Robo-3–mediated repulsive interactions guide R8 axons during *Drosophila* visual system development

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The formation of neuronal connections requires the precise guidance of developing axons toward their targets. In the *Drosophila* visual system, photoreceptor neurons (R cells) project from the eye into the brain. These cells are grouped into some 750 clusters comprised of eight photoreceptors or R cells each. R cells fall into three classes: R1 to R6, R7, and R8. Posterior R8 cells are the first to project axons into the brain. How these axons select a specific pathway is not known. Here, we used a microarray-based approach to identify genes expressed in R8 neurons as they extend into the brain. We found that Roundabout-3 (Robo3), an axon-guidance receptor, is expressed specifically and transiently in R8 growth cones. In wild-type animals, posterior-most R8 axons extend along a border of glial cells demarcated by the expression of Slit, the secreted ligand of Robo3. In contrast, *robo3* mutant R8 axons extend across this border and fasciculate inappropriately with other axon tracts. We demonstrate that either Robo1 or Robo2 rescues the *robo3* mutant phenotype when each is knocked into the endogenous *robo3* locus separately, indicating that R8 does not require a function unique to the Robo3 paralog. However, persistent expression of Robo3 in R8 disrupts the layer-specific targeting of R8 growth cones. Thus, the transient cell-specific expression of Robo3 plays a crucial role in establishing neural circuits in the *Drosophila* visual system by selectively regulating pathway choice for posterior-most R8 growth cones.

A striking feature of the insect visual system is the organization of neurons into parallel, interconnected layers and orthogonal columns that contain the axonal and dendritic processes from many neurons (1). Columnar organization preserves the topology of visual space. This organization is achieved, in part, during development by the assembly of axons into discrete fascicles. The fly eye comprises some 750 ommatidia or simple eyes, each containing a cluster of eight photoreceptor neurons (R1–R8). R-cell axons form a topographic map of the visual world in the lamina and medulla. The R1 to R6 axons terminate in the lamina, and R7 and R8 extend through the lamina and terminate in the medulla. Axons from each ommatidium form a discrete fascicle and form connections within columnar units, referred to as cartridges and columns in the lamina and medulla, respectively. The orderly assembly of cartridges and columns relies upon the precise spatiotemporal pattern of R-cell innervation.

Two features of early eye development facilitate the orderly assembly of the visual system. First, individual rows of ommatidia are recruited sequentially, following a wave of differentiation beginning at the posterior margin of the eye primordium or eye disk and progressing anteriorly across it. As new ommatidia form, the R cells within them extend axons into the brain. Thus, the wave of ommatidial formation is converted into sequential innervation of the brain (2). Second, R cells in the same developing ommatidium extend axons within a single fascicle sequentially, beginning with R8, followed in a pairwise fashion by R2/R5, R3/R4, and R1/R6, and finally after a lag, R7. The axons of R1 to R6 terminate in the lamina, but those of R7 and R8 project through the lamina and into the underlying medulla. The axons of lamina neurons (L1–L5), from the same cartridge, fasciculate with the R7/R8 axon pair from a topographically matched ommatidium, as they project into the medulla. As a consequence of this pattern of neuronal differentiation, R8 cells at the most posterior edge of the eye primordium navigate a unique pathway into the brain (Fig. 1 *A* and *B*). The molecular mechanisms that guide these earliest projecting, posterior R8 axons are not known.

Here we show that the cell-surface receptor Roundabout 3 (Robo3) is required for the proper guidance of posterior R8 growth cones. The Robo proteins are Ig domain, containing type I transmembrane proteins, which play evolutionarily conserved roles in axon guidance (3). *Drosophila* has three Robo paralogs (Robo1, Robo2, and Robo3) that transduce repulsive signals in response to a secreted ligand, Slit (4–8). In addition, Robo2 can promote attraction in certain contexts, although the attractive ligand to which it responds is not known (9). The extracellular domains of Robo receptors comprise five conserved Ig domains and three fibronectin type III repeats. The cytoplasmic domains of Robo receptors are more divergent, but contain some combination of four conserved motifs (3). Unique roles of Robo receptors during embryonic development rely on structural features specific to different paralogs and different patterns of expression (9).

In this article, we identify *robo3* in a microarray-based molecular screen as a gene expressed specifically and transiently in R8 neurons at an early stage of differentiation. In *robo3* mutants, R8 axons in the posterior region of the eye disk take abnormal paths into the developing visual system leading to abnormal fascicle and column formation. We show that Robo3 prevents posterior R8 growth cones from crossing Slit-expressing glial cells, which separate axon tracts in the lamina from parallel tracts extending into deeper regions of the medulla. We demonstrate that it is the unique spatiotemporal pattern of Robo3 expression, rather than biochemical functions distinct from Robo1 and Robo2, that is crucial for its function in R8. These findings demonstrate that R8 cells elaborate a unique guidance program distinct from later differentiating R cells.

Results

Molecular Screen for R8-Specific Cell Surface Molecules. As a first step toward identifying specific guidance receptors expressed in R8 but not in other R cells at an early stage of retinal development, we compared gene expression in mutant eye discs lacking R8 neurons with mutant eye discs containing additional R8 neurons using a microarray-based approach (see *SI Materials and Methods* for detailed description). R8 and R7 neurons do not form in eye discs lacking the Senseless (Sens) transcription factor, but additional R8- and R7-like neurons form in eye discs in which the wild-type Runt (Run) transcription factor is ectopically expressed in R1 to R6 neurons. The Run [G163R] mutant was used as a control and does not cause targeting defects when mis-

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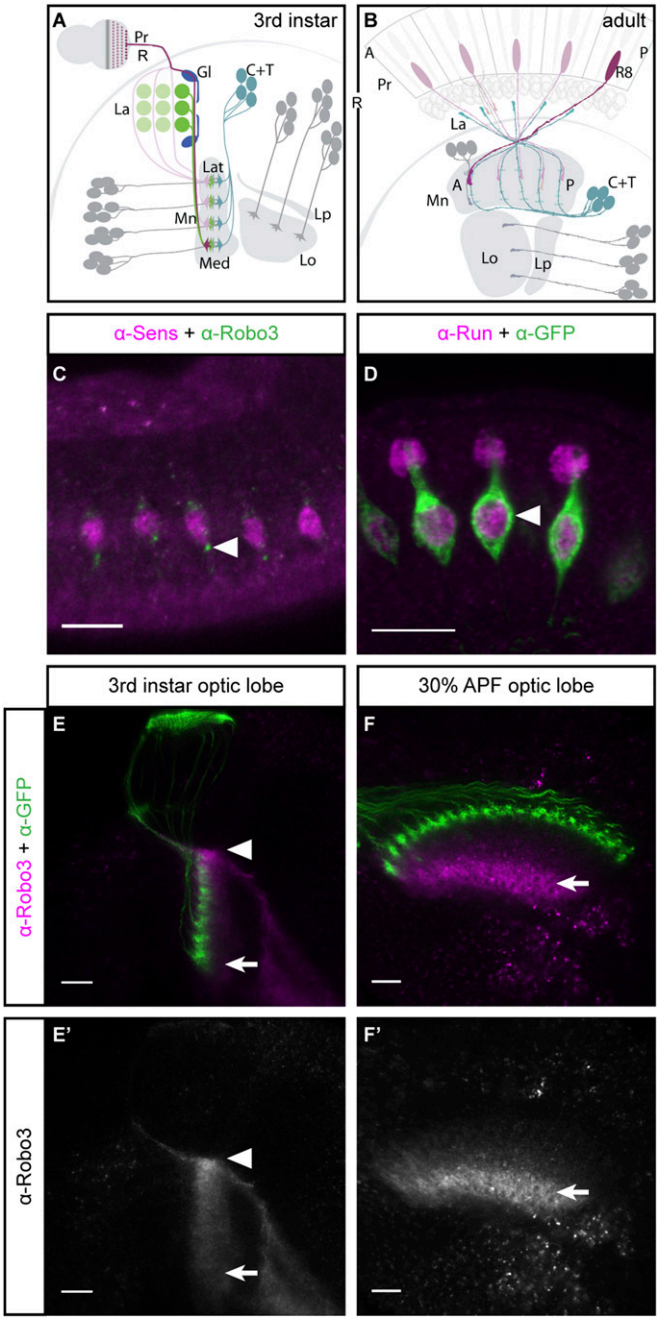

Fig. 1. Robo3 is expressed specifically in the R8 growth cones. (*A* and *B*) Schematic representations of the developing 3rd instar optic lobe (*A*) and the adult optic lobe (*B*), showing the relative orientation of the lamina and medulla neuropils and projections of the posterior R8 axons. R, R cells; La, lamina; Gl, glia; Lo, lobula; Lp, lobula plate; Med, medial; Lat, lateral; Mn, medulla; A, anterior; P, posterior; C+T, C2, C3, T2, and T3 neurons. (*C*) A third instar eye disc costained with antibodies against Robo3 protein (green) and Sens (magenta). An apico-basal section through the eye disc reveals punctate Robo3 staining around the Sens positive nucleus. As shown in Fig. 2*B*, staining was not observed using the antibody to Robo3 in *robo3*^{3} mutant tissue. (*D*) A third instar, *robo3-GAL4/UAS-CD8-GFP*, eye disc costained with antibodies against GFP protein (green) and Run (magenta) reveal Run positive basal nuclei surrounded by membrane localized GFP (white arrowhead). (*E* and *E'*) Robo3 is expressed in third instar R8 growth cones as they enter the optic lobe, but down-regulated shortly after the R8 axons enter the medulla neuropil. Arrowhead and arrow indicate the youngest and oldest part of the medulla neuropil, respectively. (*F* and *F'*) Robo3 expression is not detectable in the R8 axons by 30% APF at which time all the R8 axons have reached the R8 temporary layer. Residual Robo3 staining is observed in the growth cones of a group of optic lobe neurons that project into more proximal regions of the developing medulla neuropil (white arrow in *F* and *F'*). (All scale bars: 10 μm.) See *SI Materials and Methods* for a list of genotypes.

expressed in R1 to R6 photoreceptors (Fig. S1 *A–E* and *SI Materials and Methods*). Genes with altered expression patterns were identified and changes for selected genes were confirmed using quantitative PCR (Table S1). Several genes encoding proteins with known roles in R8 and R7 targeting were identified in this screen (Table S1). Moreover, the gene encoding the Roundabout-3 (Robo3) guidance receptor was the most significantly altered gene in both the *sens* mutant and ectopic Run-expressing eye discs (Table S1). This initial step identified genes enriched in R7/R8 cells versus R1 to R6. Additional studies, described below, established that Robo3 was selectively expressed in R8.

Robo3 Is Expressed Specifically in R8 Growth Cones. Three lines of evidence indicate that Robo3 is specifically expressed in developing R8 neurons at an early stage of neuronal differentiation. First, a Robo3-specific monoclonal antibody (*SI Materials and Methods*) stains in a punctate pattern surrounding the R8 nucleus; the R8 nucleus was identified by its basal location within the developing eye disk and coexpression of Sens and Run (Fig. 1*C* and Fig. S2*A*). This perinuclear pattern may reflect expression within vesicles or within the endoplasmic reticulum (Fig. 1*C* and Fig. S2*A*). Second, an HA-tagged version of Robo3 expressed from the endogenous Robo3 locus was detected in R8 using an anti-HA antibody (9), in a similar pattern to that observed for anti-Robo3 antibody (Fig. S2*B*). Third, an enhancer trap line that comprises a GAL4 containing a P-element inserted 972 bp upstream of the *robo3* transcription start site drives expression of a cell surface-localized GFP reporter (*UAS-CD8-GFP*) selectively in the R8 cell body (Fig. 1*D*). Thus, these results identify Robo3 as a cell-surface receptor selectively expressed in the R8 neuron.

To assess whether Robo3 is expressed in R8 growth cones, we stained developing third-instar optic lobes with the anti-Robo3 monoclonal antibody. R8 growth cones were marked by CD8-GFP expressed using the GAL4/UAS system with an R8-specific GAL4 transgene (*sens-GAL4*). R8 neurons from the posterior edge of the developing eye disk enter the medulla first followed by axons from R8 neurons located more anteriorly (Fig. 1*A*). At early stages of optic lobe development, R8 growth cones are aligned along the medio-lateral axis of the brain at the extreme distal edge of the developing medulla neuropil (see Fig. 1*A* for orientation). This distal medulla layer is referred to as the R8-temporary layer; the medial axons (closest to the central brain) are the oldest R8 axons (i.e., first to enter the brain) and the lateral axons are the youngest (i.e., most recently arriving R8 axons). The targeting of all R8 photoreceptors to the temporary layer is complete by early pupa. During a second phase of targeting, occurring between 50 and 60 h after puparium formation (APF), R8 axons extend further to reach their final layer in the medulla (10). Strong Robo3 staining overlapped with the youngest R8 axons in the R8 temporary target layer (white arrowheads in Fig. 1*E*). Furthermore, a graded pattern of staining was observed within this layer with a marked decrease in Robo3 staining observed in the oldest R8 axons at medial edge (white arrow in Fig. 1*E*). As each additional row of axons is 1.5 to 2 h younger than its medial neighbor, Robo3 expression is largely lost from R8 growth cones many hours before extending from the temporary to its permanent target layer (Fig. 1 *F* and *F'*). Additional Robo3 staining, however, persists in other neurons projecting into more proximal layers of the medulla (arrow in Fig. 1*F*). Thus, these data are consistent with the transient expression of Robo3 protein in R8 growth cones.

Defects in Axon Pathways in *robo3* Mutant Optic Lobes. To critically assess the role of Robo3 in R8 growth cones, we examined optic lobes of a previously identified allele, *robo3*^{1}, but we observed no defects. As previous reports have suggested that this allele retains

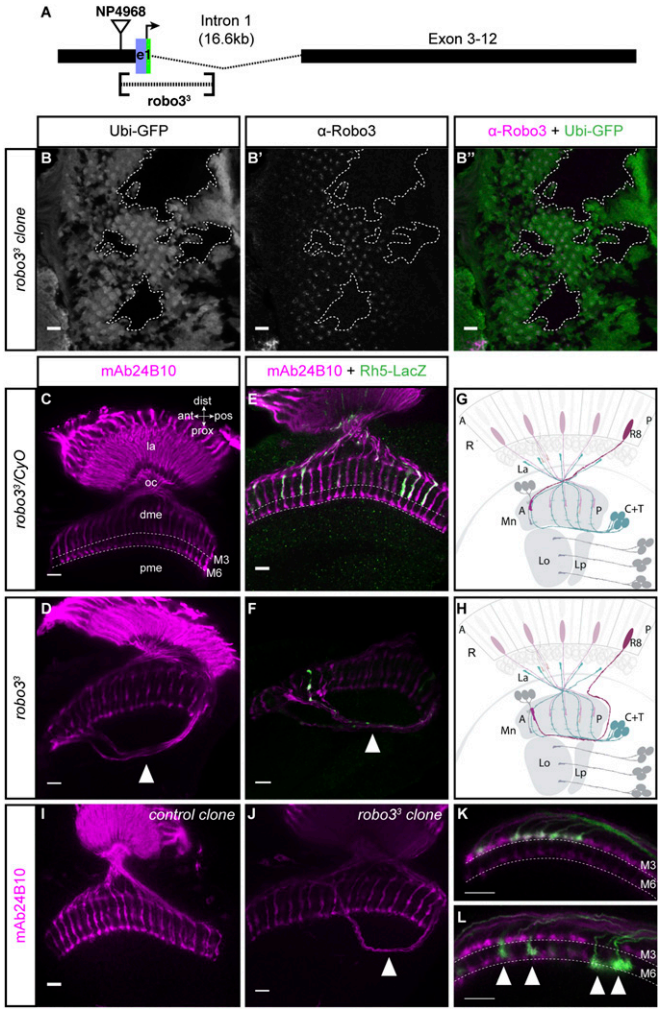

Fig. 2. Defects in axon pathways in *robo3* mutant optic lobes. (*A*) Schematic of the *robo3* locus and the position of the NP4968 P-element insertion (*robo3-GAL4*). The *robo3*^{3} allele was generated by imprecise excision of the NP4968 P-element and deletes exon1, which contains both the transcriptional and translational start sites for *robo3*. (*B* and *B*″) *robo3*^{3} mutant clones in a 3rd instar eye disc. The mutant patches lack GFP expression and are demarcated by the dotted white lines. GFP negative patches also lack Robo3 protein staining (*B*′ and *B*″). (*C* and *D*) Adult optic lobes stained with the mAb-24B10 antibody. (*C*) A *robo3*^{3} heterozygous optic lobe shows the wild-type organization of visual neuropils. la, lamina; oc, optic chiasm; dme, distal medulla; pme, proximal medulla. The R8 axons terminate in the M3 layer and the R7 axons terminate in the M6 layer (dashed lines). (*D*) Posterior photoreceptor axons take an aberrant path to the proximal medulla in *robo3*^{3} homozygous optic lobes (white arrowhead). (*E* and *F*) Adult optic lobes from *robo3* heterozygous (*E*) or *robo3* homozygous animals (*F*) in the background of the *Rhodopsin-5-LacZ* (Rh5-LacZ) transgene costained with mAb-24B10 (magenta) and -galactosidase (green) antibodies. Misprojecting *robo3*^{3} mutant fascicles contain R8 axons (white arrowhead in *F*). As previously reported, *Rh5-LacZ* (like the endogenous gene) is only expressed in some 30% of wild-type R8 neurons. -Galactosidase staining is not continuous and thus projections appear punctate. (*G* and *H*) Schematic representations of wild-type (*G*) and *robo3*^{3} mutant (*H*) optic lobes, highlighting the normal and aberrant paths taken by the posterior R8 axons. R, R cells; La, lamina; Gl, glia; A, anterior; P, posterior; Mn, medulla; C+T, C2, C3, T2, and T3 neurons. (*I* and *J*) Adult optic lobes from animals with the whole eye rendered mutant by mitotic recombination of a control, wild-type, chromosome (*I*) or a *robo3*^{3} mutant chromosome (*J*). *robo3*^{3} mutant photoreceptor axons take an aberrant path to the proximal medulla (white arrowhead in *J*). (*K* and *L*) Thirty percent APF optic lobes containing control or MARCM mutant R8 clones with persistent Robo3 expression, colabeled with anti-GFP (green) and mAb-24B10 (magenta) antibodies. *sens-GAL4* was used in conjunction with MARCM to promote persistent expression of *UAS-robo3* in R8 (green). Persistent expression of Robo3 beyond early pupal stages causes R8 axons to extend through the R8 temporary layer and, instead, terminate in deeper layers close to the R7 temporary layer (arrowheads in *L*). Control clones show wild-type R8 targeting (*K*). (Scale bars: 10μm.) See *SI Materials and Methods* for a list of genotypes.

some activity, we sought to identify null alleles via P-element–mediated imprecise excision (6, 11). Four new *robo3* mutant alleles were isolated by mobilizing a P-element inserted 1,035 bp from the translation start site. Each deletes the transcription and translation start site and is protein-null (Fig. 2 *A* and *B*) (data shown only for *robo3*^{3} allele). All four *robo3* mutant alleles are viable both as homozygotes and *in trans* to a deficiency for the region. All further characterization of the *robo3*-null mutant phenotypes described in this study was performed with the *robo3*^{3} allele.

R-cell axons exhibit a striking phenotype in all *robo3*^{3} mutant optic lobes. In wild-type adult optic lobes, R-cell axons from the oldest ommatidia, located at the posterior edge of the eye, enter through the posterior lamina, traverse the optic chiasm, and project into the anterior-most medulla; R-cell axons from the anterior region of the eye extend through the anterior lamina and the optic chiasm before entering the posterior medulla. All wild-type R-cell axons enter the medulla neuropil from its distal face (Fig. 2 *C* and *G*). Posterior R-cell axons were selectively affected in all *robo3*^{3} mutant adult brains examined ($n > 30$ brains) (Fig. 2 *D* and *H*). These axons bypass the optic chiasm and instead project along the posterior edge of the medulla neuropil, turn anteriorly, typically projecting for variable distances along the proximal face of the medulla before entering the medulla neuropil (arrowhead in Fig. 2*D*). These misprojecting photoreceptor bundles contain axons expressing an R8-specific marker (Fig. 2 *E* and *F*). In summary, Robo3 is required for normal R-cell innervation of the posterior medulla.

Robo3 Is Required for Posterior R8 Axon Guidance. To test whether Robo3 phenotype reflects its function in R cells or in other cells in the optic lobe, we generated clones of mutant tissue scattered throughout the eye surrounded by heterozygous and homozygous wild-type cells. No mutant phenotypes were observed. As only posterior R cells exhibited defects in homozygous animals and mutant clones in the most posterior region of the eye disk were infrequent, we sought to generate clones that would include the posterior edge of the eye disk. As methods for selectively generating such clones are not available, we used a variation on targeting mitotic recombination in the eye primordium to ensure that all cells in the eye discs, including cells along the posterior edge, were rendered homozygous-mutant (see *SI Materials and Methods* and refs. 12 and 13). In these animals, posterior R cells extending into an otherwise wild-type optic lobe projected aberrantly to the distal medulla, as in homozygous *robo3*^{3} mutant animals (Fig. 2 *I* and *J*). Furthermore, the misprojecting *robo3*^{3} mutant posterior axons often disrupt the normal columnar organization of the medulla (Fig. 2 *F* and *H*). However, the organization of R8 axons projecting from more anterior regions of the eye disk is largely unaffected by loss of Robo3 (Fig. S3).

To assess whether rapid down-regulation of Robo3 is required for R8 targeting to the temporary layer, we prolonged the expression of Robo3 in R8 neurons beyond early pupal stages (20% APF) using the *sens-GAL4* driver coupled with the MARCM (mosaic analysis with a repressible cell marker) technique. This process resulted in 84% of the R8 axons ($n = 169$) bypassing their temporary layer and instead projecting into the R7 temporary layer (Fig. 2 *K* and *L*). Thus, the restricted expression of Robo3 in R8 neurons during a precise developmental time window, the precise level of Robo3 expression in R8 or both are important in controlling R8 projections into the developing optic lobes. Taken together, these data support the view that Robo3 is required in posterior R8 axons to select the appropriate pathways to their targets in the optic lobe.

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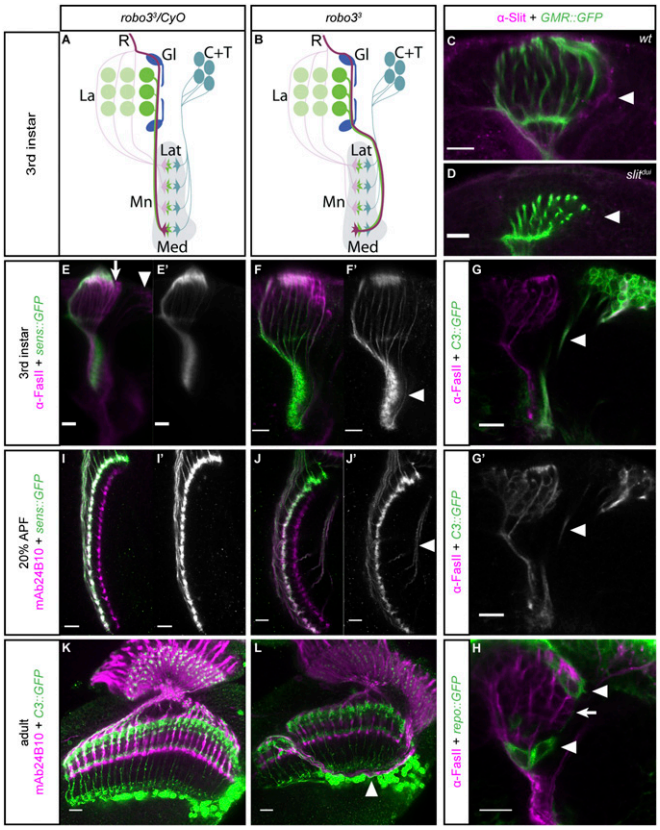

Fig. 3. Posterior R8 axons take an inappropriate pathway into the medulla. (*A* and *B*) Schematic of the relationship between the posterior R8 axons (red), glial cells (dark blue), and C+T axons (light blue) in early third-instar optic lobes of wild-type (*A*) or *robo3*^{3} mutant (*B*) animals. C+T, C2, C3, T2, and T3 neurons; Gl, Glia; La, Lamina; Lat, Lateral; Med, Medial; Mn, Medulla; R- R Cells. (*C* and *D*) Third-instar larval optic lobes from *GMR-GFP* flies in a wild-type (*C*), or *slit*^{dui} homozygous mutant background (*D*) (anti-Slit, magenta; anti-GFP, green). Slit expression is detected at the posterior margin of the lamina in wild-type optic lobes (arrowhead in *C*). Slit expression is lost in *slit*^{dui} homozygous mutant optic lobes (arrowhead in *D*). (*E* and *F*) Third-instar larval optic lobes from *sens-GAL4, UAS-CD8-GFP* flies colabeled with anti-FasII and GFP antibodies showing the relationship of the earliest arriving R cell and the C+T axons. FasII labels photoreceptors, lamina neurons, and C+T axons. (*E* and *E*′) Wild-type R8 axons project along glial-cell boundaries but never cross them but *robo3*^{3} mutant posterior R8 (*F* and *F*′) axons cross glial-cell boundaries and project along the paths taken by the C+T axons (arrowhead in *F*′). (*G*, *G*′, and *H*) Glial cells mark the posterior boundary of the lamina and separate posterior R8 axons from C+T axons (arrowhead in *H*). Third-instar optic lobes from *C3-GAL4, UAS-CD8-GFP* larvae (*G* and *G*′) and *repo-GAL4, UAS-CD8-GFP* larvae (*H*) showing the relationship of the earliest arriving R cell axons (FasII, magenta), glia (anti-GFP, green in *H*), and the C3 axons (anti-GFP, green in *G*). (*I* and *J*) Inappropriately projecting posterior R8 axons can be visualized in *robo3*^{3} mutant pupal optic lobes (arrowheads in *J* and *J*′). Twenty-percent APF optic lobes with *sens-GAL4/ UAS-CD8-GFP* in WT (*I* and *I*′), or *robo3*^{3} mutant backgrounds (*J* and *J*′); R-cell axons, anti-24B10 (magenta); R8 axons, anti-GFP (green). (*K* and *L*) Inappropriately projecting posterior R8 axons grow along the C3 axons tracts before entering the distal medulla (arrowhead in *L*). *robo3*^{3} heterozygous (*K*) or homozygous (*L*) adult optic lobes that also contain the *C3-Gal4/ UAS-CD8GFP* transgenes. R-cell axons, mAB-24B10 (magenta); C3 cell bodies and axons, anti-GFP (green). (Scale bars, 10 μm.)

Robo3 Mutant R8 Axons Inappropriately Cross Slit-Expressing Glial Cells. To assess how R8 projection defects emerge during development, we analyzed *robo3*^{3} mutants carrying a marker for developing R8 axons in third-instar larval and pupal brains. In wild-type, posterior R8 axons project along glial cells that separate the posterior regions of the developing lamina from C+T lobula neurons (only the C3 subset are labeled in Fig. 3 *G*, *K*, and *L*). In wild-type, these neurons project into the proximal face of the medulla (Fig. 3 *A* and *E*). In *robo3*^{3} mutants posterior R8 axons cross the glial-cell boundary and follow C+T axons into the medulla (Fig. 3 *B* and *F*). This inappropriate crossing of R8 axons in *robo3*^{3} mutants is clearly apparent at about 20 h APF after more axons have grown in, resulting in a further separation of the axon tracts (Fig. 3 *I* and *J*) and in adult optic lobes (Fig. 3 *K* and *L*). Interestingly, the Robo3 ligand Slit is expressed in a domain overlapping these glial cells (Fig. 3 *C*, *G*, and *H*). Thus, Slit may repel R8 growth cones through Robo3, thereby preventing them from extending across the glial-cell boundary.

To test whether Slit is required to guide posterior R8 axons, we assessed the projections of R cells in animals that lack Slit selectively in the developing visual system. *Slit* mutant homozygotes do not survive embryogenesis, precluding the assessment of phenotypes in the third instar and adult optic lobes. However, a previously described P-element insertion in the first intron of the *slit* locus (*slit*^{dui}) exclusively disrupts expression of Slit protein in the optic lobes, presumably by selectively disrupting the enhancer elements that control optic lobe expression (*slit*^{dui}) (Fig. 3*D*) (11). R-cell axons do not form an optic chiasm in *slit*^{dui} mutants (Fig. S4). This phenotype is similar to, but more severe than, the loss of Robo3. This finding reflects, at least in part, the loss of Robo3-mediated repulsion in R8, as well as the loss of Slit, working through other Robo receptors affecting migration of lobula neurons, as previously described by Garrity and colleagues (11). In summary, these data support a model in which Slit, produced by glial cells, acts through Robo3 to control posterior R8 axon pathfinding within the developing optic lobes.

R7 and Lamina Axons Follow Misdirected R8s in Robo3 Mutants. To assess whether other axons that fasciculate with R8 in wild-type followed misdirected R8s into the medulla in *robo3*^{3} mutants, we used markers for different R-cell subclasses and for lamina monopolar neurons. In wild-type, R1 to R6 and R7 axons form a fascicle with R8, as these axons project from the eye disk into the developing optic lobe. R1 to R6 axons terminate in the lamina but R7 follows the R8 axons into the medulla. The axons of lamina monopolar neurons (L1–L5) from each developing lamina cartridge fasciculate with the R7/R8 axon pair from an ommatidium in the same topographic location and project into a column in the developing medulla. As in wild-type, R1 to R6 axons terminate in the lamina in *robo3*^{3} mutant flies (Fig. S5 *A* and *B*). The axons of posterior R7 and posterior lamina monopolar neurons take an aberrant route to the medulla, similar to mutant R8 axons (Fig. S5 *C*–*F*). As Robo3 protein is only detected in the R8 cell, we conclude that defects in R7 and lamina axon projections in *robo3*^{3} mutant optic lobes are a secondary consequence of the loss of Robo3 protein in the R8 cell.

Robo3 Function Can Be Rescued by the Other Robo Paralogs. To assess whether the function of Robo3 in R8 is dependent on unique biochemical properties distinct from Robo1 and Robo2 or is a reflection of its unique spatial temporal pattern of expression, we assessed R-cell axon projections in two knock-in alleles in which an HA-tagged *robo1* or *robo2* coding sequence replaced *robo3*. A third knock-in allele in which a HA-tagged *robo3* coding sequence replaced endogenous *robo3* was used as a control (9). HA-tagged proteins in all three knock-in alleles were expressed in a pattern similar to wild-type Robo3 protein in the R8 cell (Fig. 4 *A*–*C*). Slight variations in the amounts of HA staining were observed in the R8 cells, either because of differences in genotypic backgrounds or differences in the stability of the HA tagged proteins (Fig. 4 *A*–*C*). However, R-cell projections in all three genotypes were indistinguishable from wild-type (Fig. 4 *D*–*F*). This result is similar to the finding that either Robo1 or Robo2 can functionally replace Robo3 in promoting pathway selection by growth cones in the developing embryo (9). Thus, the Robo3 requirement for R8 axon guidance is not dependent on structural features of the Robo3 protein that are

different from those in Robo1 and Robo2, but instead depends on the precise spatiotemporal pattern of its expression.

Discussion

Using a microarray-based molecular screen as a starting point, we identified the early and transient expression of Robo3 in R8 growth cones. We demonstrated through loss of Robo3 a specific axon guidance choice point at an early stage of optic lobe innervation. In the absence of Robo3, posterior R8 growth cones inappropriately extend across Slit-expressing glial cells joining axon fascicles of the C+T lobula neurons, instead of remaining alongside the glial process as they extend into the lamina. This early repulsive function of Robo3 plays a crucial role in segregating axons and thereby contributes to the orderly assembly of columnar units comprising the fly visual system.

Robo3 Expression in R8s Is Regulated and Transient. Our microarray data, coupled with antibody staining and the identification of a *robo3-Gal4* enhancer trap, identified Robo3 as an R8-specific guidance receptor. Robo3 expression is transient in the R8 growth cone and prolonging Robo3 expression in R8 axons results in defects in R8 targeting. The microarray analysis suggests that restricted Robo3 expression during early stages of R8 differentiation occurs downstream of the transcription factors Sens and Run. However, the expression of both Sens and Run persists beyond the expression of Robo3. For example, during mid- to late-pupal development, Sens regulates both targeting of R8 axons to their final target layer in the medulla and the expression of R8-specific opsins (14). Therefore, other mechanisms must exist to control the expression of Robo3 in R8.

The importance of the tightly regulated expression of Robo receptors is emerging as a central theme in axon guidance. Indeed, previous studies have revealed a set of discrete post-translational mechanisms controlling Robo functions both in vertebrates and invertebrates. For example, commissural axons in the fly embryo express Robo1 transcripts before crossing the midline, but Robo1 protein in these axons is sequestered into endosomes by the action of Commissureless protein, thereby preventing precocious repulsion from the midline and, thus, allowing these axons to cross (15, 16). Subsequent up-regulation of Robo1 prevents them from recrossing. In vertebrates, alternative splicing of a divergent Robo receptor Rig1/Robo3, perhaps coupled with translational regulation, governs the switch from midline attraction to repulsion (17).

The regulated expression of Robo3 in the R8 photoreceptors is similar to its expression in the chordotonal neurons in the embryonic peripheral nervous system (18). Sens is activated downstream of Ato in both chordotonal and in R8 neurons, suggesting that a conserved transcriptional program regulates Robo3 expression in these neurons (19). In a broader sense, these findings raise the possibility that conserved regulatory cassettes exist, which link specific transcriptional hierarchies controlling neuronal differentiation with specific constellations of downstream guidance receptors controlling wiring specificity.

Posterior R8 Axon Guidance Plays a Crucial Role in Axon Fascicle Organization. Posterior-most R8 neurons face at least three different guidance choices as they extend from the eye disk into the developing optic lobe. These early choices have a profound effect on later aspects of visual system assembly. First, R8 axons must navigate to the posterior of the eye disk and enter the optic stalk. This process is facilitated, in part, by retinal basal glial cells at the posterior edge of the eye disk (20, 21). If glial cells are displaced anteriorly, R-cell axon fascicles project away from the optic stalk rather than toward it (21). Although it seems likely that this directional choice relies upon R8, it is not known whether posterior growth requires R8-specific functions or whether all retinal neurons are endowed with this function.

Second, R8 axons from each ommatidium must possess molecular mechanisms to retain their individuality. As the R8 axons extend down the optic stalk they form a tight fascicle. Fasciculation is transient, however, because R8s defasciculate as they exit the optic stalk. R8 defasciculation relies on two cell surface receptors, Flamingo (Fmi) and Golden Goal (Gogo) that are expressed in the R8 growth cones as they exit the optic stalk (22–24). Fmi and Gogo mediate repulsive interactions between R8 axons, and thus play a key role ensuring that columns remain as separate modules (22, 24). These repulsive interactions between R8 axons of adjacent columns also explain why axons from later-born (anterior) R8 neurons are not affected in *robo3*^{3} mutant optic lobes; only the posterior R8 axons traverse through the optic lobe with access to the glial cell boundaries that separate them from the C+T axons.

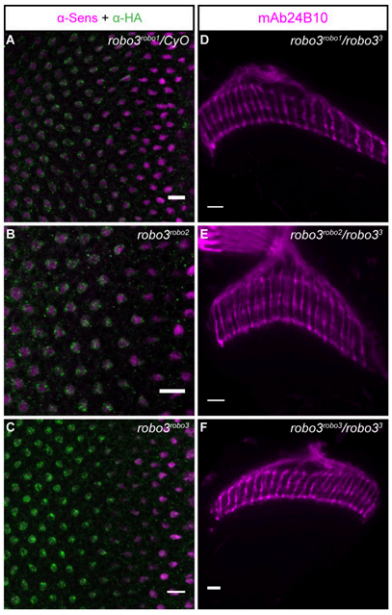

Fig. 4. Both *robo1* and *robo2* rescue the *robo3* R8 axon guidance defects. (*A–C*) Representative eye discs from *robo3*^{robo1} (*A*), *robo3*^{robo2} (*B*), and *robo3*^{robo3}(*C*) third-instar larvae, costained with antibodies against HA and Sens. In each case HA staining colocalizes around the R8 nucleus labeled by Sens. The superscript indicates the coding sequence knocked into the endogenous *robo3* locus. (*D–F*) Robo1 and Robo2 receptors can substitute for Robo3 to prevent posterior R8 axons from joining C+T axon tracts. Adult optic lobes from *robo3*^{robo1}/*robo3*^{3} (*D*), *robo3*^{robo2}/*robo3*^{3} (*E*), and *robo3*^{robo3}/*robo3*^{3} (*F*) animals stained with the mAb-24B10 antibody. (Scale bars, 10 μm.)

Third, in this article we demonstrate that posterior R8 axons rely upon Robo3 to prevent inappropriate fasciculation with C+T lobula neurons. This process requires early, transient, and specific expression of Robo3 in R8 growth cones and is likely to require the reciprocal expression of Slit in the glial cells that these posterior R8 axons encounter when they enter the optic lobe. Thus, the posterior R8 axons are unique because they navigate a choice point that is not encountered by later arriving, more anterior R8 axons. The *robo3*^{3} mutant phenotype described here is reminiscent of the loss of another Ig receptor encoded by the *irregular chiasm*/*roughest* (*irre-C*) locus (25). Whether IrreC acts in the same molecular pathway as Robo3 and, indeed, whether it acts in photoreceptor growth cones or lamina neurons is not known.

In summary, Fmi, Gogo, and Robo3 play crucial roles in R8s in regulating fascicle organization, which provides the structural basis for columnar organization of the visual system. Although Fmi and Gogo mediate interactions between axons of the same class of cells (R8s), Robo3 prevents axons from one class of neurons (R8s) from inappropriately associating with a different class of axons (C+Ts) projecting into the same neuropil along a different pathway. Given the cellular complexity of columns (e.g., medulla columns comprise more than 50 axons from many different neuronal subclasses) and the stereotyped organization of axons and synaptic connections within them, we speculate that many addi-

tional cell-surface proteins must act in a coordinated fashion in space and time to promote the orderly assembly of columnar units.

Robo Proteins Play Multiple Roles in Visual System Organization. Does Robo3 function in the R8 rely on Slit? This is indeed the most parsimonious model for Robo3 function in R8. Slit expression is detected around glial cells separating the posterior R8 growth cones from C+T lobula neurons. Although R8 projection defects seen in *slit* mutants are similar to those seen in *robo3* mutants, they are more severe. In contrast to *robo3*, *slit* mutant optic lobes are extremely disorganized, arguing that Slit has a broader role in neuropil organization. Indeed, Garrity and colleagues reported that mutants deficient in all three Robo proteins exhibit cell migration defects, which largely phenocopy the loss of Slit (11). They proposed that Slit provides a repellent function in the optic lobe preventing cell migration between cell populations in the lamina and lobula. However, the residual Robo3 function in the *robo3*^{1} hypomorphic allele available for study at that time masked the *robo3* phenotypes uncovered in this study. Thus, although *slit* mutants uncover a broader role for Slit-Robo signaling in many aspects of optic lobe development and patterning, the unique *robo3* mutants described here uncover a specific role for the Robo3 receptor in R8 axon guidance.

Contrasting Roles of Robo Proteins in Embryonic and Photoreceptor Axon Guidance. The repulsive role of Robo3 in R8 neurons in response to locally secreted Slit we propose here is analogous to the role of Robo1 in the guidance of ipsilateral longitudinal pioneer axons in the ventral nerve cord. Robo1 is expressed on the growth cones of ipsilateral pioneer axons and prevents these axons from crossing the midline in response to Slit secreted from midline glia (5, 8). In contrast, Robo3 is not expressed in the growth cone of commissural and longitudinal pioneer axons and is dispensable for the midline crossing during the development of the embryonic ventral nerve cord (5, 8). Thus, the function of Robo3 in posterior R8s is analogous to the function of Robo1 in embryonic ipsilateral pioneers. However, analyses of knock-in mutants indicate that Robo1 and Robo3 must be sensitive to cell-type–specific regulatory functions. Robo1 has a unique role in midline repulsion of ipsilateral pioneers as it cannot be functionally replaced by Robo2 or Robo3 (9). In contrast, as reported here, either Robo1 or Robo2 can functionally replace Robo3 in the R8s. Thus, repulsive signaling downstream of ipsilateral pioneers in the embryo is dependent on unique structural features of the Robo1 protein, but repulsion of posterior R8 axons does not depend on unique structural features of Robo3. Instead it is the unique and context-specific expression of Robo3 that allows it to determine R8 axon guidance and in a broader context function in the orderly assembly of a subset of columnar elements in the visual circuit.

Materials and Methods

Microarray Analysis. A detailed description of RNA extraction, array hybridization, and data processing is available in the *SI Materials and Methods*.

Molecular Biology. The GMR-Run construct was generated by cloning an EcoRI fragment containing the full-length Run cDNA into an EcoRI digested GMR-N1 vector (26) (a gift from Ming Guo, University of California, Los Angeles). Transgenic lines were generated by germline injections as previously described (27).

For Immunohistochemistry and image analysis, and for genetics, see *SI Materials and Methods*.

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