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glial cells missing and *gcm2* Cell Autonomously Regulate Both Glial and Neuronal Development in the Visual System of *Drosophila*

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Summary

The transcription factors Glial cells missing (Gcm) and Gcm2 are known to play a crucial role in promoting glial-cell differentiation during *Drosophila* embryogenesis. Our findings reveal a central function for *gcm* genes in regulating neuronal development in the postembryonic visual system. We demonstrate that Gcm and Gcm2 are expressed in both glial and neuronal precursors within the optic lobe. Removal of *gcm* and *gcm2* function shows that the two genes act redundantly and are required for the formation of a subset of glial cells. They also cell-autonomously control the differentiation and proliferation of specific neurons. We show that the transcriptional regulator Dachshund acts downstream of *gcm* genes and is required to make lamina precursor cells and lamina neurons competent for neuronal differentiation through regulation of epidermal growth factor receptor levels. Our findings further suggest that *gcm* genes regulate neurogenesis through collaboration with the Hedgehog-signaling pathway.

Introduction

One essential step in building a functioning nervous system is the correct specification of different neuronal and glial-cell types. In the vertebrate central nervous system, common neural progenitor cells have been shown to first produce neurons and then to give rise to glial cells in regionally restricted domains (reviewed in Rowitch, 2004). In the *Drosophila* embryonic nervous system, neurons and glial cells are either derived from committed precursors, which exclusively produce neuronal or glial progeny, or from neuroglioblasts with mixed neuronal and glial daughter cells (reviewed in Jones, 2001). This raises the important question of what mechanisms direct the decision of precursor cells to give rise to neuronal or glial progeny.

In the vertebrate spinal cord, the transition from neurogenesis to gliogenesis is regulated by extrinsic signals (e.g., Sonic hedgehog or Bone morphogenetic proteins) and a combination of intrinsic determinants such as basic Helix-loop-Helix (bHLH) and homeodomain-containing transcription factors, as well as Sox family members (reviewed in Rowitch, 2004). In the *Drosophila* embryo, the choice to produce neuronal or glial progeny depends on two key transcriptional regulators with glial-fate promoting activity, Glial cells missing (Gcm) and its closely related homolog Gcm2 (Jones et al., 1995; Hosoya et al., 1995; Vincent et al., 1996; Kammerer and Giangrande, 2001; Alfonso and Jones, 2002). Gcm transcription factors are highly conserved from flies to vertebrates, and homologs have been identified in a wide range of species including zebrafish, *Xenopus*, chick, mouse, and humans (reviewed in Wegner and Riethmacher, 2001; Hashemolhosseini and Wegner, 2004). Gcm family members are characterized by the Gcm motif, a zinc-containing, sequence-specific DNA binding domain that recognizes a conserved octameric nucleotide sequence (Akiyama et al., 1996; Schreiber et al., 1997; Cohen et al., 2003). In *Drosophila*, loss-of-function analyses have shown that *gcm* mutant embryos fail to develop most glial cells and instead form neurons. Conversely, ectopic expression of *gcm* or *gcm2* promotes the generation of excess glial cells at the expense of neurons (Jones et al., 1995; Hosoya et al., 1995; Vincent et al., 1996; Kammerer and Giangrande, 2001; Alfonso and Jones, 2002). Although removal of *gcm* alone results in the loss of most neurectoderm-derived glia, only lack of both *gcm* and *gcm2* prevents the formation of all glial cells, indicating that the two genes have partially redundant functions (Kammerer and Giangrande, 2001; Alfonso and Jones, 2002). One key target of *gcm* and *gcm2* is the glial-specific gene *reversed polarity* (*repo*) encoding a Pou-domain transcription factor (reviewed in Jones, 2005). The central role of *gcm* as glial-fate promoting gene also extends to stages of postembryonic nervous system development. *gcm* has been shown to control glial-cell fate determination in sensory organ precursors, which give rise to thoracic mechanosensory sensilla (Fichelson and Gho, 2003). Similarly, peripheral glial cells in the wing imaginal disc fail to develop in the absence of *gcm* (Van De Bor et al., 2000). However, the role of *gcm* genes in the central nervous system during postembryonic development is not known.

The visual system of *Drosophila* is characterized by intricate interactions between photoreceptor axons (R-cell axons) and glial cells in the optic lobe during the third instar larval stage (reviewed in Clandinin and Zipursky, 2002; Chotard and Salecker, 2004). The compound eye of *Drosophila* represents an array of about 750 identical subunits, called ommatidia, each containing eight R-cells (R1–R8). R1–R6 axons project to the first optic ganglion, the lamina, whereas R7 and R8 axons terminate in two sublayers within the second optic ganglion, the medulla. R1–R6 growth cones stop between a subset of glial cells, the rows of epithelial and marginal glia; a third row of medulla glia is found beneath the marginal glia (Winberg et al., 1992). Our previous studies have revealed that these glial cells act as transient intermediate targets and are required for R1–R6 growth cones to terminate in their appropriate target layer, until all R-cell axons have entered the projection field and the future postsynaptic target neurons have differentiated (Poeck et al., 2001).

Neurons and glial cells in the lamina are derived from separate precursor cell populations. Lamina neurons

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are generated by neuroblasts in the outer proliferation center (OPC), which give rise to lamina precursor cells (LPCs). These are equivalent to ganglion mother cells in other parts of the central nervous system and divide once to generate lamina neurons. Development of lamina neurons is controlled by two anterograde extrinsic cues, Hedgehog (Hh) and the epidermal growth factor (EGF)-like ligand Spitz, which are both provided by incoming R-cell axons to coordinate the proliferation and differentiation of pre- and postsynaptic partners (Huang and Kunes, 1996, 1998; Huang et al., 1998). Epithelial and marginal glial cells originate from two superficial dorsal and ventral glial precursor cell (GPC) areas and migrate into the R-cell projection field to form one row above and one row below R1–R6 growth cones terminating in the lamina plexus (Perez and Steller, 1996a; Dearborn and Kunes, 2004). The regulation of glial-cell formation and migration in the optic lobe by extrinsic and intrinsic factors is not well understood. There is evidence that glial-cell development is controlled by an as yet unidentified R-cell-derived signal (Perez and Steller, 1996a; Huang and Kunes, 1998; Suh et al., 2002). Moreover, *nonstop* encoding a ubiquitin-specific protease has been shown to be required in glial cells or their precursors to mediate their migration into the lamina (Poeck et al., 2001).

To gain further insights into the intrinsic control of gliogenesis in the optic lobe, we set out to determine the function of *gcm* genes during larval development. Here, we show that both transcriptional regulators, known for their glial-fate promoting activity in the embryonic nervous system, unexpectedly play a central role in controlling neuronal development in the lamina. We demonstrate that *gcm* and *gcm2*, in addition to their function in regulating the development of epithelial and marginal glia, are cell-autonomously required to promote the differentiation and proliferation of lamina neurons.

Results

Gcm and Gcm2 Are Expressed in Glial and Neuronal Precursor Cells in the Optic Lobe

To determine the role of Gcm and Gcm2 in the third instar optic lobe, we examined their expression pattern. We converted the *lacZ*-expressing enhancer trap line *rA87*, which is known to reflect *gcm* expression, into a *gcm-Gal4* line by the P element replacement strategy (Jones et al., 1995; Sepp and Auld, 1999). This driver induces expression of reporter genes in a pattern identical to that of the original P element (cf., Figures 1B–1F). In the embryonic nervous system, the *rA87* P element has been reported to specifically label glial progenitors and their offspring (Jones et al., 1995; Hosoya et al., 1995; Vincent et al., 1996). However in the visual system, we detected expression not only in specific glial subtypes but, surprisingly, also in the lineage that gives rise to lamina neurons.

Epithelial and marginal glial cells are derived from two superficial dorsal and ventral GPC areas (Perez and Steller, 1996a; Dearborn and Kunes, 2004) (Figure 1A). *gcm* enhancer trap lines specifically label two clusters of cells within GPC areas located closest to the R-cell projection field. These include cells that have differentiated and hence express the glial differentiation marker Repo (Halter et al., 1995), as well as mitotically active cells positive for phospho-Histone H3 (Figures 1B and 1C). Staining was also detected in epithelial and marginal glial cells. In addition, some medulla neuropil glial cells were labeled, but not medulla glial cells or those adjacent to the lobula neuropil (Figures 1D and 1E). Comparison of findings from lineage analysis experiments with the expression pattern of enhancer trap lines indicates that *gcm* is expressed within a population of committed precursors within the larger GPC areas, which give rise to epithelial and marginal glial cells (Figure S1 available with this article online).

Lamina neurons are derived from neuroblasts in the OPC and are the product of two mitotic divisions, which occur on either side of a groove on the surface of the optic lobe called the lamina furrow. Neuroblasts located at the anterior of the lamina furrow divide asymmetrically to produce LPCs. A second division takes place in LPCs posterior to the lamina furrow and generates differentiated lamina neurons (Figure 1A). Their cell bodies subsequently become arranged in columns separated by bundles of R-cell axons. The enhancer trap lines displayed reporter gene expression within this neuronal lineage, labeling LPCs located posterior to the lamina furrow as well as differentiated lamina neurons L1–L5 (Figure 1F).

Consistent with the expression pattern of the enhancer trap lines, we detected *gcm* mRNA in clusters of cells at the margins of GPC areas and in LPCs (Figures 1G and 1H). A similar distribution and level of expression were found with *gcm2* probes (Figures 1J and 1K). Colocalization of *gcm* and *gcm2* mRNA with the early neuronal differentiation marker Dachshund (Dac) (Mardon et al., 1994; Huang and Kunes, 1996) confirms their expression in LPCs posterior to the lamina furrow (Figures 1H′–1I′ and 1K′–1L′). Finally, we assessed the protein expression pattern with an antibody against Gcm (Akiyama-Oda et al., 1998). Nuclear localized Gcm was found in a subset of cells within the GPC areas and in LPCs (Figures 1M–1N′). In summary, these findings show that *gcm* and *gcm2* are expressed not only in glial but also in neuronal precursors in the lamina.

A Genetic System to Induce Loss-of-Function Clones Only in the Target Area

To analyze the function of genes in glial cells and neurons in the optic lobe independently from their role in eye development, we have devised a FLP/FRT-based genetic approach that induces clone formation solely in the target area. First, we substituted *P{PZ}A8*, an insertion into the *lamina ancestor* (*lama*) gene (Perez and Steller, 1996b), with a Gal4 enhancer trap line by the P element replacement strategy (Sepp and Auld, 1999). The resulting *lama-Gal4* line recapitulates the previously described expression pattern of the *lacZ* P element (Perez and Steller, 1996b). It is active in GPC areas, in neuroblasts in the OPC and in LPCs, and their respective glial and neuronal progeny (Figures 2B–2C). We also detected *lama-Gal4* activity in second instar larval eye imaginal discs and in some differentiated R-cells within third instar larval eye discs (Figures 2A and 2O). The *lama-Gal4* line was recombined with a *UAS-FLP* transgene to drive recombinase expression.

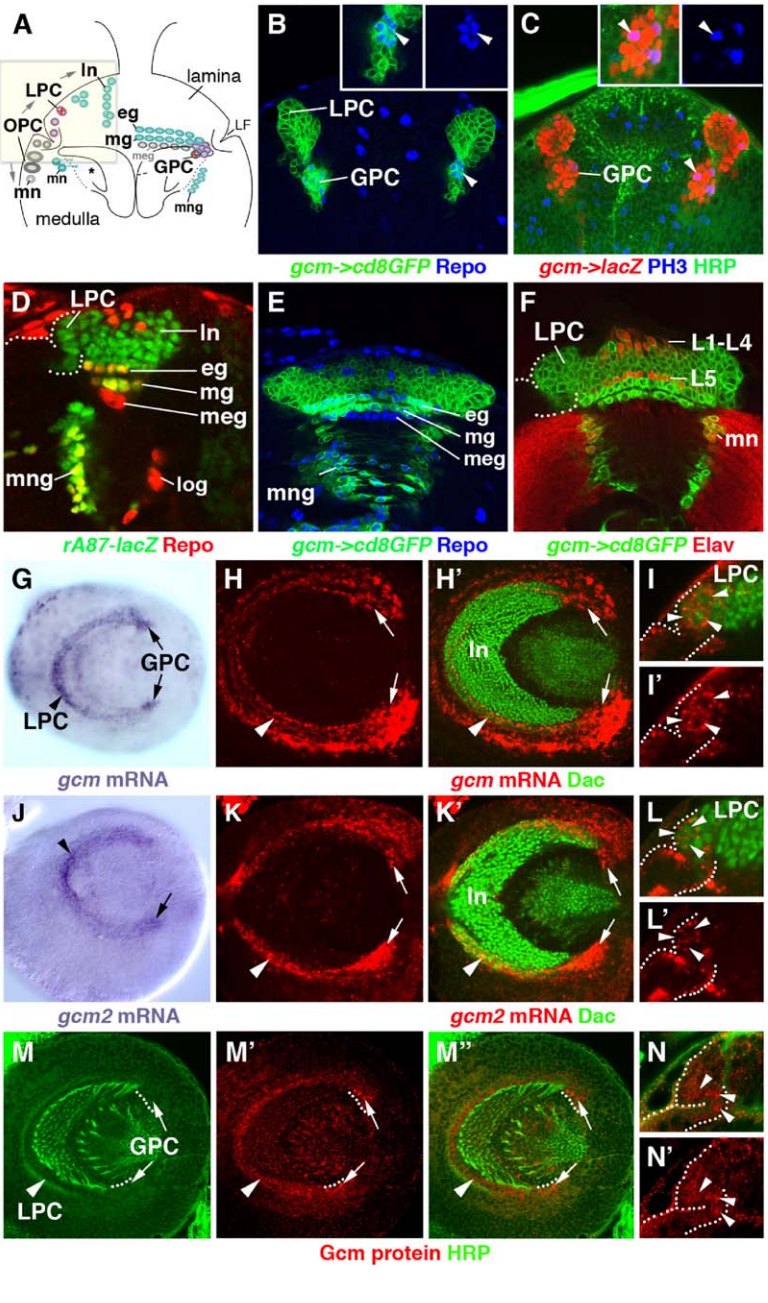

Figure 1. *gcm* and *gcm2* Are Expressed in Glial and Neuronal Precursors within the Optic Lobe

(A) The schematic diagram illustrates the origins of lamina and medulla neurons as well as epithelial and marginal glial cells in the third instar larval optic lobe. In the outer proliferation center (OPC), neuroblasts closest to the lamina furrow (LF) give rise to lamina precursor cells (LPC), which in turn divide to generate lamina neurons (In). Other neuroblasts in the OPC produce medulla neurons (mn). Epithelial (eg) and marginal glial cells (mg) originate from dorsal and ventral glial precursor cell (GPC) areas. Medulla neuropil glial cells (mng) and medulla glial cells (meg) border the medulla neuropil (asterisk). The scheme further summarizes the expression pattern of *gcm* genes in the optic lobe within neurons (left) and glial cells (right). Cells that exhibit reporter-gene expression are shown in green, and cells that were labeled by in situ hybridization are highlighted in purple. The area highlighted in yellow corresponds to the region of the optic lobe shown in panels I, I′, L, L′, N and N′.

(B–F) *rA87* drives *lacZ* expression (green in [D]). *gcm-Gal4* drives expression of *UAS-cd8GFP* (green in [B], [E], and [F]) and *UAS-nuclear lacZ* (red in [C]). Cell membranes were stained with anti-HRP (green in [C]), glial cells with anti-Repo (blue in [B] and [E]; red in [D]), mitotic cells with anti-phospho-Histone H3 (blue in [C]), and lamina neurons with the late neuronal differentiation marker anti-Elav (red in [F]). The enhancer trap lines *rA87* and *gcm-Gal4* indicate expression of *gcm* in clusters of cells within GPC areas that have accumulated at the margins of the lamina. Labeled cells include Repo-positive glial cells (arrowheads, [B] and insets) and mitotically active phospho-Histone H3-positive cells (arrowheads, [C] and insets). *rA87* and *gcm-Gal4* drive expression in epithelial, marginal, and some medulla neuropil glia, but not in medulla glia or lobula glia (log) (D and E). Enhancer trap lines reveal *gcm* expression in lamina precursor cells (LPCs), differentiated lamina neurons L1–L5 and some medulla neurons (mn) (F).

(G–L′) In situ hybridization shows that *gcm* (purple in [G]; red in [H]–[I′]) and *gcm2* (purple in [J]; red in [K]–[L′]) are strongly expressed in parts of GPC areas (arrows) and in LPCs (arrowheads). Colocalization of *gcm* or *gcm2* mRNA (red) with the early neuronal differentiation marker anti-Dachshund (green) confirms the expression of *gcm* and *gcm2* in LPCs (arrowheads) (H′–I′ and K′–L′).

(M–N′) Labeling with an anti-Gcm antibody reveals nuclear expression of Gcm protein (red) in cells at the margins of GPC areas (arrows) and in LPCs (arrowheads). Membranes were visualized with anti-HRP (green). Frontal (B, C, E, F, I, I′, L, L′, N, and N′), horizontal (D), and lateral (G–H′, J–K′, M, and M″) views of the optic lobe. These different views are illustrated in schematic drawings in Figure S3.

To prevent FLP recombinase activity and, thus, clone formation in the retina, we next introduced a transgene expressing the Gal4-antagonist Gal80 (Lee and Luo, 1999) under the control of a 3.5 kb eye-specific enhancer element from the *eyeless* (*ey*) gene (Bello et al., 1998). To test Gal80 activity, we examined eye imaginal discs of flies carrying an *actin-Gal4 UAS-cytoplasmic lacZ* recombinant chromosome. Although β-Galactosidase is found abundantly in the entire eye disc of control animals, expression is specifically blocked in R-cells in the presence of *ey-Gal80* (Figures 2D and 2E).

Finally, for studies on chromosome arm 2L, a recombinant *FRT40A* chromosome was generated, which also carries a ubiquitously expressed clonal marker (green fluorescent protein under the control of a Ubiquitin promoter [*Ub-GFP*]) and the cell-lethal mutation *cyclin E*. This enables us to visualize somatic clones by the absence of GFP expression. Using a homologous wild-type FRT chromosome as control, we determined that large somatic clones were reliably induced in GPC areas of each animal; at least 80% of glial cells in the projection field undergo mitotic recombination.

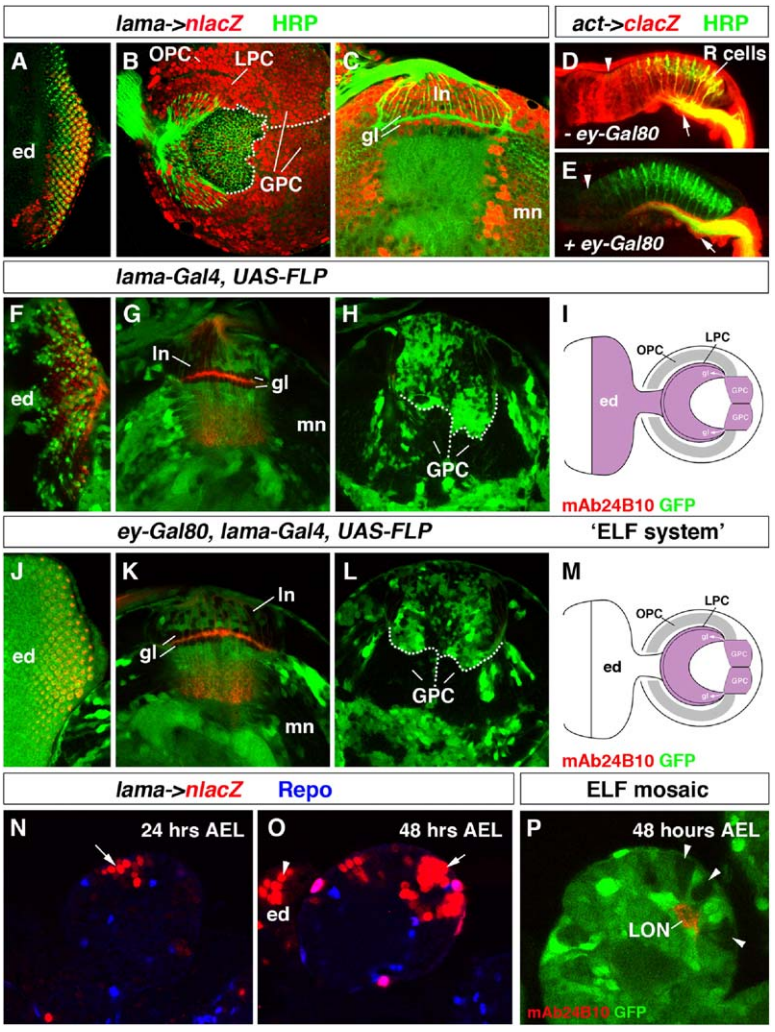

Figure 2. Genetic Approach to Generate Clones in the Target Area

(A–C) *lama-Gal4* drives expression of *UAS-nuclear lacZ* (red) in R-cells within the eye disc (ed) (A), in neuroblasts in the outer proliferation center (OPC), in lamina precursor cells (LPCs), glial precursor cell (GPC) areas (B), as well as in lamina glial cells (gl), lamina neurons (ln), and medulla neurons (mn) (C). Neuronal membranes were visualized with anti-HRP (green in [A]–[E]).

(D and E) The efficiency of the *ey-Gal80* transgene was assessed in flies carrying a *act-Gal4 UAS-cytoplasmic lacZ* recombinant chromosome. In control animals, β-galactosidase (red) is present in all cells in the eye disc (D). In the presence of *ey-Gal80*, expression of β-galactosidase is blocked in the eye field (E). Only glial-cell processes (arrow) in the underlying axonal layer are labeled. White arrowheads indicate the position of the morphogenetic furrow.

(F–M) Somatic clones were visualized by the absence of *Ubiquitin-GFP* expression (green), and R-cell axons were labeled with mAb24B10 (red). (F–I) *Ub-GFP cycE FRT40A/+ FRT40A; lama-Gal4 UAS-FLP mδ/+*. Large somatic clones are induced in the eye imaginal disc (F), in glial cells, lamina and medulla neurons (G), as well as in GPC areas (H). (J–M) *ey-Gal80/w or Y; Ub-GFP cycE FRT40A/+ FRT40A; lama-Gal4 UAS-FLP mδ/+*. In the presence of *ey-Gal80*, clone formation is efficiently suppressed in the eye (J) without interfering with mitotic recombination in the target area (K–M).

(N and O) *lama-Gal4, UAS-nuclear lacZ*. *lama-Gal4* induces expression of β-Galactosidase (red) within the optic lobes of first and second instar larvae (arrows) and in eye imaginal discs (ed) of second instar larvae (arrowhead). There is little overlap with the glial-specific marker anti-Repo (blue) at these stages.

(P) *ey-Gal80/w or Y; Ub-GFP cycE FRT40A/+ FRT40A; lama-Gal4 UAS-FLP mδ/+*. The larval optic neuropil (LON) marks the position of the optic lobe. The ELF system induces clones (arrowheads) at early larval stages well before lamina neurons and glia begin to proliferate and differentiate.

Because the *lama* enhancer also drives expression in neuronal precursor cells, optic lobes showed additional clones of variable size in lamina and medulla neurons (Figures 2F–2I). When crossed into this genetic background, *ey-Gal80* efficiently prevented mitotic recombination in the eye because of the early activity of the *eyeless* enhancer without interfering with clone formation in the optic lobe (Figures 2J–2M). Consistent with the observation that the *lama-Gal4* enhancer drives expression of *lacZ* in the optic lobe from the first instar larval stage onward, we detected clones in second instar larval optic lobes, well before lamina neurons and glia begin to proliferate and differentiate (Figures 2N–2P).

To summarize, this approach, which we named "ELF system" (*ey-Gal80, lama-Gal4, UAS-FLP* system) allows us to efficiently generate mosaic animals in which heterozygous R-cell axons innervate a target area containing large homozygous clones of glial cells, lamina and medulla neurons, as well as their precursors.

gcm and *gcm2* Are Both Redundantly Required for Gliogenesis in the Optic Lobe

Analysis of the complete loss-of-function allele $gcm^{\Delta P1}$ suggested that *gcm* is a central regulator of gliogenesis because the majority of glial cells are lost in homozygous mutant embryos (Jones et al., 1995). However, the removal of *gcm* function in third instar larval optic lobes with this allele and the ELF system unexpectedly did not interfere with glial-cell development. As in wild-type, epithelial and marginal glial cells homozygous mutant for *gcm* expressed the differentiation marker Repo and migrated in large numbers to their characteristic positions adjacent to R-cell growth cones in the lamina (Figures 3A–3A″ and 3D–3D″). Using the small homozygous viable deficiency *Df(2L)gcm2* to remove the entire open-reading frame of *gcm2*, Alfonso and Jones (2002) have previously demonstrated that loss of *gcm2* alone does not affect gliogenesis in embryos. Consistently, we also did not detect any glial defects

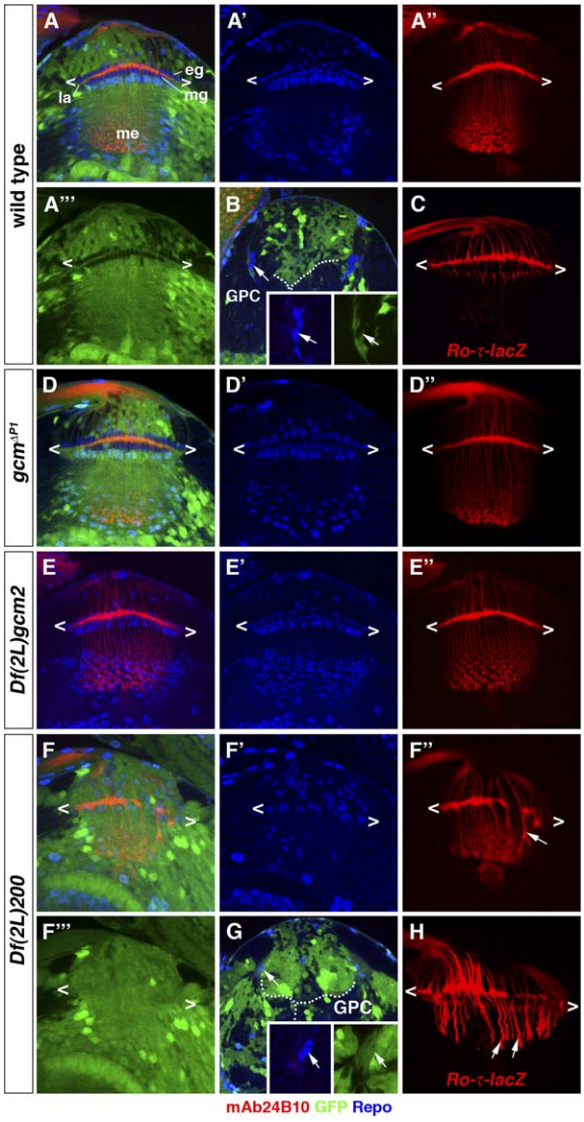

Figure 3. *gcm* and *gcm2* Are Both Required for Gliogenesis in the Lamina

Loss-of-function analysis with the ELF system. R-cell axons are labeled with mAb24B10 (red), and glial cells with the differentiation marker Repo (blue). Somatic clones lack expression of GFP (green). (A–C) Wild-type. (A–A‴) Repo-positive epithelial (eg) and marginal (mg) glial cells migrated in large numbers above and below the lamina plexus (la, brackets) formed by R1–R6 growth cones. (A‴) Glial cells, which underwent mitotic recombination and, thus, are GFP negative, are found alongside the lamina plexus (brackets). (B and insets) Small clones of glial cells accumulate at the dorsal and ventral margins of glial precursor cell (GPC) areas (arrows). (C) R2–R5 axons were stained with *Ro-τ-lacZ* (red). Labeled axons terminate in the lamina (brackets). (D–D″) In $gcm^{\Delta P1}$ mosaic animals, mutant epithelial (eg) and marginal glial (mg) cells migrate alongside the lamina plexus (brackets) and express the differentiation marker Repo. (E–E″) In homozygous mutant *gcm2* animals, glial-cell development is normal. (F–H) *Df(2L)200* target mosaics. (F–F‴) Glial-cell development is affected, and R-cell projection pattern formation is disrupted in the lamina (brackets) and the medulla (arrow). (F‴) Exclusively heterozygous cells are found alongside the lamina plexus (brackets). (G and insets) Only heterozygous glial cells accumulate at the margins of GPC areas (arrows). (H) R2–R5 axons, visualized with *Ro-τ-lacZ* (red), fail to stop in the lamina and instead project to the medulla (arrows). (A–H) Frontal view.

in *gcm2* homozygous mutant larval optic lobes (Figures 3E–3E″).

To address the question as to whether the two *gcm* genes may act redundantly, we turned to the deficiency *Df(2L)200*, a 120 kb deletion that removes both *gcm* and *gcm2* (Alfonso and Jones, 2002). In control animals, many wild-type epithelial and marginal glial cells, which had undergone mitotic recombination and thus lacked GFP expression, were found within the R-cell projection field (Figures 3A–3A‴). However in *Df(2L)200* target mosaics, only heterozygous GFP-positive, but not mutant GFP-negative, glial cells bordered the lamina plexus and expressed Repo (Figures 3F–3F‴). Similarly, within GPC areas where glial cells normally accumulate in small groups at the most dorsal and ventral margins before migrating into the R-cell projection field, homozygous mutant glial cells were rarely detected (Figures 3B and 3G). Thus, lack of both *gcm* and *gcm2* severely disrupts the formation of glial cells in the lamina.

The presence of exclusively heterozygous glial cells within the R-cell projection field raises the possibility that an efficient compensatory mechanism is in place ensuring that the correct amount of glial cells is generated even when proliferation and differentiation of many cells within GPC areas are severely disrupted. This effectively guarantees that sufficient glial cells are present to act as intermediate targets and to promote stopping of R1–R6 axons in the lamina. In some *Df(2L)200* mosaic animals, this compensatory mechanism was overcome as indicated by the irregular rows of glial cells alongside the lamina plexus and associated defects in R-cell target layer selection (Figures 3A and 3F). In such animals, many R2–R5 axons, visualized with the genetic marker *Ro-τ-lacZ*, fail to terminate in the lamina and instead project to the medulla (Figures 3C and 3H). In summary, in contrast to their partially redundant function in the embryonic nervous system, *gcm* and *gcm2* are both redundantly required in promoting gliogenesis in the optic lobe. Our findings also confirm the critical role of glial cells in regulating R1–R6 target-layer selection.

gcm and *gcm2* Are Not Sufficient to Induce Excess Glial-Cell Formation in the Lamina

In the embryo, either *gcm* or *gcm2* is sufficient to promote glial-cell differentiation (e.g., Jones et al., 1995; Alfonso and Jones, 2002). To address whether this also applies to the optic lobe, we used the *lama-Gal4* driver to misexpress *gcm* or *gcm2* in LPCs and GPC areas, as well as in differentiated lamina neurons and glial cells. Although such larvae were unhealthy and frequently arrested at the second instar stage, ectopic expression of *gcm* or *gcm2* in neuronal and glial precursor cells did not produce any additional glial cells at the expense of neurons within the lamina at the level of the R-cell projection field or within GPC areas (data not shown). We then misexpressed *gcm* and *gcm2* by the *actin-Gal4 "FLP-Out"* system in conjunction with the *ey-FLP* transgene (Ito et al., 1997; Newsome et al., 2000). This leads to persistent and high levels of expression in the eye disc, as well as in most neuronal and glial lineages within the optic lobe, with the exception of medulla glia and lobula cells (Figures 4A and 4A′). Overexpression of *gcm* and *gcm2* at high levels frequently

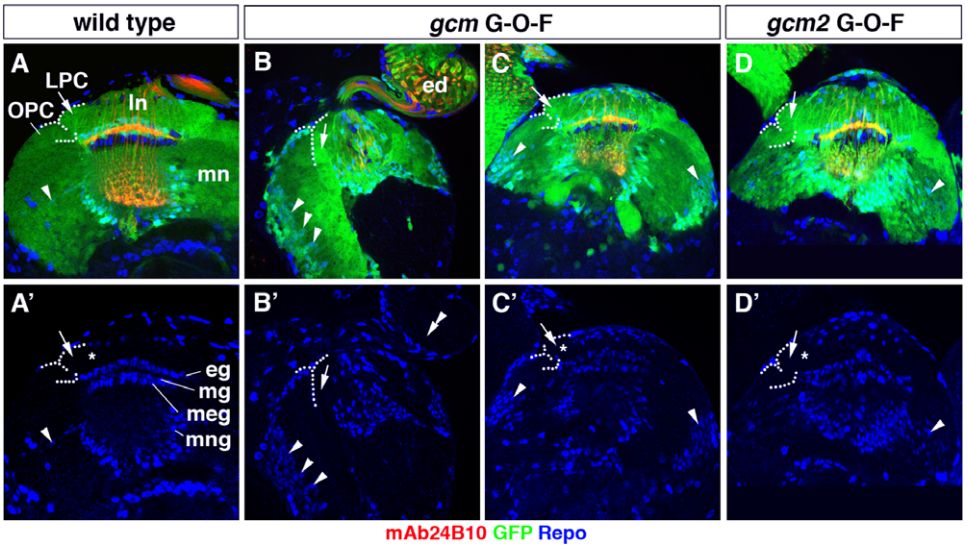

Figure 4. *gcm* and *gcm2* Are Not Sufficient to Generate Ectopic Glial Cells in the Lamina

Gain-of-function analysis. Glial cells were labeled with anti-Repo (blue), and R-cell axons were visualized with mAb24B10 (red). (A and A′) Wild-type: *ey-FLP/w or* Y*; act>>Gal4 UAS-GFP/+*. This combination of transgenes drives expression of GFP (green) in the eye, in neuroblasts in the outer proliferation center (OPC), lamina precursor cells (LPC), lamina neurons (ln) and medulla neurons (mn), as well as in epithelial, marginal, medulla neuropil (mng), and medulla cortex glia (arrowhead), but not in medulla glia (meg). (B–D and B′–D′) Misexpression of high and persistent levels of *gcm* and *gcm2* with this approach interferes with eye-disc (ed) development and consequently with R-cell projection-pattern formation. It does not convert R-cells (double arrowhead), LPCs (arrows), or lamina neurons (asterisks) into glial cells. However, misexpression results in the formation of ectopic glial cells within the medulla cortex (arrowheads). The position of ectopic glial cells close to the proximal border of the medulla suggests that the lineage of medulla neurons derived from the OPC has been affected. (A–D′) Frontal view.

interfered with the formation of R-cells in the eye and, thus, with optic lobe development (Figures 4B and 4B′). In those animals, which could be analyzed because of less severe R-cell defects, again no excess glial cells were seen within the eye or the lamina (Figures 4C–4D′). Overexpression did, however, produce ectopic glial cells within the medulla cortex (Figures 4B–4D and 4B′–4D′). Taken together, these findings indicate that although *gcm* and *gcm2* are not sufficient to induce ectopic gliogenesis within the lamina neuron lineage, they are sufficient to produce excess glial cells within the medulla.

gcm and *gcm2* Are Required for the Differentiation and Proliferation of Lamina Neurons

Because *gcm* and *gcm2* are expressed in LPCs as well as glial precursor cells, we next sought to determine the potential of both transcription factors to control neurogenesis in the optic lobe. In wild-type, the transcriptional regulator and early neuronal differentiation marker Dac is expressed in LPCs and in postmitotic lamina neurons. In $gcm^{\Delta P1}$ target mosaics and in *Df(2L)gcm2* homozygous mutant animals, this expression pattern was unchanged (Figures 5A–5C′). However, in *Df(2L)200* mosaic animals, only heterozygous, but not homozygous, mutant LPCs expressed Dac in the lamina (Figures 5D and 5D′). The loss of *gcm* genes in LPCs, therefore, interferes with early neuronal differentiation.

Moreover, we did not detect homozygous mutant lamina neurons integrated into columns in *Df(2L)200* mosaic animals, suggesting that the proliferation of lamina neurons is equally affected. BrdU and phospho-Histone H3 markers highlight three zones of mitotically active cells in the OPC, at the level of LPCs, and in the inner proliferation center (IPC) in wild-type (Figures 5E–5G). By contrast, in *Df(2L)200* mosaic animals, we did not detect LPCs in S phase or in mitosis, whereas proliferation markers were found in the OPC and IPC (Figures 5J–5L). Miranda, a coiled-coil protein involved in asymmetric cell division, is a reliable marker for neuroblasts and ganglion mother cells in the embryonic central nervous system (Ikeshima-Kataoka et al., 1997). In wild-type optic lobes, Miranda is strongly expressed in neuroblasts in the OPC and at lower levels in LPCs (Figure 5H). A similar pattern was detected in target mosaic animals lacking *gcm* and *gcm2* function (Figure 5M). Also, expression of the proneural bHLH protein Asense, a general marker for neuronal precursors, was not affected in neuroblasts within the OPC of *Df(2L)200* mosaic animals when compared to wild-type (Wallace et al., 2000) (Figure S2). This further supports our observation that lack of *gcm* and *gcm2* does not interfere with the formation and mitotic divisions of neuroblasts in the OPC but rather affects LPCs and the second division producing lamina neurons. Finally, we determined the level of apoptosis with an antibody against activated Caspase 3. In wild-type, no expression of activated Caspase was detected in LPCs, whereas an increased number of labeled cells was found in *Df(2L)200* target mosaic animals (Figures 5I and 5N). This suggests that the lack of *gcm* and *gcm2* in LPCs impairs their ability to undergo mitosis and differentiate (Figures 5O and 5P), and perhaps, as a result, a cell death program is activated. Alternatively, *gcm* and *gcm2* may have an additional function in promoting cell survival.

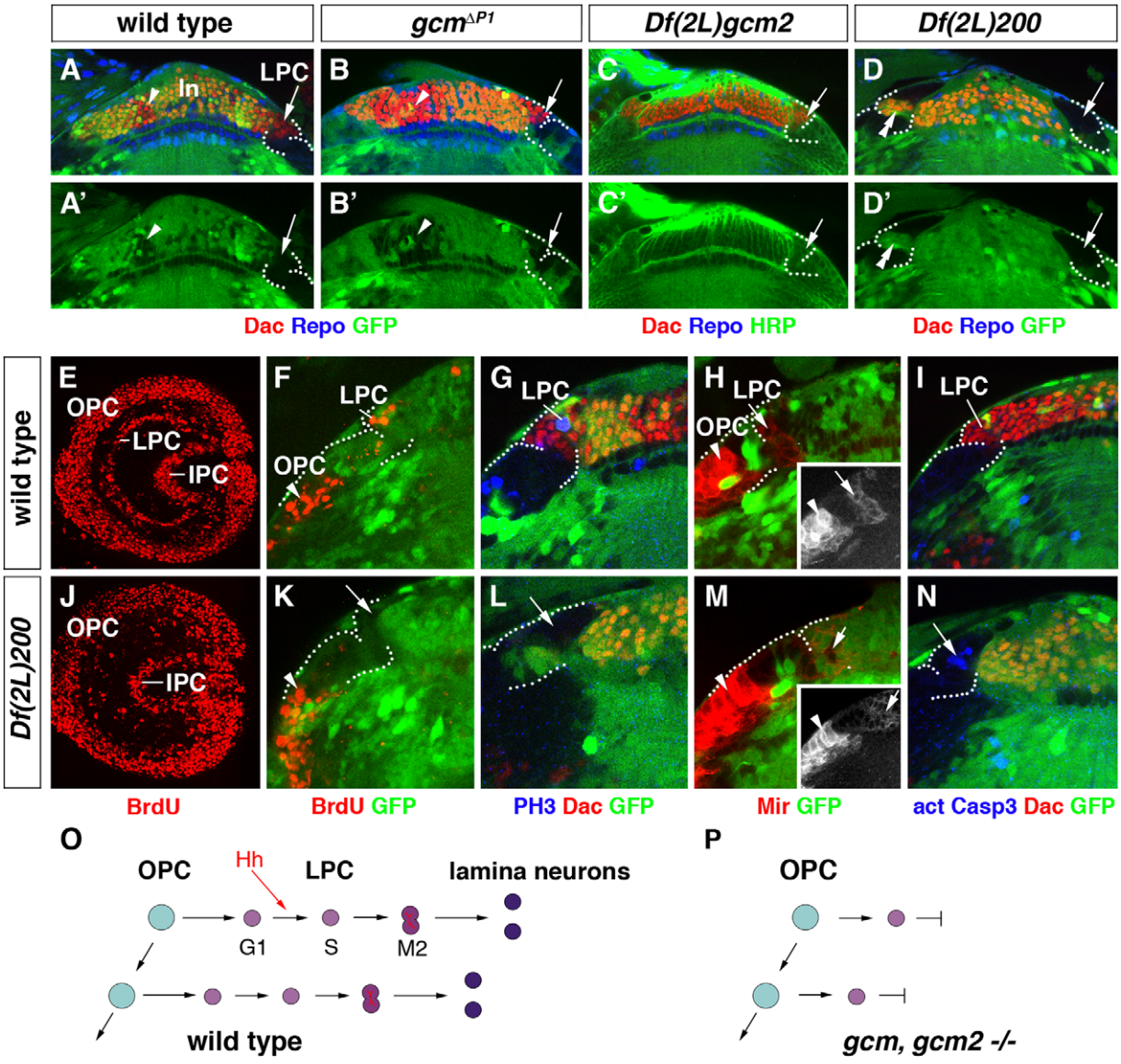

Figure 5. *gcm* and *gcm2* Are Required for Neuronal Differentiation and Proliferation in the Lamina

Clones in the target area were generated with the ELF system and visualized by the absence of GFP expression (green). Neuronal membranes were labeled with anti-HRP (green in [C] and [C′]). Glial cells were stained with anti-Repo (blue in [A]–[D]). (A–C′) As in wild-type (A and A′), lamina neurons develop normally in $gcm^{\Delta P1}$ target mosaics (B and B′) or in *Df(2L)gcm2* homozygous mutant animals (C and C′). Expression of the early neuronal differentiation marker Dachshund (red) is found in lamina precursor cells (LPCs, arrows) posterior to the lamina furrow and persists in lamina neurons (In, arrowheads) arranged in columns. (D and D′) LPCs homozygous for *Df(2L)200* fail to express Dac (arrows). Mutant lamina neurons are not found within columns. Only heterozygous cells express Dac (double arrowheads). (E–I) Wild-type. (J–N) *Df(2L)200* target mosaic animals. (E, F, J, and K) Cells in S phase were visualized with anti-BrdU (red). In wild-type (E), three zones are labeled: the outer proliferation center (OPC), LPCs, and the inner proliferation center (IPC). In *Df(2L)200* mosaic animals (J), labeling of LPCs is diminished. In wild-type (F), cells in S phase are found in the OPC and LPC. In *Df(2L)200* mosaic animals (K), labeled cells are found in the OPC (arrowhead) but not in the LPC area (K, arrow). (G and L) Cells in mitosis were visualized with anti-phospho Histone H3 (blue), LPCs, and lamina neurons with anti-Dachshund (red). In wild-type, one cell undergoes mitosis in the LPC area (G). In mutants (L), LPCs fail to divide (arrow). (H, M, and insets) Neuroblasts in the OPC (arrowhead) and LPCs (arrows) are labeled with anti-Miranda (red in [H] and [M] and white in insets). Expression of this marker in mutants (M) is similar to wild-type (H). (I and N) Cells undergoing apoptosis were visualized with an antibody against activated Caspase 3 (blue) and neurons with anti-Dachshund (red). In wild-type (I), no staining is detected within the LPC area. However, in mosaic animals (N), dying cells are found (arrow). (O and P) The schemes illustrate the similar requirements of *gcm* genes and Hedgehog (Hh) signaling in regulating neuronal development. (E and J) Lateral views. All other panels show frontal views.

Rescue Experiments Reveal that *gcm* Is Cell-Autonomously Required in the Lamina Neuron Lineage

The deficiency *Df(2L)200* removes both *gcm* and *gcm2* and also the open reading frames of 13–15 other predicted genes (Alfonso and Jones, 2002). To exclude that the loss of any of these additional genes was responsible for the observed defects, we assessed the ability of exogenous *gcm* to rescue the *Df(2L)200* phenotypes. Rescue experiments were conducted by the FLP/FRT-based approach called MARCM (mosaic analysis with a repressible cell marker) (Lee and Luo, 1999).

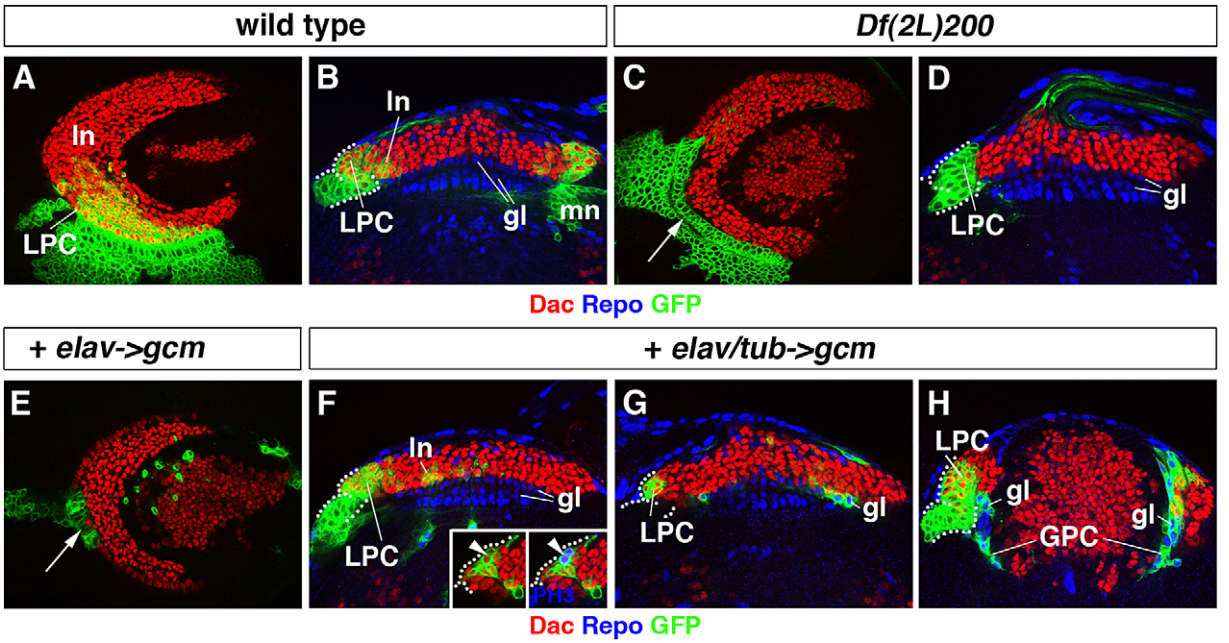

Figure 6. Overexpression of *gcm* Cell Autonomously Rescues Neuronal and Glial Defects in *Df(2L)200* Mosaics

Lamina neurons and glia were labeled with anti-Dachshund (red) and anti-Repo (blue), respectively. MARCM clones were visualized with GFP (green). (A and B) *w hs-FLP^{122} elav-Gal4^{c155} UAS-cd8GFP/w; tubP-Gal80 FRT40A/+ FRT40A; tubP-Gal4/+*. Wild-type MARCM clones include Dac-expressing lamina precursor cells (LPC) and lamina neurons (In). They do not include Repo-positive glial cells (gl) (B). mn, medulla neurons. (C and D) *w hs-FLP^{122} elav-Gal4^{c155} UAS-cd8GFP/w; tubP-Gal80 FRT40A/Df(2L)200 FRT40A; tubP-Gal4/+. Df(2L)200* homozygous mutant cells in the LPC area do not express Dac (arrow). The clone does not include Repo-positive glial cells (D). (E) *w hs-FLP^{122} elav-Gal4^{c155} UAS-cd8GFP/w; tubP-Gal80 FRT40A/Df(2L)200 FRT40A; UAS-gcm/+*. Overexpression of *gcm* in *Df(2L)200* homozygous mutant clones with *elav-Gal4^{c155}* fails to restore Dac expression (arrow). (F–H) *w hs-FLP^{122} elav-Gal4^{c155} UAS-cd8GFP/w; tubP-Gal80 FRT40A/Df(2L)200 FRT40A; tubP-Gal4/UAS-gcm*. Overexpression of *gcm* in *Df(2L)200* homozygous mutant clones with *elav-Gal4^{c155}* and *tubP-Gal4* rescues Dac expression in LPCs and lamina neurons. Dac-positive neuronal clones include mitotic-active cells (arrowheads) labeled with phospho-Histone H3 (blue in [F] insets). Glial formation is equally rescued as indicated by the presence of Repo-positive glial cells (gl) within the R-cell projection field (G) and at the margins of glial precursor cell (GPC) areas (H). (A, C, and E) Lateral view; (B, D, and F–H) frontal view.

This enabled us to generate clones of GFP-labeled cells that express *gcm* at the same time as becoming homozygous mutant for *Df(2L)200*. Two different driver combinations were used: *elav-Gal^{c155}* alone or *elav-Gal^{c155}* and *tubulin-Gal4* (*tubP-Gal4*) together (Lee and Luo, 1999). *elav-Gal^{c155}* is mostly active in postmitotic lamina neurons, whereas *tubP-Gal4* drives high levels of expression in all cells including neuroblasts in the OPC.

As a control, we generated clones that were made homozygous mutant for *Df(2L)200* with MARCM. Consistent with our previous findings with the ELF system, *Df(2L)200* MARCM clones did not express Dac in the lamina when compared to wild-type (wild-type: 21/21 clones express Dac) (Figures 6A and 6B) (*Df(2L)200*: 0/20 clones express Dac) (Figures 6C and 6D). Because defects were detected in animals, in which clones had been induced solely in the lamina neuron but not in the glial lineage, this confirms that the neuronal phenotype is cell autonomous and not an indirect consequence of impaired glial-cell development.

We then repeated this experiment but simultaneously expressed *gcm* in homozygous mutant cells. Overexpressing *gcm* in clones with *elav-Gal4^{c155}* failed to restore Dac expression (n = 6) (Figure 6E). However, both neuronal and glial defects were rescued in clones with *elav-Gal4^{c155}* and *tubP-Gal4* as drivers (Figures 6F–6H). Neuronal clones in the lamina expressed Dac (30/30 clones) and also included mitotically active cells that were positively labeled with phospho-Histone H3 (10/10 clones) (Figure 6F). Glial-cell clones showed expression of Repo and were found in their characteristic positions adjacent to R-cell growth cones in the lamina (14/14 clones) (Figures 6G and 6H).

Taken together, these rescue experiments demonstrate that the phenotypes observed in *Df(2L)200* homozygous mutant clones are caused by the loss of *gcm* and *gcm2* function and that expression of *gcm* alone is sufficient to overcome the lack of both genes. Furthermore, our findings show that *gcm* and *gcm2* are cell-autonomously required to promote neurogenesis.

Dachshund Acts Downstream of *gcm* Genes to Control Neuronal Differentiation but Not Proliferation

R-cell-derived Hh promotes the mitotic divisions of LPCs (Huang and Kunes, 1996). It also induces the expression of Dac and the EGF receptor. The latter enables postmitotic lamina neurons to respond to the second R-cell-axon-derived signal Spitz, which induces further maturation of lamina neurons including the expression of the late differentiation marker Elav (Robinow et al., 1988; Huang et al., 1998). Our findings revealed that in the absence of *gcm* and *gcm2*, LPCs fail to express Dac and to divide. Moreover, onset of Dac

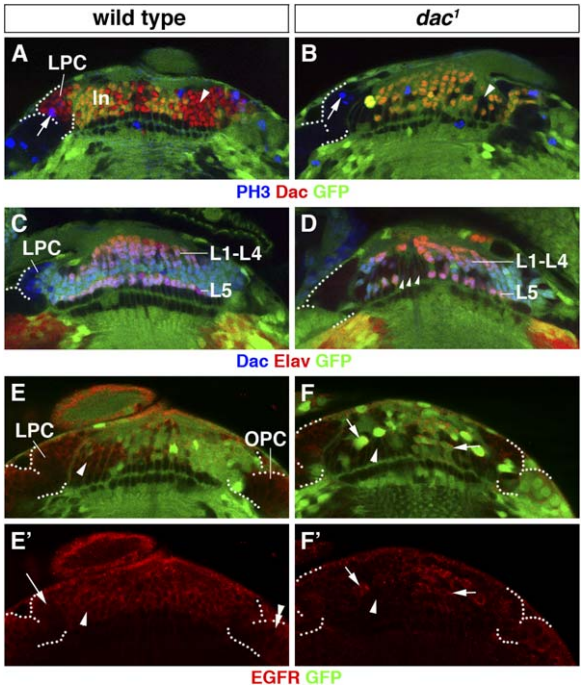

Figure 7. *gcm* Genes Mediate Neuronal Differentiation through Dachshund

Wild-type (A, C, E, and E′) and *dac*^{1} (B, D, F, and F′) target mosaic clones are generated with the ELF system. Clones lack GFP expression (green). (A and B) Lamina precursor cells (LPCs) and lamina neurons (In) are labeled with Dachshund (red), and cells undergoing mitosis with phospho-Histone H3 (blue). As in wild-type (A), *dac*^{1} mutant LPCs undergo mitosis (arrows) and are integrated into columns (arrowheads). (C and D) Whereas in wild-type (C), mature lamina neurons L1–L5 coexpress Dac (blue) and Elav (red), *dac*^{1} mutant lamina neurons fail to express this late neuronal differentiation marker (arrowheads) (D). (E–F′) In wild-type (E and E′), EGF receptor (red) is expressed at increasing levels in neuroblasts in the outer proliferation center (OPC, double arrowhead), in LPCs (arrow), and in lamina neurons (arrowhead). In *dac*^{1} target mosaic animals (F and F′), EGF receptor staining is strongly reduced in mutant lamina neurons (arrowhead), when compared to adjacent heterozygous cells (arrows). (A–F′) Frontal view.

expression precedes mitotic divisions in LPCs. One possible model is that neuronal differentiation and proliferation are both mediated by Dac. Alternatively, both processes are regulated separately. Although the role of Dac as a member of the retinal determination network is well understood in the eye, its function in regulating lamina development is not known. We therefore investigated *dac* function in the lamina independently from its function in the eye with the phenotypic null-allele *dac*^{1} (Tavsanli et al., 2004) and the ELF system. Phospho-Histone H3 labeling revealed that *dac*^{1} mutant LPCs were able to undergo mitosis. Furthermore, homozygous mutant lamina neurons were integrated into columns in the same way as wild-type cells (Figures 7A and 7B). However, lamina neurons lacking *dac* failed to express Elav (Figures 7C and 7D). This indicates that *dac* is necessary for the differentiation and maturation of lamina neurons.

dac could be responsible for EGF receptor upregulation because activation of this pathway is known to be necessary and sufficient for Elav expression (Huang et al., 1998). Therefore, we examined the levels of EGF receptor immunoreactivity in *dac*^{1} mutant lamina neurons. Consistent with previous studies (Huang et al., 1998), higher levels of EGF receptor expression were detected in LPCs and in differentiated lamina neurons in wild-type mosaic animals (Figures 7E and 7E′). In contrast, homozygous mutant lamina neurons within the R-cell projection field of *dac*^{1} target mosaics exhibited severely reduced staining (Figures 7F and 7F′). Because we detected some immunoreactivity in mutant LPCs, we infer that *dac* is necessary to upregulate and maintain EGF receptor expression in lamina neurons.

Together, these findings demonstrate that *dac* does not control divisions of LPCs but is required to promote differentiation of lamina neurons through regulation of EGF receptor expression. This, in conjunction with our observation that *gcm* and *gcm2* are required in LPCs to express Dac, can explain how *gcm* genes contribute to the differentiation of lamina neurons. However, it cannot account for their requirement in regulating LPC proliferation.

gcm Genes Cooperate with the Hedgehog-Signaling Pathway

LPCs lacking *gcm/gcm2* fail to divide and to express Dac and eventually undergo apoptosis. Because these phenotypes are identical to the defects observed in the absence of Hh signaling in the lamina (Huang and Kunes, 1996, 1998), we next sought to examine whether *gcm* genes cooperate with the Hh-signaling pathway to mediate neuronal differentiation and proliferation. Hh signaling may induce expression of *gcm* genes, which in turn regulate neurogenesis. Alternatively, *gcm* genes may contribute to the activation of Hh signaling, which then promotes neuronal differentiation and proliferation.

To distinguish between these possibilities, we tested whether *hh* acts upstream of *gcm* and *gcm2* to induce their expression. Third-instar larvae, homozygous for the hypomorphic eye-specific allele *hh*^{1}, form about 12 rows of R-cell clusters within eye imaginal discs, but R-cell axons lack Hh and thus fail to trigger neurogenesis (Huang and Kunes, 1996). In situ hybridization with RNA probes against *gcm* and *gcm2* showed that both genes were transcribed in the absence of Hh signaling in the optic lobe (Figures 8A and 8B). To examine further whether Hh might induce *gcm* expression, we examined β-galactosidase levels in the *rA87* enhancer trap line in *eya*^{1}/*eya*^{1} animals lacking all R-cells and, thus, Hh activation. In a finding consistent with the in situ hybridization data, we observed that expression of this *gcm* reporter was not blocked (data not shown). Moreover, *gcm* overexpression in lamina and glial precursor cells in a *hh*^{1}-homozygous mutant background did not rescue the generation of lamina neurons (Figures 8C–8E). This suggests that *hh* does not regulate the expression of *gcm* or *gcm2* and that *gcm* genes cannot bypass the lack of Hh signaling.

We therefore considered the alternative possibility that *gcm* and *gcm2* modulate Hh signaling. We first determined whether *gcm* genes could control the expression of the zinc-finger nuclear factor Cubitus interruptus (Ci), which is a main effector of Hh signal transduction (reviewed in Ingham and McMahon, 2001). It is also known as a component whose expression can be

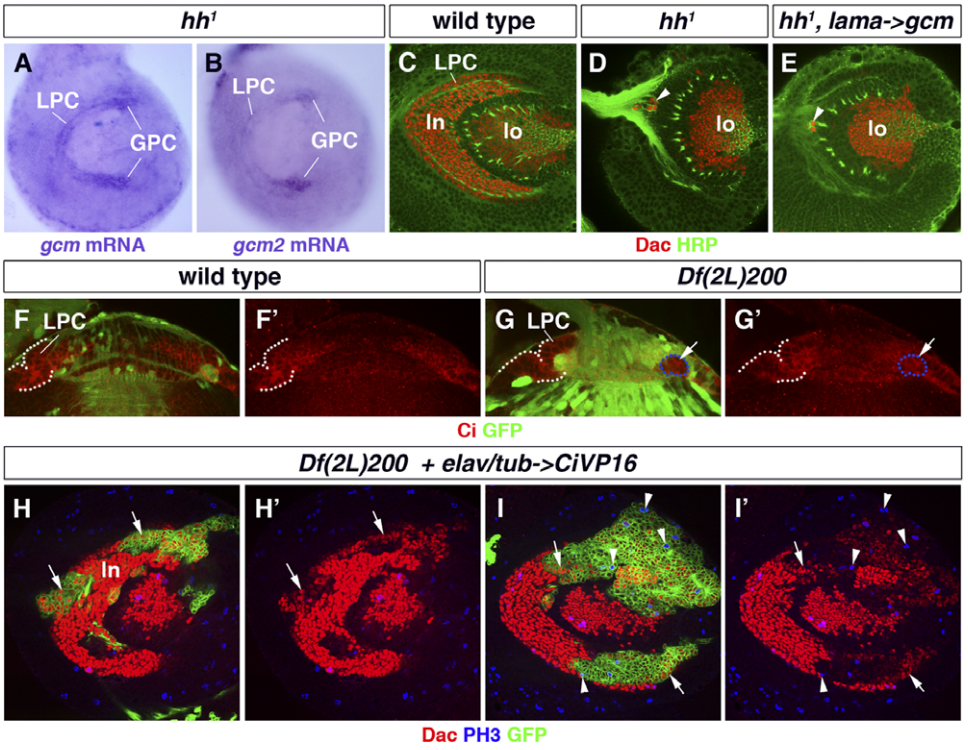

Figure 8. *gcm* Genes Mediate Neurogenesis in the Lamina in Collaboration with the Hedgehog-Signaling Pathway

(A and B) In *hedgehog*^{1} (*hh*^{1}) mutant animals, R-cell axons fail to induce neurogenesis in the optic lobe. In situ hybridization labeling reveals that although Hh is absent, *gcm* and *gcm2* mRNA (purple) are expressed in lamina precursor cell (LPC) areas and glial precursor cell (GPC) areas, albeit at a slightly reduced level, probably because of the disrupted LPC development in this genetic background.

(C–E) R-cell axons are labeled with anti-HRP (green), and lamina neurons with anti-Dachshund (red). Overexpression of *gcm* with *lama-Gal4* in *hh*^{1} mutants does not rescue Dac expression in LPCs and lamina neurons (ln) (arrowheads). Io, lobula. Wild-type (C), *hh*^{1} (D), *UAS-gcm/+; lama-Gal4 hh*^{1}*/hh*^{1} (E).

(F–G′) Wild-type and *Df(2L)200* target mosaics are generated by the ELF system. Somatic clones lack GFP expression (green). Ci expression levels (red) in LPCs are indistinguishable in wild-type and *Df(2L)200*-homozygous mutant clones (arrows).

(H–I′) *w hs-FLP*^{122} *elav-Gal4*^{c155} *UAS-cd8GFP/w; tubP-Gal80 FRT40A/Df(2L)200 FRT40A; tubP-Gal4/UAS-CiVP16*. MARCM clones express GFP (green). Overexpression of activated full-length Ci partially restores Dac expression to variable levels in *Df(2L)200* mutant clones, which include LPCs and lamina neurons (arrows). Clones comprise mitotically active, phospho-Histone H3-positive cells (blue, arrowheads). Some clones had tumor-like features; they were abnormally large, displayed a convoluted epithelial-like structure, and extended into the OPC or dorsal and ventral areas adjacent to the lamina field, underscoring the powerful mitogenic effect of Ci. (A–E and H–I′) Lateral view; (F–G′) frontal view.

regulated by a transcription factor acting outside the canonical Hh pathway, i.e., Engrailed (Schwartz et al., 1995). In the absence of Hh, Ci is proteolytically cleaved and converted into a transcriptional repressor. In the presence of Hh, cleavage is inhibited, enabling the full-length activator form of Ci to promote the transcription of target genes (reviewed in Ingham and McMahon, 2001). This activator form of Ci has been shown to directly mediate the transcriptional response to R-cell-derived Hh in the lamina (Huang and Kunes, 1998). Labeling with the antibody 2A1, which recognizes full-length Ci (Motzny and Holmgren, 1995), showed that the onset and levels of Ci expression in LPCs remained unchanged in *gcm* and *gcm2* homozygous mutant clones: as in wild-type, increasing Ci immunoreactivity was detected in the cytoplasm of LPCs as they progressed through the lamina furrow (Figures 8F–8G′). Thus, *gcm* genes do not simply regulate levels of Ci.

To test whether lack of *gcm* and *gcm2* function may affect Ci activity, we next attempted to rescue the neuronal differentiation and proliferation defects in *Df(2L)200* homozygous mutant clones by overexpressing activated Ci (*UAS-CiVP16*) (Larsen et al., 2003). In 35 of 39 *gcm/gcm2* mutant clones overexpressing CiVP16 in LPCs and lamina neurons, we observed the partial recovery of Dac expression ranging from low to strong levels despite the lack of *gcm/gcm2* function. Clones included mitotically active, phospho-Histone H3-positive cells, indicating that the proliferation of LPCs has been equally rescued (Figures 8H–8I′). Thus, constitutive activation of Hh signaling can partially bypass the requirement of *gcm* genes. Taken together, these observations suggest that *gcm* and *gcm2* cooperate with the Hh pathway to regulate neurogenesis in the lamina.

Discussion

In this study, we provide evidence that the key determinants of embryonic gliogenesis, *gcm* and *gcm2*, acquired an extended role in the postembryonic nervous system of *Drosophila* and not only promote glial development but also the differentiation and proliferation of neurons in the lamina. We propose that *gcm* genes positively regulate neurogenesis via interaction with the Hh-signaling pathway.

gcm and *gcm2* Are Redundantly Required to Promote Gliogenesis in the Optic Lobe

Our genetic analysis shows that *gcm* genes control gliogenesis in the postembryonic visual system, as they do during embryogenesis. Removal of *gcm* and *gcm2* function in the optic lobe prevents the formation of epithelial and marginal glial cells. These findings confirm that *gcm* genes play a similar role in the optic lobe in initiating glial differentiation as previously established in the embryonic nervous system (reviewed in Jones, 2001) (e.g., Akiyama-Oda et al., 1999). However, we uncovered one major difference. In the optic lobe, *gcm* and *gcm2* are both redundantly required, whereas *gcm* plays a more prominent role than *gcm2* in controlling gliogenesis during embryonic development (Kammerer and Giangrande, 2001; Alfonso and Jones, 2002). One likely explanation for this disparity regarding the relative requirements of *gcm* and *gcm2* is that they are expressed at different levels in the embryonic and larval nervous system. This is supported by the following observations. In both the embryonic nervous system and the optic lobe, *gcm* and *gcm2* are expressed in a largely similar pattern. However, *gcm2* transcripts have been detected at a significantly lower level than *gcm* in the embryo (Kammerer and Giangrande, 2001; Alfonso and Jones, 2002), whereas the levels of *gcm* and *gcm2* transcripts detected in the optic lobe appear to be largely similar. Moreover, high levels of expression of *gcm* alone can rescue phenotypes caused by the loss of both factors in the optic lobe. Although Gcm2 has been shown to be a less potent transcriptional activator than Gcm in vitro, both nuclear factors are likely to have similar binding specificities because of the high degree of homology within the Gcm motif (69% identity), enabling them, in principle, to compensate for each other (Kammerer and Giangrande, 2001; Alfonso and Jones, 2002). Consistently, ectopic expression of either of them is sufficient to induce excess glial formation in the embryonic nervous system (Kammerer and Giangrande, 2001; Alfonso and Jones, 2002) or within the medulla cortex in the larval optic lobe.

gcm Genes Promote Neurogenesis in the Optic Lobe

Our study presents two lines of evidence that *gcm* genes also play a central role in mediating neuronal differentiation and proliferation in the visual system. First, *gcm* genes are expressed in the lamina neuron lineage, which is known to solely give rise to neurons but not glial cells (Perez and Steller, 1996a). Second, LPCs homozygous mutant for *gcm* and *gcm2* fail to express the early neuronal differentiation marker Dac and to undergo S phase or mitosis and consequently do not generate lamina neurons. They are, however, not required for the initial formation of neuroblasts in the OPC.

This role of *gcm* genes in mediating neurogenesis is unexpected because the onset of Gcm expression is considered to be a key step in initiating gliogenesis in the embryonic nervous system (reviewed in Jones, 2005). Analysis of adjacent cis-regulatory DNA sequences indicate that distinct enhancer modules control the transcription of *gcm* in embryos (Ragone et al., 2003; Jones et al., 2004). Interestingly, this includes enhancer elements, which promote gene expression specifically in glial lineages, as well as distinct regulatory sequences, which can drive expression more widely in the central nervous system. These regulatory elements are usually thought to mediate general neuronal repression in the embryo (Jones et al., 2004), but it is not known whether they are also active during larval stages. *gcm* and *gcm2* then could only be expressed in the lamina neuron lineage if LPCs lack a potential repressor that normally prevents transcription of *gcm* genes in a neuronal context. Alternatively, a larval optic lobe-specific neuronal module may promote expression of *gcm* genes in LPCs.

Previous studies have shown that Gcm controls gliogenesis in the embryo through activation of a "proglial" transcriptional program and suppression of neuronal target genes. Gcm promotes terminal glial differentiation by inducing the expression of Repo, the ETS domain transcription factor PointedP1 and the RGS protein Locomotion defects. In parallel, it also induces the expression of Tramtrack, which in conjunction with Repo, represses the transcription of neuronal differentiation genes (Giesen et al., 1997; Granderath et al., 2000; Yuasa et al., 2003). To positively regulate neuronal development in one lineage and glial development in another, Gcm proteins most probably have to work in concert with different cofactors in each cellular context. This could then lead to the induction of a different set of transcriptional downstream regulators, which subsequently determine neuronal and glial fates. By analogy, *gcm* proteins could function in a similar way as the bHLH protein Olig2 in the vertebrate nervous system, which has been shown to be cell-autonomously required for the specification of both neuronal and glial lineages (reviewed in Rowitch, 2004). Olig2 regulates neuronal and glial development through complex interactions with different transcription factor partners: it initially promotes the generation of motor neurons in conjunction with the bHLH factor Neurogenin2 and subsequently mediates the generation and maturation of oligodendrocytes together with other transcriptional regulators including the Sox family member Sox9 and the homeodomain-containing nuclear factor Nkx2.2 (Novitch et al., 2001; Mizuguchi et al., 2001; Zhou et al., 2001; Lee et al., 2005).

Dachshund Mediates Neuronal Differentiation through the Regulation of EGF Receptor Levels

To explore the mechanisms by which *gcm* genes mediate neuronal development in the optic lobe, we examined the role of Dac because its expression depends on both the activation of the Hh pathway (Huang and Kunes, 1996) and on *gcm* and *gcm2* function. Our genetic analysis added two findings to our understanding as to how Hh and EGF signaling work in concert to regulate neurogenesis in the lamina (see Huang et al., 1998). We show that (1) *dac* is not required for cell divisions of LPCs and (2) that expression of *dac* is necessary for the upregulation and maintenance of EGF receptor expression in lamina neurons to promote their further maturation. This is consistent with findings in the developing eye imaginal disc, demonstrating that Dac promotes early progression of the morphogenetic furrow and aspects of R-cell specification but is not required for cell proliferation (Mardon et al., 1994). In the eye, genetic interaction assays have previously

established a link between Dac and EGFR signaling because *dac* mutant alleles were identified as suppressors of the dominant-active EGFR allele Ellipse (Mardon et al., 1994), although the precise mechanism underlying this interaction is unclear. Our findings present evidence for one possible mechanism by demonstrating that Dac controls EGF receptor levels in the optic lobe and, in this way, makes LPCs and their progeny competent for neuronal differentiation. In *Drosophila*, processing of EGF ligands by Rhomboids rather than the regulation of the receptor itself has been considered to be a limiting step in EGF receptor signaling (reviewed in Shilo, 2003). In the rodent retina, both ligand and receptor levels have been reported to mediate different cellular responses such as proliferation and cell-fate specification (Lillien, 1995; Lillien and Wancio, 1998). Therefore, regulating receptor levels by Dac represents an additional mechanism to modulate activity of the EGF receptor pathway in the optic lobe of flies. *gcm* genes can contribute to neuronal differentiation through induction of Dac. Their role in promoting mitotic divisions of LPCs, however, must involve another mechanism. Indeed, our genetic analysis suggests that *gcm* genes regulate both developmental processes through interaction with the Hh-signaling pathway.

gcm Genes Collaborate with the Hh-Signaling Pathway

That *gcm* genes work in concert with the Hedgehog-signaling pathway is supported by the following findings. First, the loss-of-function phenotypes of *gcm/gcm2* and *hh* share three characteristics because in their absence, LPCs neither enter S phase nor express the neuronal differentiation marker Dac, and show increased levels of apoptosis (Huang and Kunes, 1998; C. Chotard, W. Leung, and I. Salecker, unpublished data). Second, *gcm/gcm2* loss-of-function phenotypes can be partially rescued by overexpressing activated full-length Ci in cells homozygous mutant for *gcm* and *gcm2*. One possible explanation for the partial rescue is that levels of activated Ci need to be under a tight spatially and temporally control to trigger a normal cellular response. Thus, overexpressing activated Ci at high amounts with our approach may have compromised the ability of *gcm* and *gcm2* homozygous mutant LPCs to express normal levels of Dac or to divide at the correct rate.

Our epistasis analysis supports a model in which *gcm* genes interact with the Hedgehog pathway upstream of Ci. Because loss of *gcm* and *gcm2* function does not interfere with the general expression of Ci in LPCs, one possible mechanism is that *gcm* genes may indirectly affect the production of activated Ci. In the zebrafish embryo, the Zinc-finger protein Iguana/Dzip1 has recently been implicated in regulating the balance between activator and repressor forms of the vertebrate homologs of Ci, Gli1, and Gli2, possibly by modulating their nuclear activity or import (Sekimizu et al., 2004; Wolff et al., 2004). Perhaps *gcm* and *gcm2* act in an analogous manner and regulate the production or subcellular localization of activated Ci by promoting the expression of another member of the Hh-signaling pathway. Alternatively, *gcm* genes may act in parallel and cooperate with Ci at the DNA level of common target genes. The dissection of the precise mechanism underlying the genetic interaction of *gcm* genes and the Hh pathway will require additional genetic analysis in the future.

Gcm genes mediate neuronal differentiation in collaboration with the Hh pathway through induction of Dac. Proliferation is likely regulated by controlling a component of the cell-cycle machinery, such as Cyclin E (Huang and Kunes, 1998). Indeed, in the eye and wing imaginal discs, Ci has been shown to directly promote entry into S phase by inducing increased transcription of Cyclin E. Moreover, three consensus Ci binding sites have been found within the 5′ regulatory region of *cyclin E* (Duman-Scheel et al., 2002).

gcm Genes Play a Diverse Role in Mediating Differentiation and Proliferation

In *Drosophila*, Gcm and Gcm2 have also been shown to act as specific transcriptional regulators outside the nervous system, i.e., in the hematopoietic system (Lebestky et al., 2000; Alfonso and Jones, 2002) and in tendon cells at segmental borders of the epidermis (Soustelle et al., 2004). In the hematopoietic system, *gcm* genes appear to play a similar dual role as in the visual system. *gcm* and *gcm2* are required to promote the differentiation of plasmatocyte precursors into plasmatocytes and then macrophages (Bernardoni et al., 1997; Lebestky et al., 2000; Alfonso and Jones, 2002). Because the number of plasmatocyte precursors is reduced in homozygous mutant embryos, *gcm* genes have also been suggested to control their proliferation (Alfonso and Jones, 2002).

Although vertebrate and *Drosophila* Gcm transcription factors share a high degree of sequence similarity within the *gcm* motif, vertebrate Gcm proteins appear to play a more significant role in placenta, parathyroid gland, and pharyngeal arches development than in the nervous system (Anson-Cartwright et al., 2000; Günther et al., 2000; Okabe and Graham, 2004). So far, Gcm transcripts have only been detected at low levels in the developing mammalian nervous system (Kim et al., 1998), and loss of Gcm1 function does not significantly reduce the number of astrocytes (Iwasaki et al., 2003). However, overexpression of Gcm1 in the mouse neocortex can trigger the formation of ectopic astrocytes (Iwasaki et al., 2003), and transient ectopic expression of Gcm1 in mesenchymal tail-bud cells of mice intriguingly induces the formation of ectopic neural tubes during embryogenesis (Nait-Oumesmar et al., 2002). This suggests that at least some aspects of *gcm* function are conserved. Our results in *Drosophila* indicate that Gcm transcription factors have a more versatile role in the developing nervous system than previously thought. This includes the regulation of gliogenesis as well as of neuronal proliferation and differentiation. It may be that these wider aspects of Gcm function are also conserved between vertebrates and flies.

Experimental Procedures

Molecular Biology

The *ey-Gal80* transgene was constructed by subcloning a 3.5 kb *eyeless* enhancer fragment and a noninducible *hsp70* promoter fragment (obtained from the *ey-tTA* vector) (Bello et al., 1998), as

well as a fragment containing the *Gal80* open-reading frame and SV40 polyA region (obtained from the *tubulin 1αP-Gal80* vector) (Lee and Luo, 1999) into pCaSpeR 3. The *UAS-gcm2* transgene was obtained by subcloning a 2.5 kb fragment containing *gcm2* cDNA (Alfonso and Jones, 2002) into the pUAST vector (Brand and Perrimon, 1993). Transgenic flies were generated by a standard microinjection approach.

Genetics

Generation of gcm-Gal4 *and* lama-Gal4 *Lines*

gcm-Gal4 and *lama-Gal4* lines were generated by the P element replacement strategy (Sepp and Auld, 1999) to substitute *P{PZ}gcm^{rA87}* (Jones et al., 1995) and *P{PZ}A8* (Perez and Steller, 1996b) with Gal4 *(PGawB)* enhancer trap insertions. The efficiency was 16% for *gcm-Gal4* (7/43 insertions) and 10% for *lama-Gal4* (3/30 insertions).

gcm, gcm2, *and* dac^1 *Loss-of-Function Analysis*

Glial cells and target neuron clones were generated with the ELF system: *y w ey-Gal80; Ub-GFP cycE^{AR95} FRT40A/Gla Bc; lama-Gal4 UAS-FLP mδ. mδ-lacZ* is used as a marker for R4 axons (Cooper and Bray, 1999). This stock was crossed to: (1) *FRT40A* (control), (2) *gcm^{ΔP1} FRT40A/Gla Bc*, (3) *Df(2L)200 FRT40A/Gla Bc*, (4) *Df(2L)200 FRT40A/Gla Bc*; *Ro-τ−lacZ*, and (5) *dac^1 FRT40A/Gla Bc.* Larvae were raised at 25ºC to consistently obtain large clones. Because of the expression of *lama-Gal4* in wing imaginal discs, our ELF system also induces efficiently clones in the wings; this does not interfere with studies in the visual system. The efficiency of the *ey-Gal80* trangene was tested by crossing it to an *act-Gal4 UAS-cytoplasmic lacZ* recombinant chromosome. The *gcm2* loss-of-function phenotype was assessed in larvae homozygous mutant for *Df(2L)gcm2* (Alfonso and Jones, 2002). *gcm^{ΔP1}*, *Df(2L)gcm2*, and *Df(2L)200* lines were kindly provided by B. Jones.

gcm *and* gcm2 *Gain-of-Function Analysis*

Ectopic expression of *gcm* and *gcm2* in glial and neuronal precursor cells in the optic lobe was achieved with the following crosses: (1) *UAS-gcm*; *UAS-gcm*, or *UAS-gcm2* x *lama-Gal4* and (2) *UAS-gcm* or *UAS-gcm2* x *y w ey-FLP; act>y$^+$>Gal4 UAS-GFP*.

Rescue and Genetic Interaction Analysis

The following strains were built: *w hs-FLP^{122} elav-Gal4^{c155} UAS-cd8GFP; tubP-Gal80 FRT40A* and *w hs-FLP^{122} elav-Gal4^{c155} UAS-cd8GFP; tubP-Gal80 FRT40A; tubP-Gal4/Tm6B* (the *w hs-FLP^{122} elav-Gal4^{c155} UAS-cd8GFP* recombinant chromosome was kindly provided by T. Clandinin). These stocks were crossed to the following lines: (1) *FRT40A* (control), (2) *Df(2L)200 FRT40A/Gla Bc*, (3) *Df(2L)200 FRT40A/Gla Bc; UAS-gcm*, and (4) *Df(2L)200 FRT40A/Gla Bc; UAS-CiVP16* (*UAS-CiVP16* was kindly provided by C. Alexandre) (Larsen et al., 2003). To further test possible interactions between *gcm* and the *Hh*-signaling pathway, we set up the following crosses: (1) *P{PZ}gcm^{rA87} eya^1/Gla Bc* x *eya^1* and (2) *UAS-gcm; hh^1* x *lama-Gal4 hh^1*.

Immunostaining and In Situ Hybridization

The following primary antibodies were used for immunolabeling of third instar larval eye-brain complexes: mouse mAb24B10 (1:75; Developmental Studies Hybridoma Bank [DSHB]), rabbit anti-Repo (1:500; kindly provided by J. Urban), mouse anti-Repo (1:10; DSHB), rat anti-Gcm (1:100; kindly provided by Y. Hotta), mouse anti-Dac (1:25; DSHB), rat anti-Elav (1:25; DSHB); FITC-conjugated goat anti-HRP (1:200; Cappel), mouse anti-Miranda (1:25; kindly provided by F. Matsuzaki), rabbit anti-Asense (1:500; kindly provided by A. Jarman), rabbit anti-GFP (1:200; Molecular Probes), mouse anti-β-galactosidase (1:300; Promega), mouse anti-BrdU (1:100; Becton Dickinson), goat anti-EGFR (1:75; Santa Cruz Biotechnology), rabbit anti-phospho-Histone H3 (1:200; Upstate Biotechnology), rabbit anti-cleaved Caspase 3 (1:75; Cell Signaling Technology), and rat anti-Ci (1:20; kindly provided by P. Thérond). Secondary antibodies were supplied from Jackson ImmunoResearch Laboratories: goat anti-mouse, anti-rabbit, and anti-rat F(ab′)$_2$ fragments coupled to FITC, Cy3, or Cy5 (1:200 for FITC and Cy5 and 1:400 for Cy3), as well as donkey anti-goat F(ab′)$_2$ fragments conjugated to Cy3 (1:400).

For in situ hybridization and immunostainings, *gcm* and *gcm2* cDNA fragments (subcloned into pBluescript SK[+]; kindly provided by B. Jones) were used as templates to synthesize digoxigenin-labeled sense and antisense riboprobes with T3 or T7 RNA polymerases (Roche). After fixation and proteinase K treatment, larval brains were incubated in hybridization buffer containing digoxigenin-labeled riboprobes at 60ºC overnight and with sheep anti-digoxigenin antibody conjugated to alkaline phosphatase (1:2000; Roche) or sheep anti-digoxigenin conjugated to Horseradish Peroxidase (1:1000; Roche) at room temperature for 1 hr. Labeled probes were detected with NBT/BCIP (Roche) or Tyramide Signal Amplification (TSA, PerkinElmer), respectively. Brains stained with TSA were postfixed and incubated in mouse anti-Dac antibody. As secondary antibody, goat anti-mouse F(ab′)$_2$ fragments coupled to FITC were used (1:200; Jackson ImmunoResearch Laboratories).

Details of protocols are available upon request. Images were collected with a Bio-Rad/Zeiss Radiance2100 confocal laser scanning microscope. 3D image analysis was performed with Volocity software.

Supplemental Data

Supplemental Data include three figures and Supplemental Results and can be found with this document online at http://www.neuron.org/cgi/content/full/48/2/237/DC1/.

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