7 axons, *Syt-GFP* is primarily localized between the M4 and M6 layers of the medulla. (B, B') *Syt-GFP* is predominantly localized between M4 and M6 in *ttk69* mutant R7 axons. (C, C') *dsyd-1* mutant R7 axons mislocalize *Syt-GFP* to all layers of the medulla and display reduced bouton size and retractions from the M6 layer. (D, D') *imp-α3* mutant R7 axons extend into neighboring columns, but correctly localize *Syt-GFP*. (E, F) *Syt-GFP* is mislocalized in *imp-α3, dsyd-1* double mutant R7 axons, and double mutant R7s invade neighboring terminals (arrow, F). (G) Quantitation of axon defects in C-F.

localization (Figures 3G and 5D). *syd-1, imp-α3* double mutants mislocalized *Syt-GFP* and frequently failed to form boutons at the M6 layer (Figures 5E and 5E'). Double mutant R7 growth cones also extended into neighboring columns, even in instances when axons terminated prior to the M6 layer (Figure 5F). These results (quantitated in Figure 5G) suggest *ttk69* mutant R7 axon overgrowth is independent of presynaptic assembly.

Ttk69 acts upstream of the Activin pathway effector dSmad2

Canonical Activin signaling was previously shown to downregulate the intrinsic motility of R7 growth cones (Ting et al., 2007). R7s lacking the Activin receptor Baboon, or the downstream transcription factor dSmad2 correctly reach the M6 layer of the medulla but extend axons into adjacent columns. The lateral invasion phenotype observed in these R7 axons is similar to, although less severe, than that of *ttk69* mutant axons, suggesting that Ttk69 may act upstream of or in parallel to Activin signaling.

To determine whether Ttk69 and Activin signaling function either in parallel or independently to restrict R7 axon growth, I removed Ttk69 from *babo* mutant R7s. If Ttk69 and Babo function in the same pathway to restrict R7 axon growth, I would expect to observe a similar frequency of R7 axon invasion whether I removed Ttk69 alone, or both Ttk69 and Babo. I generated homozygous wild type or *babo* mutant R7s with ey-FLP/MARCM and labeled them with *mCD8-GFP* (Figures 6A and 6B). To remove Ttk69 protein expression, I expressed a *ttk-RNAi* transgene in either wild type or *babo* mutant R7s. In wild type R7s, expression of *ttk-RNAi* resulted in axon invasion similar to the *ttk69* allele (Figure 6C). *babo* mutant R7s expressing *ttk-RNAi* invaded neighboring terminals at the same frequency as wild-type R7s expressing *ttk-RNAi* (Figure 6D). This

result (quantitated in Figure 6E) indicates that Ttk69 and Activin signaling likely function in the same pathway to regulate R7 axon growth.

The output of Activin signaling can be assessed by measuring nuclear accumulation of the effector molecule dSmad2 (Figure 6F). When expressed in R7s, the fluorescent intensity of Flag-tagged dSmad2 is significantly decreased in the nuclei of *babo* mutants at 50 hr APF (Ting et al., 2007). To test if Ttk69 is promoting Activin signaling, I expressed *FLAG-dSmad2* in GMR-FLP/MARCM generated wild type, *imp-α3* and *ttk69* mutant R7s. The levels of nuclear Flag-dSmad2 were significantly decreased in *imp-α3* and *ttk69* mutant R7s compared to wild type (quantitated in Figure 6G). These results indicate Ttk69 is required for Activin signaling and place Ttk69 upstream of dSmad2. If Ttk69 directly promotes dSmad2 localization or expression, it's possible overexpression of dSmad2 could circumvent the requirement for Ttk69 and rescue *ttk69* mutant axons. However, expressing Flag-dSmad2 in *ttk69* mutant R7s did not rescue *ttk69* mutant axon overgrowth (data not shown).

I hypothesized that Ttk69 also acts independently of Activin signaling to regulate R7 growth cone morphology. In contrast to *ttk69* mutants, R7 axons mutant for the Activin receptor Babo (generated with ey-FLP) invaded neighboring terminals less frequently (12.8% versus 21%, Figure 1H) and the remaining non-invasive *babo* mutant axons had wild-type sized terminal boutons (Figures 1D and IH). Collectively, the increased frequency of axon overgrowth and expanded terminal size of *ttk69* mutant R7s constitute an R7 phenotype distinct from that of loss of Activin signaling.

If these phenotypic differences are due to Activin-independent functions of Ttk69, I predicted that Ttk69 would be sufficient to restrict R7 axon growth even in the absence

of Activin signaling. To test this, I overexpressed Ttk69 in R7s that lack Babo. R7s expressing *UAS-Ttk69* under control of the Chp-Gal4 driver failed to reach the M6 medulla layer (11.5%, Figure 6H). R7 axons expressing a *Babo-RNAi* transgene under control of Chp-Gal4 correctly reached the M6 layer but invaded neighboring columns (Figure 6I). When I expressed both Ttk69 and *Babo-RNAi*, a proportion of R7 axons failed to reach the M6 layer (9.1%), while others invaded neighboring columns (Figure 6J). Since Ttk69 was sufficient to induce premature axon termination in the absence of Babo, I conclude that Babo is not absolutely required for Ttk69 function. However, a small amount of Babo protein may still be present in R7s that express *Babo-RNAi*, which could contribute to Ttk69-induced axon termination. In addition, I observed that Ttk69 is not able to fully rescue the loss of Babo in R7s, further supporting the conclusion that Activin signaling and Ttk69 have non-overlapping functions within R7 axons.

Our results strongly suggest Ttk69 functions within canonical Activin signaling to regulate R7 axon extension. I also show that Ttk69 functions independently of Activin signaling to restrict R7 axon growth. These observations point to a broader role for Ttk69 in R7 growth cone regulation and hint that other targets lie downstream of Ttk69.

Discussion

I have shown that the transcription factor Ttk69 is necessary and sufficient to restrict R7 axon growth such that R7 axons form a retinotopic map in the medulla. While Ttk69 has previously been described as a repressor of neuronal development, I have uncovered a novel role for Ttk69 as a regulator of R7 axon growth.

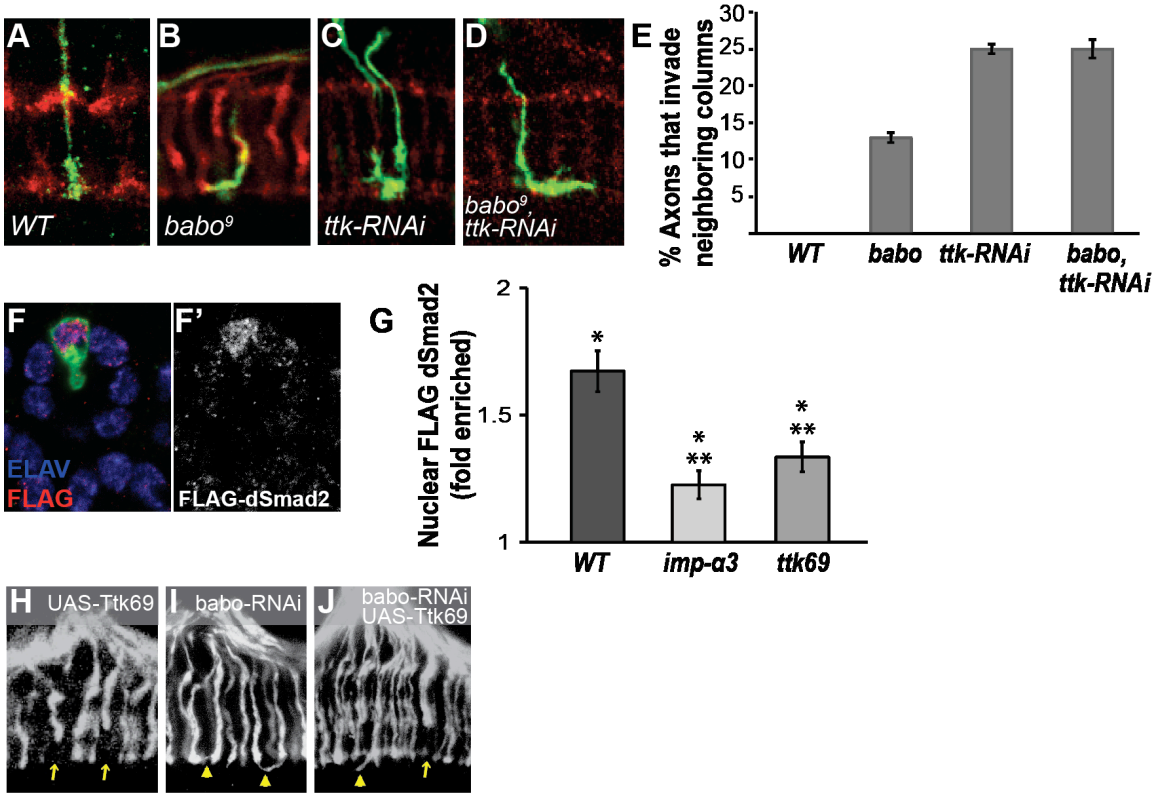

Figure 6. Ttk69 acts upstream of the Activin pathway effector dSmad2.
(A-D) Individual homozygous R7s created with ey-FLP were labeled with *mCD8-GFP* and medullas were dissected at 48 hr APF. (A) Wild type (B) *babo* (C) *ttk-RNAi* (D) *babo, ttk-RNAi*. (E) Quantitation of A-D. There is no difference in the frequency of R7 axon invasion between *babo* mutant R7s that express *ttk-RNAi* and wild type R7s that express *ttk-RNAi*.
(F) *UAS-FLAG-dSmad2* was expressed in individual mutant R7s created with GMR-FLP and labeled with *mCD8-GFP*. (G) Quantitation of nuclear FLAG-dSmad2 in wild type, *imp-a3* and *ttk69* mutant R7 nuclei at 50 hr APF. The accumulation of FLAG-dSmad2 that occurs in wild-type R7 nuclei is reduced by a statistically significant amount in *imp-α3* and *ttk69* mutant R7s (single asterisk, $p < 0.005$). The difference between *imp-α3* and *ttk69* mutant R7s is not statistically significant (double asterisk, $p>0.5$).
(H-J) R7s were labeled with *EB1-GFP* (green) under control of Chp-Gal4 and dissected as adults. (H) Wild-type R7s overexpressing *UAS-Ttk69* fail to reach the M6 layer (arrows). (I) Wild-type R7s expressing *UAS-baboRNAi* reach the correct target layer but invade neighboring axon terminals (arrowheads). (J) Wild-type R7s co-expressing *UAS-Ttk69* and *UAS-baboRNAi* fail to reach the M6 layer (arrow), but also extend into neighboring columns (arrowhead).

R7 growth cone motility is regulated transcriptionally

I showed that Ttk69 is expressed in the nucleus of R7 neurons and nuclear localization of Ttk69 is required for R7 axon growth to occur properly, confirming a likely role for Ttk69 as a transcriptional regulator of axon growth. Transcription factors that control axon growth frequently also regulate neuron specification and polarity, making it difficult to determine their distinct role in each process. For example, in the *Drosophila* visual system, the Zn-finger transcription factor Hindsight (Hnt) regulates neuronal polarity and morphology as well as axon pathfinding. However, a recent study was able to uncouple the axonal defect in *hnt* mutants from differentiation defects (Oliva and Sierralta, 2010). Similarly, I found that the axon overgrowth that results from loss of Ttk69 is independent of R7 neuronal fate specification.

R7 neurons normally extend axons that pause at a temporary layer in the medulla, which allows the remaining R7 axons to arrive and fill out the retinotopic columns of the medulla. R7 axon pausing also coincides with further development of the medulla neuropil, which may help coordinate eventual R7 synaptic target selection (Ting et al., 2005). I found that *ttk69* mutant R7s correctly extend axons to the medulla, but their growth cones do not remain paused. During the period when wild type R7 axons are paused, *ttk69* mutant growth cones abnormally extended lateral projections toward neighboring R7 axon terminals. Notably, these abnormal projections continue to grow longer throughout development, even after wild type growth cones have transitioned to synaptic boutons. The exact nature of *ttk69* mutant R7 overgrowth remains unclear, in part because of speculation surrounding the process that transforms R7 growth cones into synaptic boutons. After pausing at the R7-temporary layer in the medulla, R7 growth

cones ultimately extend 3-5 μm further into the medulla before transitioning to boutons (Ting et al., 2005). It is unknown whether this final extension is an active or passive process, the latter conceivably facilitated by adhesive target interactions pulling the growth cone and stretching the axon as the medulla neuropil expands. Our results suggest that the default R7 state is to restrict growth during this period and support a passive model of R7 axon extension. Furthermore, although *ttk69* mutant growth cones extended lateral projections into neighboring axon terminals before axon extension was complete, axons that had crossed into a neighboring terminal did not continue their extension in the invaded column (Figure 3). Therefore, it appears that growth cone projections do not drive R7 axon extension during this final step.

The timing of Ttk69 expression is critical to R7 axon growth

Ttk69 is sufficient to repress neural fate and must be removed from neuronal precursors, yet is later expressed in R7 axons after they have extended to the medulla. The *ttk69* mutant and gain of function phenotypes highlight the importance of this dynamic Ttk69 expression in R7 neurons. R7s that lack Ttk69 extend axons to the medulla normally, but fail to restrict growth cone motility once within the medulla. On the other hand, R7s that prematurely express Ttk69 become stuck upon entering the medulla and fail to reach their target. Therefore, precise temporal expression of Ttk69 is essential for proper R7 axon growth. Analogously, the tramtrack-related transcription factor Sequoia is expressed at different times in either R7 or R8 neurons in order to regulate axonal targeting to distinct layers of the medulla. (Petrovic and Hummel, 2008).

I hypothesized that temporal expression of Ttk69 is regulated both early and late by the presence or absence of the Phyl/Sina complex that targets Ttk69 for ubiquitylation (Li et al., 2002). However, expressing RNAi constructs directed towards this complex did not result in premature Ttk69 expression. There is evidence that ubiquitin-mediated degradation of Ttk69 is regulated by a developmental switch that is balanced by Phyl/Sina and the deubiquitylating enzyme Ubp64 (Bajpe et al., 2008), in which case merely removing Phyl/Sina may not be sufficient to prematurely reverse Ttk69 degradation.

Ttk69 promotes canonical Activin signaling to regulate R7 growth cone motility

The restriction of R7 axons to individual columns in the medulla is accomplished by extrinsic and intrinsic mechanisms. Repulsive interactions, mediated in part by the adhesion molecule Turtle (Ferguson et al., 2009), prevent R7 axons from invading neighboring columns. Distinct from repulsive interactions, canonical Activin signaling was identified as an intrinsic regulator of R7 axon growth that is required to prevent R7 axons from invading neighboring terminals (Ting et al., 2007).

Here I have presented evidence that Ttk69 promotes Activin signaling as part of the intrinsic regulation of R7 growth cone motility. Nuclear accumulation of the Activin effector dSmad2 is reduced in R7s mutant for the Activin receptor Babo, indicating that dSmad2 is an output of Activin signaling (Ting et al., 2007). I found that loss of *ttk69* also reduces nuclear dSmad2 accumulation, suggesting that Ttk69 is required for transduction of Activin signaling. In addition, I found that abnormal R7 growth cone

extensions caused by loss of Ttk69 are not enhanced by loss of Babo, further suggesting Ttk69 functions within the Activin pathway.

However, the method by which Ttk69 promotes Activin signaling is unknown. Our genetic analysis indicates that Ttk69 acts upstream of dSmad2, however I found that overexpressing dSmad2 is not sufficient to rescue loss of Ttk69. It is possible that overexpressed dSmad2 is not adequately phospho-activated by Babo and therefore unable to rescue *ttk69* mutant R7s, but an additional possibility is that dSmad2 is not the functional output of Activin signaling in R7 neurons. Canonical Activin signaling is transduced by dSmad2 and the co-Smad Medea (Newfeld and Wisotzkey, 2006), but loss of dSmad2 is reported to cause a much weaker R7 axon growth defect than loss of Babo (Ting et al., 2007), and we found that loss of Medea has no effect on R7 axon growth (data not shown).

Investigating the relationship between Ttk69 and Activin signaling has also revealed that Ttk69 likely acts via an Activin-independent mechanism to restrict R7 axon growth. I found that Activin signaling is not required for Ttk69 expression in R7 neurons and, even in the absence of Babo, Ttk69 misexpression is sufficient to arrest R7 axon growth. Future studies to identify the downstream targets of Ttk69 will lend insight into its role in Activin and Activin-independent signaling pathways.

What are the downstream targets of Ttk69?

I show that the growth cones of *ttk69* mutant R7 axons are abnormally large and extend into neighboring axon terminals. Growth cone motility is driven by coordination of microtubules and filamentous actin in the axon shaft and growth cone (Tanaka et al.,

1995). Therefore, molecules that participate in microtubule and actin cytoskeleton organization are reasonable targets of Ttk69 regulation. The MAP1B-like protein Futsch is required for microtubule organization in *Drosophila* (Roos et al., 2000) and mutations in *tramtrack* lead to ectopic expression of the Futsch antigen, 22C10, in the somatic and visceral musculature of the mesoderm (Giesen et al., 1997; Hummel et al., 2000). While Ttk69 may regulate Futsch in R7 growth cones, I did not observe 22C10 expression in wild type or *ttk69* mutant R7 cell bodies or axons (data not shown).

Because the DNA-binding specificity of Ttk69 is known, I was able to screen the *Drosophila* genome for Ttk69 binding sites and compile a list of putative Ttk69 transcriptional targets (Table 1). Utilizing MotifMap's Motif Search tool and the Genomatix MatInspector tool, I screened both the entire *Drosophila* genome and a database of *Drosophila* promoter regions, respectively, for Ttk69 binding sites (Xie et al., 2009; Cartharius et al., 2005). Motif Search yielded 1047 genes and MatInspector yielded 1410 genes, with 573 overlapping genes found in both data sets. Of these 573, 36 genes contained multiple binding sites (Table 1), and two genes were identified which have been experimentally verified as targets of Ttk69 (*ftz* and *eve*). However, this analysis lacks functional significance and our results do not indicate transcriptional function. The modENCODE project has compiled Chromatin immunoprecipitation (ChIP) data that offers a functionally relevant survey of Ttk69 binding (Roy et al., 2010) and, recently, two groups published microarray expression profiles in response to Ttk69 knockdown and misexpression (Reddy et al., 2010; Rotstein et al., 2011). These emerging data sets highlight a list of genes across several gene ontology categories and signaling pathways that may be under Ttk69 transcriptional control. However, these studies were not

conducted in R7 neurons, but rather in S2 cells, whole embryos, and a tracheal cell population. Nonetheless, recent reports suggest Ttk69 regulates cellular rearrangements and the cytoskeleton during tracheal tube formation (Araújo et al., 2007), epithelial morphogenesis (French et al., 2003), and cell shape regulation during oogenesis (Boyle and Berg, 2009), which indicates Ttk69 may act on similar targets in R7s and other cell types. Therefore, the results of Ttk69 transcriptome studies in other cell types may yet be instructive in understanding the role of Ttk69 in R7 growth cone motility. Future research will explore these potential downstream targets by taking advantage of the robust phenotype of *ttk69* mutant R7 axons and the powerful genetic tools available to examine R7 axons.

Gene	Molecular Function	Biological Process
AP-2	Transcription Factor	Proboscis, limb development
Bx	Transcription Factor	Leg, wing morphogenesis, phagocytosis
Ets65A	Transcription Factor	Transcriptional regulation
eve	Transcription Factor	Axon guidance, cell fate specification
pdm3	Transcription Factor	Transcriptional regulation
cngl	Cation channel	Unknown
Gr43a	Chemoreceptor	Sensory perception of taste
jeb	[LDL receptor-like]	Axon guidance
Nmdar2	Glutamate receptor	Ion transport
Ptp99A	Transmembrane receptor, tyrosine phosphotase	Axon guidance
rad	Calcium ion binding; phospholipase A2	Regulation of heart contraction
Slob	Protein binding; protein kinase	Synaptic transmission
Task6	Potassium channel	Potassium ion transmembrane transport
trp	Calcium channel	Sensory perception, calcium ion transmembrane transport, G-protein signaling
CG31646	[Immunoglobulin-like]	Unknown
CG34393	Guanyl-nucleotide exchange factor	Small GTPase mediated signal transduction
CG5226	Neurotransmitter transporter	Neurotransmitter transport
CG5549	Neurotransmitter transporter	Neurotransmitter transport
CG7918	G-protein coupled Ach-receptor	G-protein coupled Ach receptor signaling pathway
Rbp9	mRNA binding	Establishment of blood-brain barrier
shep	mRNA binding	Gravitaxis
CG1544	Oxoglutarate dehydrogenase	Tricarboxylic acid cycle
CG9235	Carbonate dehydratase	Unknown
m6	Unknown	Cell fate specification
malpha	Unknown	Sensory organ development, Notch signaling, cell fate specification
Mst33A	Unknown	Unknown
sty	Unknown	Tracheal development, photoreceptor development
CG13636	Unknown	Unknown
CG30419	Unknown	Unknown
CG31055	Unknown	Unknown
CG34109	Unknown	Neurogenesis
CG5139	Unknown	Unknown
CG5217	Unknown	Unknown

Table 1. Putative transcriptional targets of Ttk69
The *Drosophila* genome was analyzed for regions that contain aTtk69 DNA binding site. Genes, or predicted genes, that contained more than one hit either upstream of or within their intergenic sequence are listed above.

CHAPTER III

HOW DOES TTK69 REGULATE R7 AXON GROWTH?

In Chapter II, I described my work showing that the BTB/POZ zinc-finger transcription factor Ttk69 regulates a specific stage of R7 axon growth. In particular, I demonstrated that Ttk69 is required for the final step of R7 axon development, as R7s prepare to extend from a temporary layer to their final target layer. This final extension not only brings R7 axons to their synaptic targets, but also involves the transition from a motile growth cone to a synaptic bouton. While *ttk69* mutant R7 axons appear to correctly assemble the components necessary for synapse formation at the appropriate target layer, they fail to successfully transition out of a growth mode, and thus presumably fail to form synapses. While the failure to phototax toward UV light suggests a failure of synapse function, it is possible that *ttk69* mutant R7s form synapses but sensory processing is muddled by extra or inappropriate synapses. Since Ttk69 appears to regulate an essential, but poorly understood component of axon growth and synapse formation, determining the downstream targets of Ttk69 is of significant biological and clinical importance. I have already linked Ttk69 to Activin signaling, but the higher frequency of axon overgrowth and larger bouton size of *ttk69* mutant R7s compared to loss of Activin signaling components suggests Ttk69 is also acting through an Activin-independent pathway to regulate R7 axon growth.

In this chapter I will explore preliminary evidence that suggests a MAP kinase cascade functions downstream of or in concert with Ttk69. In addition, I will propose a

molecular mechanism for Ttk69 regulated growth cone motility based on studies in R8 photoreceptor axons and the use of live imaging.

Does Ttk69 Act Through JNK Signaling?

The MAP kinase kinase kinase Wallenda (Wnd), a homolog to the vertebrate dual leucine zipper kinase (DLK), has a demonstrated role in synaptic growth and axonal regeneration in *Drosophila* and the nematode *Caenorhabditis. elegans* (Collins et al., 2006; Hammarlund et al., 2009). In both studies, loss of Wnd was found to have no effect on neuronal development as Wnd is normally post-transcriptionally degraded. However, overexpression of Wnd is sufficient to promote synaptic overgrowth at the *Drosophila* neuromuscular junction and sufficient to induce exuberant axon regeneration in severed *C. elegans* motor neurons. When I overexpressed Wnd in individual homozygous wild-type R7 neurons, R7 axons extended into neighboring columns similar to *ttk69* mutant R7 axons (data not shown). Similar to loss of Activin signaling, the bouton size of non-invasive R7s was not increased compared to wild type. Because Wnd overexpression exhibits the same phenotype as loss of Ttk69, and Ttk69 has been shown to function as a transcriptional repressor when regulating neuronal fate, I hypothesized that Ttk69 might be required to degrade Wnd expression during R7 axon extension. If this hypothesis is valid, I would expect removal of Wnd to rescue or partially rescue the *ttk69* mutant R7 axon phenotype. Future work will attempt to answer this question.

As a MAPKKK, Wnd sits at the top of a signaling cascade that culminates in the phosphoactivation of a MAP kinase (MAPK), which then acts on a substrate molecule. At the neuromuscular junction, Wnd acts through the MAPK JNK (Basket [Bsk] in

Drosophila) and the transcription factor Fos (Collins et al., 2006). If Ttk69 is acting as a repressor of Wnd, Bsk or Fos, removal of one of these proteins from R7s would be expected to suppress *ttk69* mutant R7 axon overgrowth. Fos was removed from R7s by expressing a dominant negative version that contains the DNA binding and dimerization domains but lacks the transcriptional activation domain (Eresh et al., 1997). Individual homozygous *ttk69* mutant R7s that express Fos^{DN} partially rescue the *ttk69* mutant phenotype ($p>0.05$, Sasha Feoktistov, unpublished data). This result suggests that the transcription factor Fos may act downstream of Ttk69. If removal of Wnd and Bsk also at least partially rescues the *ttk69* mutant R7 axon overgrowth defect, it would confirm that Ttk69 indeed functions as a repressor of Wnd signaling. To validate this claim, future work will seek to demonstrate that loss of *ttk69* leads to an increase in Wnd levels in the R7 cell body or axon.

In a recent study, Oliva and Sierralta expressed a dominant negative Bsk transgene in R7s with the eye-specific driver GMR-Gal4 and observed that R7 axons extended into neighboring columns (Oliva and Sierralta, 2010). This result is surprising considering our observation that overexpressing Wnd causes R7 axon overgrowth and loss of Fos rescues *ttk69* mutant overgrowth. In light of this report and my data, it is possible that Bsk may not function as a MAPK downstream of Wnd to regulate R7 axon growth. In *C. elegans*, Wnd has been shown to act through the MAPK p38 (Nakata et al., 2005). Future work will determine whether Ttk69 functions via Wnd signaling, and if so, which MAPK is relaying the kinase cascade downstream of Wnd.

What Is the Mechanism of *ttk69* Mutant Overgrowth?

Despite identifying two potential pathways with which Ttk69 interacts to regulate R7 axon growth, the molecular basis of the *ttk69* mutant phenotype is still unknown. Activin and Wallenda signaling are both transduced by nuclear effectors of gene expression and do not intuitively offer any insight into the molecules directly regulating the transition from R7 growth cone to synaptic bouton. However, observations from the *ttk69* mutant phenotype provide preliminary evidence to support a model in which Ttk69 is required to regulate microtubule assembly and disassembly in the motile growth cone.

I have shown that R7 axons that lack Ttk69 extend projections into neighboring columns and exhibit oversized growth cones that do not transition to synaptic boutons. I also examined R8 axons that lack Ttk69 expression. I expressed a *ttk-RNAi* transgene exclusively in R8 neurons with the R8 specific driver 2-80-Gal4, and I expressed *ttk-RNAi* in both R7 and R8s with the R neuron driver Chaoptin-Gal4. In both experiments, R8 axons that lacked Ttk69 failed to extend to their final target layer, the M3 layer of the medulla. Rather, these R8 axons remained in their temporary layer in the medulla. I propose that the distinct phenotypes of R7 and R8 axons are due to the differing mechanisms that regulate axon extension and targeting in each neuron.

R8 neurons are the first to differentiate in the developing retina and also the first to extend axons from the retina to the brain. Upon entering the medulla, R8 axons provide an anterograde signal to the medulla neurons that results in proper expression of the cell-adhesion molecule Flamingo in both R8s and medulla neurons (Bazigou et al., 2007). Flamingo expression is required for proper targeting of R8s to the M3 layer and restriction of R8s to individual columns. Flamingo expression is also required for R8s to

correctly pause at the R8-temporary layer. The transmembrane protein Golden Goal is also required for R8 pausing, and migration away from the temporary layer can be prevented by prolonged expression of Golden Goal, suggesting it is a key regulator of R8 axon anchoring at the temporary layer (Lee et al., 2003; Tomasi et al., 2008). After pausing, R8 growth cones extend filopodia toward the M3 layer, while leaving the bulk of their growth cone paused in the temporary layer (Ting et al., 2005). A homophilic interaction between R8 filopodia and target neurons in M3 is mediated by the cell surface molecule Capricious, and ensures R8 axons stop at the M3 rather than continuing on to the M6 (Shinza-Kameda et al., 2006).

Aside from the additional adhesion molecules that are required for proper R8 but not R7 extension, the most significant difference in R7 and R8 extension is the complex level of regulation at the R8 temporary layer. Therefore, I hypothesize that a defect in growth cone regulation could result in R8 axons becoming stuck at the temporary layer. I found that loss of Ttk69 results in R8 axons that do not extend from the temporary to final target layer.

Is the *ttk69* R8 phenotype mechanistically distinct from the *ttk69* R7 phenotype? In chapter III, I described the evidence that suggests R7 axon extension from the paused layer to the final target layer is a passive process. ttk69 mutant R7 axons have overgrown growth cones that do not appear to drive axon extension. Rather, I hypothesize that *ttk69* mutant R7 growth cones that have extended into neighboring columns are stuck and then pulled along in the same position as the R7 axon passively extends. If this hypothesis is correct, then R7 and R8 axons share a similar *ttk69* loss of function phenotype

To address this hypothesis, we have begun analyzing R7 and R8 growth cones in live animals. Given the dynamic nature of growth cone motility, studying axon extension in real-time is the logical next step toward understanding Ttk69 regulated axon growth. The periphery of a growth cone contains a mesh network of F-actin and long F-actin bundles that form the lamellipodia and filopodia, respectively. Within the axon, bundles of microtubules extend pioneer microtubules that travel along F-actin bundles into the growth cone (Lowery and Van Vactor, 2009). To visualize R7 and R8 axons, we are expressing an assortment of fluorescently tagged proteins under control of the R neuron specific driver Chaoptin-Gal4. The protein EB1 binds to microtubule plus ends that are in a growth state, therefore expressing EB1-GFP allows us to measure microtubule growth rate based on the movement GFP puncta (Tirnauer et al., 1999; Mimori-Kiyosue et al., 2000). In addition, we will be expressing fluorescent tags to label the actin cytoskeleton as well as the axon cell membrane.

Initial results suggest that R7 axons that lack Ttk69 have larger growth cones that appear disorganized and express diffuse levels of EB1-GFP compared to wild type (Sasha Feoktistov, unpublished data). Prelimary analysis suggests these larger growth cones also exhibit a slower rate of EB1-GFP puncta movement. Wild type growth cones extend and retract filopodia as they probe their surroundings. Meanwhile, growth cones that lack Ttk69 extend filopodia but fail to efficiently retract these filopodia, resulting in projections that are stuck. While this work is very preliminary, it suggests a model in which Ttk69 is required for microtubule disassembly to retract probing filopodia. Further analysis with other fluorescent markers of growth cone components will undoubtedly allow us to expand upon this model. Whether this model also applies to R8 growth cone

extension is unknown, as we have yet to analyze R8 growth cones under live imaging conditions. Future experiments will also focus on comparing fixed-tissue studies with live imaging analysis to assay the candidacy of putative Ttk69 targets.

CHAPTER IV

CONCLUSIONS

My research has focused on understanding a single step of neural connectivity, the transition from axon extension to synapse formation. Significant advances have been made in understanding the cellular machinery that drives axon extension and identifying the guidance cues that provide directionality to the growth cone. However, the details of how extrinsic guidance cues are translated to signaling events, which then regulate cytoskeletal interactions in the growth cone, are less clear. Additionally, both the scope and potential role of intrinsic instructions are poorly understood.

At sites of paused growth cones, for example, a reorganization of the microtubule and actin network that drives growth cone motility is often observed in the form of 'microtubule looping', a process mediated by various families of microtubule-associated proteins (MAPs). Wnt signaling promotes microtubule looping by depleting the MAP APC (Purro et al., 2008), whereas overexpression of members of the CLASP family of MAPs can lead to looping and prevention of growth cone extension (Mimori-Kiyosue et al., 2005; Lee et al., 2004). It is clear that tight control of the various factors involved in microtubule organization is essential to coordinate growth cone behavior and thus neural connectivity. My work on a transcriptional regulator required to restrict axon growth suggests a role for intrinsic regulation at the level of gene expression. Further study of Ttk69 and its downstream targets should lead to a greater understanding of how neurons coordinate cytoskeletal machinery at the growth cone in response to both developmental

timing and extrinsic cues. I presented evidence that Activin signaling is one such pathway, and I am pursuing genetic and live imaging studies in R7s to identify more regulators of axon growth.

A second, and less expected, finding from this study is in regard to medullar layer formation in the *Drosophila* optic lobe. While R7 axons do not appear to be involved in the signaling that directs development of the medulla neuropil or the decision of which medullar column to innervate, the pattern of R7 axon extension in wild type and *ttk69* mutant R7s suggests an apparent mechanism for layer-specific connections within the medulla. I observed that *ttk69* mutant R7 fully extend axons within a single column, despite the fact that mutant growth cones often invade neighboring terminals long before axons have fully extended. This suggests that R7 axons passively extend by the growth of the medullar environment they inhabit. The fact that R7 axons extend en masse to their synaptic target layer, despite arriving in the medulla sequentially, adds further weight to this premise. While R8 axons don't appear to extend in a similar fashion, this unexpected R7 extension may represent a common method of axon growth and targeting.

CHAPTER V

MATERIALS AND METHODS

UV/Visible Light-Choice Assay

The effects of mutations on R7 function were assayed by creating mosaic animals having homozygous mutant R7s (with GMR-FLP and PAN-R7-tetanus toxin and testing their preference for UV light in a UV/Vis choice test as described in Lee et al., 2001).

Genetics

Homozygous wild-type, $ttk1^{e11}$, $imp\text{-}\alpha3^{D93}$, or $syd1^{w46}$ R7s were created by GMR-FLP induced mitotic recombination between FRT82 chromosomes (Lee et al., 2001). Homozygous $babo^{9}$ R7s were created with ey-FLP and FRT42. Homozygous cells were labeled by the MARCM system (Lee and Luo, 1999) with act-Gal4 UAS-mCD8-GFP or UAS-Synaptotagmin (Syt)-GFP. Mosaic animals lacking R7s were males hemizygous for sev^{v1}. The following stocks were used to express transgenes in R7s: UAS-Ttk69 (#7361, Bloomington); UAS-Ttk88 (#7360, Bloomington); UAS-FLAG-dSmad2 (a generous gift from Chi-Hon Lee); UAS-ttk-RNAi (v101980, VDRC); UAS-babo-RNAi (v106092, VDRC).

Histology

Fixation was in 4% PLP at room temperature for 20 minutes. Antibody staining was as described in Lee et al. (2001). Confocal images were collected on a Leica SP2

microscope and analyzed with ImageJ software. The following concentrations of antibodies were used: mouse anti-Pros mR1A, 1:500 dilution (C. Doe, University of Oregon); chicken anti-GFP, 1:1000 dilution (Abcam); mouse anti-FLAG M5, 1:200 dilution (Sigma); mouse anti-Chaoptin 24B10, 1:200 dilution; rat anti-Elav 7E8A10 1:5 dilution (Developmental Studies Hybridoma Bank); rabbit anti-Ttk69, 1:500 dilution; rabbit anti-GFP, 1:10,000 dilution, phalloidin conjugated to Alexa Fluor 555 1:10 dilution, all secondary antibodies (goat IgG coupled to Alexa Fluor 488, 555, or 633), 1:250 dilution (Molecular Probes); rabbit anti-Ttk69, 1:500 dilution.

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