R7 Photoreceptor Axon Growth Is Temporally Controlled by the Transcription Factor Ttk69, Which Inhibits Growth in Part by Promoting Transforming Growth Factor- β /Activin Signaling

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Work on axon growth has classically focused on understanding how extrinsic cues control growth cone dynamics independent of the cell body. However, more recently, neuron-intrinsic transcription factors have been shown to influence both normal and regenerative axon growth, suggesting that understanding their mechanism of action is of clinical importance. We are studying axon targeting in the *Drosophila* visual system and here show that the BTB/POZ zinc-finger transcription factor Tramtrack69 (Ttk69) plays an instructive role in inhibiting the growth of R7 photoreceptor axon terminals. Although *ttk69* mutant R7 axons project to the correct medullar target layer, M6, their terminals fail to remain retinotopically restricted and instead grow laterally within M6. This overgrowth is not caused by an inability to be repelled by neighboring R7 axons or by an inability to recognize and initiate synapse formation with postsynaptic targets. The overgrowth is progressive and occurs even if contact between *ttk69* mutant R7 axons and their normal target layer is disrupted. Ttk69 is first expressed in wild-type R7s after their axons have reached the medulla; *ttk69* mutant R7 axon terminal overgrowth begins shortly after this time point. We find that expressing Ttk69 prematurely in R7s collapses their growth cones and disrupts axon extension, indicating that Ttk69 plays an instructive role in this process. A TGF- β /Activin pathway was shown previously to inhibit R7 axon terminal growth. We find that Ttk69 is required for normal activation of this pathway but that Ttk69 likely also inhibits R7 axon growth by a TGF- β /Activin-independent mechanism.

Introduction

The growth cone, a specialized structure at the axon tip, advances, turns, or halts by regulating cytoskeletal actin and microtubules (Vitriol and Zheng, 2012). Growth cones are acutely responsive to their environment and can interpret extrinsic guidance cues without instruction from the cell body. In addition to the four major families of classical guidance cues, many other extrinsic factors, including members of the TGF- β family, can influence axon growth (Kolodkin and Tessier-Lavigne, 2011). Past efforts to promote axon regeneration in the CNS have therefore focused on understanding the extrinsic inhibitory signals that prevent injured axons from regrowing. However, recent work has shown that neuron-intrinsic transcription factors also contribute to the varying abilities of axons to grow both during normal development and after injury (Sun and He, 2010; Moore

and Goldberg, 2011). Manipulation of these factors can promote axon growth even in the presence of environmental inhibitors, highlighting the potential clinical importance of understanding their properties.

We are studying axon targeting using the R7 photoreceptor neurons in the *Drosophila* visual system. Each wild-type retina contains ~750 R7s, each of which is paired with an R8 photoreceptor neuron (Wolff and Ready, 1993). R7 axons extend to their final target layer in the medulla, M6, in two main steps (Astigarraga et al., 2010; Hadjieconomou et al., 2011). First, each R7 axon extends along the previously extended R8 axon to reach the medulla and pauses at a temporary target layer located just beyond the already paused R8 axon terminal. Next, the R7 axon terminals move slowly forward—whether by active migration or because of passive displacement by intercalating neuronal processes remains unclear—until ultimately they form a non-overlapping, retinotopic array in the M6 layer. Previous work has shown that components of a TGF- β /Activin signaling pathway, including the Activin receptor Baboon (Babo), are required to prevent overgrowth of R7 axon terminals during the second phase of targeting (Ting et al., 2007): *babo* mutant R7 axon terminals correctly reach their final M6 target layer but extend laterally within M6, resulting in overlap with adjacent wild-type R7 terminals.

Here we show that the transcription factor Tramtrack69 (Ttk69) plays an instructive role in inhibiting R7 axon growth. Like loss of Babo, loss of Ttk69 causes R7 axon terminals to ex-

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tend laterally within M6. We show that Ttk69 is not required for R7 axons to repel one other or to recognize their synaptic targets, suggesting that Ttk69 instead controls the intrinsic ability of R7 axon terminals to grow. The timing of Ttk69 expression is critical: Ttk69 is specifically expressed in R7s only after their axons have paused in the medulla, *ttk69* mutant R7 terminal overgrowth begins shortly afterward, and premature Ttk69 expression causes R7 growth cone collapse. Although we find that Ttk69 is required for normal Activin signal transduction in R7s, we provide evidence that Ttk69 also acts independently of Activin. Together, our results suggest that R7 axon growth is controlled by multiple pathways whose effects are temporally coordinated by expression of a transcription factor.

Materials and Methods

Genetics. Homozygous wild-type (*FRT82*), *ttk*, *importin- α 3* (*imp- α 3*), and *syd-1* mutant R7s were generated and labeled using *GMR-FLP* and MARCM (mosaic analysis with a repressible cell marker) (Lee and Luo, 1999; Lee et al., 2001). When two mutations are on different chromosomes, generating double-mutant R7s using *GMR-FLP*/MARCM is difficult because of the large number of transgenes that must be present, some on specific chromosome arms. For that reason, we chose to use a mutation in *syd-1* (on chromosome 3) and not mutations in *Ncad*, *LAR*, or *Liprin- α* (all on chromosome 2) to disrupt contact between *ttk69* mutant R7 axons and their target layer. However, because *syd-1* is <100 kb away from *ttk* (<http://flybase.org>; McQuilton et al., 2012), we chose not to create a *syd-1*, *ttk69* double-mutant chromosome but instead disrupted Ttk69 in *syd-1* mutant R7s by creating an *imp- α 3*, *syd-1* double-mutant chromosome. Homozygous wild-type (*FRT42*) and *babo* mutant R7s were generated and labeled using *ey3.5-FLP* (from Iris Salecker, MRC National Institute for Medical Research, London, UK) and MARCM. In all MARCM experiments, homozygous cells expressed either *UAS-mCD8-GFP* or *UAS-Synaptotagmin* (*Syt*)-GFP, along with any other *UAS* transgenes present, under the control of *actin* (*act*)-*Gal4*. Flies containing the *UAS-GFP* constructs and *act-Gal4* were obtained from the Bloomington *Drosophila* Stock Center. Because *GMR-FLP*/MARCM induces mitotic recombination during the final cell division that generates R7s, homozygous R7s inherit Gal80 protein from their heterozygous parents, causing a delay in their expression of transgenes under control of *Gal4/UAS* (Miller et al., 2009).

In addition to the transgenes used in the MARCM experiments (mentioned above), we also used the following: *UAS-ttk69* and *UAS-ttk88* (both from the Bloomington *Drosophila* Stock Center), *UAS-ttk-RNAi* (#101980), *UAS-Ncad-RNAi* (#1093), *UAS-babo-RNAi* (#106092), *UAS-phyllipod* (*phyl*)-*RNAi* (#35469), and *UAS-seven in absentia* (*sina*)-*RNAi* (#100691) (all from the Vienna *Drosophila* RNAi Center), *UAS-Dcr-2* (Dietzl et al., 2007), *UAS-FLAG-dSmad2* (Ting et al., 2007), *chaoptin* (*chp*)-*Gal4* (a generous gift from S. L. Zipursky, University of California, Los Angeles, CA), and *UAS-GI^{DN}* (Allen et al., 1999). We used the following mutations: *ttk^{le11}* (which we refer to as “*ttk69*”; Xiong and Montell, 1993), *imp- α 3^{D93}* (Mason et al., 2003), *babo⁹* (Zheng et al., 2003), *syd-1^{w46}* (Holbrook et al., 2012), *sev^{V1}* (Mullins and Rubin, 1991), and deficiencies spanning the right arm of chromosome 3 (Bloomington *Drosophila* Stock Center). All animals were raised at 25°C except for those analyzed in Figure 7, which were raised at 29°C. The *UAS-Dcr-2* transgene was included in all RNAi experiments except those in Figure 6A–C; we found that the *UAS-ttk-RNAi* transgene fully eliminates detectable Ttk69 expression even in the absence of *UAS-Dcr-2*. All *sevenless* (*sev*) mutant animals analyzed were male, *UAS-ttk88* and *UAS-FLAG-dSmad2* experiments were performed with females, and all other experiments were performed with animals of either sex.

Image acquisition and analysis. Brains and retinas were dissected, fixed, and stained by standard methods (Holbrook et al., 2012). Confocal images were collected on a Leica SP2 microscope and analyzed with Leica or Fiji software (<http://fiji.sc/Fiji>; Schindelin et al., 2012). All quantifications were performed blind.

To quantify nuclear FLAG–dSmad2 levels, we determined the anti-FLAG fluorescence intensities within all GFP-labeled R7 nuclei in a field of view (typically two to four) and divided each by the fluorescence intensity within a single non-GFP-expressing (and therefore non-FLAG–dSmad2-expressing) R7 nucleus in the same field to calculate a “fold enriched.” Anti-Elav staining was used to identify the boundaries of nuclei. To quantify Ttk69 levels in R7s, we determined the average anti-Ttk69 fluorescence intensity within at least 10 R7s per retina and corrected for slight differences in gain settings between samples by calibrating the gain using the same fluorophore and otherwise consistent detector settings.

We used the following antibodies: mouse anti-Chp (24B10; 1:200) and rat anti-Elav (7E8A10; 1:5) from the Developmental Studies Hybridoma Bank; mouse anti-FLAG (M5; 1:200) from Sigma-Aldrich; mouse anti-Pros (mR1A; 1:500) from C. Doe (University of Oregon, Eugene, OR); rabbit anti-Ttk69 (1:500) from A. Travers (Medical Research Council Laboratory of Molecular Biology, Cambridge, UK); chicken anti-GFP (1:5000) from Abcam; and rabbit anti-GFP (1:5000), Alexa Fluor 555-conjugated Phalloidin (1:10) and all secondary antibodies (goat IgG coupled to Alexa Fluor 488, Alexa Fluor 555, or Alexa Fluor 633; 1:250) from Invitrogen.

Results

Ttk69 is required to prevent overgrowth of R7 axon terminals

To identify genes that regulate R7 axon growth and targeting, we performed a genetic screen using mosaic animals whose R7 neurons were homozygous for randomly mutagenized chromosome arms. We identified three lethal, noncomplementing mutations on 3R that caused R7 terminals to overlap with their neighbors. We mapped these by performing complementation tests with a standard set of deficiencies (Bloomington *Drosophila* Stock Center) and ultimately found that they failed to complement loss-of-function mutations in the *ttk* gene for lethality. We therefore examined whether loss of *ttk* caused an R7 axon defect. By 60 h after puparium formation (APF), wild-type R7 axons terminate in non-overlapping, approximately spherical boutons in the M6 layer of the medulla (Fig. 1A). In contrast, $20.8 \pm 0.70\%$ ($n = 9$ brains) of *ttk^{le11}* mutant R7 axon terminals extended laterally within the M6 layer, resulting in overlap with adjacent wild-type R7 terminals (Fig. 1B,E). Some *ttk^{le11}* mutant R7s extended beyond their immediate R7 neighbors while remaining in M6 (data not shown), whereas others extended back toward the M3 layer along an adjacent R7 axon (Fig. 1B). *ttk^{le11}* mutant R7 axons also occasionally extended short branches at the M1 or M3 layers (Fig. 1C). We noticed that even those *ttk^{le11}* R7 axon terminals that remained spherical had abnormally large terminals (Fig. 1C,F). We conclude that *ttk* is required to prevent overgrowth of R7 axon terminals within the medulla.

The *ttk* locus encodes two transcription factor isoforms, Ttk69 and Ttk88, which share a common BTB/POZ domain but have different zinc fingers and consequently different DNA-binding specificities (Read and Manley, 1992). The *ttk^{le11}* allele contains a deletion within a Ttk69-specific exon, completely eliminating Ttk69 expression but leaving Ttk88 expression intact (Lai and Li, 1999). To confirm that the Ttk69 isoform is required for proper R7 axon growth, we tested the ability of each isoform to rescue the *ttk* mutant R7 axon defect. We found that overexpressing Ttk88 had no effect on *ttk^{le11}* mutant R7s (Fig. 1E) but that overexpressing Ttk69 prevented *ttk^{le11}* mutant R7 axon overgrowth (Fig. 1E; Ttk69 overexpression also caused a gain-of-function phenotype, as described below). We conclude that Ttk69 is required to prevent R7 axon overgrowth and will refer to *ttk^{le11}* as *ttk69*.

Figure 1. Ttk69 is required to prevent overgrowth of R7 axon terminals. **A–C**, Medullas of mosaic animals, created by *GMR-FLP* and MARCM, at 60 h APF. **D**, Medulla of a mosaic animal, created by *ey3.5-FLP* and MARCM, at 60 h APF. R7s homozygous for the specified chromosome arm express mCD8–GFP (green). All R7 and R8 axons are labeled with anti-Chp (red). **A**, Wild-type (*FRT82*) (WT) R7 axons terminate at the M6 layer of the medulla in spherical boutons (chevrons). **B**, *ttk^{1e11}* mutant R7 axons terminate in the M6 layer but often extend laterally (arrows), resulting in overlap with neighboring R7 axon terminals. Some *ttk^{1e11}* mutant R7 axons extend not only laterally but also return along an adjacent R7 axon toward the M3 layer (arrowheads). **C**, Even those *ttk^{1e11}* mutant R7 terminals that remain spherical are abnormally large (chevron). *ttk^{1e11}* mutant R7 axons occasionally have lateral branches at the M1 or M3 layer ($8.3 \pm 0.7\%$; $n = 9$; arrowheads point to an M3 example). **D**, *babo* mutant R7 axons also extend laterally within M6, resulting in overlap with neighboring R7 terminals (arrow), but those *babo* mutant R7 terminals that remain spherical are of normal diameter. **E**, Quantification of R7 axon extension within M6, scored as the frequency with which individual homozygous R7 axons, created by *GMR-FLP*/MARCM, overlapped with neighboring R7 axon terminals. If neighboring R7 axons were absent (see below), “overlap” was scored as overlap with the M6 regions directly below neighboring R8 axon terminals in M3. Error bars represent SEM. Zero percent of wild-type (*FRT82*) R7 axon terminals extend laterally within M6, whether neighboring R7 axons are present (WT; $n = 5$) or have been removed by means of the hypomorphic *sev¹* mutation ($n = 6$). The frequency with which *ttk^{1e11}* mutant R7 axon terminals extend laterally within M6 ($20.8 \pm 0.70\%$; $n = 9$) is enhanced by removing neighboring R7 axons ($53.3 \pm 2.2\%$; $n = 9$). Expression of the Ttk69 isoform rescues *ttk^{1e11}* mutant R7 terminal overlap ($0.8 \pm 0.4\%$; $n = 13$), but expression of Ttk88 does not ($21.9 \pm 2.0\%$; $n = 8$). Individual homozygous wild-type (*FRT82*) R7 axons expressing *ttk-RNAi* extend laterally at a frequency ($18.7 \pm 0.77\%$; $n = 14$) similar to that at which *ttk^{1e11}* mutant R7 axons do so. The same is true of *imp-α3* mutant R7 axons ($22.3 \pm 1.0\%$; $n = 10$). **F**, Quantification of the width of R7 axon terminals that remain spherical. Error bars represent SEM. Both *ttk^{1e11}* ($2.69 \pm 0.10 \mu\text{m}$ in width; $n = 9$) and *imp-α3* ($2.84 \pm 0.10 \mu\text{m}$; $n = 10$) mutant R7 terminals are significantly larger than either wild-type ($1.58 \pm 0.05 \mu\text{m}$; $n = 5$) or *babo* ($1.67 \pm 0.12 \mu\text{m}$; $n = 7$) mutant terminals. $***p < 0.00001$. There is no significant difference (n.s.) between wild-type and *babo* R7 terminals ($p = 0.7$) or between *ttk^{1e11}* and *imp-α3* terminals ($p = 0.2$). **G, H**, Mosaic animals created by *GMR-FLP*/MARCM in which R7s homozygous for *imp-α3*

Ttk69 is required in the nucleus and specifically affects R7 axon terminal morphology

Ttk69 has been shown previously to act as a transcription factor that represses neuronal development (Read and Manley, 1992; Guo et al., 1995; Giesen et al., 1997; Badenhorst, 2001; Badenhorst et al., 2002). Although some transcription factors have been found to have additional functions outside the nucleus, we did not observe Ttk69 expression in R7 axons (data not shown), and we found that excluding Ttk69 from R7 nuclei by disrupting the nuclear importer Imp-α3 precisely phenocopied loss of Ttk69 (Fig. 1E–H). We conclude that Ttk69 is likely functioning as a conventional transcription factor to regulate R7 axon terminals.

Loss of Ttk69 from the entire retina has been shown previously to disrupt adult R neurons (Lai and Li, 1999). We therefore examined whether the *ttk69* mutant R7 axon defect might be secondary to a failure to differentiate properly. By 60 h APF, wild-type R7 cell bodies have undergone a change in apical–basal polarity and consequently have a characteristic wedge shape (Fig. 2A; Wolff and Ready, 1993). In addition, they express the neuronal marker Elav (Fig. 2A'), the photoreceptor marker Chp (Fig. 1A), and the R7 fate marker Prospero (Pros; Fig. 2A''), a combination of markers that is unique among retinal cells (Kauffmann et al., 1996). We found that *ttk69* mutant R7 neurons exhibited all the characteristics of normal R7 neurons (Figs. 1B, 2B–B'') and so conclude that loss of *ttk69* does not grossly alter R7 differentiation.

Loss of Ttk69 from non-neuronal cone cells has been shown previously to transform them into R7 neurons (Siddall et al., 2009). Our *GMR-FLP*/MARCM method of creating *ttk69* mutant R7s also creates *ttk69* mutant cone cells and, as a consequence, causes some ommatidia to contain extra R7 neurons (Fig. 2B–B''). We found that $49.2 \pm 3.5\%$ ($n = 7$ retinas) of ommatidia that contained at least one *ttk69* mutant R7 (whether the R7 itself or a transformed cone cell) also contained at least one additional R7 neuron. These extra R7s are indistinguishable

express mCD8–GFP (green). **G, G'**, At 48 h APF, Ttk69 protein (red) is excluded from the nuclei of *imp-α3* mutant R7s (arrows; compare with wild-type R7s, arrowheads). **H**, At 60 h APF, *imp-α3* mutant R7 terminals extend laterally within M6 (arrows). Those *imp-α3* mutant R7 terminals that remain spherical are abnormally large (chevron). Scale bars, $5 \mu\text{m}$. n equals number of brains scored.

from the native R7s in shape and in their expression of R7 markers (Fig. 2*B–B''*). However, because we cannot unambiguously trace the origin of each *ttk69* mutant R7 axon, we were concerned that these ectopic R7s might somehow be abnormal and exclusively responsible for the observed axon morphology defect. To test this, we needed to remove Ttk69 from R7s without disrupting any non-neuronal cells. To do so, we used the R neuron-specific driver *chp-Gal4* to drive expression of a *ttk-RNAi* transgene (Fig. 2*C–F'*). We found that this technique specifically eliminated Ttk69 expression from R neurons and therefore did not result in the formation of extra R7 neurons (Fig. 2*F, F'*). However, R7 axon terminals consequently extended laterally within the M6 layer, causing overlap with adjacent R7s at a frequency similar to that observed for individual *ttk69* mutant R7 axons ($21.8 \pm 2.6\%$; $n = 9$ brains; Fig. 2*E*). We conclude that Ttk69 is normally required in R7 neurons to prevent abnormal axon terminal growth. In additional support of this conclusion, we found that using *GMR-FLP/MARCM* to drive expression of the *ttk-RNAi* transgene in individual homozygous wild-type (*FRT82*) cells did not cause non-neuronal cells to become extra R7s [data not shown; presumably this is because of the delay in expression (see below and Materials and Methods)] but did cause R7 axon terminals to extend laterally within M6 ($18.7 \pm 0.77\%$; $n = 14$ brains; Fig. 1*E*).

ttk69 mutant R7 axons are still responsive to repulsion by their neighbors

Previous studies have shown that wild-type R7 axons will extend laterally within M6 if two conditions are met: (1) there is at least one extra R7 in the same ommatidium, causing multiple R7 axon terminals to occupy the M6 space normally reserved for one (a condition we will refer to as “crowding”); and (2) neighboring R7 axons have been removed (Ashley and Katz, 1994). Under these circumstances, crowded R7 axon terminals will specifically extend into M6 regions lacking R7 innervation, leading to the conclusion that R7 axons are mutually repulsive but that a cell-intrinsic mechanism is normally sufficient to prevent R7 axon overlap (Ashley and Katz, 1994). As mentioned in the previous section, *ttk69* mutant R7 axons can be crowded, satisfying condition 1. One possibility, therefore, is that *ttk69* mutant R7 axons extend laterally within M6 because they are unable to detect the presence of adjacent R7 axons, satisfying condition 2. If this were the case, we would predict that removing adjacent R7 axons would have no effect on the ability of *ttk69* mutant terminals to overlap with

Figure 2. Ttk69 specifically affects R7 axon terminal morphology. *A, B*, Retinas of mosaic animals, created by *GMR-FLP/MARCM*, at 60 h APF. Homozygous R7s express mCD8-GFP (green). *A–A''*, Wild-type (*FRT82*) (WT) R7 cell bodies (arrow) express the neuronal marker Elav (blue; *A'*) and the R7 fate marker Pros (red; *A''*). The *GMR-FLP/MARCM* technique also creates homozygous R1 and R6 neurons (arrowhead), which express Elav but not Pros. *B–B''*, Native R7s that are homozygous for *ttk69* (*ttk1e11*; arrow) correctly express Elav (*B'*) and Pros (*B''*). Cone cells homozygous for *ttk69* are transformed into R7s (arrowheads) and also express Elav and Pros. Transformed cone cells can be positioned anywhere within the ommatidium. In this example, two transformed cone cells flank the native, wild-type R7 (double arrowhead), displacing the latter into a slightly different focal plane. *C*, Medulla of a wild-type adult animal in which R1–R8 neurons express EB1-GFP (white) under the control of *chp-Gal4*. The R7 axons terminate in non-overlapping boutons in M6. *D, D'*, Retina from a wild-type 60 h APF pupa in which R1–R8 neurons express EB1-GFP under the control of *chp-Gal4*. All R neurons express Ttk69 (*D*), and each ommatidium contains exactly one R7 neuron, as judged by Pros expression (*D'*). *E*, Medulla of a wild-type adult animal in which R1–R8 neurons express both EB1-GFP (white) and *ttk-RNAi* under the control of *chp-Gal4*. R7 axons in these animals extend laterally within M6, resulting in overlap ($21.8 \pm 2.6\%$; $n = 7$ brains; arrow), or have abnormally large terminals (chevrons). *F, F'*, Retina from a 60 h APF pupa in which R1–R8 neurons express both EB1-GFP and *ttk-RNAi* under the control of *chp-Gal4*. Ttk69 protein is absent from all R neurons (*F*), but each ommatidium still contains exactly one R7, as judged by Pros expression (*F'*); the asterisk indicates an ommatidium in which the Pros-positive R7 is present but out of the plane of focus. Scale bars, 5 μ m.

their neighbors. However, when we used a hypomorphic *sev* allele to eliminate most R7s, those *ttk69* mutant R7 axons that remained were significantly more likely to extend laterally within M6 (Fig. 1*E*). We conclude that *ttk69* mutant R7 axons are still responsive to repulsion by adjacent R7 axons.

ttk69 mutant R7 axons correctly recognize their synaptic target layers

In addition to being repelled by their neighbors, R7 axons presumably also receive cues from their synaptic targets. Although disrupting presynaptic assembly within R7s does not cause their

Figure 3. *ttk69* mutant R7 axons correctly identify their synaptic target layer yet still overgrow if contact with their target layer is disrupted. **A–F'**, Medullas of mosaic animals, created by *GMR-FLP/MARCM*, at 60 h APF. R7s homozygous for the specified chromosome arm express Syt–GFP (green). All R7 and R8 axons are labeled with anti-Chp (red). **A, A'**, Wild-type (*FRT82*) (WT) R7s primarily localize Syt–GFP to the region of the axon below the M3 layer (dashed line). **B, B'**, *syd-1* mutant R7s mislocalize Syt–GFP to regions of the axon that lie within and above the M3 layer. *syd-1* mutant R7 axons often fail to contact M6. In *ttk69* (*ttk^{1e11}*; **C, C'**) and *imp-α3* (**D, D'**) mutant R7 axons Syt–GFP is primarily localized below M3, as in wild type. **E–F'**, *imp-α3, syd-1* double-mutant R7 axons mislocalize Syt–GFP yet extend laterally within M6. **F, F'**, Even double-mutant R7s that fail to contact M6 can still extend laterally (arrowheads). **G**, Quantification of R7 axons that either extend within or fail to contact M6. Error bars represent SEM. Loss of *imp-α3* greatly increases the frequency with which *syd-1* mutant R7 axons contact M6 [only $9.5 \pm 1.3\%$ ($n = 19$) of *imp-α3, syd-1* R7 axons fail to contact M6 compared with $20.0 \pm 1.6\%$ ($n = 13$) of *syd-1* mutant axons]. Loss of *syd-1* moderately decreases the ability of *imp-α3* mutant axons to extend laterally [$15.8 \pm 1.0\%$ ($n = 19$) of *imp-α3, syd-1* R7 axons overlap with neighboring R7 axons compared with $21.6 \pm 0.93\%$ ($n = 11$) of *imp-α3* mutant axons]. Similarly, disrupting *ttk* with *ttk-RNAi* greatly increases the frequency with which *syd-1* mutant R7 axons contact M6 [only $10.3 \pm 0.84\%$ ($n = 13$) of *ttk-RNAi*-expressing *syd-1* mutant R7 axons fail to contact M6], and loss of *syd-1* moderately decreases the ability of *ttk-RNAi*-expressing R7s to extend laterally [only $15.0 \pm 0.99\%$ ($n = 13$) of *ttk-RNAi*-expressing *syd-1* mutant R7 axons extend within M6 (the percentage of *ttk-RNAi*-expressing wild-type R7 axons that do so is redisplayed from Fig. 1E for ease of comparison)]. Finally, loss of *ttk69* likewise greatly increases the frequency with which *Ncad-RNAi*-expressing R7 axons contact M6 [only $5.5 \pm 0.44\%$ ($n = 14$) of *Ncad-RNAi*-expressing *ttk69* (*ttk^{1e11}*) mutant R7 axons fail to contact M6 compared with $16.2 \pm 3.4\%$ ($n = 7$) of *Ncad-RNAi*-expressing wild-type R7 axons]. *Ncad-RNAi* does not significantly affect the frequency with which *ttk69* mutant R7 axons extend laterally [$23.6 \pm 1.3\%$ ($n = 14$) of *Ncad-RNAi*-expressing *ttk69* mutant R7 axons overlap with neighboring R7 axons (the percentage of *ttk69* mutant R7 axons that do so is redisplayed from Fig. 1E for ease of comparison); $p = 0.12$]. Error bars represent SEM. * $p < 0.01$ and *** $p < 0.0001$. Scale bar, 5 μm . n indicates number of brains scored.

axons to extend laterally (Clandinin et al., 2001; Lee et al., 2001; Maurel-Zaffran et al., 2001; Choe et al., 2006; Hofmeyer et al., 2006; Astigarraga et al., 2010; Holbrook et al., 2012), it remained possible that the *ttk69* mutant R7 axon defect might nonetheless

be caused by an inability to recognize synaptic targets or might reflect a failure to transition from axon growth to synaptogenesis. Indeed, although wild-type R7 terminals lack filopodia and are instead smooth, ellipsoid boutons by 60 h APF (Fig. 1A), *ttk69* mutant R7 terminals failed to form normal boutons and instead were irregular in shape and had short, filopodia-like extensions (Fig. 1B). To distinguish whether *ttk69* mutant R7 axons recognize their synaptic targets, we examined their ability to localize the synaptic vesicle marker Syt–GFP. In wild-type R7 axons, Syt–GFP is localized primarily below the M3 layer, consistent with the known locations of presynaptic sites (Fig. 3A, A'; Takemura et al., 2008). However, R7s lacking the presynaptic assembly proteins Syd-1 or Liprin-α mislocalize Syt–GFP to puncta along the length of the axon (Fig. 3B, B'; Holbrook et al., 2012). We found that *ttk69* mutant R7s correctly localized Syt–GFP below the M3 layer (Fig. 3C, C'), although the amount of Syt–GFP in this region appeared to be increased in *ttk69* mutant R7 axons (Fig. 3C, C'), perhaps because of their expanded size. This result suggests that *ttk69* mutant R7s correctly identify their synaptic targets and can at least partly assemble synapses. In addition, *ttk69* mutant R7 axons never extended outside the layers within which they normally form synapses: in particular, most remained within M6, and those that instead turned back along adjacent R7 axons never extended beyond M4 (Fig. 1B). This result suggests that the overgrowth of *ttk69* mutant R7 terminals is restricted to regions that contain their normal synaptic targets.

ttk69 mutant R7 axons still overgrow if contact with their M6 target layer is disrupted

The restriction of *ttk69* mutant R7 axon terminals to regions containing R7 synaptic targets suggested that their ability to overgrow might require contact with M6 or the ability to form synapses. To test this, we set out to disrupt the latter processes in R7s lacking Ttk69. Loss of Ncad, LAR, Liprin-α, or Syd-1 reduces the size of R7 terminal boutons and causes many to fail to contact M6 (Clandinin et al., 2001; Lee et al., 2001; Maurel-Zaffran et al., 2001; Choe et al., 2006; Hofmeyer et al., 2006; Holbrook et al., 2012). For technical reasons, we

were unable to create R7s doubly mutant for *ttk69* and *Ncad*, *LAR*, or *Liprin-α* (see Materials and Methods). Instead, we took the following three approaches to disrupt both *ttk69* and contact with M6: (1) we created *imp-α3, syd-1* double-mutant

R7s [loss of *imp-α3* eliminates nuclear Ttk69 expression and exactly phenocopies all aspects of the *ttk69* mutant R7 axon defect (Fig. 1*E–H'*), including normal localization of Syt–GFP (Fig. 3*D, D'*)]]; (2) we disrupted Ttk69 protein in *syd-1* mutant R7s by causing the latter to express *ttk-RNAi*; and (3) we disrupted Ncad protein in *ttk69* mutant R7s by causing the latter to express *Ncad-RNAi*. We obtained similar results in all three cases. As expected, loss of *syd-1* from *imp-α3* mutant R7s disrupted their ability to localize Syt–GFP and to maintain contact with M6 (Fig. 3*E–G*), as did loss of *syd-1* from *ttk-RNAi*-expressing R7s (Fig. 3*G*) or causing *ttk69* mutant R7s to express *Ncad-RNAi* (Fig. 3*G*). However, even those *imp-α3*, *syd-1* double-mutant R7 axons that failed to contact M6 were able to extend laterally (Fig. 3*F, F'*), suggesting that *ttk69* mutant R7 axon overgrowth does not require contact with synaptic targets. In addition, significantly more *imp-α3*, *syd-1* double-mutant and *ttk-RNAi*-expressing *syd-1* mutant R7 axons made contact with M6 than did *syd-1* single-mutant R7 axons (Fig. 3*G*), suggesting that loss of Ttk69 from *syd-1* mutant R7s may cause their axons to overgrow not only laterally but also toward M6. Similarly, more *Ncad-RNAi*-expressing *ttk69* mutant R7 axons made contact with M6 than did *Ncad-RNAi*-expressing wild-type R7 axons (Fig. 3*G*), again indicating that loss of Ttk69 causes R7 axons lacking Ncad to overgrow toward M6. However, we do note that loss of *syd-1* from *imp-α3* mutant R7s and loss of *syd-1* from *ttk-RNAi*-expressing R7s reduces the frequency with which their axons extend laterally (Fig. 3*G*), indicating that, although not absolutely required, stable contact with M6 may facilitate R7 axon overgrowth.

Ttk69 is first expressed in wild-type R7s after their axons extend into the medulla; *ttk69* mutant R7 axon terminal overgrowth begins shortly afterward

We have so far shown that loss of Ttk69 causes R7 axon terminals to overgrow, that this overgrowth does not require contact with the normal R7 synaptic target layer, and that this overgrowth is not caused by an inability to be repelled by neighboring R7 axons or to recognize the appropriate synaptic targets. Together, these results indicate that Ttk69 is required to downregulate some R7-intrinsic mechanism that promotes axon terminal growth. We next wanted to determine whether Ttk69 might also be sufficient to downregulate R7 axon growth and so play an instructive rather than simply permissive role in this process. To do so, we first determined the time course of Ttk69 expression to compare it with that of wild-type R7 axon outgrowth. Previous work has shown that Ttk69 is absent from cells as they differentiate into R neurons during larval development (Li et al., 1997) but is present at high levels in all R neurons at 40 h APF (Lai and Li, 1999). If Ttk69 is indeed instructive rather than merely permissive, one prediction is that it should be absent from R7 axons that are actively extending. We therefore expected that R7s would not express Ttk69 as they initially extend to their temporary target layer. Because R7s are specified by a wave of differentiation that passes across the developing retina (Wolff and Ready, 1993), the initial extension of R7 axons occurs sequentially rather than simultaneously and is complete by ~30 h APF (Ting et al., 2005). Indeed, we found that Ttk69 was still undetectable in R7s at 24 h APF, when most R7 axons have completed their initial extension into the medulla (Fig. 4*A, A', L*). However, by

30 h APF, Ttk69 was expressed at high levels in all R7s (Fig. 4*B, B', L*). Ttk69 levels then decreased but persisted throughout the second phase of R7 axon targeting (Fig. 4*C–E', L*).

The absence of Ttk69 from R7s whose axons are actively extending is consistent with the possibility that Ttk69 might be an instructive signal for R7s to decrease axon growth. If such a model were true, we would also predict that *ttk69* mutant R7 axons should exhibit increased growth shortly after Ttk69 would normally be expressed. Indeed, we found that wild-type and *ttk69* mutant R7 axons were indistinguishable at both 24 h APF (data not shown) and 30 h APF (Fig. 4*F, I*). However, by 36 h APF, $10.1 \pm 0.8\%$ ($n = 10$ brains) of *ttk69* mutant R7 axon terminals overlapped with their neighbors (Fig. 4*G, J, M*), and by 42 h APF, this overlap was as frequent as that observed at 60 h APF (Fig. 4*H, K, M*). The average length of *ttk69* mutant lateral extensions continued to increase between 48 and 60 h APF (Fig. 4*M*). Together, these results are consistent with a model in which the timing of Ttk69 expression coincides with the second phase of R7 targeting, during which Ttk69 inhibits R7 axon growth.

Premature Ttk69 expression disrupts R7 axon extension

To directly test whether Ttk69 might be sufficient to inhibit R7 axon growth, we wanted to drive expression of Ttk69 prematurely, before the R7 axons have reached their temporary target layer. However, expressing Ttk69 in R7 precursors before they are committed to a neuronal fate has been shown previously to prevent their differentiation as R7s (Li et al., 1997; Siddall et al., 2009). To avoid this outcome, we therefore needed to delay ectopic expression of Ttk69 until after the R7s are specified. We chose not to use a global driver, such as the heat shock promoter, because that would result in Ttk69 induction simultaneously in all R7s despite their varying developmental ages; at any given time point, many would not yet be committed to the neuronal fate. Instead, we took advantage of the same *GMR-FLP/MARCM* system that we use to generate and label individual homozygous R7s. We have found that *GMR-FLP/MARCM* causes each homozygous R7 to express UAS-driven transgenes ~10–15 h after its recruitment as an R7 and causes their axons to be GFP labeled before they have reached their temporary target layer (Ting et al., 2005; Miller et al., 2009). We therefore used *GMR-FLP/MARCM* to express UAS-*ttk69* in wild-type R7s. By 24 h APF, most wild-type R7 axons have reached their temporary target layer in the medulla, although some have not (Fig. 5*A*; Ting et al., 2005). Although R7s forced to express Ttk69 prematurely can still reach their temporary target layer, we noticed that some appeared to terminate instead in the R8 temporary target layer and had abnormally thin terminals (Fig. 5*B, B'*). This result suggested that premature Ttk69 might collapse R7 growth cones and so inhibit axon growth.

To test whether premature Ttk69 actually prevents R7 axons from reaching their targets, we next examined *GMR-FLP/MARCM/UAS-ttk69* R7s at 48 h APF, by which time all wild-type R7 axons have reached their temporary target layer and have begun their slow advance to their final destination, M6. We found that $17.6 \pm 1.3\%$ ($n = 9$ brains) of wild-type R7 axons forced to express Ttk69 were absent from their normal target layer and instead terminated in the same layer as R8 axons (Fig. 5*C*); some of these apparently stalled axons extended thin

Figure 4. Ttk69 is normally first expressed in R7s after their axons extend into the medulla, and *ttk69* mutant R7 axon overgrowth begins shortly afterward. **A–E'**, Wild-type pupal retinas in which the R neurons are outlined by Phalloidin (red) and R7 nuclei express Pros (blue). Anti-Ttk69 is in green. **A, A'**, At 24 h APF, Ttk69 protein is absent from all R neurons including R7s (arrow). **B–E'**, From 30 h until at least 60 h APF, Ttk69 is expressed in all R neurons, including R7s (arrows; quantified in **L**). **F–K**, Pupal medullas in which homozygous R7 axons express mCD8–GFP (green). At 30 h APF, wild-type (**F**) and *ttk69* (*ttk^{1e11}*) mutant (**I**) R7 axon terminals are indistinguishable. By 36 h APF, *ttk69* mutant R7 axons extend laterally within the M6 layer (**J**). By 48 h APF, *ttk69* mutant R7 axons have begun to extend back toward M3 (**K**, arrowhead). Quantified in **M**. **L**, Quantification of Ttk69 protein expression in wild-type R7 nuclei. Previous work has shown that Ttk69 protein is absent from third-instar larval R7s, suggesting that the 9.60 ± 1.5 ($n = 15$) units of fluorescence we observe at that time point is caused by nonspecific background staining. At 24 h APF, Ttk69 protein remains absent from R7s (9.97 ± 2.3 units; $n = 11$). However, by 30 h APF, R7s express a high level of Ttk69 (155 ± 17 units; $n = 10$). This level drops somewhat by 36 h APF (75.2 ± 6.0 units; $n = 14$) and further decreases by 60 h APF (57.6 ± 2.2 units; $n = 14$). The differences between 30 h APF and the later time points are highly significant, as is the difference between 48 and 60 h APF. The difference between 36 and 48 h APF (93.5 ± 4.5 units; $n = 14$) is not ($p = 0.02$). $*p < 0.01$ and $***p < 0.0001$. **M**, Quantification of *ttk69* (*ttk^{1e11}*) mutant R7 axon extension over time. Error bars represent SEM. At 36 h APF, $10.1 \pm 0.8\%$ ($n = 10$) of *ttk69* mutant R7 axons overlap with neighboring wild-type R7 axons. The frequency of *ttk69* mutant R7 axon terminals that overlap with their neighbors peaks at 42 h APF ($21.2 \pm 1.1\%$; $n = 11$) and remains unchanged at 48 h APF ($21.6 \pm 1.8\%$; $n = 10$) and 60 h APF (data for the latter time point is redisplayed from Fig. 1E for ease of comparison). The average length of the *ttk69* R7 axon terminal extensions continues to increase even after the frequency of overlap has peaked: the difference between 36 h APF (2.9 ± 0.08 μm ; $n = 10$) and 48 h APF (4.5 ± 0.3 μm ; $n = 10$) is significant, as is the difference between 48 and 60 h APF (5.7 ± 0.1 μm ; $n = 9$). $**p < 0.001$ and $***p < 0.0001$. Scale bars, 5 μm . *n* indicates number of brains or retinas scored.

filopodia-like processes toward their normal target layer (Fig. 5C). Even those Ttk69-expressing R7s that did reach the correct target layer had abnormally thin terminals. We observed similar results when we expressed Ttk69 in *ttk69* mutant R7s (Fig. 5D). Because Ttk69, when expressed earlier, can transform R7 precursors into non-neuronal cells, we examined whether *GMR-FLP*/MARCM-mediated Ttk69 expression altered R7 fate. We found that Ttk69-expressing R7s underwent their normal change in polarity and continued to express the photoreceptor-specific marker Chp (Fig. 5C), the neuron-specific marker Elav (data not shown), and the R7-specific marker Pros (Fig. 5F–F'), indicating that other aspects of R7 development were unaffected. In addition,

tional support of the specificity of the effect of Ttk69, using *GMR-FLP*/MARCM to express the Ttk88 isoform had no effect on R7 growth (Fig. 5E), despite also being able to transform R7 precursors into non-neuronal cells if expressed earlier (Li et al., 1997; Tang et al., 1997). Together, these results suggest that premature Ttk69 expression can collapse R7 growth cones and prevent them from advancing and that Ttk69 does so independently of its effect on neuronal differentiation.

Ttk69 is required for normal Activin signaling in R7s

Previous work has shown that the Activin receptor Babo is required to downregulate R7 axon growth during the second phase

of targeting (Ting et al., 2007). We therefore wanted to test whether Ttk69 might act upstream or downstream of one or more components of this pathway. If Ttk69 controlled R7 axon growth independently of Activin signaling, we would predict that disrupting both Ttk69 and the Activin pathway would have a more severe effect on R7s than disrupting either alone. The most effective way to eliminate Activin signaling in R7s is to create large clones of *babo* mutant tissue using *ey3.5-FLP/MARCM* (Ting et al., 2007). Consistent with previous reports, we found that $12.8 \pm 0.68\%$ ($n = 9$ brains) of *babo* mutant R7 axon terminals overlapped with their neighbors (Figs. 1D, 6C). We found it technically easiest then to disrupt Ttk69 protein production in *babo* mutant clones by causing these clones to express a *UAS-ttk-RNAi* transgene. Expressing *ttk-RNAi* in wild-type clones using *ey3.5-FLP/MARCM* completely eliminated detectable Ttk69 in R7s (data not shown) and caused their axon terminals to overgrow at approximately the same frequency as *ttk69* mutant R7 terminals ($25.5 \pm 0.66\%$; $n = 14$ brains; Fig. 6A, C), suggesting that this method completely disrupts Ttk69 function. We found that the frequency of axon overgrowth was not increased by expressing *ttk-RNAi* in *babo* mutant R7s ($25.4 \pm 1.3\%$; $n = 12$ brain; Fig. 6B, C), suggesting that Ttk69 and Babo likely function in the same pathway to regulate R7 axon growth.

We next wanted to test directly whether Ttk69 might act upstream or downstream of the Activin/Babo pathway. We found that Ttk69 is expressed at normal levels in *babo* mutant R7 nuclei during the second phase of R7 axon targeting (data not shown), suggesting that Ttk69 is not regulated by Babo. We therefore tested whether, instead, Activin signaling might be regulated by Ttk69. The output of Activin signaling can be assessed by measuring nuclear accumulation of the effector molecule dSmad2. A previous comparison of wild-type and *babo* mutant R7s expressing a *UAS-FLAG-dSmad2* transgene showed that loss of *babo* significantly decreased the amount of FLAG-tagged dSmad2 present in R7 nuclei (Ting et al., 2007). We therefore tested whether loss of *ttk69* might have a similar effect. We expressed *UAS-FLAG-dSmad2* in wild-type, *ttk69*, and *imp- α 3* mutant R7s and quantified the amount of nuclear FLAG-tagged dSmad2 in each case (Fig. 6D, D'). We found that loss of either *ttk69* or *imp- α 3* significantly decreased nuclear dSmad2 compared with wild-type (Fig. 6E). We conclude that Ttk69 is required for normal Activin pathway signaling in R7s.

Ttk69 can also inhibit R7 axon growth independently of the Activin pathway

Although these results indicate that Ttk69 restricts R7 axon growth by promoting Activin pathway activity, disrupting the

Figure 5. Premature Ttk69 expression disrupts R7 axon extension. **A–E**, Pupal medullas in which homozygous R7s, created by *GMR-FLP/MARCM*, express both mCD8–GFP (green) and the *UAS* transgene indicated. All R7 and R8 axons are labeled with anti-Chp (red). **A**, By 24 h APF, most wild-type (*FRT82*) R7 axons have reached their temporary target layer in the medulla. **B, B'**, At 24 h APF, wild-type R7 axons forced to express Ttk69 prematurely can have abnormally thin terminals (arrow), although some appear indistinguishable from wild type (chevron). **C**, At 48 h APF, $17.6 \pm 1.3\%$ ($n = 9$) of wild-type R7 axons forced to prematurely express Ttk69 fail to contact their target layer (arrow). **D**, Forced Ttk69 expression rescues the overgrowth of *ttk69* (*ttk^{let1}*) mutant R7 axons (see also Fig. 1E) but also prevents $14.3 \pm 1.6\%$ ($n = 10$) from contacting their target layer (arrows). **E**, Forced Ttk88 expression neither rescues *ttk69* mutant R7 axon overgrowth (see also Fig. 1E) nor prevents their axons from contacting their target layer. **F–F''**, At 48 h APF, the cell bodies of wild-type R7s that express Ttk69 prematurely (arrow) are normal in shape and express the R7 fate marker Pros. Scale bars, 5 μ m. n indicates number of brains scored.

Activin pathway in R7s does not cause as severe a defect as loss of Ttk69. *babo* mutant R7 axon terminals overlap with neighboring terminals less frequently [$12.8 \pm 0.68\%$, $n = 9$ (Fig. 6C) compared with $20.8 \pm 0.70\%$, $n = 9$ (Fig. 1E); $p < 0.00001$] and have shorter extensions [$2.6 \pm 0.2 \mu$ m ($n = 7$) at 60 h APF compared with $5.7 \pm 0.1 \mu$ m ($n = 9$; Fig. 4M); $p < 1 \times 10^{-8}$], and those *babo* mutant R7 terminals that do not overlap with their neighbors have terminals of normal size (Fig. 1D, F). This comparison suggests that Ttk69 might additionally inhibit R7 axon growth independently of the Activin pathway. To test this directly, we examined whether premature Ttk69 misexpression could inhibit R7 axon growth in the absence of Babo. We chose to disrupt Babo expression using a *UAS-babo-RNAi* transgene, because expressing Ttk69 in *babo* mutant clones using *ey3.5-FLP/MARCM* causes very early expression of Ttk69 and so blocks R7 differentiation (data not shown). We expressed both *UAS-babo-RNAi* and *UAS-ttk69*

Figure 6. Ttk69 is required for normal Activin signaling in R7s. **A, B**, Medullas of mosaic animals, created by *ey3.5-FLP/MARCM*, at 48 h APF. R7s homozygous for the specified chromosome arm express mCD8–GFP (green). All R7 and R8 axons are labeled with anti-Chp (red). **A**, Wild-type (*FRT82*) (WT) R7 axons that express *ttk-RNAi* extend laterally and overlap with neighboring R7 terminals at a frequency similar to that of *ttk69* mutant R7 axons. **B**, *babo* mutant R7 axons that express *ttk-RNAi* also extend laterally and overlap with neighboring R7 terminals. **C**, Quantification of R7 axon extension within M6. Error bars represent SEM. *babo* mutant R7 axons overlap with their neighbors significantly less often ($12.8\% \pm 0.68$; $n = 9$) than wild-type R7 axons expressing *ttk-RNAi* (25.5 ± 0.66 ; $n = 14$). However, removing *babo* from R7s that express *ttk-RNAi* does not significantly increase the frequency of this defect ($25.4 \pm 1.3\%$; $n = 12$). **D, D'**, A 50 h APF retina in which homozygous wild-type (*FRT82*) R7s (created by *GMR-FLP/MARCM*; arrows) express mCD8–GFP (green) and FLAG–dSmad2 (red). All R neuron nuclei are labeled with anti-Elav (blue). Unlabeled R7 nuclei (arrowheads) do not express FLAG–dSmad2. **E**, Quantification of FLAG–dSmad2 levels in mutant R7 nuclei at 50 h APF. Error bars represent SEM. FLAG–dSmad2 staining in *ttk69* (*ttk^{1e11}*; 1.48 ± 0.06 ; $n = 10$) and *imp-α3* (1.37 ± 0.06 ; $n = 10$) mutant R7 nuclei is significantly reduced compared with that in wild-type R7 nuclei (1.82 ± 0.08 ; $n = 10$). Scale bars, 5 μm . *n* indicates number of brains or retinas scored. * $p < 0.01$ and *** $p < 0.0001$.

using *chp-Gal4*, which initiates expression in R neurons after their specification as neurons (data not shown). We found that expressing *babo-RNAi* alone caused R7 axon terminals to overlap with their neighbors at the same frequency as *babo* mutant R7 terminals ($13.9 \pm 0.9\%$; $n = 8$; Fig. 7A,D). Expressing Ttk69 alone using *chp-Gal4* caused R7 axons to stop short of their final target layer only slightly less often than *GMR-FLP/MARCM/UAS-ttk69* R7 axons [$11.5 \pm 0.6\%$, $n = 11$ (Fig. 7B,D) compared with $17.6 \pm 1.3\%$, $n = 9$ (Fig. 5C)] and did not disrupt their neuronal identity (data not shown). We therefore examined the effects of expressing Ttk69 and *babo-RNAi* simultaneously. We found that forced Ttk69 expression caused only a mild reduction in the frequency with which *Babo*-lacking R7 axons extended laterally within M6 (Fig. 7C,D), suggesting that Ttk69 acts upstream of *Babo* to promote Activin signaling. However, if this were the only mechanism by which Ttk69 restricts R7 axon growth, then disrupting *Babo* should similarly prevent Ttk69 from causing premature arrest of R7 axons. Instead, disrupting *Babo* did not significantly decrease the frequency with which Ttk69-expressing R7s failed to reach M6 (Fig. 7D). Together with the observed difference between the *ttk69* and *babo* loss-of-function phenotypes, these results suggest that Ttk69 can inhibit R7 axon growth independently of the Activin pathway.

Discussion

Ttk69 inhibits axon growth during the second phase of R7 axon targeting

We have shown that Ttk69 is both necessary and sufficient to prevent R7 axons from extending. Because *ttk69* mutant R7s remain responsive to repulsion by their neighbors and can initiate synapse assembly with the appropriate target layers, we hypothesize that Ttk69 regulates the intrinsic ability of R7 axon terminals to grow. Consistent with this possibility, Ttk69 regulates the morphology of R7 growth cones: loss of Ttk69 increases R7 growth cone size, whereas premature Ttk69 causes R7 growth cone collapse. We have found that Ttk69 is expressed and required specifically during the second phase of R7 axon targeting, after the R7 axons have reached the medulla. A simple model is that R7 axon terminals are normally stationary throughout this second phase, even as the layer they occupy slowly advances, and that Ttk69 is deployed to establish and maintain this stationary state. In support of this model, we note that *ttk69* mutant R7 growth cones begin extending laterally quite early in the second phase of targeting, long before they have reached M6. However, the laterally extended sections of *ttk69* mutant R7 axons always remain in the same layer as the neighboring wild-type R7 axon terminals, even as the length of the lateral extensions continues to increase. One possibility is that the entire lateral length of each *ttk69* mutant R7 axon remains motile and extends in step with wild-type R7 growth cones. However, we think it more likely

that, as the optic lobe expands, the temporary target layer to which the R7 axon terminals adhere is passively driven forward by the intercalation of additional neuronal processes, causing the R7 axons to elongate by “stretch growth” (Smith, 2009) rather than by growth cone extension. Assuming that Ttk69 does not affect the ability of R7 axons to adhere to their target layer, then the full lateral length of the *ttk69* mutant R7 axons would be pulled forward together with adjacent wild-type R7 terminals. In support of this model, we found that *ttk69* mutant R7 axons can localize synaptic vesicles normally, indicating that loss of Ttk69 does not prevent R7 axons from interacting with their targets.

Ttk69 expression in R7s must be temporally regulated

Premature Ttk69 expression prevents R7 axons from reaching their targets, and *ttk69* mutant R7 axon terminals begin to overgrow soon after the time at which Ttk69 would normally be expressed. We conclude that the temporal regulation of Ttk69 expression is critical for normal R7 axon targeting. How might this regulation be achieved? During larval development, R7s eliminate Ttk69 by initiating expression of the adaptor protein Phyl, which targets Ttk69 for ubiquitination by the RING finger protein Sina and subsequent degradation by the proteasome (Li et al., 1997; Li et al., 2002). We found that expressing Phyl in pupal R7s was sufficient to eliminate Ttk69 expression and

caused axon overgrowth (data not shown), suggesting that pupal R7s continue to express Sina. However, attempting to disrupt pupal expression of Phyl or Sina (by delayed expression of the corresponding *UAS-RNAi* transgenes using *GMR-FLP/MARCM/UAS-RNAi*) did not cause premature Ttk69 expression or R7 growth cone collapse (data not shown), perhaps because of partial redundancy with the translational repressor Musashi (Hirota et al., 1999). We therefore do not yet know whether R7s regulate both larval and pupal Ttk69 expression by the same mechanism. However, it is clear that R7s do not initiate Ttk69 expression simply based on their age: although there is at least a 38 h range of R7 ages in the pupal eye (Wolff and Ready, 1993), we found that all R7s express Ttk69 within a 6 h window. One possibility is that Ttk69 expression is initiated simultaneously in R7s by a signal from the optic lobe—a signal that would then require retrograde axonal transport for transduction to the nucleus. Although disrupting axonal transport by expressing a dominant-negative form of the Dynactin subunit Glued (Gl^{DN}) causes R7 axon to extend laterally within M6 (Ting et al., 2007), we found that R7s expressing Gl^{DN} had normal levels of nuclear Ttk69 (as judged at 48 h APF; data not shown). We conclude that Ttk69 expression in R7s is likely to be independent of target- or growth-cone-derived signals. Instead, R7 Ttk69 expression may be controlled hormonally or by signaling within the retina, or R7s may use a cell-intrinsic mechanism that does not rely solely on R7 age.

Ttk69 promotes Activin pathway activity in R7s

Our results indicate that Ttk69 restricts R7 axon growth in part by promoting Activin pathway activity. Because Ttk69 is a zinc finger transcription factor and because we find that nuclear localization of Ttk69 is required to prevent R7 axon overgrowth, the simplest model is that Ttk69 regulates the transcription of one or more Activin pathway components. Our finding that Ttk69 overexpression does not rescue Babo loss suggests that Ttk69 acts upstream of Babo. Because previous work indicated that the Activin signal may originate from the R7s themselves (Ting et al., 2007), a simple possibility is that Ttk69 promotes Activin expression. However, because loss of Ttk69 reduces but does not completely disrupt Activin pathway activity and because Ttk69 is a well-established repressor (Xiong and Montell, 1993; Reddy et al., 2010), we speculate that Ttk69 might instead repress an antagonist of the Activin ligand. We note that the locus encoding one such antagonist, Follistatin, contains a potential Ttk69-binding DNA sequence (Harrison et al., 2005; data not shown).

Canonical Activin signaling in *Drosophila* is transduced to the nucleus by the transcription factor dSmad2 (Brummel et al., 1999; Das et al., 1999; Zheng et al., 2003). Previous work found that loss of dSmad2 caused considerably less R7 axon overgrowth

Figure 7. Ttk69 also acts via an Activin-independent pathway. *A–C*, Medullas of adult animals in which R1–R8 neurons express EB1–GFP (white) and the *UAS* transgene(s) indicated under the control of *chp-Gal4*. *A*, R7 axons that express *babo-RNAi* extend laterally and overlap with neighboring axon terminals at a frequency similar to that of *babo* mutant R7 axons ($13.9 \pm 0.9\%$; $n = 8$; arrows; see Fig. 6C for comparison). *B*, R7 axons forced to express Ttk69 under the control of *chp-Gal4* fail to contact the M6 layer at approximately the same frequency ($11.5 \pm 0.6\%$; $n = 11$; arrowheads) as R7 axons forced to express Ttk69 prematurely by *GMR-FLP/MARCM* (see Fig. 5 for comparison). *C*, R7 axons that coexpress Ttk69 and *babo-RNAi* exhibit both phenotypes: lateral extension within M6 ($9.7 \pm 0.7\%$; $n = 11$; arrows) and failure to contact M6 ($9.1 \pm 1.4\%$; $n = 7$; arrowheads). *D*, Quantification of R7 axon extension within M6 and failure to contact M6. Error bars represent SEM. Expression of Ttk69 partially rescues the R7 lateral extensions caused by expression of *babo-RNAi* ($*p = 0.002$). Although expression of *babo-RNAi* may reduce the ability of Ttk69 to prevent R7s from contacting M6, the difference is not statistically significant ($p = 0.1$). Scale bar, 5 μ m. n indicates number of brains scored.

than loss of Babo (Ting et al., 2007), suggesting that Babo may additionally regulate R7 axon outgrowth by a Smad-independent mechanism. Although overexpressing regulatory Smads can compensate for reduced canonical TGF- β pathway activation, we note that overexpressing FLAG–dSmad2 did not decrease the R7 axon overgrowth caused by loss of *ttk69* (data not shown). We therefore speculate that dSmad2 may not be the only output of Activin signaling in R7 neurons and that, as in *Drosophila* mushroom body (Ng, 2008), Activin—and therefore Ttk69—may also inhibit R7 axon outgrowth by a non-canonical, Smad-independent mechanism.

Ttk69 regulates the morphology of multiple cell types

Ttk69 is broadly expressed in non-neuronal cells and is both necessary and sufficient to block the adoption of neural fates (Read and Manley, 1992; Guo et al., 1995; Giesen et al., 1997; Badenhorst, 2001; Badenhorst et al., 2002). Although cells destined to become neurons must therefore eliminate Ttk69 expression, we have here shown that R7 neurons later reexpress Ttk69 to inhibit axon growth. Might other neurons use Ttk69 to regulate neurite outgrowth? We note that the R1–R6 and R8 photoreceptor neurons also reexpress Ttk69 after their axons have reached temporary target layers in the optic lobe (Fig. 4), suggesting that Ttk69 may play such a role in these cells. In addition, loss or gain of *ttk* disrupts the morphology of dendrite arborization neurons in the peripheral nervous system (Parrish et al., 2006; Ou et al., 2008), suggesting that

Ttk69 may regulate dendrite growth. Recent work has shown that Ttk69 also regulates the morphology of several non-neuronal cell types. Loss of Ttk69 from a subset of follicle cells prevents them from undergoing a cell shape change required for proper elongation of the dorsal appendage (French et al., 2003; Boyle and Berg, 2009; Boyle et al., 2010), and loss of Ttk69 from tracheal cells disrupts their ability to elongate during intercalation (Araújo et al., 2007). Together, these results suggest that Ttk69 may play a general role in temporally coordinating pathways that regulate cytoskeleton in both neuronal and non-neuronal cells.

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