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RESEARCH ARTICLE

A transcriptional network controlling glial development in the *Drosophila* visual system

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ABSTRACT

In the nervous system, glial cells need to be specified from a set of progenitor cells. In the developing *Drosophila* eye, perineurial glia proliferate and differentiate as wrapping glia in response to a neuronal signal conveyed by the FGF receptor pathway. To unravel the underlying transcriptional network we silenced all genes encoding predicted DNA-binding proteins in glial cells using RNAi. Dref and other factors of the TATA box-binding protein-related factor 2 (TRF2) complex were previously predicted to be involved in cellular metabolism and cell growth. Silencing of these genes impaired early glia proliferation and subsequent differentiation. Dref controls proliferation via activation of the Pdm3 transcription factor, whereas glial differentiation is regulated via Dref and the homeodomain protein Cut. Cut expression is controlled independently of Dref by FGF receptor activity. Loss- and gain-of-function studies show that Cut is required for glial differentiation and is sufficient to instruct the formation of membrane protrusions, a hallmark of wrapping glial morphology. Our work discloses a network of transcriptional regulators controlling the progression of a naïve perineurial glia towards the fully differentiated wrapping glia.

KEY WORDS: *Drosophila*, Glial proliferation, Glial differentiation, FGF signalling, Transcription factors, Pdm3, Dref, Cut

INTRODUCTION

The specification of different neural cell types is a major step during the development of the nervous system. A first decision takes place on the level whether a neural cell develops into a neuron or a glial cell. Whereas in vertebrates no real master gene has been identified yet to control this lineage switch, work in *Drosophila* has revealed that activation of *glial cells missing* (*gcm*) defines glial fate (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996). *gcm* regulates the expression of a large number of target genes, including some that inhibit neuronal differentiation (Altenhein et al., 2006; Cattenoz and Giangrande, 2015; Freeman et al., 2003; Giesen et al., 1997). The exact mechanisms underlying the control of glial differentiation, however, remained elusive.

In the *Drosophila* nervous system, five main glial cell types are known: perineurial glia, subperineurial glia, wrapping, or ensheathing, glia, astrocyte-like glia and cortex glia (Awasaki et al., 2008; Edwards et al., 2012; Hartenstein, 2011; Silies et al., 2007; Stork et al., 2008). Gliogenesis has been particularly well studied during embryonic stages in which all glial cell types are identified by specific lineage markers (Beckervordersandforth et al., 2008; von Hilchen et al., 2008, 2013). A second phase of gliogenesis is observed at the onset of metamorphosis (Awasaki et al., 2008). The compound eyes are generated from the eye imaginal discs. Within each disc, ~6000 photoreceptor neurons develop that project their axons through the optic stalk into the forming visual centres of the brain (Tayler and Garrity, 2003). The photoreceptor axons are accompanied by glial cells which are born during late larval stages (Choi and Benzer, 1994; Rangarajan et al., 1999; Silies et al., 2007). Their progenitor cells are perineurial glial cells that possibly stem from the central nervous system (CNS) as the majority of the peripheral glial cells in the abdominal segments (von Hilchen et al., 2008), but this has not been carefully addressed in the developing visual system.

Three main glial cell types are found on the eye disc: perineurial glia, subperineurial glia and wrapping glia. The development of wrapping glia in the *Drosophila* eye depends on axonal contact and FGF receptor (FGFR) activity (Franzdóttir et al., 2009). Whereas low levels of FGFR activity are permissive to proliferation and migration of perineurial glia, a short peak of FGFR activity is needed to route perineurial glia towards a wrapping glial cell identity (Sieglitz et al., 2013).

To unravel the regulatory network that, in addition to the FGFR, is required for the progression from the perineurial cell towards the differentiated wrapping glial cell, we performed a glia-specific RNAi-based screen. We identified three glial-expressed transcription factors with specific roles in proliferation and differentiation. Dref activates Pdm3 in perineurial glia to control glial proliferation. Subsequent differentiation of wrapping glia is regulated by the homeodomain protein Cut, the expression of which is activated by Dref and FGFR signalling. Gain-of-function experiments show that Cut is sufficient to instruct the formation of long cell processes characteristic for wrapping glia, demonstrating that *cut* encodes a key transcription factor orchestrating wrapping glial differentiation.

RESULTS

Transcriptional regulators controlling glial cell development in the visual system

The *Drosophila* eye imaginal disc harbours ~300 glial cells (Fig. 1A). The perineurial glia migrate onto the eye imaginal disc basal to the subperineurial glia (Fig. 1B, summary of Gal4 driver strains and markers used in this study). The perineurial and the subperineurial glia can be targeted by *c527-Gal4* and *SPG-Gal4*, respectively. About 50 perineurial cells differentiate as wrapping glia to ensheathe the photoreceptor axon fascicles in the optic stalk (Fig. 1C,D). These glial cells express $Sprouty^{JacZ}$ and can be targeted using *Mz97-Gal4* (Fig. 1B). Differentiation into wrapping glia is in part controlled by FGFR signalling (Franzdóttir et al., 2009; Sieglitz et al., 2013). To identify the underlying regulatory transcriptional network we suppressed the expression of genes encoding DNA- and RNA-associated proteins specifically during glial development. To control for unspecific RNAi effects we

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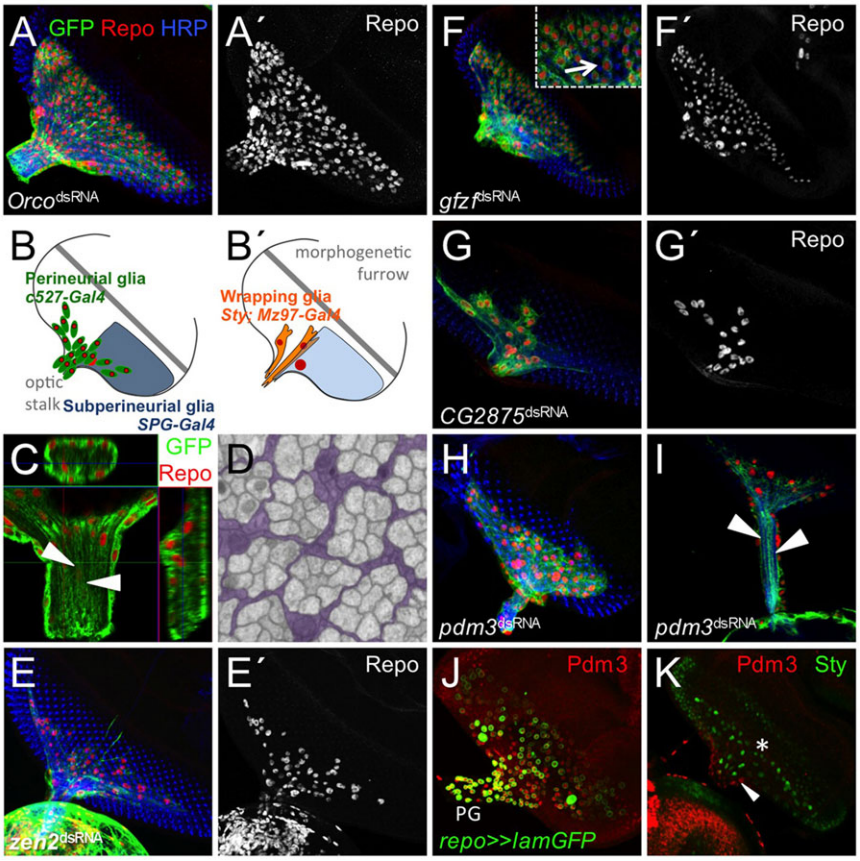

Fig. 1. Transcription factors required for proliferation of eye disc glial cells. (A) Projection of a confocal stack of a third-instar eye disc expressing *Orco*^{dsRNA} in all glial cells used as control for the RNAi experiments. Glial nuclei expressing Repo (red), glial cell membranes are shown in green (*repo>>CD8GFP*) and neuronal membranes are shown in blue (HRP staining). (A′) Glial cell nuclei are distributed evenly on the eye disc. (B,B′) Schematic views of an eye disc. Markers used in this study are indicated. (B) The basal-most layer harbours the perineurial glial cells which migrate on the subperineurial glia. (B′) The wrapping glia is located between the subperineurial glia and the disc proper and extends processes towards the optic stalk. (C) Single optical section through an optic stalk. Note the wrapping glial membranes within the stalk region (arrowheads). (D) TEM analysis of a cross-section through a wild-type optic stalk. All eight axons derived from one ommatidium are covered by processes of the wrapping glia (purple). (E,E′) Knockdown of *zen2* results in a reduction of glial cell number in the eye imaginal disc. (F,F′) Pan-glial downregulation of *gfzf* (GST-containing FLYWCH zinc-finger) results in moderately reduced glial cell number. Glial cells are sometimes detached from their neighbours (arrow in insert). (G,G′) Knockdown of *CG2875* results in few undifferentiated glial cells in the eye disc. (H) Pan-glial silencing of *pdm3* results in a reduced glial cell number. (I) Single confocal section of an eye disc with pan-glial silencing of *pdm3.* Note that wrapping glial cell processes can still be detected in the optic stalk (arrowheads). (J) Pdm3 is expressed in glial cells. High levels of expression are found in the perineurial glia (PG) in the optic stalk. (K) Pdm3 and the wrapping glial cell marker *Sprouty*^{lacZ} (Sty, asterisk) are expressed in distinct set of glial cells (arrowhead denotes Pdm3-expressing perineurial glia).

silenced the activity of the *Odorant receptor co-receptor* (*Orco*) gene, which is not expressed in the larval CNS (FlyBase; Fig. 1A,C).

For 833 genes UAS-based effector lines were available through the Vienna *Drosophila* Resource Center (VDRC), the *Drosophila* Genome Resource Center (DGRC) or the Transgenic RNAi Project (TRiP) collection in Bloomington and were crossed to a *repo-Gal4, UAS-CD8GFP* strain (see Materials and Methods; supplementary material Table S1). In 160 of the crosses, we noted lethality upon expression of the respective dsRNA in all glial cells (see supplementary material Fig. S1 for a flow diagram). For 127 of these crosses the dsRNA effector lines showed less than ten predicted off-targets. In 113 of the crosses, the knockdown animals survived until late larval or pupal stages, thus allowing histological analyses. For each genotype with surviving larvae, GFP-expressing eye disc glia of at least eight larvae were analysed (supplementary material Table S2). Glial-specific silencing of 45 genes resulted in no discernible abnormalities in the eye disc glia, whereas silencing of 68 genes resulted in penetrant phenotypes (Figs 1-3; supplementary material Table S2).

Transcriptional regulators required to adjust glial cell number

Most genes identified in our screen affected glial cell number, and only in a few cases did we note phenotypes with apparently normal glial number but impaired glial differentiation (see below). As all candidates were silenced in a pan-glial manner throughout development, phenotypes might be due to early defects. Of the 68 genes identified in this study, 54 appeared to be required for the generation of the 300 glial cells normally found in the eye disc (Fig. 1; supplementary material Table S2). Among these are genes encoding the Zen2 homeodomain protein or the zinc-finger protein Gfzf (GST-containing FLYWCH zinc-finger) (Fig. 1E,F). Silencing of 23 genes resulted in a dramatic reduction of glial cell number with less than 30 glial cells on the eye imaginal disc, e.g. *CG2875* encoding a CCAAT-binding factor (Fig. 1G). In most cases, when reduced numbers of glial cells are formed they still evenly cover the eye disc, suggesting that lateral interactions exist to ensure an even spacing of glial cells. We never noted signs of apoptotic nuclei and thus assume that the factors identified here control proliferation rather than apoptosis.

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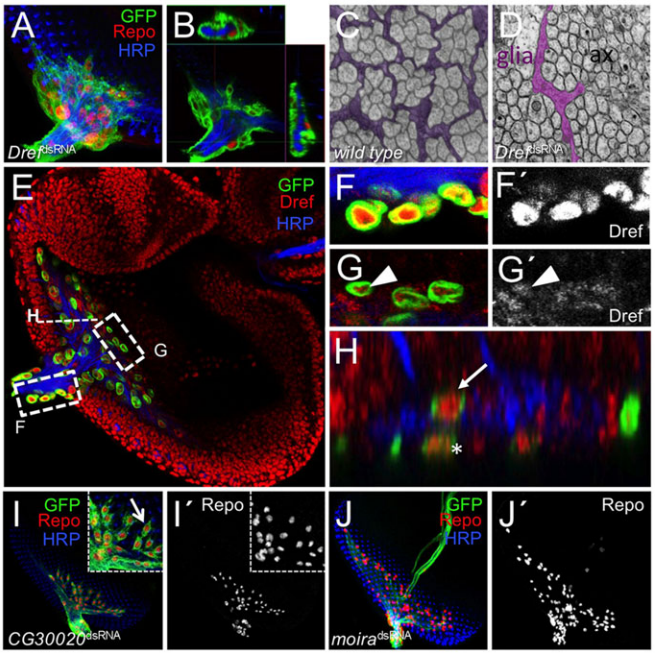

Fig. 2. Dref is required for glial proliferation and differentiation. (A,B,I,J) Glial cell nuclei are labelled with Repo (red), glial cell membranes are shown in green (*repo>>CD8GFP*) and neuronal membranes are shown in blue (HRP staining). (E-H) Dref expression is shown in red, glial nuclei are highlighted in green (*repo>>laminGFP*) and neuronal membranes are shown in blue (HRP staining). (A) Pan-glial Dref silencing (using the line *GL00532*) affects glial cell number. (B) In addition, glial silencing of Dref inhibits glial differentiation, and almost no wrapping glial cell processes could be seen in the optic stalk. The orthogonal sections highlight the absence of glial membranes within the optic stalk. (C) Transmission electron microscopic (TEM) image of a wild-type control. Note that the eight axons of each ommatidium are ensheathed by glial membranes (coloured in purple). (D) TEM image of a cross-section through an optic stalk after pan-glial *Dref* silencing. No wrapping glial cell process can be detected between photoreceptor axons (ax). The purple shading indicates one remaining glial process. (E) Dref is ubiquitously expressed in the eye imaginal disc. Boxed regions are shown in higher magnifications, as indicated. (F,F′) High levels of Dref are found in perineurial glia at the optic stalk. (G,G′) Reduced levels of Dref are found in glial cells at the migration front (arrowheads). (H) Orthogonal section of stack shown in E. Strong expression of Dref can be seen in wrapping glial cells (arrow), weak expression can be seen in migrating perineurial glia (asterisk). (I,I′) Pan-glial knockdown of *CG30020* results in very few glial cells on the eye disc, which are often migrating as single cells (arrow). (J,J′) Pan-glial knockdown of *moira* results in a reduction of glial cell number in the eye imaginal disc.

The POU domain transcription factor Pdm3 is required for glial proliferation

During glial development on the eye disc the perineurial glia divide and are able to differentiate into wrapping glia upon contact with nascent photoreceptor axons. Thus, reduced numbers of perineurial glia might result in reduced wrapping. This, however, is not necessarily the case. The knockdown of *pdm3* reduces glial cell number (using two independent RNAi constructs: 11641R-2 and 103926). The remaining wrapping glia send long processes into the optic stalk, indicating that glial differentiation is still intact (Fig. 1H, I, arrowheads). As POU domain proteins are known to be involved in gliogenesis (Umesono et al., 2002), we determined the expression of Pdm3 using previously developed antibodies (Chen et al., 2012; Tichy et al., 2008). Pdm3 is indeed expressed by glia in the eye disc that are predominantly located in the optic stalk (Fig. 1J). Pdm3 expression is not detected following glia-specific RNAi (supplementary material Fig. S2A,B). Lack of co-staining of Pdm3 expression with the wrapping glial marker *Sprouty*^{lacZ} indicates that Pdm3 is predominantly found in perineurial glia (Fig. 1K). In summary, Pdm3 is expressed in perineurial glial cells, where it is required for the expansion of the glial progenitor pool, but Pdm3 appears dispensable for differentiation into wrapping glia.

Dref controls glial proliferation and differentiation

As observed upon *pdm3* knockdown, silencing of the *DNA replication-related element binding factor* (*Dref*) results in a reduction of glial cell number (Fig. 2A). In contrast to *pdm3* suppression, however, *Dref* knockdown also affects the differentiation, as wrapping glia and no glial processes can be found in the optic stalk (Fig. 2B). Pan-glial silencing of *Dref*, using three independent dsRNA variants (*GL00532*, *GLV21507*, *JF02232*), resulted in reduced glial cell numbers with a concomitant defect of wrapping glial cell differentiation. Dref protein could not be detected in glial cells upon *repo-Gal4*-driven *Dref* knockdown (supplementary material Fig. S2C,D). To analyse the *Dref* knockdown phenotype further we performed an ultrastructural analysis. In wild-type optic stalks, ommatidial axon fascicles are always covered by processes of wrapping glia, but no such processes are detectable upon *Dref* knockdown (Fig. 2C,D). Dref shows a ubiquitous nuclear expression (Hirose et al., 1996). To test for possible glial expression we stained for Dref expression in eye discs expressing the nuclear marker LaminGFP in all glial cells (*repo-Gal4, UAS-lamGFP*) (Fig. 2E-H). All glial cells express Dref. Strong Dref expression was noted in perineurial glia along the optic stalk (Fig. 2E,F), whereas reduced levels of Dref expression were noted in the perineurial glia when they first contact photoreceptor axons (Fig. 2E,G, arrowheads). Differentiated wrapping glial cells, which can be recognized based on their position in orthogonal sections, show increased levels of Dref expression (Fig. 2H). Moreover, Dref is co-expressed with the wrapping glial marker *Sprouty*^{lacZ} (supplementary material Fig. S2E). Dref is a component of the TBP-related factor 2 complex found at promoter sites of many genes involved in proliferation and cell growth (Hirose et al., 1996; Hochheimer et al., 2002; Killip and Grewal, 2012). Corroborating the effect of Dref on glial development, we noted that knockdown of genes encoding Dref-interacting proteins, such as *CG30020* or *moira*, also resulted in similar glial phenotypes (Fig. 2I,J). In summary, Dref shows glial expression and is required for glial cell number and differentiation.

cut is expressed by wrapping glial cells

Dref is thought to interact with the homeodomain transcription factor Cut (Matsukage et al., 2008). Pan-glial silencing of *cut* results in lethality during late third-instar stage with subtle glial defects (Fig. 3A,B, and see below). Glial cell number is not affected but individual glial cells appear to have a more rounded shape (Fig. 3B″). In addition, fascicles of the photoreceptor axons appeared irregular (Fig. 3B‴, arrowheads). *cut* encodes a large homeodomain containing a transcription factor (see Fig. 5) controlling external sensory organ development in *Drosophila* embryos (Bodmer and Jan, 1987). To determine the expression pattern of Cut we used Mab2B10, specifically binding the Cut protein (Blochlinger et al., 1990). Cut is expressed by glial cells from early embryonic stages onwards (Shandala et al., 2003). Glial expression starts during embryonic stage 13 and is found in almost all neuropil glia during stage 16. In the peripheral nervous system, Cut is expressed in the glial cells ePG1, ePG5 and ePG9 that will develop into the wrapping glia (von Hilchen et al., 2013; and

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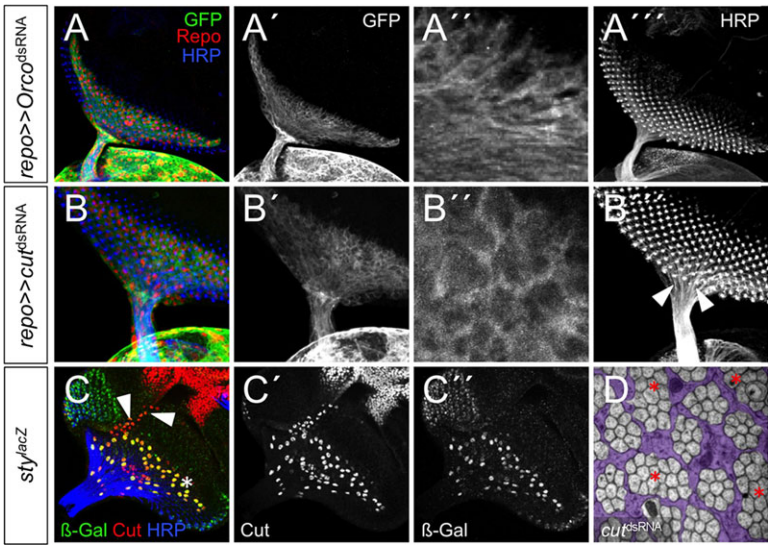

Fig. 3. Glial suppression of *cut* results in wrapping defects. (A,B) Projections of confocal stacks of third-instar eye discs. Glial nuclei are labelled with Repo (red), glial membranes are shown in green (*repo>>CD8GFP*) and neuronal membranes in blue (HRP staining). (A-A‴) Silencing of *Orco* expression in all glial cells was used as control. This does not affect glial cell number, migration or morphology. (B) Pan-glial knockdown of *cut* affects glial morphology. Glial cell number is normal but morphology of the wrapping glia appears affected (see A″,B″). (B‴) *cut* knockdown results in abnormal fasciculation of retinal axons (arrowheads). (C) Projection of confocal stacks of a third-instar eye disc showing only the glial cell layer. The wrapping glial marker *Sprouty*^{lacZ} (green) co-localizes with Cut (red) expression in wrapping glia (asterisk). The arrowheads point towards *Sprouty*^{lacZ}-negative, Cut-positive hemocytes (see supplementary material Fig. S3D). (D) TEM analysis of a cross-section through an optic stalk after pan-glial *cut* knockdown. Axon clusters are not completely separated by glial cell processes, and often axons of two clusters are fused together (red asterisks).

see supplementary material Fig. S3A,B). During third-instar stages Cut expression is found in only a few glial cells (Fig. 3C). To test the identity of the Cut-expressing cells we used the wrapping glia-specific marker *Sprouty*^{lacZ} (Franzdóttir et al., 2009). All *Sprouty*^{lacZ}-positive cells express Cut (Fig. 3C, asterisk). Similarly, glial cells expressing the wrapping glial marker *Mz97-Gal4* express Cut (supplementary material Fig. S3C). Furthermore, Cut expression is found in the subperineurial glia (supplementary material Fig. S3C,F, arrows). In addition to the glial Cut expression, we noted that small cells adhering to the eye imaginal disc express Cut but not *Sprouty*^{lacZ} (Fig. 3C, arrowheads). As these cells express the hemocyte marker Hemolectin, we conclude that these adhering cells are hemocytes (supplementary material Fig. S3D).

cut is required cell-autonomously during wrapping glial differentiation

The expression data suggest a specific role of *cut* during development of wrapping and subperineurial glial cells. To test the efficacy of the pan-glial RNAi mediated knockdown we stained for Cut expression. In knockdown animals, no Cut expression was noted in glial cells but Cut expression in cone cells was unaffected (supplementary material Fig. S4). Upon *cut* knockdown, we noted irregularities of ommatidial axon fascicles, which are indicative for differentiation defects of wrapping glia (Fig. 3B‴, arrowheads). To corroborate this notion we performed an transmission electron microscopy (TEM) analysis. Here, we frequently found ommatidial fascicles that are not separated from each other by glial membranes (Fig. 3D, red asterisks). These data indicate that Cut is expressed by wrapping glia and is required for their normal differentiation.

To test the role of *cut* in glial differentiation further we performed a MARCM analysis. For these studies we used *cut*^{C145}, a well-established null allele, and two glial-specific *repoflp* sources (Silies et al., 2007; Stork et al., 2014). Wild-type MARCM cell clones were generated as control for each of the strains. Wrapping glial cells were identified based on their characteristic elongated morphology and their association with axons (Fig. 4A-D). In 67% of all *repoflp1c*-induced wild-type MARCM clones we noted wrapping glial cells (n=41 eye discs). Within the optic stalk, the wrapping glial cells normally generate extensions projecting towards the inner optic stalk (Fig. 4B,D). By contrast, when we generated *cut*^{C145} mutant cell clones, only 33% of the eye imaginal discs exhibited glial cells with a wrapping glial-like morphology (Fig. 4E-J, n=44 eye discs). In addition, the differentiation of these cells seemed impaired and wrapping glial processes did not invade the centre of the optic stalk (Fig. 4F,H, arrowheads). Overall, the number of wrapping glial-like cells was greatly reduced, whereas the number of perineurial glial cells was increased (Fig. 4K). Similar results were obtained using the *repoflp6-2* construct. Mutant wrapping glial clones appeared in the same ratio and showed an incomplete differentiation (n=48 wild-type and n=34 mutant eye discs). Thus, genetic analysis shows that *cut* acts cell autonomously to regulate wrapping glial cell development.

Cut is able to induce wrapping glial-like differentiation

The experiments described above suggest that *cut* functions as an important regulator of wrapping glial differentiation. *cut* encodes at least four different isoforms with >2174 amino acids. All proteins are characterized by the presence of three evolutionarily conserved so-called Cut repeats (C1-C3) and a homeobox-type DNA-binding domain (Fig. 5A; Blochlinger et al., 1990, 1991). To test further the functional properties of *cut*, we performed gain-of-function studies. We generated a full-length UAS-based expression construct, allowing expression of isoform Cut^{PA}. In addition, we generated several Cut deletion constructs lacking either one of the Cut-repeats or the homeodomain, all inserted into the same chromosomal landing site 86Fb (Bischof et al., 2007; and see Fig. 5A).

Expression of full-length Cut in all glial cells using *repo-Gal4* results in embryonic lethality. When we overexpressed Cut in wrapping glia of the eye imaginal disc using the *Mz97-Gal4* driver line, animals died during pupal stages but no morphological consequences were apparent in the eye disc glia (data not shown). By contrast, ectopic Cut expression in perineurial glial cells caused the formation of extensive membrane protrusions in young third-instar larvae (*c527-Gal4*, see Fig. 5B,C). The perineurial glial cells are normally spindle-shaped cells that do not generate any prominent cell protrusions. In ∼90% of late third-instar larvae (n>50), Cut-expressing perineurial glial cells form long cell protrusions and overshoot on the basal side of the eye disc (Fig. 5C, arrow). In 10% of the eye imaginal discs, perineurial glial cells developed a pronounced bipolar shape spreading over the entire eye field (Fig. 5D,E). Eye imaginal discs that showed a particularly dramatic spreading of glial processes were usually covered with relatively few glial cells (Fig. 5D). As Gal4 expression levels are known to be variable, we anticipate that in these examples

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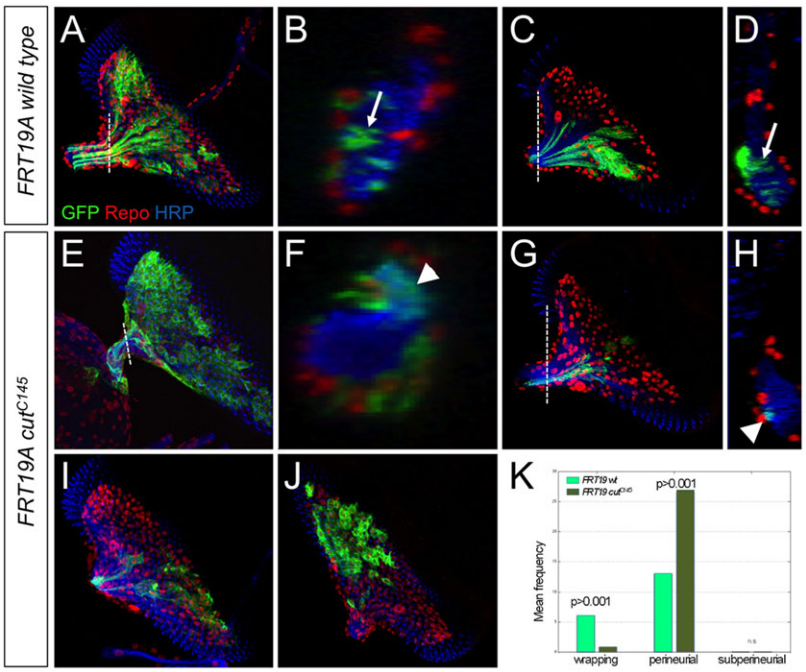

Fig. 4. *cut*^{C145} MARCM clones have fewer wrapping glial cells. Projections of confocal stacks and orthogonal sections of representative eye disc MARCM clones. Glial-specific MARCM clones were induced with *repoflp1C* and labelled with *repo>>CD8GFP* (green). Glial nuclei are shown in red and neuronal membranes in blue. The dashed lines indicate the orthogonal section shown on the right of the respective image. (A-D) Wild-type controls. In 67% of the eye disc clones, wrapping glial cells with membrane protrusions within the middle of the stalk region (B,D, arrows) can be observed. (E-J) By contrast, only 33% of the eye discs with *cut*^{C145} mutant clones contain cells with an elongated morphology, possibly resembling wrapping glial cells. Membrane protrusions never extend into the inner of the optic stalk (F,H, arrowheads). (K) Summary of cell-type frequency in wild-type and *cut*^{C145} MARCM clones. Cell types were defined by location in the eye disc and morphology.

higher levels of Cut expression cause increased spreading of glial processes. In all cases, glial cells extend their processes on the basal side of the eye imaginal disc and do not contact any neuronal membrane. Ectopic expression of Cut does not induce the expression of the wrapping glial marker *Sprouty*^{lacZ} (Fig. 5F).

Cut function requires DNA binding

To deduce the relevance of the different DNA-binding domains of the Cut transcription factor we used the deletion constructs described above (Fig. 5A). Expression of all constructs, except Cut$^{\Delta Cterm}$ and Cut$^{\Delta C3}$, in which the sequence recognized by the antibody is removed, was verified by antibody staining. Upon expression of full-length Cut in all glial cells, larvae die as first instar, whereas expression of Cut$^{\Delta Cterm}$ does not affect viability and no abnormal phenotypes were observed. Likewise, expression of Cut$^{\Delta HD}$ does not compromise normal development, suggesting that the homeodomain is crucial for function. Upon expression of Cut$^{\Delta C1}$, some escaping larvae developed until pupal stages. By contrast, expression of Cut$^{\Delta C2}$ and Cut$^{\Delta C3}$ still causes lethality, similar to what we noted following expression of full-length Cut.

To improve the analysis of the consequences of Cut overexpression we performed single-cell studies, and generated small cell clones expressing Cut using a flip-out strategy (*repoFlp6-2*, *tub>64>Gal4*, *UAS-CD8GFP*). Expression of full-length Cut (*cut*^{fl}) and Cut protein variants lacking the first or the third cut domain (*cut*$^{\Delta C1}$ or *cut*$^{\Delta C3}$) resulted in a dramatic expansion of cell morphology (supplementary material Fig. S5). We noted no phenotypic differences caused by the expression of the different deletion constructs, indicating that

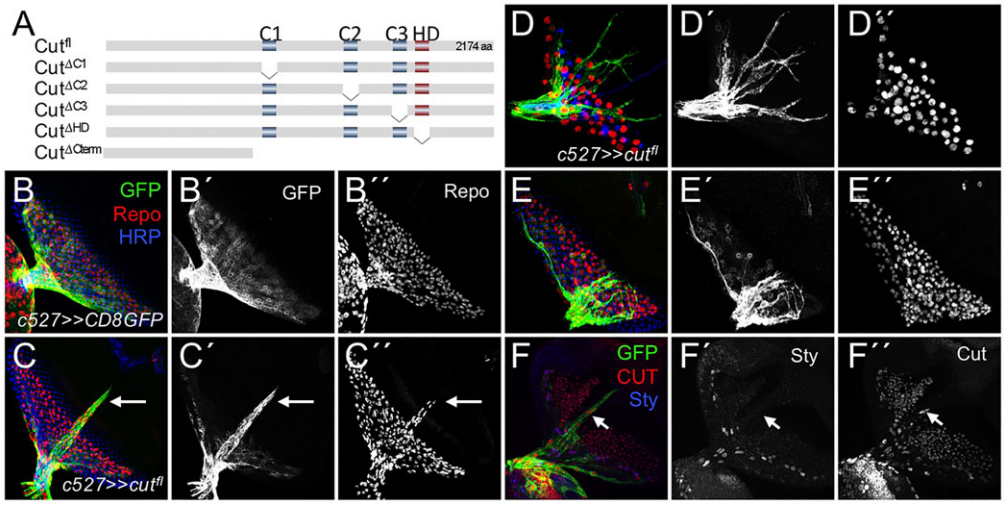

Fig. 5. Cut directs the formation of membrane protrusions. (A) Schematic representation of the Cut protein and the different constructs generated in this study. C1, C2 and C3 delineate the three Cut repeats, HD delineates the homeodomain. (B-E″) Projections of confocal stacks of a third-instar eye disc. Glial cell nuclei are labelled with Repo (red), perineurial glial cell membranes are shown in green (*c527>>CD8GFP*) and neuronal membranes in blue (HRP staining). (B) Wild-type control. (C) About 90% of the eye discs expressing full-length Cut (Cut^{fl}) in perineurial glia form long protrusions on the basal side of the eye disc (arrow). (D,E) In 10% of the imaginal discs, perineurial glial cells form elaborated cell processes. (F) Expression of Cut^{fl} in a *sty*^{lacZ} background. Sty expression is shown in blue. No activation of Sty expression is noted upon Cut expression (arrows).

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phenocritical DNA-binding sequences are not affected by these mutants. By contrast, expression of $Cut^{\Delta C2}$ or $Cut^{\Delta HD}$ is not able to redirect perineurial glial morphology (supplementary material Fig. S5). Thus, the homeodomain and the Cut repeat 2 both appear important for normal *cut* function in glial cells.

cut expression is stimulated through FGFR activation

The extent of glial differentiation is controlled by the activation of FGFR signalling (Franzdóttir et al., 2009). Initially, low levels of FGFR activation allow proliferation and migration of perineurial glia. Upon contact of perineurial glia with nascent retinal axons, strong FGFR activation controls the extent of glial differentiation. Thus, the switch to high levels of FGFR activation triggers wrapping glia differentiation (Franzdóttir et al., 2009; Sieglitz et al., 2013).

The onset of glial differentiation coincides with the onset of *cut* expression (supplementary material Fig. S6). Note that in wild-type animals cone cells express Cut at comparable levels as wrapping glial cells (Fig. 6A″). To test whether *cut* expression requires FGFR activity, we expressed a dominant-negative Heartless FGFR in all glial cells, which affects differentiation of the wrapping glia (Franzdóttir et al., 2009). Upon suppression of FGFR function, we noted a significant reduction of *cut* expression in wrapping glia in contrast to cone cells (Fig. 6B). Similar results were obtained when we silenced *dof*, which encodes a crucial component of the FGFR signalling pathway. To assay whether *cut* expression can be induced by FGFR activation we expressed a constitutively active Heartless receptor in all glial cells. This causes a tumour-like growth phenotype (Witte et al., 2009), and all glial cells ectopically activate Cut (Fig. 6C). Interestingly, the knockdown of *cut* expression does not suppress the proliferation phenotype caused by FGFR activation (supplementary material Fig. S7B). As glial *cut* expression is observed in $heartless^{AB42}$ mutant embryos (data not shown), *cut* expression can be initiated independently of FGF signalling. Still, in differentiating wrapping glia, high levels of Cut expression are maintained by high activity of the FGFR pathway.

Interactions between FGFR signalling, Cut, Dref and Pdm3

Here, we have identified three transcriptional regulators that are expressed and required during different phases of glial development. Whereas Pdm3 affects only glial proliferation and Cut is required only for glial differentiation, loss of Dref affects both glial proliferation and differentiation, suggesting that Dref is an important upstream regulator. We therefore tested whether Dref is able to regulate Cut expression. In the wild type, Cut expression in glia is comparable to Cut expression in cone cells (Fig. 6A). Upon *Dref* knockdown, reduced levels of Cut protein expression are found in the wrapping glial cells, whereas Cut expression in cone cells is not affected (Fig. 6D). By contrast, pan-glial overexpression of *Dref* did not change *cut* expression levels (Fig. 6E). However, such animals do not survive to pupal stages and the glial cells develop vesicular structures, which might correspond to an induction of apoptosis reported for *Dref* overexpression (Hirose et al., 2001).

Expression of Dref appears independent of FGF signalling, as neither the expression of a dominant-negative Heartless protein nor the expression of a constitutively activated FGFR caused alterations in the Dref expression profile (Fig. 7A-C). In addition, we found no alteration in Dref expression upon *cut* knockdown (Fig. 7D). We next tested whether Pdm3 expression is dependent on Dref. Upon pan-glial Dref knockdown, Pdm3 expression cannot be detected

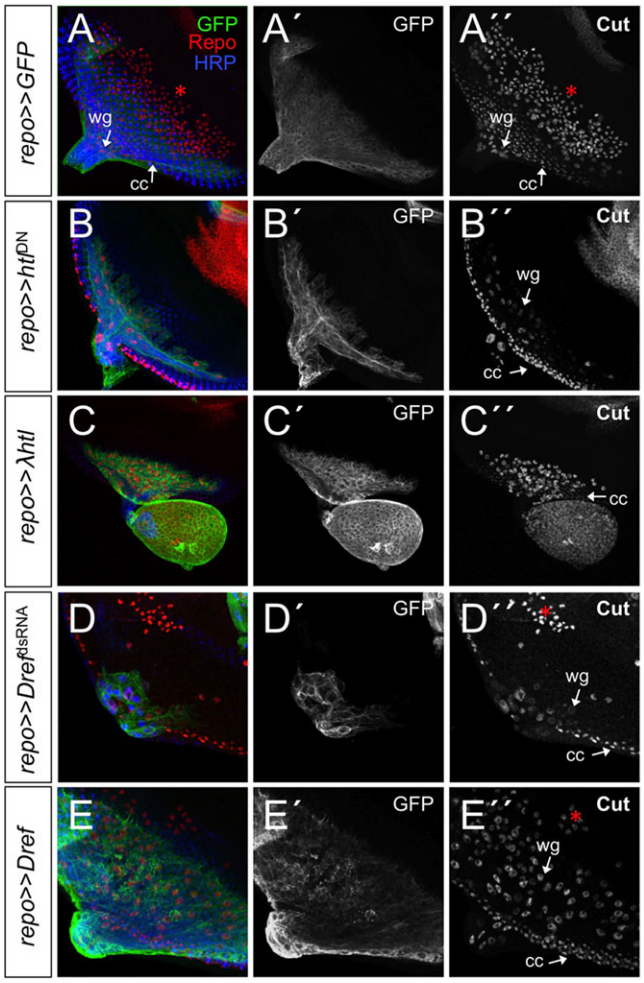

Fig. 6. Dref and FGFR signalling modulate Cut expression. Projections of confocal stacks of a third-instar eye disc. (A) Cut expression (red) in a larvae expressing GFP in all glial cells serves as wild-type control. The outlines of glial cells are labelled in green (*repo>>CD8GFP*) and neuronal membranes in blue (HRP staining). Cut expression is found in a subset of eye disc glia, the wrapping glia (wg), the cone cells (cc) and some hemocytes (red asterisk, see supplementary material Fig. S3D). Note that Cut expression levels in cone cells and wrapping glia are very similar (A″). (B) Expression of a dominant-negative FGFR in all glial cells (*repo>>htl*^{DN}) results in a reduction of Cut expression in wrapping glia. Note the difference in expression levels between wrapping glia (wg) and cone cells (cc) (arrows, B″). (C) Expression of an activated FGFR in all glial cells (*repo>>λ*) results in a pan-glial activation of Cut expression. Cut is now more strongly expressed in glial cells compared with the expression level in cone cells. (D) Expression of *Dref*^{dsRNA} in all glial cells results in a reduced number of glial cells on the eye disc. In wrapping glia (wg), Cut expression is still detectable but at reduced levels compared with cone cells (D″). (E) Upon pan-glial overexpression of Dref no change in Cut expression is noted (E″). Red asterisks (A″,D″,E″) denote hemocytes.

anymore in the perineurial glial cells (Fig. 7E,F). Pan-glial activation of *Dref* expression in turn does not result in the activation of Pdm3 (supplementary material Fig. S7). In contrast to Cut, which is induced by high levels of FGFR activity, Pdm3 expression is repressed by high levels of FGFR activity (Fig. 7G). This might suggest that Cut is able to suppress Pdm3 expression. However, upon glial *cut* knockdown, Pdm3 is still present in the perineurial glia and is not ectopically expressed by wrapping glia (see single focal plane shown in Fig. 7H). Thus, Pdm3 is only found in proliferating perineurial glial cells with high levels of Dref and low levels of FGFR activity. By contrast, Cut expression requires

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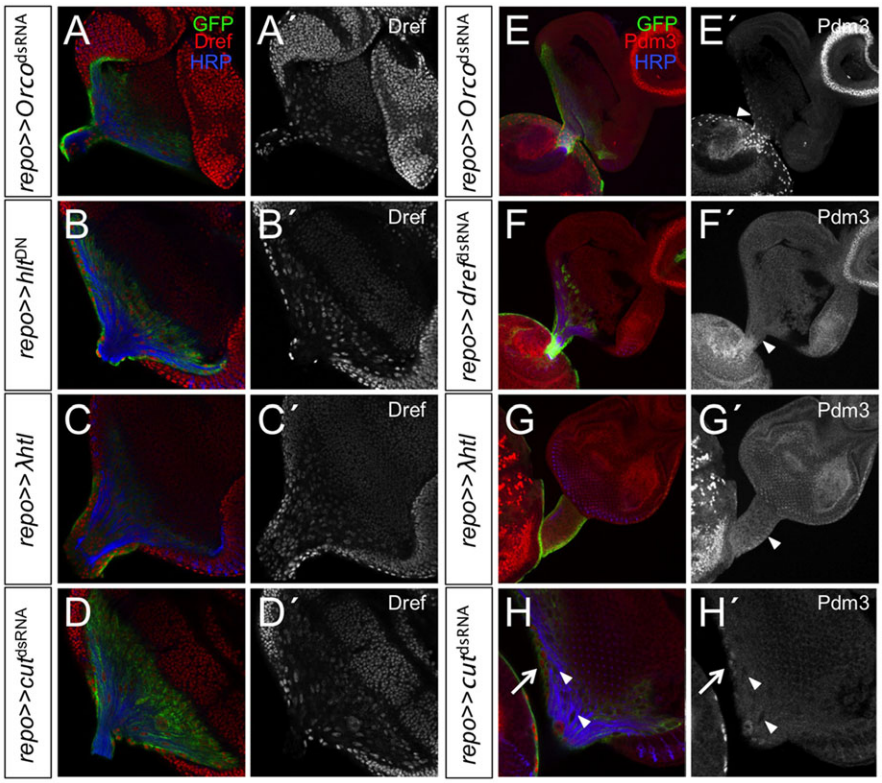

Fig. 7. Interactions between FGFR signalling, Cut, Dref and Pdm3. Third-instar eye imaginal discs stained for HRP to label neuronal cell membranes (blue), GFP to label glial cell processes (green) and Dref (red in A-D′) or Pdm3 (red in E-H′). Single channels showing Pdm3 or Dref staining are shown in grey. The genotypes are indicated on the left. (A,A′) Normal Dref expression in control animals (*Orco* knockdown in glia). (B-C′) Upon suppression or activation of FGFR signalling, no changes in Dref expression are noted in glia. (D,D′) No changes in Dref expression are noted following *cut* knockdown. (E,E′) In the control, Pdm3 expression can be detected in perineurial glial cells (arrowhead). (F,F′) Upon *Dref* knockdown, Pdm3 levels in perineurial glial cells are dramatically reduced (arrowhead). (G,G′) Upon activation of FGF signalling, glial Pdm3 expression is also suppressed (arrowhead). (H,H′) Single focal plane. No change in Pdm3 expression is noted in wrapping glial cells upon *cut* knockdown (arrowheads). Expression in perineurial glia appears normal (arrows).

high levels of FGF signalling and Dref activation and is therefore found only in wrapping glial cells (Fig. 8).

DISCUSSION

During development of the nervous system cell proliferation and differentiation need to be tightly coupled. Initially, progenitor cells divide to generate a group of cells competent to receive a specifying signal, which then prevents further proliferation and triggers terminal differentiation. Using a genome-wide RNAi-based screen we have unravelled the transcriptional machinery responsible for such a switch during gliogenesis in the *Drosophila* eye.

During embryonic development the anlage of the eye imaginal disc is formed. It is attached to the forming brain through the so-called Bolwig's nerve (Schmucker et al., 1992). A few glial cells reside along this nerve, presumably generated in the segmental nerves, as are most of the glia (von Hilchen et al., 2008). These glial

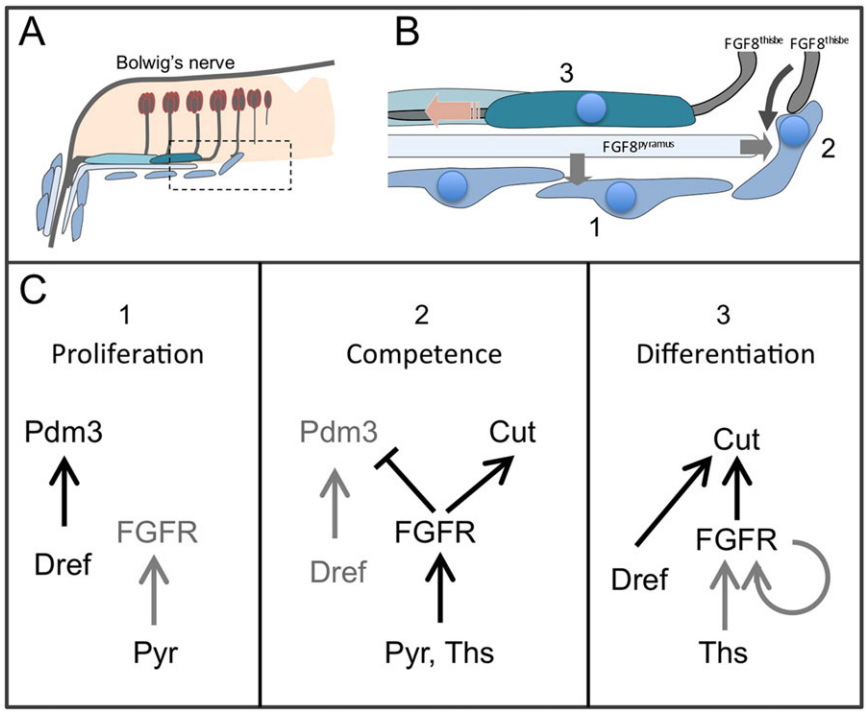

Fig. 8. Transcription factors controlling wrapping glia development. (A) Schematic view of a third-instar larval eye disc. Glial cells are depicted in blue, axons in grey and the eye imaginal disc with nascent photoreceptor neurons in red. (B) Higher magnification of the zone where perineurial glial cells become instructed to differentiate. This process is linked to a secretion of the FGF8-like ligands Thisbe and Pyramus. Three main steps can be defined: (1) migrating and proliferating perineurial glial cells; (2) glial cells contact axons in the competence zone and receive a second FGFR activation; (3) differentiating wrapping glia. (C) Transcription factors and their interactions associated with the different developmental steps. See text for details.

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cells proliferate extensively during larval stages to form ~300 glial cells within each eye imaginal disc (Choi and Benzer, 1994; Rangarajan et al., 1999; Silies et al., 2007). During the third larval stage ~50 of these cells differentiate into wrapping glia in an FGFR-dependent manner (Franzdóttir et al., 2009; Sieglitz et al., 2013; Silies et al., 2007). Here, we show that the proliferation of the glial progenitor pool requires the activity of Pdm3 and the DNA replication-related element-binding factor (Dref), which are both strongly expressed by proliferating perineurial glia. Dref was first identified as an important factor required for efficient transcription of the proliferating cell nuclear antigen (PCNA) (Hirose et al., 1993), which is a key regulator of replication (Moldovan et al., 2007). Dref protein associates with the TATA box-binding protein-related factor 2 (TRF2), which functions as a core promoter-selectivity factor that governs a restricted subset of genes co-ordinately regulated (Hochheimer et al., 2002). Interestingly, pan-glial knockdown of TRF2 also results in lethality, suggesting that the Dref/TRF2 complex is active in glia. Knockdown of *CG30020*, encoding a member of the Dref/TRF2 complex (Hochheimer et al., 2002), or *osa* and *moira*, which had been shown previously to interact with Dref (Nakamura et al., 2008), caused similar glial phenotypes in the visual system.

TRF2 targets several classes of TATA-less promoters present in more than 1000 genes, including a cluster of ribosomal protein genes (Isogai et al., 2007). Likewise, Dref was found to associate with many genes involved in protein synthesis and cell growth, and loss of *Dref* results in reduced organismal growth rates (Goodrich and Tjian, 2010; Killip and Grewal, 2012; Matsukage et al., 2008). Most likely, dividing glial cells as well as differentiating wrapping glia have an increased protein synthesis demand, which might explain the observed defects in proliferation and differentiation.

We show that expression of the transcription factor Pdm3 depends on Dref. Previously, Pdm3 has been associated with axonal pathfinding (Chen et al., 2012; Tichy et al., 2008). Our results indicate that Pdm3 also regulates cell number. In contrast to Dref, Pdm3 expression is repressed by FGFR signalling, ensuring that perineurial glia routed to differentiation do not express Pdm3 anymore (Fig. 8).

Previous work suggested that in the *Drosophila* eye imaginal disc perineurial glial cells at the anterior margin of the eye field are competent to react to a neuronal signal inducing their glial differentiation (Franzdóttir et al., 2009; Sieglitz et al., 2013; Silies et al., 2007) (Fig. 8). During this phase glial cells have reduced Dref expression but increased FGFR activity. Whereas in the absence of FGFR signalling no glial differentiation can be observed, high levels of FGF signalling trigger the expression of Cut specifically in wrapping glia. In addition to Cut, Dref is essential for proper glial differentiation. Dref is required for normal Cut expression levels but gain of Dref function is unable to activate Cut ectopically in perineurial glial cells. This requires additional FGFR signalling, indicating that two parallel molecular pathways converge on the activation of the transcription factor Cut to orchestrate wrapping glial differentiation (Fig. 8).

In the *Drosophila* PNS Cut controls the ES/ChO lineage decision (Blochlinger et al., 1990, 1991). By contrast, during glial cell development our work defined Cut as a master regulator organizing elaborated membrane growth, which is required during the wrapping of axons. Similarly, Cut instructs the morphogenesis of multi-dendritic neurons (Grueber et al., 2003). In mammals, the Cut homologues Cux1/2 also control dendritic branching, the number of dendritic spines and synapses (Cubelos et al., 2010). The number of filopodial extensions correlates to the level of Cut expression, corresponding to our findings. It was recently shown that Cut-dependent filopodia formation depends on the function of CrebA, which activates components of the secretory pathway (Iyer et al., 2013; Fox et al., 2010). Cut might not only orchestrate membrane organization through the modulation of the secretory pathway, it also directly controls cytoskeletal dynamics (Iyer et al., 2012; Nagel et al., 2012). In larval sensory *da* neurons, the actin bundling protein Fascin is necessary for a Cut-dependent induction of spiked cell protrusions (Nagel et al., 2012). However, eye disc glial cells still form long cell processes when *fascin* expression is suppressed by RNAi (data not shown). Further understanding of wrapping glial cell differentiation will require the identification of the transcriptional targets of Cut.

In conclusion, we demonstrate that the specification of wrapping glial cells in the developing visual system does not require a single lineage switch gene but rather appears as a gradual process. The specification of wrapping glia is orchestrated by a transcriptional network comprising Pdm3, Dref and Cut that is modulated by the activity of the FGFR.

MATERIALS AND METHODS

Drosophila work

All *Drosophila* work was conducted according to standard procedures. The following Gal4 strains were used: *c527-Gal4* and *Mz97-Gal4* (Hummel et al., 2002), *nrv2-Gal4* and *repo-Gal4* (Lee and Jones, 2005; Sepp and Auld, 1999). *repoflp1c* (Silies et al., 2007) resulted in glial MARCM in ~10%, whereas *repoflp6-2* (Stork et al., 2014) resulted in glial MARCM clones in almost 50% of the eye discs. For single-cell analysis we used *tub>64>Gal4* (M. Gonzalez-Gaitan, Geneva, Switzerland), cut^{C145} (L. Dobens, Kansas, USA) and *UAS-Dref* (M. Yamaguchi, Kyoto, Japan). The different *UAS-dsRNA* flies used in this study were obtained from the VDRC (Vienna, Austria), the Fly Stocks of the National Institute of Genetics (NIG-FLY, Kyoto, Japan) or the TRiP collection (Bloomington *Drosophila* Stock Center, Indiana University, Bloomington, IN, USA). All 833 screened stocks are listed in supplementary material Table S1. The list was assembled using FlyBase query builder, searching for the following terms in all fields: transcription, RNA polymerase II promoter, DNA binding, transcription factor, transcriptional regulator. All results were combined and compared with a list of 755 sequence-specific transcription factor-coding genes published by Hens et al. (2011). About 60 genes were missed by our FlyBase query compared with the list by Hens et al. (2011). To obtain strong gene silencing we employed a strain with two copies of *repo-Gal4* (Schmidt et al., 2012). We did not include *dicer2*, as this resulted in unspecific phenotypes. For confocal analysis we dissected at least eight animals. Staining of *pdm3*, *Dref* or *cut* knockdown eye imaginal discs was repeated at least three times (n>16 each time). sty^{lacZ} (Sieglitz et al., 2013), *UAS-lamGFP*, $UAS\text{-}htl^{DN}$ and *UAS-λhtl* (Franzdóttir et al., 2009). Other stocks used in this study were obtained from the Bloomington Stock Center or the *Drosophila* Genome Resource Center (DGRC) in Kyoto, Japan.

Immunohistochemistry and TEM analyses

Fixation and preparation of tissues for immunohistochemistry was performed as described (Yuva-Aydemir et al., 2011). Anti-Repo and anti-Cut antibodies were obtained from DSHB. The following other antibodies were used: Anti-Pdm3 [1:100; Chen et al., 2012; Tichy et al., 2008; kindly provided by W. Grueber (Columbia University, NY, USA) and C.-T. Chien (Taipei, Taiwan)]; anti-Dref (1:1000; Hirose et al., 1996); anti-β-Gal (1:1000; Cappel, MP Biomedicals, 08559762); anti-GFP (1:1000; Molecular Probes, A11122); anti-HRP649 (1:500; Jackson ImmunoResearch Laboratories, 115-035-205). Specimens were analysed using a Zeiss710 LSM; orthogonal sections were taken using the Zeiss LSM imaging software. Electron microscopy analyses were performed as described (Stork et al., 2008).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
A.-C.B. designed experiments, performed the screen and studied the role of Pdm3, Dref and Cut. S.S. designed experiments and analysed the genetic relationship of Pdm3, Dref, Cut and FGF signalling. A.-C.B. and S.S. participated in writing the manuscript. T.M. performed the TEM analysis; C.K. conceived experiments and wrote the manuscript.

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Supplementary material
Supplementary material available online at
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