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 (Beckvordersandforth 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 (Sieg litz 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 Sproutylinz 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
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 S1). 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). Pan-glial downregulation of gzf (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 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.
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 (GL00332, 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
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 Sproutylox (Franzdóttir et al., 2009). All Sproutylox–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 Sproutylox (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 cutC145, a well-established null allele, and two glial-specific repoHFL 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 repoHFL–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 cutC145 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 repop6–2 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; Blochliger 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 CutPA. In addition, we generated several Cut deletion constructs lacking either one of the Cut-repeats or the homeodomain, all inserted into the same chromosomal sources (Silies et al., 2007; Stork et al., 2014). Wild-type MARCM cell clones were established null allele, and two glial-specific sources (Silies et al., 2007; Stork et al., 2014). Wild-type MARCM cell clones were generated 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 Sproutylox (green) co-localizes with Cut (red) expression in wrapping glia (asterisk). The arrowheads point towards Sproutylox−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).
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 SproutylacZ (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 CutΔCterm and CutΔC3, 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 CutΔCterm does not affect viability and no abnormal phenotypes were observed. Likewise, expression of CutΔHD does not compromise normal development, suggesting that the homeodomain is crucial for function. Upon expression of CutΔC1, some escaping larvae developed until pupal stages. By contrast, expression of CutΔC2 and CutΔC3 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 (cutfl) and Cut protein variants lacking the first or the third cut domain (cutΔC1 or cutΔC3) 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. 4. cutC145 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 cutC145 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 cutC145 MARCM clones. Cell types were defined by location in the eye disc and morphology.

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 (Cutfl) 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 Cutfl in a stylacZ background. Sty expression is shown in blue. No activation of Sty expression is noted upon Cut expression (arrows).
phenocritical DNA-binding sequences are not affected by these mutants. By contrast, expression of Cut<sup>ΔC2</sup> or Cut<sup>ΔHD</sup> 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<sup>AB42</sup> mutant embryos (data not shown), cut expression can be initiated independently of FGFR 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 FGFR 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 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
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
cells proliferate extensively during larval stages to form ∼300 glial cells within each eye imaginal disc (Choi and Benzer, 1994; Rangarajan et al., 1999; Silles 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; Silles 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-selecitvity 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 (Isoai 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 Tijian, 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; Silles et al., 2007). 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 FGFR 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 filipodia 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 M97-Gal4 (Hummel et al., 2002), nrv2-Gal4 and repo-Gal4 (Lee and Jones, 2005; Sepp and Auld, 1999). repoP1pc (Silies et al., 2007) resulted in gial MARCM in ∼10%, whereas repoP6p-2 (Stork et al., 2014) resulted in gial MARCM clones in almost 50% of the eye discs. For single-cell analysis we used tub64>Gal4 (M. Gonzalez-Gaitan, Geneva, Switzerland), cut45 (L. Dobens, Kansas, USA) and UAS-Dref (M. Yamaguchi, Kyoto, Japan). The different UAS-deRNA 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+FR (Sieglitz et al., 2013), UAS-lumGFP, UAS-hhtP and UAS-lhlt (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 (Yuya-Aydemir et al., 2011). Anti-Reto 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. Graeber (Columbia University, NY, USA) and C.-T. Chien (Taipei, Taiwan)); anti-Dref(1:1000; Hirose et al., 1996); anti-dlGal (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.
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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 http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.119750/-/DC1

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