DPP signaling controls development of the lamina glia required for retinal axon targeting in the visual system of *Drosophila*

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Accepted 12 August 2005

Development 132, 4587-4598
Published by The Company of Biologists 2005
doi:10.1242/dev.02040

Summary

The *Drosophila* visual system consists of the compound eyes and the optic ganglia in the brain. Among the eight photoreceptor (R) neurons, axons from the R1-R6 neurons stop between two layers of glial cells in the lamina, the most superficial ganglion in the optic lobe. Although it has been suggested that the lamina glia serve as intermediate targets of R axons, little is known about the mechanisms by which these cells develop. We show that DPP signaling plays a key role in this process. *dpp* is expressed at the margin of the lamina target region, where glial precursors reside. The generation of clones mutant for *Medea*, the DPP signal transducer, or inhibition of DPP signaling in this region resulted in defects in R neuron projection patterns and in the lamina morphology, which was caused by defects in the differentiation of the lamina glial cells. *glial cells missing/glial cells deficient* (*gcm*; also known as *glide*) is expressed shortly after glia precursors start to differentiate and migrate. Its expression depends on DPP; *gcm* is reduced or absent in *dpp* mutants or *Medea* clones, and ectopic activation of DPP signaling induces ectopic expression of *gcm* and REPO. In addition, R axon projections and lamina glia development were impaired by the expression of a dominant-negative form of *gcm*, suggesting that *gcm* indeed controls the differentiation of lamina glial cells. These results suggest that DPP signaling mediates the maturation of the lamina glia required for the correct R axon projection pattern by controlling the expression of *gcm*.

Key words: *dpp*, Visual system, Glia, *Drosophila*

Introduction

The mechanisms by which each axon is navigated to the correct destination and establishes synapses with its correct partner is a central issue for the formation of a functional nervous system. Glial cells present in the nervous system support the growth and survival of the neurons, and, in some cases, the glial cells act as a platform for the migration of neuronal cell precursors (Rakic, 1988). In addition to their well-known supportive role for neuronal cells, glial cells may also play active roles in axon navigation and synapse formation (reviewed by Chotard and Salecker, 2004). In the embryonic central nervous system of *Drosophila melanogaster*, midline glia have been shown to control the guidance of commissural axons, a process mediated by *roundabout* (*robo*) receptors expressed in neurons, as well as *slit* in the midline glia and *commissureless*, which regulates ROBO (Tear et al., 1996; Brose et al., 1999; Kidd et al., 1998; Kidd et al., 1999). In addition, in mammals it has recently been shown that thrombospondins secreted from astrocytes promote synaptogenesis in cultured retinal ganglion cells (Christopherson et al., 2005).

The visual system of *Drosophila* provides a powerful genetic tool with which to address the molecular and cellular mechanisms underlying these processes. The visual system comprises a pair of compound eyes and optic ganglia, which are the visual processing centers of the brain (reviewed by Meinertzhagen and Hanson, 1993; Clandinin and Zipursky, 2002; Tayler and Garrity, 2003). A repeated unit of the compound eye, the ommatidium, contains eight photoreceptor neurons (R neurons). Axons from photoreceptor R7 and R8 connect to a deeper target site known as the medulla. By contrast, R1-R6 neurons stop between two layers of lamina glial cells at the bottom of the ganglion layer known as the lamina (Winberg et al., 1992). R axons provide signals, Hedgehog (Hh) and Spitz, to the lamina neuron precursors (LPCs) and induce LPC differentiation into lamina neurons, the future synaptic partners of the R axons (Huang and Kunes, 1996; Huang and Kunes, 1998; Huang et al., 1998). The lamina glia play a crucial role in the guidance of R1-R6 growth cones, by serving as an intermediate target of R1-R6 axons (Poeck et al., 2001). It has also been shown that R axons play an

important role in the migration of lamina glia from the progenitor domain (Suh et al., 2002). These glia arise from dorsal and ventral marginal progenitor zones and migrate into the lamina target region.

Although both the importance of lamina glia for R axon targeting and the mechanism for their migration have been analysed (Dearborn and Kunes, 2004), little is known about the signaling mechanisms that regulate the development of these glial cells. We show that *decapentaplegic* (*dpp*) plays a key role in the process. DPP is a homolog of BMP and is a member of the TGFβ superfamily, which is widely conserved among species and which plays central roles in diverse cellular and molecular processes (reviewed by Hogan, 1996; Massague and Chen, 2000). In *Drosophila*, DPP provides positional information by acting as a morphogen (Lecuit et al., 1996; Nellen et al., 1996) (reviewed by Tabata and Takei, 2004). In the vertebrate nervous system, signaling mediated by BMP family proteins operates in diverse processes during development. For example, BMP promotes the differentiation of astroctytes from multipotent progenitor cells, but inhibits the differentiation of neuronal and oligodendroglial cell types (reviewed by Mehler et al., 1997).

In the *Drosophila* visual system, *dpp* is expressed in dorsal and ventral marginal zones via induction by *wingless* (*wg*) (Kaphingst and Kunes, 1994). An optic lobe mutant for *dpp* shows defects in the neuropile in the medulla cortex. These defects are presumably caused by a failure in neuronal cell fate specification of cells in the outer proliferation center (OPC), i.e. in the neuroblast populations (Kaphingst and Kunes, 1994). However, brains from animals with the same *dpp* mutant allele also lack retinal axon input. In turn, this affects multiple steps of visual system development and makes it difficult to address the other roles of DPP signaling in the visual system.

We examined the role of DPP signaling in the visual system using clonal analysis and the targeted expression of inhibitors specific to signaling. We show that *dpp* is required for the establishment of correct R-axon projection patterns via the control of lamina glia development. The expression of *glial cells missing*/*glial cells deficient* (*gcm*; also known as *glide*) is the earliest marker for lamina glia differentiation (Dearborn and Kunes, 2004). *gcm*-positive cells, as well as mature glial cells, are greatly reduced in *dpp* mutant animals and in animals with clones mutant for the intracellular components of DPP signaling. Conversely, overactivation of DPP signaling induces ectopic expression of *gcm* and the production of mature glial cells. Taken together, these data suggest that DPP signaling is required for R axon projection, as DPP signaling mediates the production of lamina glia, the intermediate targets of the R axons, via the control of *gcm* expression.

Materials and methods

Fly stocks

The following mutant and transgenic strains were used in this study. *y w* flies were used as wild-type controls. *dpp-lacZ* is an enhancer-trap allele of *dpp* (Tabata and Kornberg, 1994). *optomotor-blind* (*omb*)-*Gal4* contains a P element carrying *Gal4* in the promoter region of the *omb* gene (Lecuit et al., 1996). The *NP6099-Gal4* driver is expressed in the lamina precursor cells and developing lamina neurons; expression is hardly detected in the mature lamina neurons (Hayashi et al., 2002) (D.U., S.M. and T.T., unpublished). *UAS-GFP* encodes a cytoplasmic form of GFP and *UAS-GFPnls* encodes a nuclear-tagged form of GFP (Lee and Luo, 1999). *UAS-Daughters against dpp* (*Dad*) has been used in a variety of tissues to specifically block processes mediated by DPP signaling (Tsuneizumi et al., 1997; McCabe et al., 2004; Kawase et al., 2004). *UAS-thickveins* (*tkv*)^{Q253D} has an amino acid replacement in *tkv* and acts as a constitutive-active form of the receptor (Wiersdorff et al., 1996). *UAS-glide*^{DN} and *UAS-glide*$^{N7\text{-}4DN}$ have been described by Soustelle et al. (Soustelle et al., 2004). *gcm-lacZ*^{r87} is a reporter that has a P element insertion containing *lacZ* in the promoter of the gene (Jones et al., 1995; Vincent et al., 1996; Badenhorst, 2001). *sine oculis*^{1} (*so*^{1}) is a spontaneous allele that lacks eye formation almost completely (Kunes et al., 1993). *hh*^{bar3} is an eye-specific regulatory allele (Lee et al., 1992; Heberlein et al., 1993; Huang and Kunes, 1996; Huang and Kunes, 1998). The *dpp*^{d6} and *dpp*^{d12} alleles are described as disc-specific regulatory alleles, but defects in the brain have also been reported (Kaphingst and Kunes, 1994). *Medea*^{8} is identified as a null allele and *Medea*^{4} as a strong hypomorph (Wisotzkey et al., 1998); they showed an indistinguishable phenotype in the experiments described here.

Clonal analysis

Medea^{4} and *Medea*^{8} mutant clones were generated by the *FLP-FRT*-mediated mitotic recombination system, as previously described (Xu and Rubin, 1993), in the *Minute* mutant background; *y w*; *Medea*^{4} *FRT82B/TM6B* or *y w*; *Medea*^{8} *FRT82B/TM6B* flies were crossed to *y w hsflp*^{122}*; FRT82B Ubi-GFP M (3)*^{w124}. The progeny were heat shocked at the first instar at 37°C for 60 minutes, and dissected in the late third instar. Overexpression using the *GAL4-UAS* system was performed at 29°C except for for *UAS-glide*^{DN}, which was performed at 25°C.

Immunohistochemistry

Immunohistochemistry was performed as described previously (Huang and Kunes, 1996; Takei et al., 2004). Rat anti-DAC and rat anti-β-Galactosidase (β-GAL) were raised against synthetic peptides (Hokudo) and diluted 1:100 and 1:4000, respectively. Anti-Cyclin A was a gift from S. B. Selleck and was diluted 1:200 (Nakato et al., 2002). Mouse monoclonal anti-β-GAL (Promega) was diluted 1:250. The mouse monoclonal antibodies anti-Caoptin (referred to as mAb24B10 throughout the text), anti-CUT and anti-REPO were provided from the Developmental Studies Hybridoma Bank and were used at a 1:10 dilution. Specimens were mounted with Vectashield Mounting Media (Vector Laboratories) and viewed on a Zeiss LSM510 confocal microscope.

Results

dpp is expressed in the lamina glia precursor region in the optic lobe

dpp is expressed in the dorsal and ventral margins of the posterior region of the optic lobe, adjacent to the cells expressing *wg*, which induces *dpp* expression (Kaphingst and Kunes, 1994; Song et al., 2000) (Fig. 1B). Glial cells in the lamina target region arise from these regions and migrate into the lamina target region as they contact R axons (Perez and Steller, 1996; Dearborn and Kunes, 2004) (Fig. 1B,C). Axons from R1-R6 neurons stop between two rows of glial cell layers, the epithelial and marginal layers, and form the lamina plexus. The third row of glial cells, the medulla glia, is located just beneath the marginal glia (Fig. 1C). The homeodomain protein REPO is expressed in these glial cells (Xiong et al., 1994; Halter et al., 1995).

We compared the expression pattern of *dpp-lacZ*, an enhancer-trap allele of *dpp* (Tabata and Kornberg, 1994), with

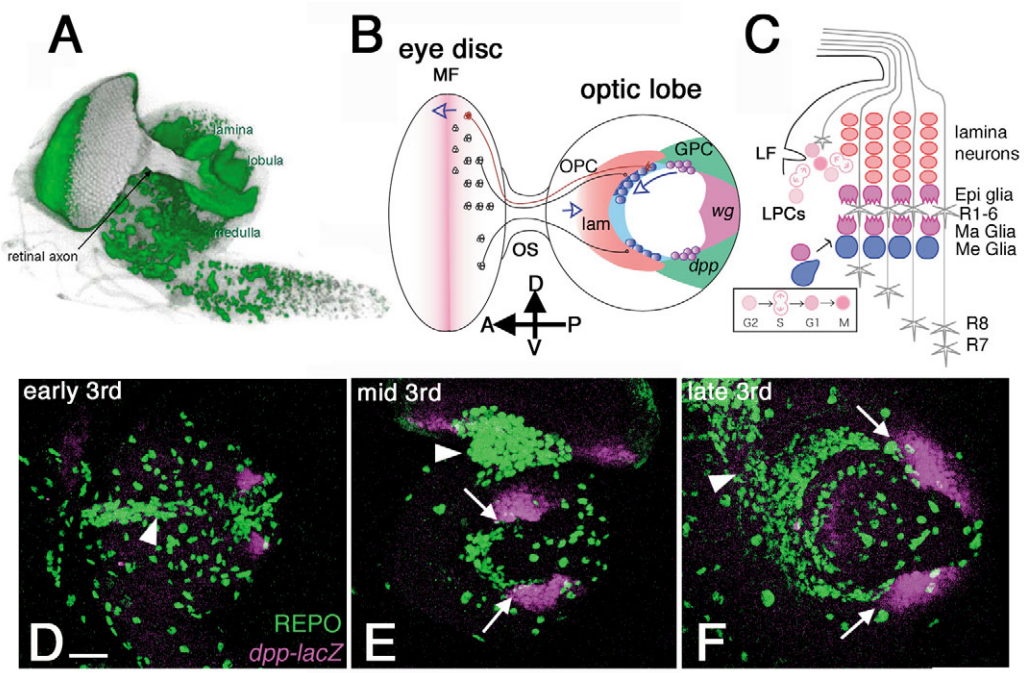

Fig. 1. Expression of *dpp* in the developing visual system of the third instar larvae of *Drosophila.* Unless otherwise noted, all specimens are viewed from a lateral perspective. (A) Developing visual system of the third instar larvae. R axons (white) and DAC-positive neural cell precursors (green) are labeled. Developing optic ganglia (the lamina, the medulla and the lobula) are shown. (B) Schematic illustration of the *Drosophila* visual system, including the expression domains of *dpp* and *wg*. MF, morphogenetic furrow; OS, optic stalk; lam, lamina; OPC, outer proliferation center; GPC, glial cell precursors. *wg* is expressed at the posterior-most domain (magenta) and *dpp* is expressed in the dorsal and ventral margin (green). Differentiating lamina glia migrate from the posterior domain to the lamina target region. (C) Schematic illustration of the developing visual system viewed from the horizontal perspective. Axons from R1-R6 neurons induce the differentiation of lamina neurons from lamina neuron precursors (LPCs) arrested at G1 phase in the lamina furrow (LF). Their growth cones stop between rows of epithelial (Epi) and marginal (Ma) glia. Axons from R7 and R8 proceed to the deeper target region in the medulla. (D-F) Three-dimensional image of the optic lobe in the third instar larvae. Glial cells are labeled with anti-REPO antibody (green); the *dpp* expression domain is visualized by anti-β-Galactosidase (β-GAL) antibody in the presence of *dpp-lacZ* (magenta). (D) Early third instar; (E,F) progressively older stages. (D) REPO-positive glial cells form the optic stalk (arrowhead). (E) The number of glial cells in the optic stalk is increased and the structure becomes thickened (arrowhead). (F) The optic stalk locates at the center of the lamina target region (arrowhead). During development, *dpp-lacZ* expression is observed just posterior to the margin of the lamina glia (arrows in E and F). Scale bar: 50 μm.

the expression pattern of REPO (Fig. 1D-F). At a stage prior to glia differentiation and migration, expression of the *dpp* reporter is detected in the dorsal and ventral margins of the lamina target region (Fig. 1D). *dpp* continues to be expressed at the margins of the lamina target region throughout the third larval instar (Fig. 1E,F).

Medea activity is required in the glial cell lineage for R axon targeting and lamina formation

The role of DPP signaling in optic lobe development was examined by generating clones mutant for *Medea*, which encodes a transcription factor downstream of TGFβ-mediated signaling (Das et al., 1998; Wisotzkey et al., 1998; Hudson et al., 1998; Muller et al., 2003). We observed defects in R axon projections and lamina morphology in two independent alleles of *Medea* mutants, both of which are considered to be strong alleles (Wisotzkey et al., 1998) (Fig. 2B-E; n=8). Such defects were observed only when large clones were generated in the posterior-dorsal or ventral domains, which presumably include glial cell progenitors (Fig. 2B,D).

When clones were induced in other regions (i.e. the OPC, lamina or medulla), R axon targeting defects were not observed. In addition, neither CUT expressed in the medulla (Fig. 2G,H; n=19) nor DAC expressed in the lamina (Fig. 2I,J; n>20) were affected in these clones, suggesting that DPP signaling is not required for the development of these cell types. Furthermore, Cyclin A accumulation in G2 to early M phase of the cells in the outer proliferation center (OPC) and the first row of the lamina neurons (Nakato et al., 2002) was not affected (Fig. 2L,M; n=16), suggesting that DPP signaling is not required for cell cycle progression in the OPC and lamina neuron precursors.

These results suggest that *Medea* activity is required in the posterior marginal cells for correct R axon targeting. Because this region includes glial cell progenitors and lamina glia have been shown to play an important role in R axon targeting, we assume that these defects could be due to a failure in some aspect of lamina glia development.

Inhibition of DPP signaling in the lamina glia causes defects in R axon projection

To further address the direct role for DPP signaling in optic lobe development, we analysed the antagonizing effects of DPP signaling by targeted expression of the negative regulator *Daughters against dpp* (*Dad*) (Tsuneizumi et al., 1997). We overexpressed *Dad* in various domains within the optic lobe

using several *Gal4* drivers. Defects in the pattern of R axon projection were observed when *optomotor-blind* (*omb*; *bifid* – FlyBase)-*Gal4* was used as a driver (Fig. 3B,F). The penetrance of the phenotype was about 40% (n=41). The expression pattern of Dachshund (DAC), a marker for the differentiating lamina neurons, was also impaired, and the lamina failed to form the regular crescent shape (Fig. 3C,G). *omb* is expressed in large posterior domains, including in glial cell progenitors and in mature glia in the target region (Huang and Kunes, 1998; Dearborn and Kunes, 2004) (Fig. 3A). Together, these data suggest that DPP signaling is required in the glial cell lineage for the differentiation/migration of lamina glial cells, and DPP loss causes the defect of R axon targeting.

Although *omb* is also expressed in the retinal basal glia, and in the dorsal and ventral margins of the eye disc, we did not observe significant defects in R axon projections when DPP signaling was blocked in these cells. Lamina glia serve as intermediate targets of R1-R6 axons and thus are required for the establishment of the correct R axon projection pattern (Poeck et al., 2001). Therefore, we examined the structure of the lamina glia layer and the morphology of the cells to investigate whether they are affected by *Dad* overexpression. We found that the lamina glia layers were indeed defective in these brains. Epithelial, marginal and medulla glia layers were not clearly distinguishable, cells were not regularly spaced (Fig. 3K) and there was a defect in the cell shape. Although epithelial and marginal glia have a cuboidal shape with many small processes (Poeck et al., 2001) (Fig. 3J,J′), the glial cells failed to develop their characteristic cuboidal shape and look irregular in these mutant brains (Fig. 3K,K′). Taken together, these results suggest that DPP signaling controls the development and formation of the regular array of lamina glial cells.

gcm is expressed from an early step of lamina glia differentiation

The above results show that blocking DPP signaling can disrupt the formation of the lamina glia layers. Defects in either differentiation or cell migration of the glial cells can cause these phenotypes. It has been reported that when migration of

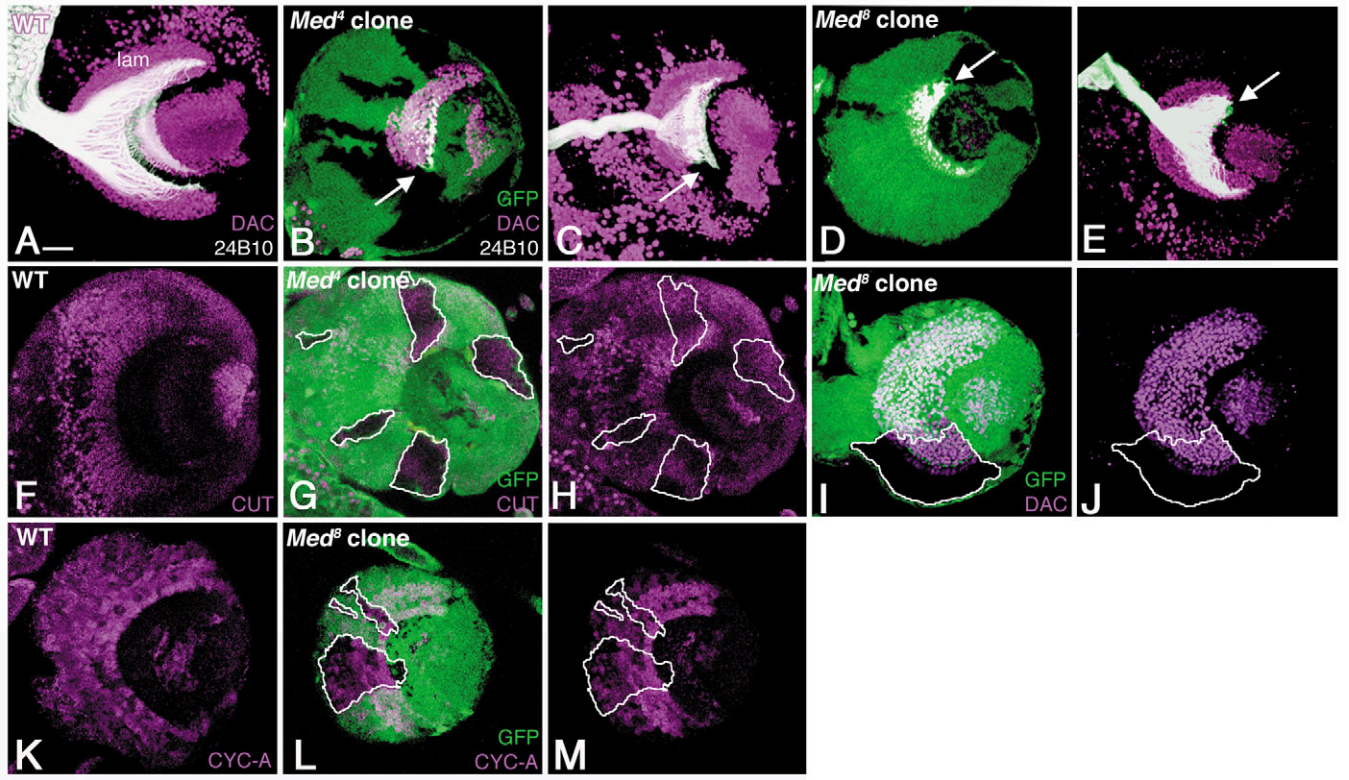

Fig. 2. *Medea* is required for R axon projection patterning. Mutant clones can be identified by the absence of GFP (green); outlined in white in G-J,L,M. R axons are detected with mAb24B10 (white) and developing lamina neurons with an anti-DAC antibody (magenta) except where noted. (A) Three-dimensional image of a wild-type optic lobe with the characteristic crescent-shaped lamina (lam). (B,C) Confocal image of an optic lobe with *Medea*^{4} mutant clones. (B) A clone in the posterior-ventral domain (arrow). R axons (white dots) are not observed in mutant clones. (C) Three-dimensional image of the specimen shown in B. There is no R-axon projection in the posterior-ventral region where the clone is present, and the lamina appears to be truncated (arrows in B and C). (D,E) Confocal image of an optic lobe with *Medea*^{8} mutant clones. (D) Confocal image showing a clone in the posterior-dorsal region (arrow). R axon projection is not seen in the mutant clone. (E) Three-dimensional image of the specimen shown in D. The R-axon innervation pattern and the lamina morphology are compromised near the border of the mutant clone (arrow). (F) Confocal image of a wild-type optic lobe with anti-CUT antibody staining (magenta). (G,H) Confocal image of an optic lobe with *Medea*^{4} clones in the medulla stained with anti-CUT antibody (magenta). CUT expression is not affected in the mutant clone (shown alone in H). (I,J) Confocal image of an optic lobe with *Medea*^{4} clones in the OPC and the lamina. DAC expression is not affected in the mutant clone (shown alone in J). (K) Confocal image of a wild-type optic lobe stained with anti-Cyclin A. (L,M) Confocal image of an optic lobe with *Medea*^{4} clones in the OPC, stained with anti-Cyclin A antibody (magenta). No clear defect is observed in Cyclin A expression in the mutant clone (shown alone in M). Scale bar: 50 μm.

the lamina glia is defective, REPO-positive glia get stuck in the progenitor domain (Poeck et al., 2001; Suh et al., 2002). However, we did not observe such 'stuck' cells when DPP signaling was blocked (data not shown). These results led us assume that DPP signaling is required for differentiation rather than for migration of the glial cells.

To further distinguish between these possibilities, first we followed the process by which the progenitor cells differentiate into lamina glia. The *gcm* gene is expressed in the glial cell precursors and acts as a genetic switch to promote glial cell fate by directly activating the expression of glia-specific genes in the embryo (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996; Akiyama et al., 1996; Egger et al., 2002) (reviewed by Granderath and Klambt, 1999; Van De Bor and Giangrande, 2002). In the developing optic lobe, the onset of *gcm* expression marks the onset of lamina glia differentiation (Dearborn and Kunes, 2004). During migration towards the neuropile, *gcm*-positive glia start to express REPO, which is known to be downstream of *gcm* (Dearborn and Kunes, 2004).

gcm expression was analysed using an enhancer-trap line,

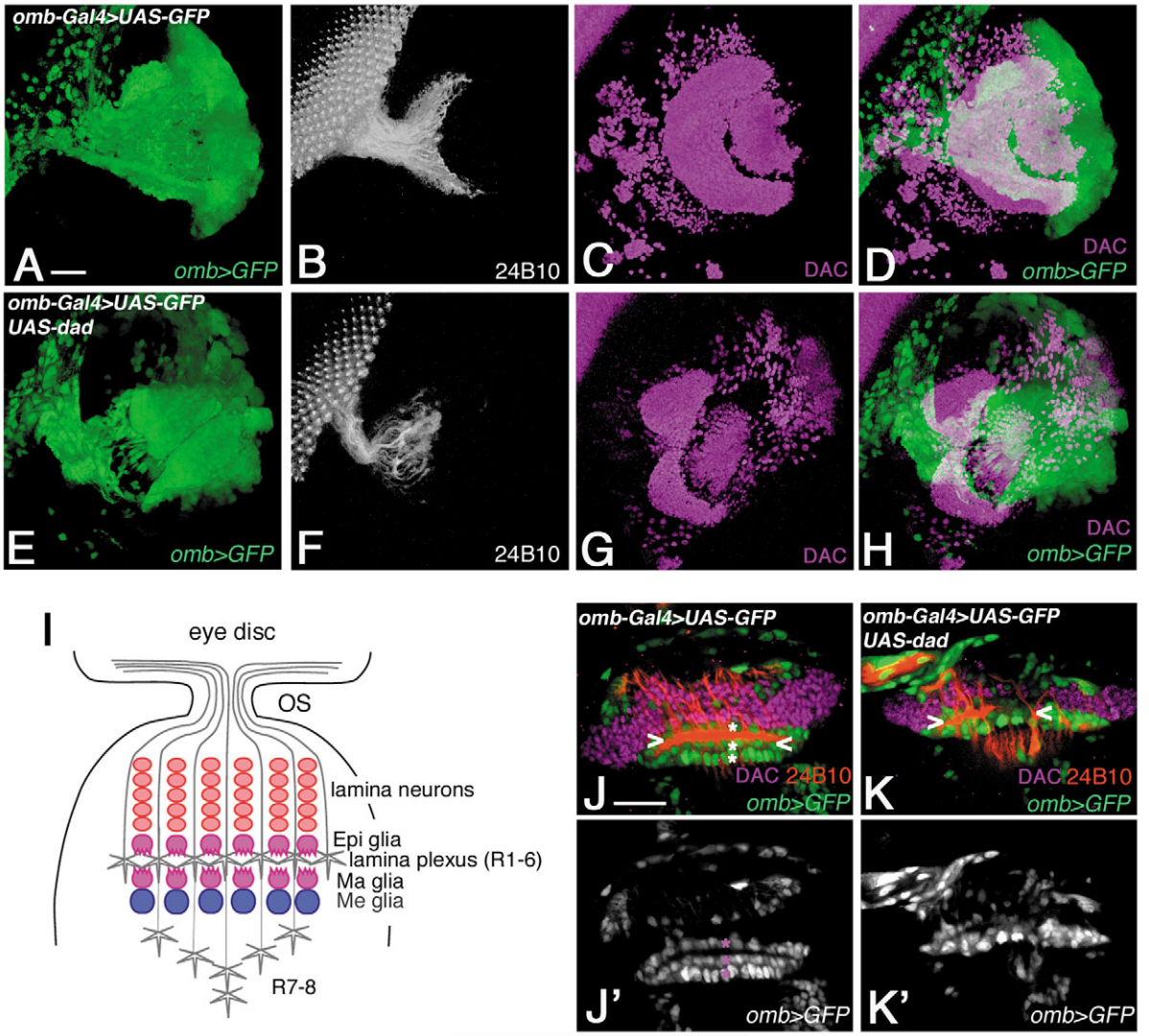

Fig. 3. Inhibition of DPP signaling causes defects in R axon projection patterns and lamina morphology. (A-D) Three-dimensional images of a wild-type optic lobe. (A) Expression of the *omb-Gal4* driver, visualized by *UAS-GFP* (green). (B) R axons visualized by mAb24B10 (white). (C) Developing lamina neurons visualized by anti-DAC antibody (magenta). (D) Merged image of A and C. (E-G) Three-dimensional images of an optic lobe in which *UAS-Dad* and *UAS-GFP* expression are induced by *omb-Gal4*. (E) Expression pattern of *omb-Gal4* visualized with *UAS-GFP*. (F) R axon projections visualized by mAb24B10; the normal crescent-shaped pattern is disrupted. (G) Lamina neurons visualized by anti-DAC antibody; the normal crescent shape is compromised. (H) Merged image of E and G. (I) Schematic illustration of the visual system viewed from the coronal perspective. Epi glia, epithelial glia; Ma glia, marginal glia; Me glia, medulla glia. (J) Confocal image of the wild-type optic lobe carrying *omb-Gal4*, *UAS-GFP* and *UAS-GFPnls*, viewed from the coronal perspective. R axons are visualized by mAb24B10 (red) and lamina neurons are visualized by anti-DAC antibody (magenta). GFP-positive glial cells are shown alone in J′. The growth cones of R axons form the lamina plexus (arrowheads) between the rows of epithelial and marginal glial cells (two asterisks at the top in J,J′). (K,K′) Confocal image of the optic lobe, in which *Dad* expression is induced by *omb-Gal4*. Alignment of the growth cones of R axons is irregular and growth cones do not form a clear plexus structure (K, arrowheads). Epithelial, marginal and medulla glia layers are not clearly distinguishable and the shape of the cells is irregular. GFP-positive glial cells are shown alone in K′. Scale bars: 50 μm.

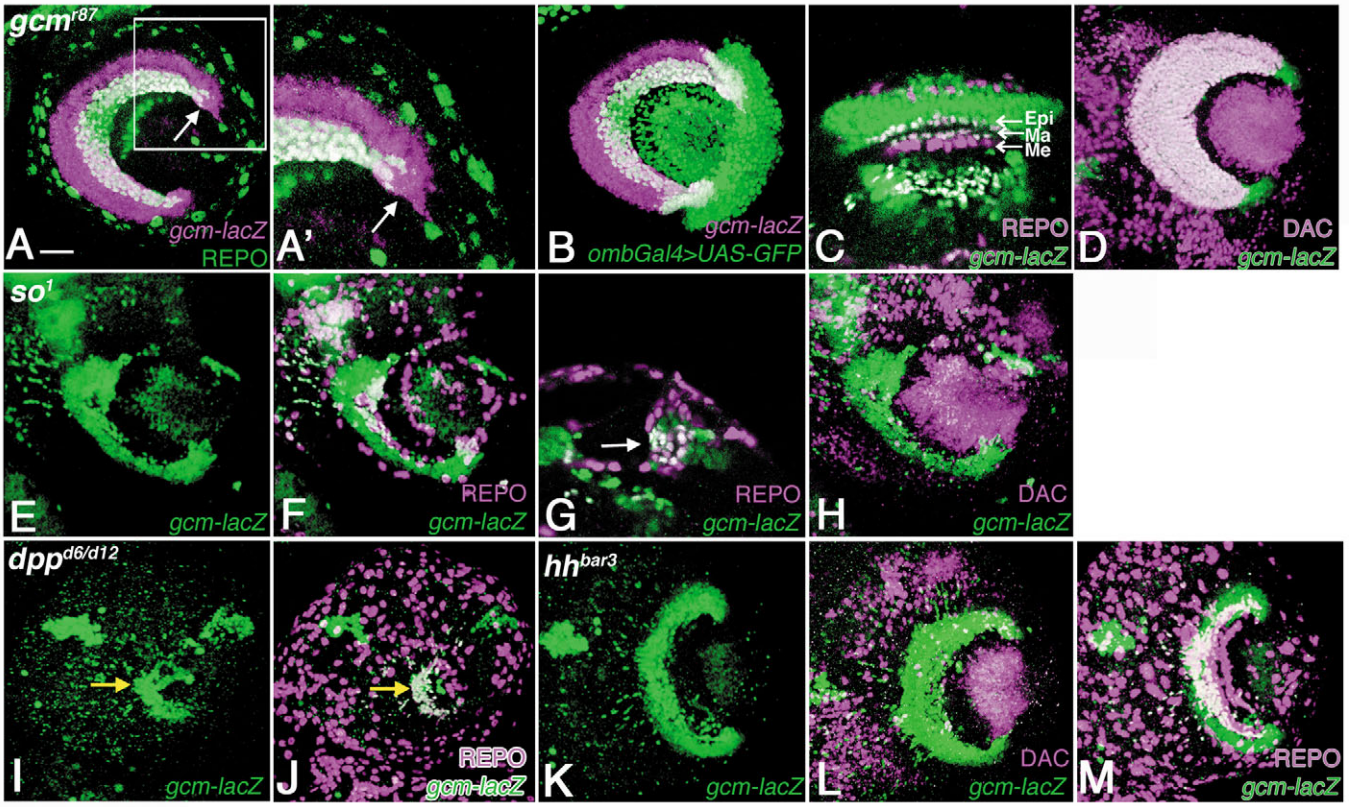

Fig. 4. Expression of the *gcm-lacZ*^{rA87} reporter during development of the optic lobe and its dependence on DPP signaling. (A) Three-dimensional image of the expression of the *gcm-lacZ*^{rA87} reporter in the visual system (revealed by anti-β-GAL antibody, magenta), together with REPO expression (revealed by anti-REPO antibody, green). (A′) Magnified image of the boxed area of A. Expression of β-GAL is seen in the glial precursor cells just before the entry into the lamina target region and the onset of REPO expression (arrow in A,A′). (B) Expression of *gcm-lacZ* (magenta), and *omb-Gal4* and *UAS-GFPnls*. (C) Confocal image of the optic lobe with *gcm-lacZ*, viewed from the coronal perspective. *gcm-lacZ* expression is visualized by anti-β-GAL antibody (green) and glia are shown by anti-REPO antibody (magenta). Expression of β-GAL can be observed in the lamina region and in the epithelial and marginal glia, but not in the medulla glia (arrows in C). (D) Three-dimensional view showing the expression of *gcm-lacZ* (green) in lamina neurons, revealed by co-labeling with DAC (anti-DAC antibody, magenta). (E) Three-dimensional image of *so*^{1} mutant brain carrying *gcm-lacZ*, visualized by anti-β-GAL antibody (green). A significant population of β-GAL-positive cells is observed. (F) Glial cells visualized by anti-REPO antibody (magenta) in the same specimen shown in E; many β-GAL-positive cells have migrated and express REPO, However, some REPO-negative (and DAC-negative; H) cells are also present. (G) Confocal image of the coronal view of the same specimen as in E and F. REPO-positive glia fail to form the normal three-layer structure (arrow). (H) Anti-DAC antibody staining of the same specimen shown in E-G. No DAC-positive cells are detected, and thus no R axon innervations, in this specimen. (I) Three-dimensional image of *gcm-lacZ* expression in the brain from *dpp*^{d12}/*dpp*^{d6} mutant animals (anti-β-GAL antibody, green). A severe reduction of β-GAL-positive cells is observed compared with in the *so*^{1} mutant brain (yellow arrow). (J) Glial cells (visualized by anti-REPO antibody, magenta) in the same specimen as in I. Most of the cells expressing *gcm-lacZ* are REPO-positive glia (yellow arrow). (K) Three-dimensional image of *gcm-lacZ* expression in a brain mutant for *hh*^{bar3} (visualized by anti-β-GAL antibody, green). *gcm-lacZ* expressing cells form an almost normal crescent shape; however, expression in the presumptive lamina region is decreased. (L) Anti-DAC antibody staining of the *hh*^{bar3} mutant brain (magenta), shown together with *gcm-lacZ* (green). No DAC-positive cells are detected. (M) Double staining with anti-β-GAL (green) and anti-REPO (magenta) antibody in the same specimen as in K. Most of the β-GAL-positive cells are glia; however, REPO- and DAC-negative cells were also present. Scale bar: 50 μm.

gcm-lacZ^{rA87}, which has been shown to faithfully reflect the expression pattern of endogenous *gcm* in the embryo (Jones et al., 1995; Vincent et al., 1996). β-GAL signal was observed in the glia lineage, including in precursors at the margin of lamina target region, which is virtually the same pattern as that described by Dearborn and Kunes (Dearborn and Kunes, 2004) when using an anti-GCM antibody (Fig. 4A,A′). β-GAL signal was detected in mature epithelial and marginal glia (Fig. 4C), which were also positive for *omb-Gal4* (Fig. 4B). When cells migrate into the targeting region, REPO expression is observed (Fig. 4A,A′). In addition, *gcm* expression is seen in the developing lamina neurons (Fig. 4D). With these markers, lamina glia differentiation can be monitored right from the stage when cell fate is specified.

gcm expression in the lamina glia depends on *dpp* activity in the optic lobe

To address whether *gcm* expression depends on R axon input or factors in the optic lobe, we analysed *gcm* expression in brains mutant for *sine oculis* (*so*). In this mutant, eye formation is defective (indeed, the eyes are almost completely absent) and, thus, there is no R axon projection into the optic lobe

(Kunes et al., 1993; Pellez and Steller, 1996). *gcm* expression was observed together with REPO in this mutant background (Fig. 4E,F). In addition, glial cell migration occurred to some extent, although these glial cells fail to form the normal three-layer structures (Fig. 4G). This indicates that R axon innervations are necessary for the lamina glia to efficiently migrate and form regular three-layer structures, a finding that is consistent with previous reports (Perez and Steller, 1996; Huang and Kunes, 1998; Suh et al., 2002; Dearborn and Kunes, 2004). In addition, glia differentiation and migration also depends upon a factor within the brain.

To address whether this factor in the brain is indeed *dpp*, we analysed the brains of *dpp* loss-of-function mutants (Kaphingst and Kunes, 1994) (dpp^{d6}/dpp^{d12} carrying *gcm-lacZ*). A severe reduction in the number of *gcm-lacZ* expressing cells was observed in these brains (Fig. 4I). These cells co-express REPO, indicating that they are glial cell populations (Fig. 4J). There is no R axon input in brains with this allelic combination of *dpp*, as this combination disrupts the normal contribution of *dpp* to the eye formation (Wiersdorff et al., 1996; Pignoni and Zipursky, 1997; Curtiss and Mlodzik, 2000). However, the decrease of *gcm*- and REPO-expressing cells was more prominent than that in the so^{1} mutant, which also lacks R axon innervations (Fig. 4E,F,I,J). Together, these data suggest that the further reduction in the number of *gcm*-expressing cells is due to a reduction of *dpp* activity. In addition, *gcm* expression in these mutant brains was not completely lost, possibly because they are disc-specific regulatory mutants that might still have residual *dpp* activity.

As previously mentioned, Hh induces the differentiation of lamina neurons. DAC-positive lamina neurons fail to differentiate in hh^{bar3}, the eye-specific allele of *hh* (Heberlein et al., 1993; Huang and Kunes, 1998) (Fig. 4K,L). We observed that *gcm* expression and REPO-positive glia differentiation and migration were still observed in hh^{bar3} mutant brains (Fig, 4K,M), which is consistent with results reported by Huang and Kunes (Huang and Kunes, 1998), who observed REPO expression in hh^{bar3} mutant brains. Conversely, *gcm* expression in the lamina neuron appeared to be significantly reduced (Fig. 4M). This result suggests that *gcm* expression in the lamina depends on Hh, and that its expression is controlled independently in these two cell populations.

We also observed that there are *gcm*-positive cells that are neither REPO- nor DAC-negative, which are not present in the target region of the wild-type brain. These cells could be glia-type cells that remain in an immature state, as it has been shown that R axon innervation plays a role in the terminal differentiation of glial cells (Perez and Steller, 1996). It is also possible that these are fated to the lamina but fail to differentiate because of a lack of R axon innervations.

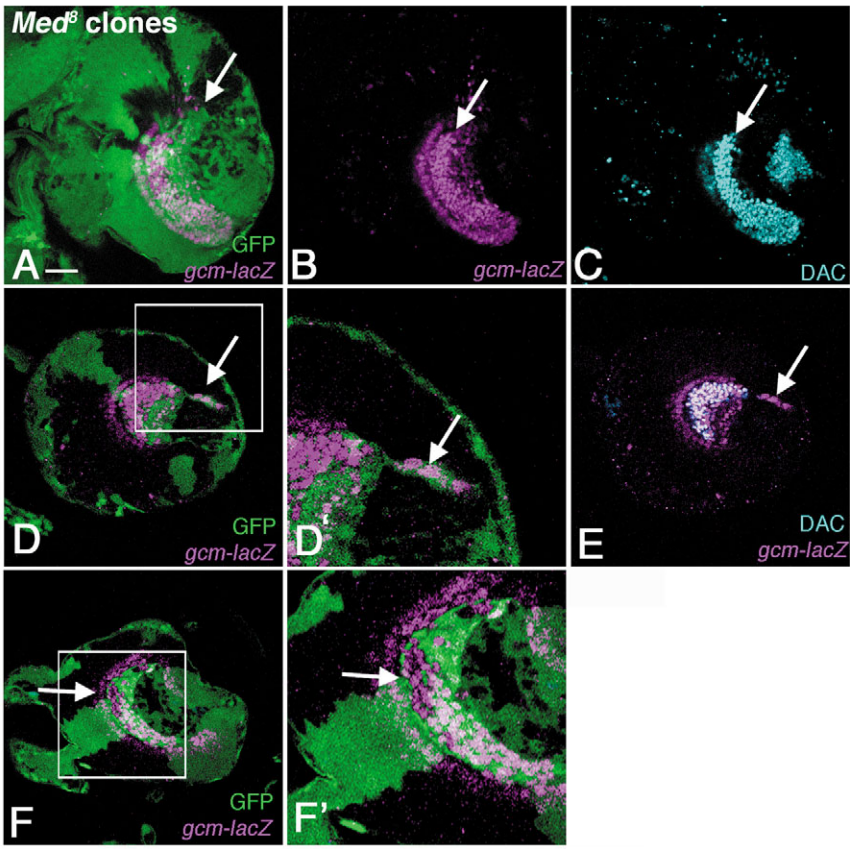

Fig. 5. *Medea* is required autonomously for the expression of *gcm*. (A-C) Confocal image of an optic lobe expressing the *gcm-lacZ*^{rA87} reporter and containing *Medea*^{8} mutant clones at the posterior-dorsal domain. Reporter expression visualized with anti-β-GAL antibody (magenta); lamina neurons visualized with anti-DAC antibody (blue). (A) The position of the clone (arrow) and the expression pattern of *gcm-lacZ* revealed by anti-β-GAL antibody (magenta) are shown. (B,C) β-GAL (magenta, B) and DAC (blue, C) expression in the same specimen as in A. Expression of *gcm-lacZ* and DAC are compromised near the border of the clone (arrows in A-C). (D,E) Confocal image from another specimen. (D) The border of wild-type and *Medea*$^{-/-}$ cells coincides the border of *gcm*-expressing cells (arrows in D). (D′) Magnified image of the boxed area in D. (E) Double labeling with anti-DAC (blue) and anti-β-GAL (magenta) antibodies in the same specimen as in D. The migrating β-GAL-positive cells (arrow) are negative for DAC, indicating these are glial cells. (F) Confocal image of the optic lobe with *Medea*^{8} clones inside the lamina target region. A small clone is visible in the lamina plexus region (arrow in the boxed area in F, a magnified image is shown in F′). *gcm-lacZ* is expressed (anti-β-GAL, magenta) in the clone inside of the lamina target region (arrow in F′). Scale bar: 50 μm.

Cell-autonomous requirement of *Medea* activity for the expression of *gcm* in the lamina glia

To further address the requirement of DPP signaling in lamina glia development, *gcm* expression in *Medea* mutant clones was examined. When large clones were generated in the posterior domain, defects in *gcm-lacZ* expression were observed (Fig. 5, n=10). In the specimen shown in Fig. 5A-C, defects in the expression of *gcm* in the lamina were observed as well (Fig. 5B). In such cases, DAC expression was also defective and appeared truncated near the edge of the mutant clone (Fig. 5C). Conversely, *Medea* mutant clones generated in the OPC and

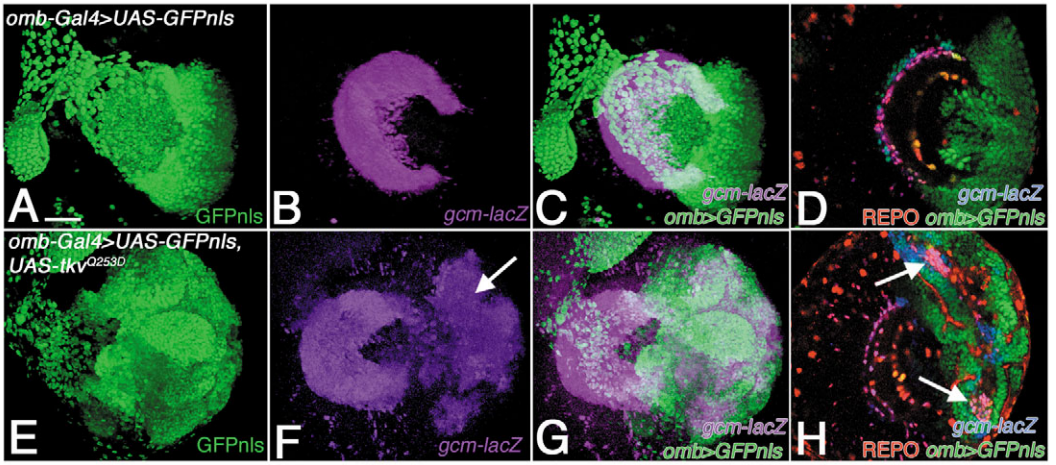

Fig. 6. Activation of DPP signaling in the posterior domain results in the ectopic induction of *gcm* and REPO-positive glial cells. (A-C) Three-dimensional image of a wild-type optic lobe carrying the *omb-Gal4* driver, with *UAS-GFPnls* (green, A) and *gcm-lacZ* expression visualized using anti-β-GAL antibody (magenta, B). (C) Merged image of A and B. (D) Confocal image of the wild-type optic lobe showing *gcm-lacZ* (anti-β-GAL, blue), *UAS-GFPnls* driven by *omb-Gal4* (green), and REPO expression (anti-REPO, red). (E-G) Three-dimensional image of the optic lobe carrying the *omb-Gal4* driver, with *UAS-GFPnls* (green, E), and *gcm-lacZ* visualized by anti-β-GAL antibody (magenta, F). (G) Merged image of E and F. An ectopic cluster of β-GAL-expressing cells is seen in the posterior domain (arrow in F). (H) Confocal image of the optic lobe with tkv^{Q253D} expression by *omb-Gal4*. REPO expression (anti-REPO, red), *gcm-lacZ* (anti-β-GAL, blue), and *UAS-GFP* driven by *omb-Gal4* (green) are shown. REPO-positive glial cells are visible inside of the domains that ectopically express *gcm-lacZ* (arrows). Scale bar: 50 μm.

lamina do not affect DAC expression (Fig. 2I,J) or *gcm-lacZ* expression (not shown). Therefore, we conclude that it is likely that this defect in the lamina is indirectly caused by defects in the development of lamina glial cells, rather than by a direct effect on lamina development. In the specimen shown in Fig. 5D,E, only a small number of wild-type cells expressed *gcm-lacZ* in the migratory route from the presumptive progenitor zone, and no mutant cells did. These results suggest that DPP signaling is required autonomously in these cells for the expression of *gcm*.

When small clones were generated inside the lamina target region, the mutant cells expressed *gcm-lacZ* (Fig. 5F). This suggests that once the glial cells migrate into the lamina target region, the DPP signal is no longer required.

Ectopic activation of DPP signaling in the posterior domain induces *gcm* and REPO expression

The above results suggest that *dpp* is required for *gcm* expression and for differentiation of the lamina glia lineage. We next investigated whether ectopic activation of DPP signaling induces glia differentiation. To this end, we expressed *UAS-*tkv^{Q253D}, a constitutively active form of *tkv* (Wiersdorff et al., 1996), with an *omb-Gal4* driver. An ectopic *gcm* expression domain was induced in these brains (Fig. 6F; n=15), and REPO-positive cells were induced inside of the ectopic *gcm* domain (Fig. 6H), indicating that overactivation of DPP signaling can induce ectopic glia differentiation. Thus, cells in the *omb* domain are able to express *gcm* in response to the *dpp* signal, and subsequently develop into glial cells when they receive higher-than-endogenous levels of the signal.

By contrast, when tkv^{Q253D} was expressed in the OPC, neither *gcm* nor REPO expression was induced (data not shown). This suggests that only cells in the posterior domain of the optic lobe are competent to become glia in response to DPP signaling.

gcm controls differentiation of the lamina glia

From the above results, we conclude that *dpp* controls *gcm* expression in lamina glia progenitors. Given the role of *gcm* in the embryo for the determination of glial cell fate, it is likely that *gcm* controls the differentiation of lamina glial cells as well. The *gcm* gene exists as a cluster with the homologous gene *gcm2*, which may act redundantly in some contexts (Kammerer and Giangrande, 2001). We made use of the *UAS-*$glide^{DN}$ construct, which encodes a chimeric protein that combines (1) a DNA-binding domain that is highly conserved between *gcm* and *gcm*2, and (2) the repressor domain of Engrailed, which renders the chimeric protein capable of blocking the function of both *gcm* and *gcm*2 (Soustelle et al., 2004). When $glide^{DN}$ was induced via *omb-Gal4*, R axon projection defects associated with defects in lamina morphology were observed (Fig. 7F,G; n>20). In these brains, very few REPO-positive cells can be seen in the presumptive lamina target region (Fig. 7H), indicating that the differentiation of the lamina glia is compromised. This defect was not observed with *UAS-*$glide^{N7\text{-}4DN}$, which carries a point mutation in the DNA-binding domain that makes the protein unable to bind DNA (Soustelle et al., 2004). These data confirm that the phenotype observed with the *UAS-*$glide^{DN}$ is the result of the specific blocking of *gcm* function rather than a non-specific effect of the chimeric protein. Taken together, the results suggest that *gcm* is indeed required for lamina glia differentiation.

As mentioned, *gcm* is also expressed in the lamina precursors/neurons. To investigate the function of *gcm* in the lamina, we expressed *UAS-*$glide^{DN}$ in the lamina using *NP6099-Gal4* (Hayashi et al., 2002) (D.U., S.M. and T.T.,

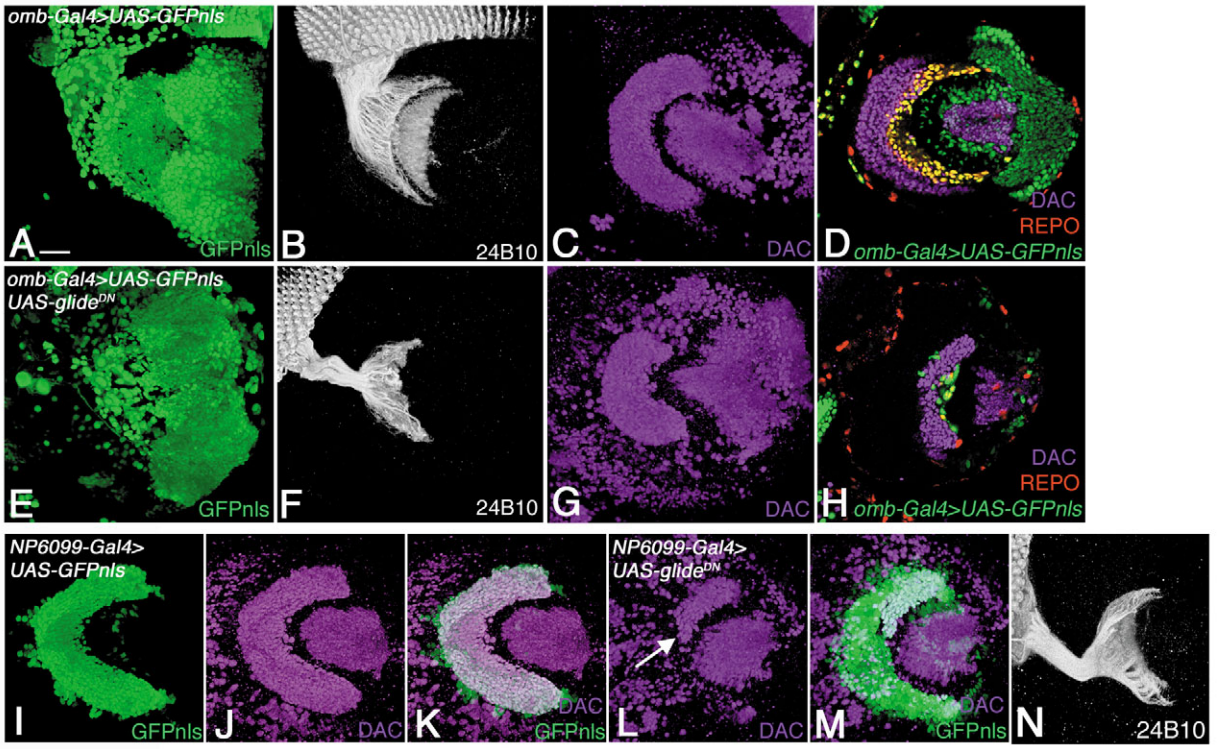

Fig. 7. *gcm* controls differentiation of the lamina glia and lamina neurons. (A-C) Three-dimensional image of a wild-type optic lobe. (A) The expression pattern of *omb-Gal4* visualized by *UAS-GFPnls* (green). (B) R axons are revealed by mAb24B10 (white). (C) The lamina neurons are visualized by anti-DAC antibody (magenta). (D) Confocal image of the lamina target region in the wild-type optic lobe. Lamina neurons are visualized with anti-DAC (magenta), mature glial cells in the target region are visualized with anti-REPO (red) and *omb-Gal4* expression is visualized by *UAS-GFPnls* (green). Mature glial cells are doubly positive for REPO and GFP, shown in yellow. (E-H) Three-dimensional image of the optic lobe with expression of *UAS-glide*^{DN} driven by *omb-Gal4*. (E) Expression of the driver is visualized with *UAS-GFPnls* (green). (F) mAb24B10 staining in the same specimen (white); the R-axon projection pattern is compromised. (G) Anti-DAC staining in the same specimen (magenta). The lamina is smaller than in wild type and the morphology is defective. (H) Confocal image showing the lamina target region in the optic lobe expressing *glide*^{DN} with the *omb-Gal4* driver. Lamina neurons, glial cells and *omb*-expressing cells are visualized as in D. There is a smaller number of glial cells in the target region than in wild type. (I) Three-dimensional image of the wild-type optic lobe, in which the expression pattern of *NP6099-Gal4* is visualized by *UAS-GFPnls*. (J) Anti-DAC staining of the same specimen as in I. (K) Merged image of I and J, showing that *Gal4* expression coincides with the developing lamina neurons. (L) Three-dimensional image of the optic lobe where *UAS-glide*^{DN} expression is induced in the developing lamina neurons by *NP6099-Gal4*. A reduction of DAC-positive cells is observed (arrow). (M) The same specimen as in L, shown with expression of the driver (green). (N) mAb24B10 staining (white) in the same specimen as in L and M. R axons project to the presumptive lamina target region with an irregular pattern. Scale bar: 50 μm.

unpublished) (Fig. 7I-K). A severe reduction in the number of DAC-positive lamina cells was observed in this situation (Fig. 7L,M; n=12). In such specimens, R-axon projection to the presumptive lamina target field is still observed, although the pattern is not completely normal (Fig. 7M). *glide*$^{N7\text{-}4DN}$ did not cause such defects, again confirming the specificity of the phenotype. These data suggest that *gcm* activity is also required for lamina neuron differentiation.

Discussion

The establishment of the correct R axon projection patterns is crucial for the construction of the visual system in *Drosophila*. The complex set of events that controls axon projection includes interplay between axons and their targets: the R axons need to be positioned for establishing contacts with their synaptic targets and, conversely, the lamina relies on the R axons as a source of factors required for their own development (Huang and Kunes, 1996; Huang et al., 1998). The lamina glia play a crucial role in this process by serving as intermediate targets for R1-R6 axons (Poeck et al., 2001). Here, we have shown that *dpp*, which is expressed in the dorsal and ventral marginal regions in developing lamina target region, plays a key role in the development of the optic lobe by inducing the expression of *gcm* to initiate a genetic program for lamina glia differentiation (summarized in Fig. 8).

dpp regulates the differentiation of lamina glial cells directly

wg at the posterior-most domain induces the expression of *dpp* and *omb* (Huang and Kunes, 1998; Song et al., 2000). Some *wg*-expressing cells extend projections towards the lamina target region. These cells extend scaffold axons along which the lamina glia migrate (Dearborn and Kunes, 2004). Thus, it was possible that the *wg* signal is involved in the migration and/or differentiation of lamina glia. However, partial elimination of Wg activity with a *wg*^{ts} allele does not cause a specific defect in glia migration (Dearborn and Kunes, 2004).

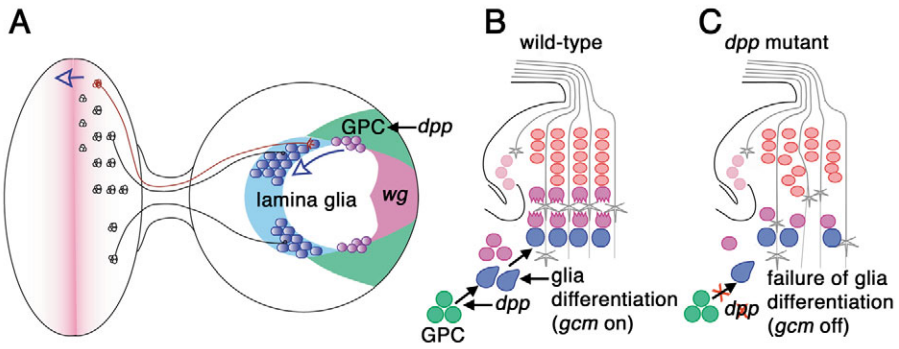

Fig. 8. Model showing how DPP signaling controls differentiation of the lamina glia. (A) *dpp* expression (green) is induced by *wg* (magenta) in the lamina glia progenitor domains, then *dpp* induces the expression of *gcm* in GPCs (purple), triggering the lamina glia (blue) differentiation program. (B) As glia migrate, they contact R axons, leading to the formation of the lamina plexus by the R1-R6 growth cones. Signals from R axons facilitate the migration and maturation of the lamina glia, leading to formation of the correctly layered structure. (C) In the absence of DPP signaling, *gcm* expression is not induced and lamina glia fail to differentiate. R axons fail to find their intermediate partner and mis-target, resulting in an irregular induction of lamina neurons and thus the characteristic crescent-shaped lamina fails to form.

Therefore, *wg* may play a role in organizing domains in the visual cortex by activating/repressing various genes, rather than contributing to the generation of specific cell types (Song et al., 2000).

We have shown that *Medea* is required for lamina glia development. *Medea* encodes a co-SMAD and mediates a range of DPP/BMP/TGFβ signaling events (reviewed by Mehler et al., 1997). In addition to *dpp*, four related genes – *glass bottom boat* (*gbb*), *screw*, *activin* and *activin2* – have been identified in *Drosophila*. GBB signals through TKV/Saxophone (SAX) and Wishful Thinking (WIT) type I and type II receptors, respectively (MacCabe et al., 2003). Activin uses Baboon as a type I receptor, and Punt and WIT as type II receptors (Zheng et al., 2003). We examined brains mutant for *gbb* and *wit*, but failed to observe any defects in lamina glia development (data not shown). Together with the results presented here, then, we conclude that it is highly likely that *dpp* is the ligand responsible for lamina glia development. However, we cannot exclude the possibility that one or more of the DPP-related ligands acts redundantly in this process.

The regulation and role of *gcm* in cells of distinct lineages

In the embryo, *gcm* initiates the specification of glial cells from neural cells of various lineages. *gcm* expression is strictly controlled to ensure the correct separation of glial versus neuronal cell fate. Analysis of the *cis*-regulatory elements of *gcm* suggests that *gcm* expression depends on multiple regulatory elements to allow the control of lineage-specific transcription and autoregulation (reviewed by Jones, 2004). Our analysis suggests that a different situation exists in the optic lobe; *gcm* is expressed in the glia and the lamina neuronal cells, and is required for the differentiation of these cell types. In addition, differentiation is controlled differently in the lamina and in the glia. In the lamina, *gcm* expression seems to be controlled by *hh*, and in the glia, by *dpp*. These results suggest that *gcm* is controlled and functioning in a different manner in the optic lobe. Uncovering the mechanisms of the control and function of *gcm* would probably prove an intriguing focus for future research.

DPP signaling in the visual system development

DPP and its vertebrate homolog BMP play crucial roles in many aspects of development by controlling patterning, cell growth and differentiation. Our analysis reveals a role for DPP signaling in lamina glia differentiation in the *Drosophila* visual system. DPP has also been reported to function in several aspects of visual center development; for instance, DPP signaling has been shown to be involved in the proliferation and migration of the subretinal glia in eye disc development, which plays an important role in the R axon navigation (Rangarajan et al., 2001; Hummel et al., 2002). In addition, Kaphingst and Kunes (Kaphingst and Kunes, 1994) reported defects in the medulla neuropile in *dpp* mutant animals, suggesting a role for *dpp* in neuronal fate specification. Furthermore, *tkv* is expressed in lamina precursor cells just ahead of the lamina furrow, where these cells meet R axons and start to differentiate (H. Tanimoto and T.T., unpublished). Although this possibility is one of the things that prompted us to look for a role of DPP signaling in lamina development, we failed to uncover any defects when *Mad* or *Medea* clones were generated in the OPC or the lamina. Moreover, *dpp* appears to be expressed in the inner proliferation center (IPC), which will form the lobula, in addition to its expression in the dorsal and ventral marginal domains. Thus, *dpp* may be required for some aspects of lobula development. Unfortunately, this cannot be easily addressed at this moment because of a lack of appropriate markers. Further study of the requirements for *dpp* in the lamina, the medulla, the lobula and other cell types could lead to a more comprehensive understanding of how DPP signaling controls differentiation and other events during development of the visual system.

We thank the Bloomington Stock Center and the Kyoto Stock Center for fly stocks. We would also like to thank S. B. Selleck for Cyclin A antibodies, and the Genetic Studies Hybridoma Bank for antibodies. We are grateful to all the members of Tabata laboratory for helpful discussions during the course of the work and, in particular, we thank Yuki Takei for valuable comments on the manuscript. We thank Yuko Maeyama for her excellent technical help. This work was supported by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan, and by Toray Science Foundation.

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