Tiling of R7 Axons in the Drosophila Visual System Is Mediated Both by Transduction of an Activin Signal to the Nucleus and by Mutual Repulsion

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SUMMARY

The organization of neuronal wiring into layers and columns is a common feature of both vertebrate and invertebrate brains. In the Drosophila visual system, each R7 photoreceptor axon projects within a single column to a specific layer of the optic lobe. We refer to the restriction of terminals to single columns as tiling. In a genetic screen based on an R7-dependent behavior, we identified the Activin receptor Baboon and the nuclear import adaptor Importin-α3 as being required to prevent R7 axon terminals from overlapping with the terminals of R7s in neighboring columns. This tiling function requires the Baboon ligand, dActivin, the transcription factor, dSmad2, and retrograde transport from the growth cone to the R7 nucleus. We propose that dActivin is an autocrine signal that restricts R7 growth cone motility, and we demonstrate that it acts in parallel with a paracrine signal that mediates repulsion between R7 terminals.

INTRODUCTION

Topographic maps, in which the spatial relationships among neurons are preserved in the arrangement of their synaptic targets, are found throughout the nervous system. Restricting the information detected by equivalent sensory neurons to distinct columns results in a succession of topographic maps and provides a means of transmitting spatial information from the periphery to more central regions of the brain with high fidelity (Kaas, 1997). The establishment of gross topographic maps is particularly well understood in the vertebrate visual system, where gradients of Eph receptors and ephrin ligands play a crucial role (reviewed in McLaughlin et al., 2003). Much less is known about the mechanisms that act on a finer scale to restrict terminals to specific columns. The precise columnar arrangement of the Drosophila visual system provides an excellent model for exploring the genetic and molecular basis for this important patterning function (Kunes and Steller, 1993).

Each unit (ommatidium) of the Drosophila eye contains three types of photoreceptor neurons, R1–R6, R7, and R8, which express different rhodopsins and project axons retinotopically into three different layers of the optic lobe (Meinertzhagen and Hanson, 1993; Ting and Lee, 2007). The R7 and R8 axons from a single ommatidium fasciculate, thereby bundling information from the same point in space into a single column. During development, the R8 axons project first, forming a retinotopic map in the medulla. The R7 axons follow the R8s but terminate in a more proximal medulla layer; the different color modalities are thereby segregated (Ting et al., 2005). The accurate processing of spatial and color information therefore presumably requires that R7 and R8 faithfully restrict their connections to the correct column and layer (Choe and Clandinin, 2005; Stavenga and Hardie, 1989).

R7 neurons are particularly amenable to genetic manipulation—for example, individual homozygous mutant R7 axons can be analyzed in otherwise wild-type animals—and therefore provide an attractive system in which to study connectivity (Lee et al., 2001). Previous work has identified several molecules required for R7 axons to terminate in the correct target layer (Choe et al., 2006; Hofmeyer et al., 2006; Maurel-Zaffran et al., 2001; Clandinin et al., 2001; Lee et al., 2001; Newsome et al., 2000).
However, even mutant R7s that project to the incorrect layer remain restricted to a single column. The molecular basis for this tiling phenomenon is not known.

To identify the molecules required for axon tiling in the visual system, we performed a genetic screen for mutations affecting R7 connectivity. We found that loss of the nuclear import adaptor, Importin-α3 (Imp-α3), of the Activin receptor, Baboon (Babo), caused R7 terminals to invade adjacent columns. We present evidence that Imp-α3 is required for nuclear import of dSmad2 in R7s in response to an Activin signal received by Babo; our results suggest further that this Activin signal is received by R7 axon terminals and originates from the R7s themselves. We demonstrate that the invasiveness of imp-α3 and babo mutant R7s is markedly increased by eliminating R7 neurons from neighboring columns. Based on these findings, we propose that R7 tiling is regulated in a redundant fashion by (1) a paracrine signal promoting repulsion between R7 terminals in adjacent columns; and (2) an autocrine signal inhibiting R7 growth cone motility through an Activin-dependent signaling pathway and nuclear import.

**RESULTS**

**imp-α3 and babo Are Required in R7 Neurons to Mediate a Normal Preference for UV Light**

To identify genes required for R7 connectivity, we conducted a behavioral screen for mutations that disrupt an R7-dependent behavior: phototaxis to UV in preference to visible light (Lee et al., 2001; Clandinin et al., 2001). As previously described, we screened mosaic animals in which R7 neurons were homozygous for a mutagenized chromosome arm while nearly all other cells were heterozygous.

In a screen of both randomly mutagenized third chromosomes and a collection of known mutations, we isolated three new alleles of imp-α3 (16-8, 17-76, and w73; see Experimental Procedures for mapping and cloning information) and identified a babo null mutation (babo0). Although only a small percentage of the wild-type mosaic flies failed to phototax to the UV light (18.9% of FRT82, n = 412; 15.0% of FRT42, n = 604), a significant proportion of mosaic flies containing FRT82 imp-α3 or FRT42 babo mutant R7s failed to do so: 44.9% (n = 414) of imp-α316-8 flies, 51.5% (n = 379) of imp-α317-76 flies, and 49.8% (n = 432) of babo0 flies phototaxed to visible light. A deletion allele of imp-α3, D93, created by Mason et al. (2003), caused a similar defect (56.8% [n = 287]). The corresponding performance indices are graphed in Figure 1; the differences between the mutants and their matching controls are statistically significant (p < 0.00001), although the difference between the two controls (FRT42 and FRT82) is not (p > 0.5). imp-α3 encodes an Importin-α, and babo encodes a type I TGF-β/Activin receptor, suggesting that nuclear import and TGF-β/Activin signaling are required autonomously for R7 function.

**The Tiling of imp-α3 and babo Mutant R7 Axon Terminals Is Disrupted**

To determine whether these behavioral defects reflected defects in R7 connectivity, we used MARCM (mosaic analysis with a repressible cell marker) to examine individual imp-α3 and babo mutant R7 axons. As previously described, we generated homozygous mutant R7s by expressing Flp recombinase under the control of the GMR promoter, and we positively labeled the mutant R7 terminals with MARCM together with the synaptic marker synaptotagmin-GFP or the membrane-tethered marker mCD8-GFP (Clandinin et al., 2001; Lee et al., 2001; Lee and Luo, 1999). In adult animals, each wild-type R7 axon labeled by the GMR-Flp/MARCM system terminates at the M6 layer of the medulla, where it forms a spherical terminus, or synaptic bouton, that is restricted spatially to a single column (0% defect [n = 181]; Figures 2A and 2A’, arrows; Figure 2D). Although imp-α3 mutant R7 axons terminated correctly in the M6 layer, a significant proportion extended laterally into columns occupied by neighboring wild-type R7s (Figures 2B, 2B’, and 2D; 29.2% [n = 137] of imp-α317-76, 18.9% [n = 217] of imp-α316-8, and 23.0% [n = 213] of imp-α3D93 mutant R7s invaded neighboring columns). The orientation of these lateral extensions appeared to...
be random. These defects in R7 column-specific targeting were fully rescued by expression of a wild-type imp-α3 cDNA, indicating that the imp-α3 mutations are solely responsible for the phenotype (0.6% were defective, n = 168; data not shown). We were also able to rescue the imp-α3 mutant R7s by expression of Drosophila importin-α1 (0.5% were defective, n = 211) or importin-α2 (4.6% were defective, n = 304), indicating that other Importin-α paralogs can fully or partially replace Imp-α3 function (data not shown).

R7s homozygous for the babo⁹ null mutation also invaded neighboring columns, although much less frequently (6% [n = 270] were defective) (data not shown). To test whether this weaker phenotype might result from perdurance of wild-type babo product already present in the heterozygous R7 precursor cells, we used ey^3.5-Flip to induce mitotic recombination earlier in eye development (Nern et al., 2005; Newsome et al., 2000). The resulting babo mutant R7s invaded neighboring columns more frequently (12.7% [n = 291] for babo⁹; 9.6% [n = 228] for babo^52) (Figures 2C and 2C0), indicating that perdurance provides a partial explanation for the weak phenotype in GMR-Flip-induced clones. Expressing either of the two Babo isoforms completely rescued the babo R7

Figure 2. The Terminals of imp-α3 and babo Mutant R7 Axons Invade Adjacent Columns
(A–C) Individual homozygous mutant R7 cells were generated with GMR-Flip- (imp-α3 and wild-type) or ey^3.5-Flip- (babo) mediated mitotic recombination; and their synaptic boutons or axons in the medulla were labeled with synaptotagmin-GFP (green) (A, A', B, B') or mCD8-GFP (green) (C, C'), with the MARCM system. R7 and R8 axons were visualized with Mab24B10 (red).
(A, A') Wild-type.
(B, B') imp-α3^316-8 mutant.
(C, C') babo⁹ mutant.
In adult flies, wild-type R7 axons formed nonoverlapping synaptic boutons (arrows) at the medulla M6 layer. The regularity of this array of R7 terminals is clear in an orthogonal section (A') (arrows). By contrast, single imp-α3 or babo mutant R7 axons terminated at the appropriate layer but extended aberrant processes into neighboring columns (B, B', C, C') (arrows). (A')–(C') are orthogonal views of (A)–(C), respectively. Scale bar in (A) represents 5 μm for (A)–(C').
(D, D') Schematic diagrams summarizing (A)–(C').

into neighboring columns.

(A) At 40 hr APF, wild-type R7 growth cones terminated in the R7-temporary layer. At 50 hr APF and found that, indeed, both imp-α3 and babo mutant R7s targeted correctly at 40 hr APF (Figures 3B and 3C) but extended laterally into neighboring columns at 50 hr APF (20.0% [n = 45] and 10.3% [n = 78], respectively) (Figures 3B' and 3C').

It is unclear why defects in R7 tiling alone, particularly at low frequency, would disrupt the ability of R7s to drive phototaxis to UV light. It is possible that these mutations cause additional defects in R7 function. One possibility is that the R7 fails to differentiate normally. However, we have found that both imp-α3 and babo mutant R7s assume correct positions in their ommatidia and express the neuronal marker Elav (see below), the photoreceptor marker Chaoptin (Figures 2B, 2B', 2C, and 2C'), and the R7 marker Prospero (Figures S1A and S1B available online). Finally, babo mutant R7s express the appropriate UV-sensitive rhodopsins, as assessed by a Rh3 antibody (Figure S1D). We therefore conclude that imp-α3 and babo are not required for R7 fate specification. Instead, it is likely that these mutations cause subtle defects in synapse formation or function in addition to the defects in tiling. Indeed, we observe that the synaptic boutons of imp-α3 and babo mutant R7s appeared to be smaller and more irregular than those of wild-type R7s (Figures 2A–2C).

Redundant Mechanisms Restrict R7 Terminals to the Correct Columns

The proportion of imp-α3 and babo mutant R7 axons that invade adjacent columns is relatively small. Previous studies by Ashley and Katz suggested that mutually repulsive interactions among R7 terminals might prevent overlap (Ashley and Katz, 1994). To determine whether imp-α3 and babo mutant R7s are still subject to repulsion by their neighbors, we tested the effect of removing the R7s adjacent to imp-α3 or babo mutant R7s. Males hemizygous for the temperature-sensitive sevenless allele, V1, lack most R7s when raised at the nonpermissive temperature 29°C (sevenless encodes a receptor tyrosine kinase that signals through the Ras pathway to induce the R7 fate). In this background, we used the GMR-Flp/MARCM method to create GFP-labeled homozygous wild-type or imp-α3 mutant R7s, and we used the ey3.5-Flp/MARCM method to create GFP-labeled homozygous wild-type or babo mutant R7s. We found that wild-type R7 axons formed morphologically normal synaptic boutons in retinotopically correct columns even in a largely empty R7 terminal field (Figures 4A, 4A', 4C, and 4C'). By contrast, removing neighboring R7s greatly increased the tendency of imp-α3 or babo mutant R7s to invade adjacent targets. Approximately 84.3% (n = 32) of isolated imp-α3 R7 terminals extended laterally into neighboring columns (as compared to 23.0% of imp-α3 R7 terminals surrounded by fully

*Figure 3. babo and imp-α3 Mutant R7 Growth Cones Invade Neighboring Columns at the Second Stage of Target Selection*

Wild-type, babo, or imp-α3 mutant R7 axons were examined before (40 hr APF [A, B, C]) and during (50 hr APF [A', B', C']) the second stage of target selection. Mutant R7s were generated as described in Figure 2 and their axons were labeled with mCD8-GFP (green) by the MARCM system. R7 and R8 axons were visualized with Mab24B10 (red).

(A) At 40 hr APF, wild-type R7 growth cones terminated in the R7-temporary layer.

(B) imp-α3 mutant. (C) babo mutant.

(A') At 50 hr APF, R7 growth cones regained motility and extended slightly deeper to their final target layer.

(B') At 40 hr APF, single imp-α3 (B) or babo (C) mutant R7 axons terminated correctly in the R7-temporary layer. At 50 hr APF, imp-α3 (B') or babo (C') mutant R7 axons (arrow) made aberrant lateral extensions into neighboring columns.

Scale bar in (A) represents 5 μm for (A)–(C).

phenotypes (Brummel et al., 1999) (data not shown), confirming that the babo mutations are responsible for the observed defects and indicating that Babo's function in R7s is not isoform specific.

We have previously shown that R7 target selection occurs in two distinct stages (Ting et al., 2005). At 17 hr after puparium formation (hr APF), newly differentiated R7 axons project into the medulla and terminate at the R7-temporary layer. At 50 hr APF, R7 growth cones simultaneously regain motility and project into their final destination layer, M6 (Figure 3A). imp-α3 and babo mutant R7 axons invade adjacent columns by extending collaterals within M6, suggesting that this defect occurs during the second stage of target selection. To test this, we examined the mutant R7 axons at 40 hr and 50 hr APF and found that, indeed, both imp-α3 and babo mutant R7s targeted correctly at 40 hr APF (Figures 3B and 3C) but extended laterally into neighboring columns at 50 hr APF (20.0% [n = 45] and 10.3% [n = 78], respectively) (Figures 3B' and 3C').

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*Neuron*

Importin-α3 Facilitates Nuclear Import of Smad2

796 Neuron 56, 793–806, December 6, 2007 ©2007 Elsevier Inc.
innervated targets), and 22.2% of these lateral extensions spanned several columns (Figures 4B and 4B'). Similarly, 75.0% (n = 76) of isolated babo mutant R7 terminals invaded neighboring columns (as compared to 12.7% of babo R7 terminals surrounded by fully innervated targets) (Figures 4D and 4D'). These results suggest that imp-α3 and babo mutant R7 axons are still responsive to repulsion by neighboring R7s, accounting for their incomplete ability to invade adjacent occupied columns and indicating that at least two pathways act in a redundant fashion to restrict terminals to columns.

Both the Babo Ligand, dActivin, and Substrate, dSmad2, Are Required in R7s for Tiling of Their Axons
The Babo receptor serine/threonine kinase has previously been shown to phosphorylate the transcription factor dSmad2 in response to dActivin, resulting in translocation...
of dSmad2 to the nucleus (Zheng et al., 2003). To determine possible sources of Activin in the visual system, we performed in situ hybridization of retinas at 40 hr APF and found that dActivin mRNA was expressed in R7 and R8 neurons (Figures 5A and 5A'). We therefore tested whether dActivin might be required in these R neurons for tiling of R7 terminals. Because dActivin mutants are not available, we used dominant-negative and RNA interference approaches to disrupt dActivin function (Zheng et al., 2003). We found that expressing a dominant-negative form (DN) or RNAi of dActivin specifically in the developing retina (with the GMR-Gal4 driver) resulted in R7 tiling defects (6.0%, n = 401) resembling those of babo mutants (Figures 5C and 5C'). Although modest, this phenotype was considerably enhanced when neighboring R7s were removed: 36% (n = 50) of R7s lacking dActivin extended laterally into unoccupied columns (Figure 5D'). By contrast, GMR-Gal4 alone (Figures 5B, 5B', and 5D) or GMR-Gal4 driving RNAi constructs for the TGF-β ligands Myoglianin or Maverick (data not shown) did not cause any detectable R7 phenotypes. We also expressed dActivinDN or dActivin RNAi in medulla neurons with various medulla drivers (including Apterous-Gal4 and ey-Gal4) and found no significant effect on R7 connectivity (data not shown). These results indicate that R7 axons are restricted to the correct column by receipt of a dActivin signal and suggest that this signal originates from the R7 and/or R8 cells.

We next tested whether the Babo substrate dSmad2 is required for R7 tiling. By using the ey3.5-Flp/MARCM method, we found that dSmad2 mutant R7 axons invaded adjacent targets (7.6% [n = 576]; Figures 5E and 5E'); the patterning of dSmad2 mutant eye discs was wild-type [data not shown]). Note that although the frequency of the phenotype is low, it is similar to that of babo and dActivin and is, again, likely to reflect redundancy; for technical reasons (both dSmad2 and sev are on the same chromosome), we did not assess the dSmad2 phenotype of R7s in a sevV1 background. The transcription factor Mad has been shown to transmit signaling of Dpp and Gbb, two TGF-β family ligands. In contrast to dSmad2, single

Figure 5. dActivin and dSmad2 Are Required in R Cells for R7 Tiling

(A, A') dActivin is expressed in R7 and R8. dActivin expression was assessed in 40 hr APF eye discs by in situ hybridization with a probe complementary to dActivin mRNA (green), R cells were visualized with GMR-mRFP (pseudo-colored in blue) and R7s with Prospero antibodies (red). In (A), the equator is marked with white lines, and in (A'), R cell bodies are outlined in white (R1–R6), red (R7), or yellow (R8) dashed lines. (A') is a high-magnification view of (A). For clarity, the red channel was omitted in (A').

(B–D') Disrupting dActivin in R7s causes tiling defects (arrows). R7 connectivity was assessed in adult flies carrying GMR-Gal4 alone (B, B', D) or GMR-Gal4 driving a dominant-negative construct of dActivin (C, C', D') in wild-type (B–C') or sevV1 (D, D') animals. Removal of neighboring R7s increased the invasiveness of R7s expressing ActivinDN (see text for quantitation). Photoreceptor axons were visualized with a GMR promoter driving a membrane-associated GFP (GFPras, green) as well as Mab24B10 (red).

(E, E') Homozygous dSmad2 mutant R7 cells were generated with ey3.5-Flp and labeled with mCD8-GFP (green) with the MARCM system. R7 and R8 axons were visualized with Mab24B10 (red). Single dSmad2 mutant R7 axons terminated at the appropriate layer but extended aberrant processes (arrows) into neighboring columns. (B'), (C'), and (E) are orthogonal views of (B), (C), and (E), respectively. Scale bars represent 8 μm in (A), 5 μm in (A'), and 5 μm in (B') for (B)–(E).
Neuron
Importin-α3 Facilitates Nuclear Import of Smad2

Mad mutant R7s, generated by GMR-Flp/MARCM, did not exhibit any detectable phenotypes (data not shown).

**Imp-α3 and dSmad2 Form Complexes in S2 Cells and Retinal Tissue**

Because of the resemblance among the dActivin, babo, dSmad2, and imp-α3 mutant R7 phenotypes and the known role of Importins in nuclear import, we set out to test whether imp-α3 might be involved in nuclear import of dSmad2 during R7 target selection. To determine whether Imp-α3 and dSmad2 form a complex, we examined whether they communoprecipitated (coIPed) from cells expressing both proteins from transgenes. To facilitate detection and immunoprecipitation, we expressed GFP-tagged Imp-α3 (Imp-α3-GFP) and myc-tagged dSmad2 (myc-dSmad2) in S2 cells. The expression level of Imp-α3-GFP was close to endogenous levels (Figure 6B). We found that myc-dSmad2 colPed with Imp-α3-GFP in the anti-GFP precipitates (Figure 6A). Conversely, Imp-α3-GFP, as well as the endogenous Imp-α3, colPed with myc-dSmad2 in the anti-myc precipitates, and the colP of endogenous Imp-α3 was dependent upon the presence of myc-dSmad2 (Figure 6B). We next examined whether dSmad2 and Imp-α3 form a physical complex in retinal tissue. We expressed Flag-tagged dSmad2 and Imp-α3-GFP in the retina by using the GMR-Gal4 driver and found that Flag-dSmad2 colPed with Imp-α3-GFP in anti-GFP precipitates (Figure 6C). Because dSmad2 antibodies suitable for immunoprecipitation and western blotting are not available, we were unable to assess whether complexes form in retinal tissue when these proteins are expressed at physiological relevant levels. Nevertheless, these results indicate that Imp-α3 and dSmad2 can form a physical complex in vivo.

**Imp-α3 and Babo Are Required for Proper Nuclear Accumulation of dSmad2 in R7s**

To test whether dSmad2 is localized to R7 nuclei during target selection and whether this localization requires imp-α3, we expressed Flag-tagged dSmad2 in MARCM clones. In this system, the expression of dSmad2 is driven by the constitutive actin promoter, thereby making it unlikely that imp-α3 affects transcription of Flag-dSmad2. In wild-type clones at 50 hr APF, Flag-tagged dSmad2 accumulated in R7 cell nuclei (Figures 6D, 6D', 6F, and 6F', quantified in Figure 6H). By contrast, dSmad2 staining was significantly reduced in the nuclei of imp-α3 mutant R7s (generated by the GMR-Flp/MARCM method) (Figures 6E, 6E', and 6H) and in the nuclei of babo mutant R7s (generated by the ey3.5-Flp/MARCM method) (Figures 6G, 6G', and 6H). The levels of nuclear dSmad2 staining were quantified in Figure 6H, and the differences between wild-type and mutants are significant (p < 0.001). By contrast, the nuclear accumulation of dSmad2 in imp-α3 or babo mutant pigment cells was largely unaffected (Figures 6E, 6E', 6G, 6G', and 6H). Together, these data indicate that dSmad2 accumulation in R7 nuclei depends on Imp-α3 and Babo, suggesting that the imp-α3 mutant R7 defect results at least in part from its disruption of the Activin/Babo/dSmad2 signaling pathway.

**Imp-α3 and dSmad2 Are Found in R Cell Axons, and R7 Tiling Requires Retrograde Axonal Transport**

To determine whether imp-α3 and dSmad2 might transduce a signal received by R7 growth cones, we determined their subcellular localizations by immunohistochemistry. First, we used an Imp-α3 antibody to stain whole-mount developing eye-brain complexes. Although the Imp-α3 antibody specifically labeled endogenous Imp-α3 in the eye discs (Figure S2), it failed to penetrate fully into the brain samples (data not shown). To resolve the penetration problem and to improve spatial resolution, we used an R cell culture system (Li and Meinertzhagen, 1995, 1997). Dissociated photoreceptor neurons from developing eye discs extend axons and growth cones within 2 days of culture (Figures 7A–7C; see Experimental Procedures for details). By using Imp-α3 antibodies, we found that endogenous Imp-α3 is located in R cell growth cones as well as in axons (Figures 7A' and 7B'). Flag-tagged dSmad2 (Figures 7A", 7B", and 7C") largely overlapped with anti-Imp-α3 staining (Pearson’s correlation coefficient, r = 0.401) (Figures 7A"' and 7B"'), unlike Nervana, a neuron-specific Na'/K'-ATPase (r = −0.32; Figures 7C"–7C"'). Both proteins were also seen in the cell bodies. The localization of both proteins to axons and growth cones suggested that Imp-α3 and dSmad2 might transduce a signal from the developing R7 terminals to the nucleus. One prediction of this model is that retrograde axonal transport would be required to carry these molecules to the cell body and therefore that disruption of the Dynein/Dynactin complex should cause R7s to invade adjacent columns. To test this, we used actin-Gal4 and the GMR-Flp/MARCM system to express a previously characterized dominant-negative form of the dynactin subunit Glued (Gid200) in R7s. Although this had only a modest effect on R7 tiling (18.1% were defective, n = 144; Figures 7D and 7D'), when Gid200 was expressed in R7s whose neighbors had been removed, 71.4% of R7s (n = 21) extended into adjacent columns (Figures 7E and 7E'). These results support a model in which the Activin signal is received by R7 growth cones and transduced to the nucleus (Figure 8).

**DISCUSSION**

Previous anatomical studies have highlighted two prominent features of neuropil organization in the fly visual system: the axons of most neuron classes arborize in characteristic layers of the brain and remain restricted either to one column or a small number of adjacent columns (Fischbach and Dittrich, 1989; Bausenwein et al., 1992). To gain insight into the developmental mechanisms that regulate these aspects of axon targeting, we have focused on the R photoreceptor neuron. In previous work, we and others have characterized mechanisms controlling the precise layer termination of R7 growth cones. In this paper, we
Figure 6. Imp-α3 Physically Associates with dSmad2 and Is Required for Nuclear Accumulation of dSmad2 in R7s

(A and B) Imp-α3 and dSmad2 form a complex in S2 cells. GFP-tagged Imp-α3 (double plus sign) and myc-tagged dSmad2 (asterisk) were expressed in S2 cells, and their physical association was assessed by reciprocal coimmunoprecipitation (coIP). In the S2 cells expressing Imp-α3-GFP and dSmad2-myc (M), WB: αMyc shows that dSmad2-myc coprecipitates with Imp-α3-GFP (double plus sign). In the S2 cells expressing Imp-α3-GFP and dSmad2-myc (M), WB: αMyc shows that dSmad2-myc coprecipitates with Imp-α3-GFP (double plus sign).

(B) In the S2 cells expressing Imp-α3-GFP and dSmad2-myc (M), WB: αImp-α3 shows that Imp-α3 coprecipitates with dSmad2-myc (asterisk). In the S2 cells expressing Imp-α3-GFP and dSmad2-myc (M), WB: αImp-α3 shows that Imp-α3 coprecipitates with dSmad2-myc (asterisk).

(C) In the S2 cells expressing Imp-α3-GFP and dSmad2-myc (M), WB: αFlag shows that Imp-α3-GFP (double plus sign) coprecipitates with FLAG-dSmad2 (asterisk). In the S2 cells expressing Imp-α3-GFP and dSmad2-myc (M), WB: αGFP shows that Imp-α3-GFP (double plus sign) coprecipitates with GFP-dSmad2 (asterisk).

(D) WT, ELAV-GFP, D': FLAG-dSmad2, E: imp-α316-8, F: WT, F': babo9, G: WT, G': babo9

(H) A.U. Nuclear FLAG-dSmad2 level in R7 and 1st pigment cells. Bar graph showing the nuclear FLAG-dSmad2 level in R7 and 1st pigment cells.

analyze the mechanisms that restrict R7 terminals to the correct columns. We demonstrate that the latter process is regulated by two partially redundant pathways: a paracrine signal that mediates repulsion between adjacent R7 axons and an autocrine Activin signal that is transduced by retrograde transport and import of the transcription factor Smad2 into the nucleus by a component of the classical nuclear import pathway, Importin-3.

R7 Terminals Are Restricted to the Correct Columns Redundantly by Activin Signaling and by Repulsion between Adjacent R7s

A prominent organizing feature of the medulla is the restriction of axons and their terminals, including those of R7, R8, and the lamina monopolar neurons (except L4), to single columns (S. Takemura and I.A. Meinerzhagen, personal communication; Millard et al., 2007; Fischbach and Dittrich, 1989). This phenomenon is similar to the tiling of processes observed in both the peripheral and central nervous systems (reviewed in Grueber and Jan, 2004). Ablation experiments in both fly and zebrafish support the view that repulsive interactions between processes of different cells of the same class prevent overlap of dendritic and axonal receptive fields (Gao et al., 2000; Sagasti et al., 2005). Consistent with this model, wild-type R7 terminals only invade adjacent columns from which R7 terminals have been removed (Ashley and Katz, 1994). However, an R7 axon does not invade even empty columns unless it is under “competitive pressure” from additional R7 axons within its own column, suggesting that a second, intrinsic mechanism also restricts R7 terminals (Ashley and Katz, 1994).

We have here presented evidence that Activin signaling is required for tiling of R7 terminals: loss of babo, dSmad2, or dActivin causes R7 axons to invade adjacent occupied targets. Because these mutant axons invade even when they are not under competitive pressure, we hypothesize that Activin affects an intrinsic property of R7 terminals such as their motility or ability to initiate synaptogenesis (Figure 8). In support of this model, we have found that virtually all R7 axons lacking babo, dSmad2, or dActivin initially extend filopodia beyond the R7-terminal layer at 17 hr APF (Figures S3B’, S3C’, and S4B), although these retract by 40 hr APF, and that expression of a constitutively active Baboon in R7s affects growth cone morphology (Figures S4C and S4C’). Although we cannot rule out models in which Activin signaling also mediates repulsion among R7 terminals, we have shown that R7s unable to respond to Activin are still partly repelled by their neighbors, indicating the existence of repulsive mechanisms that are redundant with Activin. Recent studies have demonstrated that Dscam2 mediates repulsion between L1 growth cones in a layer immediately distal to the R7 terminals (Millard et al., 2007). Because Dscam2 is not expressed in R7, other cell-surface proteins must mediate the repulsive interactions between adjacent R7 terminals.

The identification of Activin’s involvement in R7 tiling paves the way to identifying such molecules by allowing the removal of a pathway that is functionally redundant with them.

Tiling of R7 Terminals Requires Imp-3-Mediated Transport of dSmad2 to the R7 Nucleus

Members of the TGF-β superfamily have been widely implicated in regulating axon guidance and synaptogenesis by both transcription-dependent and -independent mechanisms (Packard et al., 2003; Parker et al., 2004, 2006; Butler and Dodd, 2003; Colavita et al., 1998; Nash et al., 2000; Eaton and Davis, 2005; Marques et al., 2003; McCabe et al., 2003; Marin et al., 2005; Serpe and O’Connor, 2006; Zheng et al., 2003). In this study, we found that loss of dSmad2 from R7s resembles loss of Activin, suggesting that the tiling of R7 terminals requires changes in transcription. In support of this model, we found that restriction of R7 terminals also requires imp-3, which...
we have shown is required for the accumulation of dSmad2 in R7 nuclei. Whereas some previous vertebrate studies have suggested that individual Smads are imported by Importin-α (Xiao et al., 2003) or Importin-β (Xiao et al., 2000; Kurisaki et al., 2001), others argue instead that active Smad complexes can enter the nucleus by an importin-independent mechanism (Xu et al., 2003; Chen et al., 2005). Our results provide the first genetic evidence that Smad function can require Importin-α-mediated nuclear import and may help reconcile previous results by demonstrating that different cell types import dSmad2 by different mechanisms (although R7s require

Figure 7. Imp-α3 and dSmad2 Colocalize in Photoreceptor Axons, and Retrograde Transport Is Required for R7 Tiling

(A–C”) Flag-tagged dSmad2 was expressed in third instar larval eye discs with the GMR-Gal4 driver. The subcellular localization of Flag-dSmad2 and endogenous Imp-α3 was assessed in cultured photoreceptor neurons with Flag (A”, B”, C”) (red) and Imp-α3 (A’, B’) (green) antibodies, respectively. (A”, B”) Imp-α3 and Flag signals colocalized in the growth cones (large arrows) as well as vesicle-like structures (small arrows) along the axons. (C–C”) By contrast, the neuron-specific Na+/K+-ATPase Nervana, detected by anti-HRP staining (blue), was found in the entire axon, exhibiting minimal overlap with the Flag signal. (A), (B), and (C) are bright-field images of the cultured photoreceptor neurons. (A), (B), and (C) are orthogonal views of (D) and (E). Scale bars represent 15 μm in (A) for (A’–A”) and (C’–C”) and 5 μm in (B) for (B’) and (B’). (D–E”) Blocking retrograde axonal transport affects R7 tiling. A dominant-negative form of the Dynactin subunit Glued (GipDN) was expressed in individual R7s with Actin-Gal4 and the GMR-Flp/MARCM system, and their axons were examined in adults. R7s expressing GipDN were labeled with mCD8-GFP (green), and all photoreceptor axons were visualized with Mab24B10 (red). (D, D”) R7s expressing GipDN infrequently invaded adjacent columns but (E, E”) did so more frequently when neighboring R7s were removed by sev+ (see text for quantitation). (D) and (E) are orthogonal views of (D) and (E). Scale bar represents 5 μm in (D) for (D–E”).
Importin-α3 Facilitates Nuclear Import of Smad2

**Figure 8. A Schematic Model of R7 Tiling**

The tiling of R7 terminals is regulated by two partially redundant pathways: (1) an unknown signal (blue double arrows) that mediates repulsive interactions between adjacent R7 growth cones, and (2) an Activin signal (red arrows) that regulates intrinsic R7 growth cone motility by the following mechanism. Activin, secreted from the R7 growth cone, activates its receptor Babo in R7, resulting in phosphorylation of the transcription factor, Smad2. The phosphorylated Smad2, in complexes with Imp-α3, then shuttles into the nucleus to reduce growth cone motility via transcriptional regulation of yet-to-be identified target genes.

**What Might Be the Source of the Activin Signal?**

Surprisingly, Activin appears to be required in the R neurons and likely in R7s themselves: disrupting Activin in all R neurons causes R7 terminals to invade adjacent targets, and among the photoreceptors, only R7 and R8 express Activin. Our attempt to test whether Activin is specifically required in R7s met with only partial success: we used sevenless-Gal4 (sev-Gal4) to express UAS-ActivinDN in R7s but not R8s and found that the resulting R7s temporarily overshoot their initial target layer, a phenotype also caused by loss of Babo or dSmad2 (Figure S4B). Thus, Activin can function as an autocrine effector. Unfortunately, the sev-Gal4 driver is no longer expressed by 40–50 hr APF, the time at which Activin prevents R7s from invading adjacent columns, and sev-Gal4/ActivinDN R7s appear normal at this time point (data not shown).

Nonetheless, the finding that R7s and/or R8s are the source of Activin raises two mechanistic questions. First, if the R7 or R8 neurons themselves are providing the signal and the signal is simply transduced into the R7 nucleus, why might Activin, as we argue, be secreted in the target region and received by the R7 growth cone (i.e., rather than being secreted and received by the cell body)? One possibility is that R7s use Activin to coordinate their developmental program with that of other cells within the medulla. For example, one could imagine that both R7 growth cones and their postsynaptic targets would encounter the Activin signal in the medulla at the same time, allowing them to coordinate their preparations for mutual synaptogenesis. A second question is, therefore, how might Activin signaling be coordinated with the R7 growth cones’ arrival in the medulla? We have found that the Activin-processing enzyme Tolloid-related (Tlr) is located both at the R7-temporary target layer (at 17 hr APF) and at the final R7 target layer (at 50 hr APF) (Figures S5B and S5B), and that Tlr mutants exhibit severe R7 retinotopic map defects (Figures SSC and SSC’). One possibility is therefore that the medulla localization of Tlr might confer spatial and temporal specificity on Activin expressed by R7 and/or R8.

Although Activin is also expressed in R8s, we have found no evidence that Activin affects R8 axons, as shown by the fact that neither babo mutant R8s nor R8s expressing GMR-Gal4/UAS-ActivinDN exhibited connectivity defects (data not shown). However, we cannot rule out the possibility that redundancy obscures such a role (for example, there is no straightforward method of removing adjacent R8s).

**The Relevant Transcriptional Targets of Activin Signaling in R7 Remain to Be Identified**

In the mushroom body, dActivin signaling results in upregulation of the ecdysone receptor gene, EcR-B1 (Zheng et al., 2003). Although EcR-B1 is expressed in essentially all photoreceptor neurons, three lines of evidence indicate that EcR-B1 is not the target gene of Activin signaling in imp-α3, pigment cells do not). We note that imp-α3 mutant R7s have more frequent defects in tiling than babo or dSmad2 mutant R7s, suggesting that imp-α3 may transport additional nuclear proteins that, redundantly with the Activin pathway, restrict R7 terminals.

In addition to their classical nuclear import function, Importins have been implicated in mediating retrograde transport of signals from growth cones to the nucleus (Ambron and Walters, 1996; Hanz and Fainzilber, 2004; Hanz et al., 2003). Both Imp-α3 and dSmad2 are found throughout the length of R7 axons, and we have found that, like loss of Activin signaling, disrupting retrograde axonal transport affects the intrinsic mechanism that restricts R7 terminals. These results are consistent with a model in which the Activin signal is received by R7 growth cones, and dSmad2 bound to Imp-α3 is transported through the axon and ultimately into the nucleus (Figure 8).
R7s: the expression level of EcR-B1, as judged by anti-EcR-B1 staining, was not altered in babo mutant R7 clones (Figures S6A–S6A’); forced expression of EcR-B1 did not rescue babo mutant R7 defects (Figures S6B–S6C’); and USP R7 mutants did not phenocopy babo (Figures S6D–S6E’). In the dorsal cluster of Atonal-positive neurons, Babo-mediated signaling, via an EcR-independent pathway, mediates morphogenesis and axonal extension (Zheng et al., 2006). The versatility of Activin signaling likely reflects its ability to regulate the expression of different genes in a context-dependent manner (reviewed in Sanyal et al., 2004). We speculate that dActivin signaling activates a transcriptional program that not only restricts growth cone motility once R7s are within their target layer but also promotes synaptogenesis. Such a model could explain the observed strong defects in R7-mediated behavior despite the infrequent specific defects in R7 tiling. Identifying the transcriptional targets of Activin signaling in R7s will likely provide insight into these processes.

EXPERIMENTAL PROCEDURES

UV/Vis Light-Choice Assay

The effects of mutations on R7 function were assayed by creating mosaic animals having homzygous mutant R7s (with GMR-Flp and PAN-R7-tetanus toxin as described in Lee et al., 2001) and testing their behavior in a UV/Vis choice test (also described in Lee et al., 2001). The behavioral setup is similar to that used in Reinke and Zipursky (1988) except that the light sources were replaced with UV and green light-emitting diodes. UV at 370 nm (0.15 w/cm²) and green light at 525 nm (7.8 lux) were used in all the tests. Performance index (P.I.) was calculated with the following formula: $P.I. = \frac{N_{UV} - N_{vis}}{N_{UV} + N_{vis}}$. The locus defined by the complementation group (data not shown). Shortly after our identification, an imp–3 allele, D93, generated by P element excision by D.A. Mason (Mason et al., 2003), became available to us.

Genetics

Fly stocks were maintained at 22°C on standard medium unless stated otherwise. Mutagenesis was performed with ethylmethane sulfonate according to standard procedures (Ashburner, 1989; Grigliatti, 1986).

The following stocks were used to generate imp–3 or babo R7 mosaic animals for the UV/Vis behavior assay: (1) FR742, R7-TNTE/Cyo; (2) GMR-Flp; FRT72, babo2/Cyo; (3) FRT72, R7-TNTE/TM6B; (4) GMR-Flp; FRT72, imp–3/D39/TM6B; (5) GMR-Flp; FRT72, imp–3/D39/TM6B (a gift from David S. Goldfarb); and (6) GMR-Flp; FRT72, imp–3/D39/TM6B.

For generating eye-mosaic animals used in histological analyses, ey3.5-Fip (a generous gift from Iris Salecker) was used in combination with the MARCM system. ey3.5-Fip induces eye mosaic clones but not brain clones. Single mutant R7 cells were generated by the GMR-Flp/MARC system as described previously (Lee et al., 2001). For analyzing single wild-type or mutant R7s at different developmental stages, we included the actin-Ga4 driver in the genetic scheme (Nern et al., 2005). Fly stocks that were used for these experiments are as follows. ey3.5-Fip; GMR-myr-RFP; FRT42,Citrine/Cyo; (5) UAS-babo-a; FRT42, babo3, UAS-mCD8-GFP/Cyo; (6) UAS-babo-b; FRT42, babo3, UAS-mCD8-GFP/Cyo; (7) UAS-Flag-dSmad2; FRT42, babo3, UAS-mCD8-GFP/Cyo.

For the creation of dSmad2 and usp MARCM clones: (1) FRT19A, tubP-GAL80; hs-Flp; UAS-syt-eGFP; (2) FRT19A, D1XMB688, UAS-mCD8-GFP; (3) FRT19A, I;X48 USP; and (4) ey3.5-Fip/TM6B.

For ectopic expression of various transgenes: (1) UAS-EcR-B1; (2) UAS-CmdActivin; UAS-CmdActivin; (3) UAS-HLDactivin; UAS-HLDactivin/Cyo; (4) UAS-max RNAi; (5) UAS-myo RNAi; (6) GMR-Gal4, UAS-GFPw/Cyo; (7) UAS-Flag-dSmad2; (8) pdaActivin-GAL4; (9) pdsmad2-Gal4; (10) UAS-nsGFP; (11) UAS-mCD8-GFP; (12) UAS-imp–3-GFP; (13) UAS-babo2/Cyo; and (14) sevenless-Gal4. The transgenic flies used for analyzing imp–3 rescue were generous gifts from David S. Goldfarb.

For the creation of wild-type, imp–3, and babo MARCM clones in the absence of neighboring R7s: (1) sev3, GMR-Flp; FRT82, imp–3/D39/TM6B; (2) sev2, GMR-Flp; FRT82, babo/Cyo; (3) actin-GAL4, UAS-syt-eGFP/Cyo; FRT82, tubP-GAL80/TM6B; (4) FRT42, babo3, UAS-mCD8-GFP/Cyo; ey3.5-Fip; and (5) FRT42, tubP-GAL80/Cyo; actin-GAL4, UAS-syt-eGFP.

For expressing a dominant-negative form of glued in individual R7s in the presence or absence of neighboring R7s: (1) GMR-Flp; UAS-GFPw/Cyo; FRT82/TM6B; (2) actin-GAL4, UAS-syt-eGFP; FRT82, tubP-GAL80/TM6B; and (3) sev2, GMR-Flp; UAS-mCD8-GFP/Cyo; FRT82/TM6B.

The trans-allelic combination of TlrE1 and TlrP0(2-41) was used for determining the specificity of Tir antibody and for analyzing tir mutant phenotypes. TlrE1 and TlrP0(2-41) are null and strong hypomorphic tir alleles, respectively (Serpe and O’Connor, 2006).

Molecular Identification of the imp–3 Gene

The locus defined by the complementation group (16–8, w73, 17–76) was mapped to a region of <100 kb by failure to complement available deficiencies and by male meiotic recombination. Predicted open reading frames within this region were sequenced, and only one, that of imp–3, contained sequence changes associated with the 16–8, w73, and 17–76 mutations; each resulted in a stop codon at a distinct position in the imp–3 reading frame (data not shown). Shortly after our identification, an imp–3 allele, D93, generated by P element excision by D.A. Mason (Mason et al., 2003), became available to us. 16–8, 17–76, and w73 all fail to complement D93, and all alleles cause essentially identical phenotypes.

Molecular Biology

The pUAS-dSmad2- myc, pUAS-imp–3-GFP, and pUAS-Flag-dSmad2 vectors were constructed by PCR-based cloning techniques and confirmed by sequencing. Transgenic flies were generated by standard microinjection techniques. Cloning procedures are available upon request.

Histology

Immunohistochemistry, confocal imaging, image deconvolution, and 3D image rendering were performed as described previously (Ting et al., 2005). The Imaris coloss module and ImageJ package (NIH) were used to analyze colocalization and to calculate Pearson’s coefficient of correlation (r). For the quantification of nuclear Flag-dSmad2 level, wild-type and mutant eye discs were processed in parallel for immunohistochemistry and imaged with identical laser power settings. The average intensity (0–254 AU) was calculated for each nucleus with the ImageJ package.

The following concentrations of primary antibodies were used: mAb24B10, 1:100 dilution; mouse anti-Prospor MR1A, 1:50 dilution; mouse anti-EcR-B1 AD4.4, 1:50 dilution; rat anti-Elav 7E8A10, 1:200 dilution; mouse anti-Rh3 2B1, 1:50 dilution (a generous gift from Steve Britt); rabbit anti-GFP, 1:500 dilution (Torey Pines Biolabs); mouse anti-Flag M5, 1:200 dilution (Sigma); goat anti-HRP-Cy5, 1:50 dilution.
Importin-α3 Facilitates Nuclear Import of Smad2

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Supplemental Data

Tiling of R7 Axons in the *Drosophila* Visual System Is Mediated Both by Transduction of an Activin Signal to the Nucleus and by Mutual Repulsion

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Figure S1. R7 Cell Fate Is Not Affected by *baboon* or *importin-α3*

(A, B) *imp-α3* (A) or *babo* (B) mutant R7 cells were generated and labeled with mCD8-GFP (green) using GMR-Flp or ey³⁵-Flp, respectively, and the MARCM system. R7 cell fate was examined at the mid-pupal stage using antibodies against the R7-specific marker, Prospero (red), and the pan-neuronal marker, Nervana (anti-HRP, blue). *imp-α3* and *babo* mutant R7 cells (arrows) expressed both Prospero and Nervana indistinguishably from the wild type clones. Scale bar: 5 µm in B.

(C, D) *babo* mutant R7s express an R7-specific opsin, Rh3. (C) In wild-type adults, approximately 30% of the R7s and none of the R8s express the Rh3 opsin (blue). (D) In flies in which over 90% of the retina tissue is homozygous *babo* mutant, the Rh3-expression pattern was similar to that of wild type. Scale bar: 30 µm in C.
Figure S2. The anti-Importin-α3 Antibody Specifically Labels Endogenous Importin-α3

imp-α3 mutant clones were generated in developing eye discs using ey^{3.5}-Flp and marked with a membrane-tethered GFP (mCD8-GFP, green) using the MARCM system. Antibody staining of the imp-α3 mutant clones were analyzed at 40 hr APF.

(A,A’) Anti-Imp-α3 staining (blue) was present in wild-type but not in imp-α3 mutant eye clones. Photoreceptor neurons were labeled with the anti-Chaoptin antibody (Mab24B10, red). (A’) A single-channel image of anti-Imp-α3 staining in (A). imp-α3 mutant clones are marked by red dotted lines. Scale bar; 20 µm in A.
Figure S3. *babo* or *dSmad2* Mutant R7 Axons Overshoot Their Temporary Target Layer at the First Stage of Target Selection

R7 MARCM clones (green) were generated using ey^{3-5}-Flp and the axonal targeting of wild-type (A,A’), *babo* (B,B’), or *dSmad2* (C,C’) mutant R7s were examined at 17 hr APF. Photoreceptor axons were labeled using the Mab24B10 antibody (red).

(A-A’) At 17 hr APF, single R7 cells, homozygous for a wild-type FRT42 chromosome arm, projected axons into the medulla (me). In the more mature part of the neuropil, each wild-type R7 growth cone (arrow) terminated at the R7-temporary layer approximately 6 μm below the R8 growth cone.

(B-C’) At 17 hr APF, *babo* (B,B’) and *dSmad2* (C,C’) mutant R7 growth cones (arrows) overshot the R7-temporary layers and terminated 1-2 μm below.

(A’,B’,C’) high-magnification views of (A,B,C), respectively. Scale bars: in A, 20 μm for A-C; in A’, 5 μm for A’-C’
Figure S4. Expressing a Dominant-Negative Form of Activin and a Dominant-Active Form of Babo in R7s at the First Target-Selection Stage Causes Distinct Defects in Axon Targeting and Growth Cone Morphology

(A–C’) Sevenless-Gal4 was used to drive the expression of mCD8-GFP either alone (A,A’), or together with a dominant negative form of Activin (Activin$_{DN}$; B,B’), or a dominant active form of Babo (Babo$_{DA}$; C,C’), and the R7 axons was examined at 17 hr APF. Unlike the wild-type control (A,A’), Activin$_{DN}$-expressing R7 growth cones projected just past the R7-temporary layer (arrows) but then retracted to the correct layer (B,B’). By contrast, Babo$_{DA}$-expressing R7 growth cones failed to expand properly (arrows) and some (arrowheads) remained in the layer between R7- and R8-temporary layers. (A’-C’) are high magnification views of the boxed areas of (A-C), respectively. Scale bar: in A, 5 µm for A-C; in A’, 5 µm for A’-C’. 
Figure S5. The Activin Processing Enzyme Tlr Is Localized in the R7 Target Layer during Development

(A-C’) The distribution of Tlr protein in developing optic lobe was examined in wild-type (A-B’) and tlr mutant pupae. Each eye-brain complex was labeled with GMR-mRFP (for photoreceptor axons; red) and a rabbit polyclonal anti-Tlr antibody (blue). (A,A’) At 17 hr APF, there was diffuse Tlr staining in the medulla. Tlr staining is concentrated in the R7-target and inner medulla layers at 50 hr APF (B,B’). (C,C’) Tlr staining was completely absent in tlr mutants. Note that some photoreceptor axons (arrows) overshot the R7-temporary layer at 17 hr APF, as seen in babo mutants. In addition, tlr mutants exhibited severe retinotopic map defects, suggesting that they have additional patterning defects.

Figure S6. The Ecdysone Receptor EcR and Coreceptor Ultraspiracle Are Not Downstream Targets of Babo in R7s

(A-A’’) Ecdysone receptor expression level is not affected in babo mutant R7s. babo mutant R cells were generated using ey^{3.5}-Flp-mediated mitotic recombination and labeled with mCD8-GFP (green) using the MARCM system. EcR-B1 expression was assessed using the anti-EcR-B1 antibody (red). Photoreceptors were labeled with the anti-HRP antibody (purple). (A’,A’’) high magnification view of (A). (A’’) shows the red channel (anti-EcR-B1) in (A) with the babo mutant clones marked by red dotted lines.

(B-C’’) Forced expression of the ecdysone receptor EcR-B1 fails to rescue babo mutant R7 phenotypes. R7 targeting was assessed in babo mutant R7 cells in which EcR-B1 was expressed using the MARCM system. babo mutant R7 phenotypes, i.e., overshooting the R7-temporary layer at 17 hr APF (arrow, B’) and lateral extensions in adults (arrows, C’, C’’) persisted in spite of forced expression of EcR-B1. babo mutant R7s were labeled with mCD8GFP (green) and R cell axons were visualized with Mab24B10 (red).

(D-E’’) R7s lacking the ecdysone co-receptor Ultraspiracle (Usp) do not exhibit babo-like phenotypes.

usp mutant R7 clones were generated using GMR-Flp-mediated mitotic recombination, and their axons were labeled with mCD8-GFP (green) using the MARCM system. R cell axons were visualized with Mab24B10 (red). usp mutant R7 axons targeted normally at both 17 hr APF (arrows, D,D’) and adult stages (arrows, E-E’’). Approximately 10% of the usp mutant R7 growth cones exhibited morphological defects that were not observed in babo mutant R7s (data not shown).

(A’-E’) High magnification views of (A-E), respectively. (C’,E’’) Orthogonal views of (C’,E’), respectively. Scale bars: in A, 8 µm; in A’, 5 µm; in B, 20 µm for C-E; in B’, 5 µm for C’, C’’, D’, E’, E’’.