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Temporal Control of Glial Cell Migration in the *Drosophila* Eye Requires *gilgamesh*, *hedgehog*, and Eye Specification Genes

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Summary

In the *Drosophila* visual system, photoreceptor neurons (R cells) extend axons towards glial cells located at the posterior edge of the eye disc. In *gilgamesh* (*gish*) mutants, glial cells invade anterior regions of the eye disc prior to R cell differentiation and R cell axons extend anteriorly along these cells. *gish* encodes casein kinase Iγ. *gish*, *sine oculis*, *eyeless*, and *hedgehog* (*hh*) act in the posterior region of the eye disc to prevent precocious glial cell migration. Targeted expression of Hh in this region rescues the *gish* phenotype, though the glial cells do not require the canonical Hh signaling pathway to respond. We propose that the spatiotemporal control of glial cell migration plays a critical role in determining the directionality of R cell axon outgrowth.

Introduction

Glial cells provide a variety of cues for growth cone guidance (Fitch and Silver, 1997; Lemke, 2001; Powell and Geller, 1999; Powell et al., 1997). As such, the precise positioning of these cells during development is a critical determinant of neuronal connectivity. In many cases, glial cells have a distinct developmental origin from neurons and migrate over long distances before reaching their final positions (Cameron-Curry and Le Douarin, 1995; Noll and Miller, 1993; Ono et al., 1997a, 1997b; Rogister et al., 1999). Hence, glial cell migration must be precisely coordinated, both temporally and spatially, with neuronal development.

Glial cells play important roles in establishing patterns of connectivity in the *Drosophila* nervous system (Auld, 1999; Granderath and Klämbt, 1999; Jacobs, 2000; Klämbt et al., 1991, 1996). During embryonic development, neurons extend growth cones toward and along the surface of a variety of different glial cell types (Goodman and Doe, 1993; Jacobs and Goodman, 1989; Klämbt and Goodman, 1991). Neuron-glial interactions have been intensively studied at the midline and along the longitudinal tracts (Klämbt et al., 1991; Seeger et al., 1993). Midline glial cells, for instance, provide important intermediate targets for axons that pioneer the commissures (Jacobs, 2000; Klämbt et al., 1991) and express a variety of molecules important for growth cone guidance at the midline (Kidd et al., 1999; Rothberg et al., 1990; Tear et al., 1996). Genetic ablation studies suggest that local axon-glial cell interactions regulate midline crossing (Hummel et al., 1999). During the early stage of longitudinal tract formation, pioneer growth cones make extensive contacts with glial cells along their path (Goodman and Doe, 1993; Jacobs and Goodman, 1989; Klämbt and Goodman, 1991). Nevertheless, longitudinal pioneer tracts still form in the absence of glial cells. Hence, the interactions of axons with glial cells may not be essential for guidance, but rather may serve a structural role during subsequent steps of longitudinal tract formation (Booth et al., 2000; Hidalgo and Booth, 2000; Hidalgo et al., 1995; Kinrade et al., 2001). In general, the instructive function of glial cells in axon guidance has been difficult to establish, as it has not been possible to assess guidance in response to placement of glial cells in ectopic positions.

In the developing *Drosophila* visual system, photoreceptor cell (R cell) axons contact numerous glial cell types along the pathway to the target and within the target itself (Choi and Benzer, 1994; Perez and Steller, 1996; Rangarajan et al., 1999; Tix et al., 1997; Winberg et al., 1992). These glial cells originate from different regions of the developing visual system and migrate to their final destinations where they associate with axons (Choi and Benzer, 1994; Perez and Steller, 1996; Rangarajan et al., 1999; Winberg et al., 1992). We have recently shown that a subset of glial cells in the lamina ganglion plays an essential role in determining target specificity for a subclass of R cell axons (Poeck et al., 2001).

The first glial cells encountered by extending R cell axons are retinal basal glial (RBG) cells located along the basal surface of the eye disc epithelium (Choi and Benzer, 1994). As R cells differentiate, they extend growth cones basally where they contact RBG cells and turn posteriorly toward the optic stalk. These glial cells originate in the optic stalk and migrate into the eye disc (Choi and Benzer, 1994). Migration is temporally and spatially linked to R cell development. The extension of axons from R cell clusters occurs in a sequential fashion that reflects the highly ordered pattern of R cell differentiation in the eye disc (Ready, 1989; Ready et al., 1976; Wolff and Ready, 1991). R cells in the posterior region differentiate first and additional R cell clusters form more anteriorly as a wave of differentiation sweeps across the eye disc. The leading edge of this wave is marked by a depression in the apical region of the disc epithelium called the morphogenetic furrow (MF).

Glial cells start to migrate into the eye disc as R cell differentiation at the posterior margin is initiated. During MF progression, RBG cells migrate along the basal surface up to but not past the youngest R cell axons. As Choi and Benzer (1994) first observed, RBGs appear to migrate along R cell axons thereby making a guidance role for them unlikely. Subsequent studies by Gaul and coworkers revealed that glial cells can migrate out of the optic stalk into the eye disc in the absence of axon contact and that, instead, glial cells are essential for R

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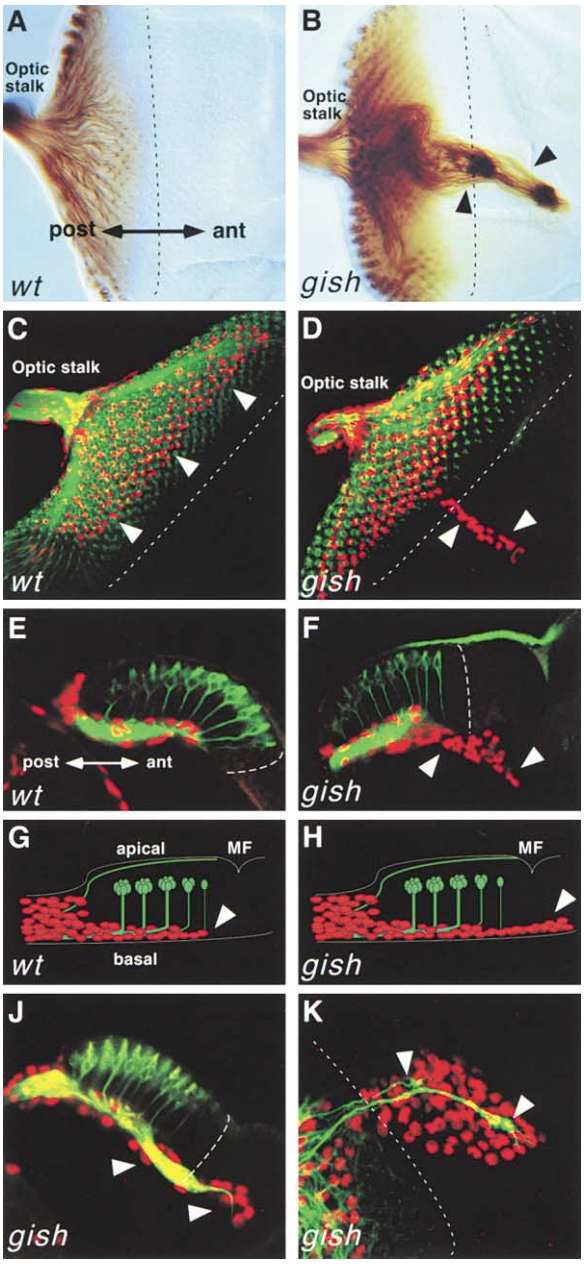

Figure 1. *gish* Regulates Glial Cell Migration and R Cell Axon Outgrowth in the Developing Eye Disc

In (A) and (B), eye discs were stained with mAb24B10. (A) In wild-type, R cells in the eye disc project posteriorly away from the morphogenetic furrow (MF, dashed line) toward and into the optic stalk. (B) In *gish* mutant larvae, a fraction of R cell axons extends anteriorly across the MF into the non-neuronal part of the eye disc (arrowheads). In (C)–(F), glial cell nuclei were stained with anti-Repo (red) and neurons with anti-HRP (green). (C) In wild-type larvae, glial cells (red) are found only in the neuronal part of the eye disc (arrowheads) posterior to the MF (dashed line). (E) Glial cells are associated with R cell axons (green) at the basal surface of the eye disc. (D) In *gish*, glial cells are located posterior and anterior (arrowheads) to the MF (dashed line). (F) Glial cells in *gish* mutants migrate along the basal surface of the eye disc across the MF (arrowheads). (G) and (H) show schematic representation of glial cell migration in wild-type (G) and *gish* mutants (H). In contrast to wild-type, where glial cells stop their anterior-directed migration along the basal surface of the eye disc upon reaching the youngest R cell axons (arrowhead in [G]), glial cells in *gish* mutants continue to migrate anteriorly (arrowhead in [H]). (J and K) Discs were stained with anti-Repo (red; glial cell nuclei) and anti-HRP (green, neurons). In *gish* mutants, anteriorly projecting R cell axons are always associated with ectopic glial cells (arrowheads). (A, B, C, D, and K), lateral view; (E, F, and J), dorsal view; anterior is to the right.

cell axons to enter the optic stalk (Rangarajan et al., 1999). These observations suggest that the precise coordination of glial cell development in the optic stalk and R cell differentiation in the eye disc is important for normal visual system development.

In the course of a genetic screen for axon guidance mutants, we identified a loss-of-function mutation that temporally uncoupled glial cell migration from R cell differentiation. As we show here, mutations in the *gilgamesh* (*gish*) locus lead to precocious glial cell migration from the optic stalk into the eye disc prior to R cell differentiation. As a result, glial cells are misplaced anteriorly in the eye disc as R cell axons extend. R cell axons frequently project along pathways demarcated by these ectopic glial cells, providing strong evidence that the positioning of glial cells plays an instructive role in regulating the directionality of R cell axon outgrowth in the eye disc. The *gish* locus encodes a casein kinase Iγ isoform and acts in conjunction with the eye specification genes, *eyeless* (*ey*) and *sine oculis* (*so*), to prevent precocious entry of glial cells into the eye disc. We also demonstrate that loss-of-function *hh* mutants exhibit precocious glial cell migration and targeted expression of Hh in the posterior region of the eye disc suppresses the ectopic glial cell migration phenotype in *gish* mutants.

Results

gilgamesh Affects Glial Cell Migration and the Directionality of R Cell Axon Outgrowth in the Developing Eye Disc

In a screen of 500 P element recessive lethal mutations that survive at least through third instar, we identified one mutation, called *gilgamesh*^{1}(*gish*^{1}), with a striking defect in the directionality of axon outgrowth (Figure 1). In late third instar larvae, wild-type R cell axons project posteriorly away from the morphogenetic furrow (MF) and toward the optic stalk (Figure 1A), whereas in 15% of the *gish* mutant discs, R cells project anteriorly past the MF (Figure 1B). A dorsal view revealed that R cells project to the basal surface of the eye disc, but then turn toward the MF (Figure 1J), rather than posteriorly as in wild-type (Figure 1E). In most cases, one or two R cell fascicles containing a small number of axons project anteriorly (Figure 1K). Anterior projections were never observed in wild-type.

In wild-type, outgrowing R cell axons are intimately associated with the RBG cells (Figures 1C and 1E). Like R cell axons, these glial cells are only found posterior to the MF (Figure 1C), where they migrate along the basal surface of the eye disc (Figure 1E). In *gish* mutants, however, glial cells were found both anterior and posterior to the MF (Figures 1D and 1F). In all homozygous *gish* mutants, at least a single cohort of glial cells extends

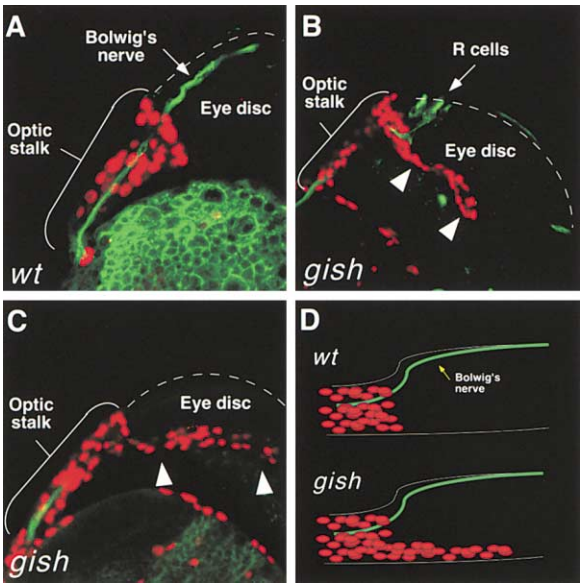

Figure 2. Glial Cells Migrate Precociously into the Eye Disc in *gish* Mutants

(A) In wild-type second instar or early third instar discs prior to R cell differentiation, glial cells (red, anti-Repo) reside in the optic stalk and contact Bolwig's nerve (green, anti-HRP) that enters the optic stalk from the apical side of the eye disc. (B) In *gish* mutant early third instar larvae, glial cells have migrated far into the eye disc (arrowheads) anterior to R cells differentiating at the posterior margin. (C) In *gish* mutants prior to R cell differentiation, glial cells exit the optic stalk and migrate along the basal surface of the eye disc (arrowheads). (D) shows schematics illustrating the precocious glial cell migration phenotype in *gish* mutants. All panels show dorsal views with anterior to the right. Dashed lines in (A)–(C) indicate the apical surface of the eye disc.

anteriorly across the MF along the basal surface of the eye disc. In most cases (about 80%; n = 50), they migrate close to the dorsoventral midline (see below) (Figure 1D). In all cases in which R cell axons projected anteriorly, they did so between ectopic glial cells and the basal surface of the eye disc (Figure 1J, n = 45; also see Figure 3F). While only 15% of late third instar eye discs showed ectopic R cell projections, ectopic glial cells anterior to the MF were observed in all eye discs (n > 200). The number of glial cells in *gish* mutant eye discs was similar to wild-type (data not shown). While most ectopic glial cells were found basally, a small fraction migrated along Bolwig's nerve on the apical surface of the disc (see below, Figure 6B).

In summary, these observations suggest that abnormal glial cell migration is the primary defect in *gish* mutants and that R cell axons follow ectopic glial cells in an anterior direction. As described below, glial cell migration defects are seen prior to third instar. Hence, the glial cells described anterior to the MF in the preceding sections may have migrated there prior to MF progression.

gish Controls the Timing of Glial Cell Migration

To determine the onset of the glial cell migration defect in *gish* mutants, we analyzed eye discs at earlier stages of development (Figure 2). In early third instar *gish* mutants, a few rows of R cells have developed and Repo-positive glial cells have migrated far into the anterior region of the eye disc (Figure 2B). Surprisingly, Repo-positive glial cells were found in *gish* mutant eye disc even prior to R cell differentiation (Figure 2C), whereas in wild-type, glial cells are found exclusively within the optic stalk prior to R cell differentiation (Figure 2A). As the number of glial cells in *gish* and wild-type were similar at this stage of development (data not shown), the abnormal distribution is likely to reflect defects in glial cell migration. Glial cells migrate out of the optic stalk and continue to migrate anteriorly as a cohort of cells. The preferential localization of ectopic glial cells to the dorsoventral midline in third instar discs is presumably a consequence of the early migration defect of these cells coupled with the later expansion of the dorsal and ventral epithelial cells in the eye disc by cell division. The penetrance of the early glial cell phenotype is 100%, with the number of glial cells migrating in the disc varying from 5 to more than 20 (n = 30). These data indicate that *gish* is required to prevent precocious glial cell migration during early eye development (Figure 2D).

R Cell Axons Follow a Subclass of Glial Cells to Ectopic Positions

Based on morphology, location and marker expression RBG cells were divided into two classes, wrapping glial (WG) and surface glial (SG) cells (Figure 3). In third instar, WG cells are intermingled with R cell axons in the eye disc, and extend long processes into the optic stalk that enwrap R cell axons (Figure 3A). SG cells are a continuation of the optic stalk and separate the R cell axons from the eye disc and the hemolymph (Figure 3B). Prior to R cell axon outgrowth, the SG cells comprise the majority of glial cells in the optic stalk. These cells form a sharp boundary between the optic stalk and the eye disc (Figure 3C). As the first R cell axons project toward the basal surface of the eye disc, they contact the surface of SG cells, turn posteriorly, and grow along them into the optic stalk (Figure 3D). The WG cells then send processes along the surface of the extending R cell axons (data not shown). In *gish* mutants, even in the absence of R cell axons, SG cells migrate precociously into the anterior region of the disc (Figure 3E). As the first outgrowing R cell axons contact them, they frequently turn anteriorly and extend along the surface of these cells (n = 40; Figure 3F). In early third instar, the anteriorly directed axon guidance phenotype exhibits a penetrance of about 50%, which is much higher than the penetrance observed at the end of larval development (~15%; see above). Presumably many of the early misrouted axons are not visible in late third instar discs because they are obscured by the axons of late differentiating axons or they may later turn and extend posteriorly. The subsequent association and elongation of the WG cells along R cell axons posterior to the MF does not appear to be affected in *gish* mutants (data not shown).

Previous studies by Rangarajan et al. (1999) showed that targeted expression of a dominant-negative form of mammalian Ras in all glial cell precursors reduces the number of glial cells entering the eye disc. We found that expression of the transcriptional repressor Tram-

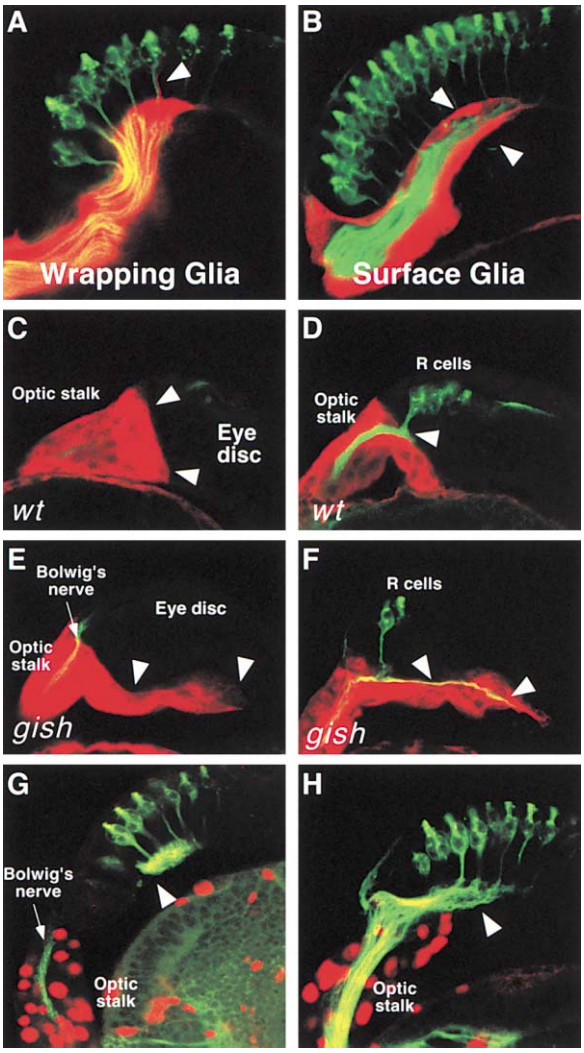

Figure 3. R Cell Axons Migrate Anteriorly along Ectopically Located Surface Glial Cells in *gish* Mutants

(A and B) Two glial cell types, wrapping glia (WG) and surface glia (SG), can be distinguished in third instar eye discs. Neurons are stained green with anti-HRP in all panels. Glia are visualized in red with *Gal4-Mz97* for WG cells (A), with *Gal4-c527* for SG cells (B–F), and with anti-Repo for all glial cell nuclei (G and H). During wild-type development, R cell axons extend along SG cells. Fascicles of axons from single ommatidia are subsequently enwrapped by WG cell processes. These processes extend from the eye disc through the optic stalk and into the brain. WG cell bodies located adjacent to the most anterior R cells frequently extend shorter processes apically along R cell axons in the epithelial layer (arrowhead in [A]). (B) A single layer sheet of SG cells surrounds the R cell neuropil in both the basal region of the eye disc (arrowheads) and the optic stalk. They do not intermingle with R cell axons. (C) Before R cell differentiation, SG cells form a sharp boundary at the transition between the optic stalk and the eye disc (arrowheads). (D) By the time the first HRP-positive R cells differentiate at the posterior margin of the eye disc, SG cells migrate a short distance into the eye disc. R cell axons grow posteriorly along the SG cells into the optic stalk (arrowheads). (E) In *gish* mutant discs, SG cells migrate prematurely prior to the onset of R cell differentiation (arrowheads). Green-stained fibers in this panel are in Bolwig's nerve and not from R cell axons. (F) As R cells differentiate, they frequently extend axons anteriorly along ectopic SG cells in *gish* mutant eye discs (arrowheads). (G and H) SG cells are necessary for R cell axon outgrowth. SG cell development was inhibited by targeted expression of TTK69.

track 69 (ttk69) in glial cell precursors led to a similar inhibition of glial cell migration without affecting their overall number or differentiation. To further analyze the role of SG cells in guiding R cell axons, we disrupted their migration through UAS-ttk69 targeted expression using the SG-specific driver *c527-Gal4* (Figures 3G and 3H). In about 20% of these animals, SG cells do not enter the eye disc and R cell axons fail to enter the optic stalk, forming a mass of R cell axons in the basal region of the disc (Figure 3G). In the remaining larvae, fewer SG cells were found in the eye disc. In these discs, R cell axons project into the optic stalk, but are highly disorganized (Figure 3H). In contrast, inhibiting WG cell differentiation by targeted expression of UAS-ttk69 using a WG-specific driver did not disrupt R cell projections into the stalk (data not shown). This is consistent with the later appearance of WG cells in the eye disc and that the processes of these cells follow the R cell axons into the stalk.

In summary, the location of the SG cells determines the directionality of R cell axon outgrowth. In *gish* mutants, SG cells migrate into anterior regions of the eye disc, thereby promoting anterior outgrowth of R cell axons. In wild-type, SG cells are located only in the posterior region and, hence, account for the initial polarity of axon outgrowth along the anteroposterior axis of the disc epithelium.

gish Encodes a Casein Kinase Iγ (CKIγ)

To clone *gish*, as well as to isolate additional alleles for phenotypic analysis, a "local hopping" P element strategy was utilized (Tower et al., 1993; Zhang and Spradling, 1993; see Experimental Procedures). This led to the identification of two P element insertions, $gish^{P144}$ and $gish^{P232}$ (Figure 4A). These *gish* alleles showed a spectrum of cell migration and guidance defects similar to that seen in $gish^{1}$ (data not shown). Precise excision of the P element in $gish^{P232}$ reverted the mutant phenotype. Phenotypic analysis of $gish^{P144}$ or $gish^{P232}$ over $gish^{1}$ did not reveal any increase in lethality or severity of the phenotype. Similarly, the glial migration phenotype of $gish^{1}$ in *trans* to a deficiency is largely indistinguishable from $gish^{1}$ homozygotes. Together these data argue that, by genetic criteria, $gish^{1}$ is a strong loss-of-function mutation.

Using plasmid rescue, DNA adjacent to the P insertion in $gish^{P232}$ was used to isolate genomic and cDNA clones (Figure 4A). A cosmid, C3, of 35 kb in length, encompassing sequences from a single family of cDNAs encoding three different isoforms of casein kinase Iγ (CKIγ), rescues the $gish^{1}$ mutant phenotype. An upstream transcription initiation site gives rise to one isoform, CKIγa. A second transcription initiation site is predicted to lie in the second intron of CKIγ and gives rise

(G) In the absence of SG cells, axons frequently form swirls, or neuromas, in the basal region of the disc (arrowhead) and do not extend into the stalk. (H) In other discs, the axons extend posteriorly into the stalk but the fibers within the basal region of these discs are highly disorganized (arrowhead). All panels show dorsal views with anterior to the right.

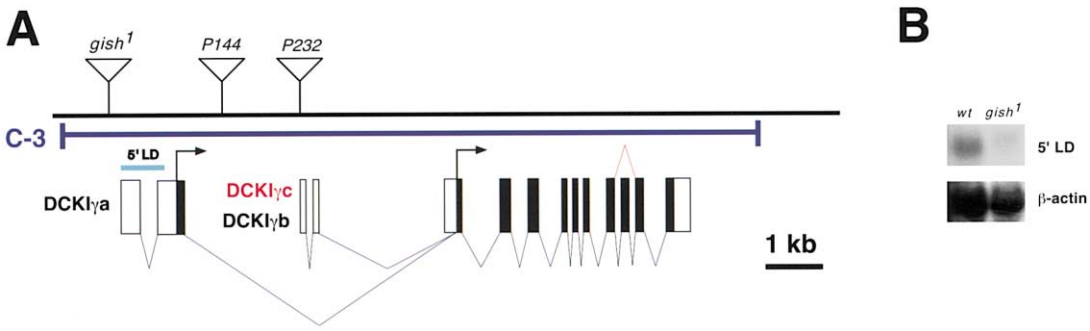

Figure 4. *Gish* Encodes Casein Kinase Iγ

(A) shows genomic organization of the *gish* locus. Exons are indicated by boxes and introns by lines. An insertion of DNA of unknown origin in $gish^{1}$ is located 5′ to the CKIγa transcript start site, whereas the P insertions in $gish^{P144}$ and $gish^{P232}$ are located upstream of the common initiation site of CKIγb and CKIγc. A genomic fragment, C3, spanning the *gish* locus rescues the $gish^{1}$ phenotype. (B) $gish^{1}$ mutants show a strong reduction in CKIγa RNA expression in adult escapers. The blot was probed with an 800 bp fragment from the 5′ UTR region of CKIγa (see [A]). β-actin served as a loading control.

to two separate transcripts, CKIγb and CKIγc, differing by a single 3′ exon (Figure 4A).

Based on the insertion sites of the P elements in $gish^{P144}$ and $gish^{P232}$ and the location of the insertion in $gish^{1}$, it is likely that the CK1γa transcription unit, but not CK1γb and CK1γc, is disrupted in these alleles. Two additional lines of evidence support this view. A CK1γa-specific probe reveals a marked reduction in CK1γ mRNA isolated from adult *gish* mutants on Northern blots (Figure 4B), and a cDNA transgene corresponding to CK1γa rescues the mutant phenotype (see below) whereas the phenotype was not rescued by CK1γb cDNA (data not shown).

Casein kinase Iγ is a member of a conserved family of serine/threonine kinases. The fly protein shares extensive sequence identity to rat CKIγ3, with an overall identity of 68%. The identity within the kinase domain is 78%. The more variable C-terminal domain of the predicted Gish protein contains a putative prenylation signal that has been shown to confer membrane localization (Wang et al., 1992).

gish Functions at the Posterior Margin of the Eye Disc to Control Glial Cell Migration

To determine whether *gish* acts within glial cells or in the eye disc cells to regulate glial cell migration, we carried out genetic mosaic experiments and targeted transgene rescue experiments (Figure 5). The entire eye disc epithelium was rendered homozygous mutant for *gish*, in an otherwise wild-type background, using FRT-dependent mitotic recombination promoted by Flp recombinase expressed under the control of the *eyeless* promoter (Newsome et al., 2000; Stowers and Schwarz, 1999). This promoter does not drive expression in the RBG cells or their precursors (data not shown). In these mosaic animals, the *gish* phenotype in late third instar eye discs ($n > 50$) was largely indistinguishable from the phenotype in *gish* homozygous larvae (Figure 5A). Similarly, in *ey*-Flp-induced mosaics in second instar larvae, glial cell migration defects were observed prior to R cell differentiation (Figure 5B). These data argue that *gish* is required in the eye disc epithelium, at or before late second instar, to control glial cell migration.

cDNA transgene experiments were undertaken to assess whether expression of *gish* in the eye disc epithelium was not only necessary, but sufficient to prevent premature glial cell migration in *gish* mutants. A full-length *gish* cDNA (CK1γa) under UAS control was specifically expressed at the posterior edge of the eye disc and along its lateral edges using the *Dpp-Gal4* driver. This rescued the migration defect in about 70% of the second instar *gish* mutant eye discs analyzed ($n = 25$), and few ectopic glial cells anterior to the MF were observed in third instar discs (Figure 5C). Conversely, targeted expression of *gish* in glial cells using different glial Gal4 driver lines did not rescue the migration phenotype (data not shown). That *gish* acts prior to R cell differentiation within the eye disc epithelium is further supported by the failure of GMR-Gal4 (which drives expression in all cells posterior to the MF) to rescue the mutant phenotype (data not shown).

Together, these data support a model in which Gish participates in a signaling pathway, within the posterior region of the eye disc, which prevents precocious migration of glial cells from the optic stalk into the basal region of the eye disc. Loss of *gish* function in this region disrupts the normal coordination between glial cell development and neuronal differentiation leading to defects in the directionality of axon outgrowth. Previous studies demonstrated that initiation of neuronal development also commences under the control of a signaling center at the posterior region of the eye disc (Dominguez and Hafen, 1997). Accordingly, we next tested the possibility that glial cell migration and neuronal differentiation are controlled by a common signaling center.

A Common Set of Genes Regulates the Initiation of Neuronal Differentiation and the Onset of Glial Cell Migration

A set of genes encoding nuclear proteins (e.g., *eyeless* (*ey*), *eyes absent* (*eya*), *sine oculis* (*so*)) and secreted factors (e.g., *hedgehog* (*hh*)) regulates the initiation of neuronal differentiation in the posterior region of the eye disc. The effect of loss-of-function mutations in these genes on glial cell migration was tested (Figure 6). As in *gish* mutants, glial cells migrated precociously out of

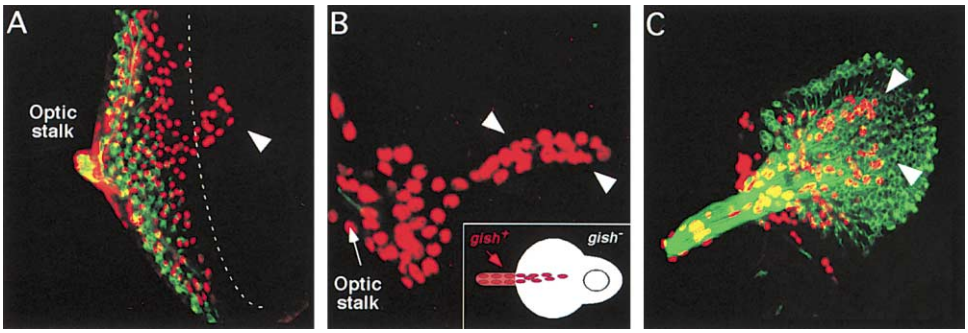

Figure 5. Gish Expression in Eye Disc Epithelial Cells Is Necessary and Sufficient to Prevent Precocious Glial Cell Migration

(A and B) Removal of *gish* function from eye disc epithelial cells using the *ey-FLP* method (see Experimental Procedures) results in third (A) and second (B) instar glial cell migration phenotypes (arrowheads) similar to *gish*^{1} homozygous mutants. Insert in (B) indicates the extend of the *gish* mutant region using *ey-FLP*. (C) Targeted expression of *gish* cDNA in the posterior region of the eye disc under the *Dpp-Gal4* driver rescues the mutant phenotype (arrowheads). Expression of *gish* in a *Dpp-Gal4* pattern also leads to a reduction in R cell development at the dorsal and ventral margins of the eye disc. All panels show lateral views. (A and C) anti-HRP for neurons (green); anti-Repo for glial cells (red).

the optic stalk in a *hh* temperature-sensitive mutation incubated at the nonpermissive temperature during first and second instar (Figure 6A). This is an early function of *hh*, as ectopic glial cells are not observed in *hh*^{1}; in this allele, the posterior eye field develops normally, but anterior progression of the MF is inhibited (Figure 6C). A similar early onset glial cell migration defect was observed in eye-specific alleles of *so* (Figures 6D–6F) and *ey* (Figure 6G). In contrast, glial cells did not migrate out from the optic stalk in an eye-specific allele of *eya* (Figure 6H), raising the possibility that *eya* is required to activate glial cell migration. As glial cells migrate out of the stalk precociously in *eya*/*gish* double mutants (Figure 6I), the production of an *eya*-dependent signal is not necessary to promote anterior migration. Hence, in contrast to the their role in R cell development, eye specification genes *ey* and *so* seem to function independent of *eya* to control the onset of glial cell migration.

These observations raise the possibility that *gish* also contributes to the genetic circuitry regulating eye specification. Indeed, while *ey*-FLP-induced clones of *gish* only lead to minor defects in MF progression during third instar stage (Figure 6M), *gish* mutant adult eyes are smaller and frequently contain a reduced number of ommatidia in the anterior region (Figure 6P). These phenotypes are frequently seen in weak alleles of eye specification genes. Furthermore, double mutants of *ey*^{1} and *gish*^{1} (Figures 6K–6L), as well as *so*^{1} and *gish*^{1} (data not shown), reveal strong synergistic effects in R cell development. The glial cell migration phenotypes in double mutants were similar in severity to the single mutants. In summary, these data argue that *gish* acts in conjunction with eye specification genes to coordinate neuronal development and glial cell migration in the eye disc.

Hh Signaling Is Required in the Eye Disc to Regulate the Onset of Glial Cell Migration

As Hh is expressed in the right place and time to function as the glial cell repellent, and as glial cells migrate precociously in *hh* mutants, we set out to assess whether Hh directly regulates glial cell migration (Figure 7). In support of this view, targeted expression of Hh at the posterior region of the second instar eye disc rescues precocious cell migration in *gish* mutants. In about 80% of the *gish* mutant larvae carrying *Dpp-Gal4* driving *UAS-hh*, premature glial cell migration was prevented in second instar (Figure 7A), and glial cells remain posterior to the MF at third instar (Figure 7B).

Does Hh directly regulate glial cell migration? To address this question, we assessed whether the canonical Hh signaling pathway is activated in glial cells and whether it is required to prevent precocious migration. *patched* (*ptc*) expression, an indicator of reception of the Hh signal, is not elevated in glial cells prior to migration into the eye disc. It is, however, induced in epithelial cells located at the most posterior edge of the eye disc immediately juxtaposed to the pre-migratory glial cells in the optic stalk (Figure 7C). In support of the importance of the posterior margin in signaling glial cells, a significant reduction in the level of *ptc-lacZ* expression was observed in this region of the second instar eye in *gish* mutants (Figure 7E). Alternatively, this reduction may simply provide a sensitive indicator that the level of Hh is reduced in *gish* mutants. As the level of Hh protein was only slightly above background in wild-type, we were not able to critically assess the level of the Hh signal in these mutants with the available reagents. Further evidence that the canonical Hh pathway is not required in glial cells came from the analysis of mutations in *smoothened (smo)* and *Cubitis interruptus (Ci)* genes encoding a Hh receptor component and a downstream transcription factor, respectively. Mosaic clones of *smo* mutant glial cells did not migrate prematurely at second instar and were found exclusively posterior to the MF (Figures 7G and 7H). Similarly, targeted expression of a dominant-negative version of Ci in glial cells did not alter early migratory behavior of eye disc glial cells (data not shown). These findings argue that Hh acts either indirectly to control glial cell migration or acts directly upon glial cells through a novel pathway independent of *ptc, smo*, and *Ci*.

Discussion

In this paper, we demonstrate that the timing of glial cell migration plays an essential role in regulating axon

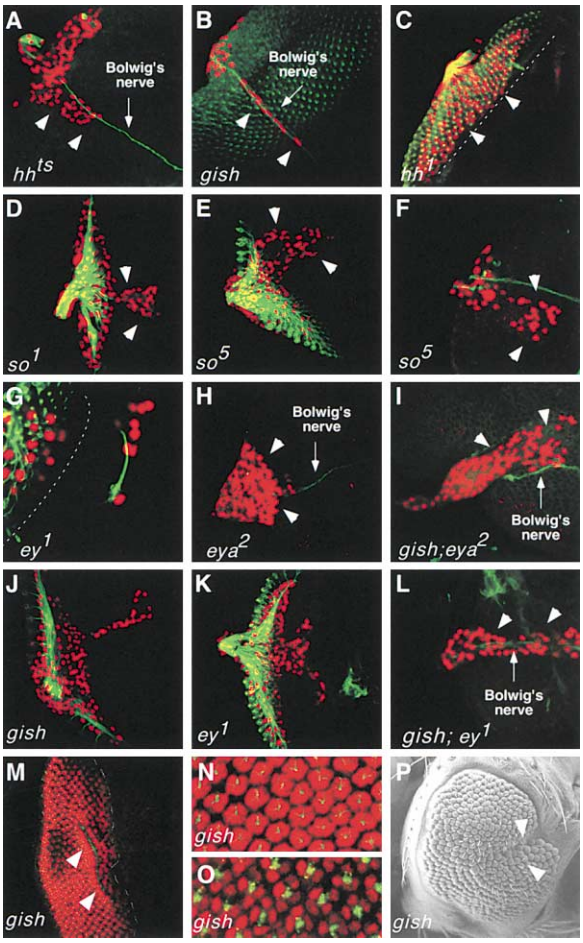

Figure 6. Genes Required for Early Eye Development Also Control the Onset of Glial Cell Migration

(A–C) *hh* is required to prevent ectopic glial cell migration. (A) Removal of *hh* during second instar using a temperature-sensitive allele leads to precocious glial cell migration out of the optic stalk (arrowheads). However, instead of choosing their normal path along the basal surface of the eye disc, glial cells in *hh* mutant larvae migrate at the apical surface next to the Bolwig's nerve. (B) In *gish* mutants, glial cells also migrate along Bolwig's nerve (arrowheads), although most glial cells migrate along the basal surface (see Figure 1F). Glial cells do not migrate along Bolwig's nerve in wild-type. (C) *hh* is not required to prevent ectopic glial cell migration during morphogenetic furrow progression in third instar. The hh^1 allele disrupts morphogenetic furrow progression but not the formation of retinal tissue in the posterior region. (D–G) Glial cell migration defects in *so* and *ey* are similar to *gish* in late second (F) and third (D, E, and G) instar discs. In (G), an R cell fascicle is seen associated with ectopic glial cells. (H and I) *eya* is not required to prevent premature glial cell migration. Double mutants (*gish*;eya^2) in (I) indicates that the failure of glial cells to ectopically migrate in *eya* does not reflect a requirement for an *eya*-dependent regulator promoting glial cell migration. (J–L) *gish* and *ey* mutant phenotypes are synergistic. (M–P) While cluster and R cell fate determination in *gish* mutant eyes appears normal, they exhibit morphological defects similar to hypomorphic *hh* alleles. (A–O) show lateral view, anterior is to the right; (N) optical section at the R cell nuclear level; (O) optical section at the cone cell nuclear level; (A–O) second or third instar eye discs; (P) adult eye; (A–L) green, anti-HRP for neurons and red, anti-Repo for glial cells. (N) Green, anti-HRP for neurons and red, anti-Elav for neuronal nuclei. (O) Green, anti-HRP for neurons and red, anti-Prospero for R7 and cone cell nuclei.

guidance in the fly visual system. Glial cells in the posterior region of the eye disc epithelium provide an intermediate target for R cell axons as they project from the eye to the brain. In *gish* mutants, glial cells migrate out of the optic stalk to more anterior regions of the eye disc prior to R cell differentiation, and as a consequence, R cell axons frequently extend anteriorly in *gish* mutants along the surface of these ectopically located glial cells. *gish* acts in combination with eye specification genes, *ey* and *so,* and the extracellular signaling protein Hh to control glial cell migration. As these genes, including *gish*, also regulate neuronal development in the eye disc, we propose that they define a signaling center in the posterior region of the eye disc which controls both neurogenesis and glial cell migration to ensure normal patterns of R cell axon outgrowth (Figure 8).

A Signal from the Posterior Eye Disc Prevents Precocious Glial Cell Migration

Through genetic mosaic analysis and transgene rescue experiments, we established that *gish* acts within the eye disc epithelium to inhibit glial cell migration. In principle*, gish* could regulate the production of a repellent preventing migration of glial cells out of the optic stalk, an attractant that promotes their close association to the posterior edge or alternatively an antagonist to an attractive signal produced by cells in the disc.

Gish belongs to the casein kinase I family of highly conserved and widely expressed enzymes (Gross and Anderson, 1998). These enzymes contain small but varied amino termini and large, highly diverse carboxy-terminal domains. Gish is most similar to mammalian CKIγ3. CKIγ3, like *gish*, is alternatively spliced which can result in kinases with different biochemical properties and functions (Zhang et al., 1996). CKIs act on proteins previously phosphorylated by other kinases. They have been shown to phosphorylate a large number of proteins in vitro. Regrettably, little evidence exists to establish a link between phosphorylation by CKI and specific developmental pathways. Recent studies have shown that casein kinase Iϵ (CKIϵ) can regulate β-catenin in the Wnt pathway in both worms and frogs (Peters et al., 1999; Sakanaka et al., 1999). Loss- and gain-of-function manipulation of Wg signaling components, however, did not disrupt glial cell migration into the eye disc (T.H. and S.L.Z., unpublished observations). This is not surprising given that CKIϵ and CKIγ differ significantly in their C-terminal regions and deletion analysis has revealed that the unique C-terminal domain is important for the interaction of CKIϵ with the axin signaling complex (Sakanaka et al., 1999). As *gish* does not encode a secreted molecule, it must act indirectly to affect signaling from the epithelium to the glial cells.

Several lines of evidence support a model in which *gish* regulates Hh signaling. First, like *gish*, *hh* is required in the posterior region of the second instar eye disc to inhibit anterior migration of glial cells. Second, *gish* mutants display defects in morphogenetic furrow progression similar to those seen for hypomorphic alleles of *hh*. And finally, the expression of Hh target genes *ptc* and *Ci* (data not shown) in the eye disc epithelium is reduced in *gish* mutants. These data suggest that *gish* acts upstream of (or in parallel to) *hh*. Although genetic

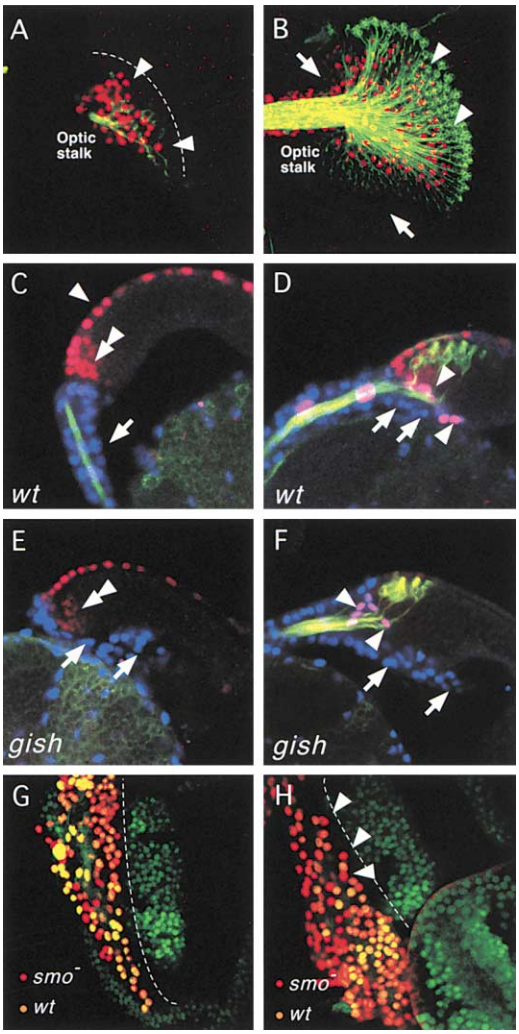

Figure 7. Hh Signaling Is Required in the Posterior Margin of the Eye Disc to Prevent Precocious Glial Cell Migration

(A and B) Targeted expression of Hh in the posterior margin of the eye disc using a *Dpp-Gal4* driver rescues the glial cell migration defect of *gish* mutants in both early (A) and late (B) third instar (arrowheads; for comparison see Figures 1 and 2 for *gish* mutants without rescue construct). Expression of Hh under *Dpp-Gal4*, like Gish, also leads to a reduction in R cell development at the dorsal and ventral margins (arrows in [B]). (C–F) Expression of the Hh target gene *patched* is selectively disrupted in the posterior region of *gish* mutants. *ptc* expression was visualized using a *ptc-lacZ* reporter. (C) In late second instar, *ptc* is expressed along the apical peripodial membrane (single arrowhead) and in a posterior band of cells juxtaposing glial cells in the eye disc (double arrowhead). In contrast, *ptc-lacZ* expression is not elevated in pre-migratory glial cells in the optic stalk (arrow). (E) In *gish* mutants, *ptc* expression in these cells is markedly reduced (double arrowheads). (D) In wild-type, as R cells differentiate and send axons into the brain, they contact glial cells and induce *ptc* expression in some of them (arrowheads). The majority of the glial cells in the eye disc do not express *ptc* (arrows). (F) In *gish* mutants, some glial cells that contact axons induce *ptc* expression as in wild-type (arrowheads). (G and H) The Hh receptor component encoded by *smoothened (smo)* is not required in glial cells for normal cell migration. Glial cells mutant for *smo* (red) were generated through mitotic recombination. As seen for wild-type (yellow/orange cells), *smo* mutant cells terminate migration at the morphogenetic furrow (dashed line in [G] and [H]). (A, B, G, and H) Lateral view; (C–F) dorsal view, anterior is to the right. (A and B) green, anti-HRP for neurons and red, anti-Repo. (C–F) Green, anti-HRP for neurons and red, anti-β-Gal for *ptc*-expressing cells and blue, anti-Repo. Pink seen in (D) and (F) represents overlap in expression of *ptc* and Repo. (G and H) Green, Ubiquitin-GFP and red, anti-Repo.

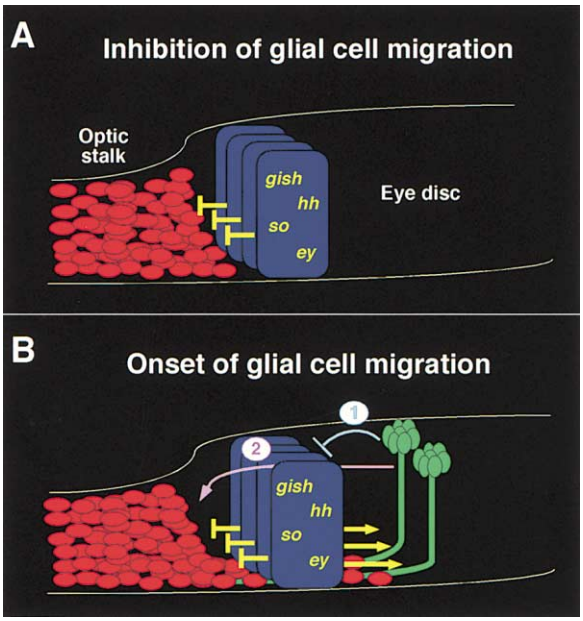

Figure 8. A Signal from the Posterior Eye Disc Regulates Glial Cell Migration and Neuronal Differentiation during *Drosophila* Eye Development

(A) A signaling center at the posterior margin of the second instar eye disc uses a set of genes to prevent precocious glial cell migration. These genes also provide patterning information to the eye epithelium. (B) As R cells begin to differentiate, they stimulate glial cell migration either indirectly by repressing the repulsive activity from the eye disc epithelium (1) or directly by increasing their motility (2).

evidence suggests that the *gish* mutants studied here are strong loss-of-function alleles, as low levels of *gish* mRNA can still be detected in homozygous *gish*[1] larvae, the epistatic relationship between *gish* and *hh* must be qualified. Indeed, Gish may function downstream from Hh and limiting levels of activity in loss-of-function alleles may be compensated by increasing the level of Hh upstream. We demonstrated through *smo* genetic mosaics, targeted expression of dominant-negative Ci, and expression of *ptc* in wild-type and mutant animals that Hh itself does not signal directly to glial cells through the canonical Hh signaling pathway to prevent precocious cell migration.

These data are consistent with two alternative models for Hh function in controlling glial cell migration in the eye disc. First, Hh may function indirectly by regulating the expression of other signals that, in turn, inhibit glial cell migration. Indeed, it is known that Hh does act in a paracrine fashion to upregulate the expression of other extracellular signals including Wnt and TGFβ family members. Conversely, Hh may act directly on glial cells, but through a novel pathway independent of *smo*, *ptc*, and *Ci*. In this context, it is interesting to note that a recent publication appearing while this paper was in

review demonstrated that Hh can inhibit integrin-dependent cell migration in neural crest progenitor cells independent of the canonical Hh pathway components (Testaz et al., 2001)

Diverse Mechanisms Regulate Temporal Aspects of Cell Migration

While the precise mechanism by which glial cell migration is regulated in the eye disc remains unclear, recent studies exemplify the range of molecular mechanisms that may underlie the temporal control of cell migration in the nervous system. In early vertebrate neurogenesis, for instance, the timing of neural crest migration is controlled by the transition of cells in the lateral neural plate from an epithelial to a mesenchymal cell population. The timing of this transition may be controlled through BMPs and their antagonists, such as noggin. While BMPs promote mesenchymal formation, noggin antagonizes it (Sela-Donenfeld and Kalcheim, 1999). Hence, during normal development, modulation of BMP signaling through an extrinsic signal may modulate the timing of neural crest migration. Alternatively, signaling pathways may also be modulated by interactions between heterologous receptors. Recent studies from Stein and Tessier-Lavigne indicate that the netrin receptor DCC is silenced through direct physical interaction with the cytoplasmic domain of a heterologous receptor, Robo (Stein and Tessier-Lavigne, 2001). The timing of the Robo receptor expression appears critical; Robo is upregulated after, but not before, the growth cone has crossed the midline. As shown recently for cerebellar granule cells by Flanagan and coworkers, migration signals can also be attenuated downstream (Lu et al., 2001). In summary, cell migration may be repressed at the level of an extracellular antagonist binding to secreted signals, through direct interaction between antagonistic cell surface components or through interactions between antagonistic intracellular signal transduction pathways.

Glial Cells Regulate Axon Pathfinding in the Eye

By preventing glial cell migration into the eye disc epithelium, Gaul and coworkers established that short-range interactions between glial cells and R cell axons are required for posterior extension of R cell axons into the optic stalk (Rangarajan et al., 1999). Here we extend these observations by demonstrating that the location of a specific subtype of glial cells is not only necessary for posterior-directed outgrowth of R cell axons, but also sufficient. Indeed, some 50% of *gish* mutant eye discs at an early stage of development contain R cell fibers that project anteriorly along the surface of misplaced glial cells. During normal development, however, more anterior R cells differentiate as a continuous wave, and glial cells migrate to a region just posterior to differentiating R cells. As a consequence, as these axons extend to the basal surface, they contact glial cells that lie posterior to them and follow them into the optic stalk.

Axon-glial cell interactions in the fly eye show interesting parallels to intra-retinal pathfinding in the vertebrate eye (Stuermer and Bastmeyer, 2000). Retinal ganglion cell axons project radially toward the optic disk, their first intermediate target in the center of the eye. Here, axons receive contact-dependent signals from glial cells to exit the retina and enter the optic nerve (Bertuzzi et al., 1999; Deiner et al., 1997). In addition, in vertebrates, a barrier at the junction between the optic nerve and the retina has been proposed to inhibit glial cell precursors from migrating into the retina (Ffrench-Constant et al., 1988). It will be interesting to assess whether the molecular strategies coordinating axon outgrowth and glial cell migration have been conserved between vertebrate and invertebrate visual systems.

Experimental Procedures

Genetics

Fly stocks were maintained on standard medium at 22°C unless stated otherwise. The mutation *gilgamesh*^{1} (*gish*^{1}) was isolated in a screen for mutations affecting R cells projections. The lethality of *gish* was mapped to the cytological region 89B9/10-22. In a "local hopping" approach (Tower et al., 1993; Zhang and Spradling, 1993) starting from the nearby insertion *l(3)jlC5*, five independent *gish* insertion mutants were isolated, from which two, *gish*^{P144} and *gish*^{P232}, were further characterized. Precise excisions of the P element in *gish*^{p232} reverted the mutant phenotype. Mutations used in this study include: *hedgehog*^{ts} (*hh*^{ts}), *hedgehog*^{1} (*hh*^{1}), *sine oculis*^{1} (*so*^{1}), *sine oculis*^{5} (*so*^{5}), *eyes absent*^{2} (*eya*^{2}), *eyeless*^{1} (*ey*^{1}), *smoothened*^{3} (*smo*^{3}), *ptc-lacZ* (*FE3*), *Mz97-Gal4* (kindly provided by J. Urban), *c527-Gal4* (kindly provided by S. Selleck), *ey-Gal4* (Newsome et al., 2000), *Dpp-Gal4* (Pignoni and Zipursky, 1997), *UAS-Ci* (DN) (Aza-Blanc et al., 1997), and *UAS-ttk69* (Giesen et al., 1997). The two main glial cell types in the eye disc were visualized by the Gal4 lines *c527* representing SG cells and *Mz97* labeling the WG cells, driving *UAS-lacZ(cyt)* expression. To analyze mosaic clones in the eye disc, flies of the following genotypes were examined: (1) yw eyFLP; P[FRT]82B E2F/P[FRT]82B *gish*; (2) yw eyFLP; P[FRT]82B Ubi-GFP/P[FRT]82B *gish*; and (3) yw hsFLP; *smo*^{3} FRT40.

To create *smo*^{3} mutant clones in the glial cells, first instar larvae of the following genotype were heat shocked 12 hr post-hatching for 60 min at 37°C, raised at 25°C, and analyzed at early and late third instar larval stage.

Molecular Biology

A 1.7 genomic fragment flanking the *gish*^{P232} insertion was isolated via plasmid rescue and used to screen a *Drosophila* genomic library. Seven independent and overlapping clones were isolated allowing to map about 28 kb of the genomic region surrounding the *gish*^{P232} insertion. A 10 kb fragment spanning the *gish*^{P232} insertion was used to screen different cDNA libraries. One ORF with three alternatively spliced transcripts was found in the 28 kb region. The longest cDNA clone obtained from the BDGP, LD04357 (CKIγa), was 2.9 kb, in which 12 exons spanned 27 kb of genomic DNA (see Figure 5A). The two other classes of alternatively spliced transcripts, 32a (CKIγb) and ed19 (CKIγc), display a different 5′ end and an alternative translational start (see Figure 5A). ed19 had the identical 3′ splice form as LD04357, but contains an exon not present in 23a.

Further molecular characterization of the three *gish* mutations showed that all of them affect the ORF defined by LD04357 (Figure 5A). *gish*^{P144} carries a P insertion 0.5 kb downstream of the coding region of LD04357 and *gish*^{P232} disrupts the first intron. The original *gish*^{1} allele carries a 7 kb insertion of uncharacterized DNA 0.5 kb upstream of the transcription start of LD04357.

Histology

R cell projections were visualized using mAb24B10 staining in combination with DAB immunohistochemistry or a FITC-coupled anti-HRP. The following concentrations of primary antibodies were used: mAb24B10 (1:100), rabbit anti-β-galactosidase (1:1000; Cappel), mouse anti-β-galactosidase (1:300; Promega), rat anti-Elav (1:25), rabbit anti-Repo (1:500; Halter et al., 1995), mouse anti-Repo (1:10; B. Johns), and FITC-conjugated goat anti-HRP (1:100; Cappel). Secondary antibodies used in this study include: HRP-conjugated goat anti-mouse antiserum (Bio-Rad), goat anti-mouse and anti-rabbit F(ab)' fragments coupled to Cy3 or FITC, and goat anti-rabbit and anti-guinea pig F(ab)' fragments coupled to Cy5 (FITC and Cy5,

1:200; Cy3, 1:400; Jackson Laboratories). For immunolabeling of whole-mount larval preparations, see Garrity et al., 1996. Fluorescent samples were visualized using a Bio-Rad MRC 1024 laser scanning confocal microscope.

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