Development 130, 2419-2428

doi:10.1242/dev.00468

Drosophila homeodomain protein REPO controls glial differentiation by cooperating with ETS and BTB transcription factors

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Accepted 10 February 2003

SUMMARY

In *Drosophila*, cell-fate determination of all neuroectoderm-derived glial cells depends on the transcription factor Glial cells missing (GCM), which serves as a binary switch between the neuronal and glial cell fates. Because the expression of GCM is restricted to the early phase of glial development, other factors must be responsible for the terminal differentiation of glial cells. Expression of three transcription factors, Reversed Polarity (REPO), Tramtrack p69 (TTK69) and PointedP1 (PNTP1), is induced by GCM in glial cells. REPO is a paired-like homeodomain protein, expressed exclusively in glial cells, and is required for the migration and differentiation of embryonic glial cells. To understand how REPO functions in glial terminal differentiation, we have analyzed the mechanism of gene regulation by REPO. We show that REPO can act as a transcriptional activator through the CAATTA motif in glial cells, and define three genes whose expression in vivo depends on REPO function. In different types of glial cells, REPO can act alone, or cooperate with either TTK69 or PNTP1 to regulate different target genes. Coordination of target gene expression by these three transcription factors may contribute to the diversity of glial cell types. In addition to promoting glial differentiation, we found that REPO is also necessary to suppress neuronal development, cooperating with TTK69. We propose that REPO plays a key role in both glial development and diversification.

Key words: *Drosophila*, Glia, Neuron, *repo*, *tramtrack*, *pointed*, *gcm*

INTRODUCTION

The two major cell types in the nervous system, neuron and glia, each comprise a large number of subtypes. In the vertebrate brain, glial cells outnumber neurons by an order of magnitude. They display a huge morphological diversity, and are involved in various functions, including supplying neurotrophic factors required for neuronal survival, the electrical insulation of axons, the formation of the blood-nerve barrier and axonal guidance. How the diversity of glial cells is generated and how these processes are linked to the cell fate choice to become a neuron or glial cell are unknown in vertebrates and invertebrates.

Glial cells in the *Drosophila* embryonic nervous system can be classified into several groups based on their location, morphology and expression of several marker genes (Ito et al., 1995). Glial cells in the central nervous system (CNS) are generated from the mesectoderm and the ventral neuroectoderm. Glial cells of mesectodermal origin are called midline glia, which unsheathe commissural axon bundles (Klämbt et al., 1991). Other CNS glia arise from glial precursors and neuroglial precursors in the ventral neuroectoderm, and consist of diverse glial types such as surface-associated glia located close to the CNS surface, cortex-associated glia that lie among the neuronal cell bodies in the cortex and neuropile-associated glia that associate with the axonal structures. Glial cells in the peripheral nervous system (PNS) are derived from either lateral neuroblasts in the ventral neuroectoderm or the dorsal epidermal anlage, and unsheathe afferent and efferent axon bundles.

In *Drosophila*, all glial cells except the midline glia require the function of the *glial cells missing* (*gcm*) gene for their cell-fate determination (Hosoya et al., 1995; Jones et al., 1995;

Vincent et al., 1996). GCM acts as a binary switch between the neuronal and glial fates; it promotes glial development, while inhibiting neuronal differentiation (Hosoya et al., 1995; Jones et al., 1995). GCM induces the expression of three transcription factors, Reversed Polarity (REPO), Tramtrack p69 (TTK69; an isoform of the *ttk* gene product) and PointedP1 (PNTP1; an isoform of the *pointed* gene product), in glial cells. Because the loss of function of any of these three factors is not as severe as the loss of GCM function, each of these factors is considered to be responsible for only a part of the GCM function. Giesen et al. (Giesen et al., 1997) proposed that glial cell differentiation is achieved by two parallel processes: the promotion of glial gene expression by PNTP1 and the suppression of neural properties by TTK69. PNTP1 is an ETS transcription factor that can activate transcription through ETS binding sites (O'Neill et al., 1994; Albagli et al., 1996; Granderath et al., 2000). It is expressed in a subset of glial cells, such as longitudinal glia and VUM glia (Klaes et al., 1994), as well as in the ventral ectoderm and tracheal cells (Mayer and Nüsslein-Volhard, 1988; Gabay et al., 1996). TTK69 is a BTB/POZ domain/zinc-finger type transcription factor that has been implicated in transcriptional repression during embryonic segmentation and eye development (Read et al., 1992; Brown and Wu, 1993; Xiong and Montell, 1993; Li et al., 1997). Its expression can be best characterized as non-neuronal; TTK69 is expressed in all cells except in the neuronal lineage, like the support cells of the sensory organ and the epidermis (Harrison and Travers, 1990; Brown et al., 1991; Read and Manley, 1992).

Although differential functions for PNTP1 and TTK69 is an attractive idea, these two factors alone cannot account for the GCM-dependent development of glial cells, because the PNTP1 and TTK69 double-mutant phenotype is different from the *gcm* mutant phenotype (Giesen et al., 1997). In addition, the pleiotropy of PNTP1 and TTK69 makes them unlikely candidates for the glial determinant. Therefore, to characterize the molecular cascades required for the differentiation of glial cells, we chose to analyze the *repo* gene, which is expressed only in GCM-positive glia (Campbell et al., 1994; Xiong et al., 1994; Halter et al., 1995). The region upstream of the *repo* transcription start site contains eleven GCM-binding consensus sequences, and GCM is necessary and sufficient to induce *repo* expression in vivo (Hosoya et al., 1995; Jones et al., 1995; Akiyama et al., 1996). Thus, *repo* is likely a direct target of GCM. REPO is a paired-like homeodomain protein that specifically binds the ATT sequence in the CAATTA motif (Halter et al., 1995). Although the expression of several glial marker genes is known to depend on *repo* activity, how REPO functions as a transcription factor in glial differentiation is as yet unknown.

We show that REPO can act as a transcriptional activator through the CAATTA motif in glial cells, and we define three genes whose expression depends on REPO function. In different types of glial cells, REPO can act alone or cooperate with either TTK69 or PNTP1 to regulate different target genes. Surprisingly, REPO also suppresses neuronal development. We propose that REPO has a cardinal function in glial identity.

MATERIALS AND METHODS

Plasmid construction, transfection and cell culture

Constructs for expression in S2 cells were made by inserting the cDNAs for *gcm*, *repo*, *repo*Δbox (with the sequence for amino acids 311-355 deleted), or the cDNA for the *pointed* P1 isoform into the pAc vector (Krasnow et al., 1989). GAL4 fusion constructs carry the GAL4 DNA binding domain (a *Hin*dIII-*Eco*RI fragment of pBD-GAL4 (Stratagene) encoding the first 147 amino acids of GAL4-) fused to a *repo* cDNA fragment, with three Myc-tags at the C terminus, in the pAc vector. The CAATTA-luc or CAGTTA-luc reporter carries two tandem copies of a 21 mer sequence, 5′-AAAGCAATTAAGCGGAACGGA-3′ or 5′-AAAGCAGTTAAGCGGAACGGA-3′ (Nelson and Laughon, 1993), upstream of the *hsp70* minimal promoter, fused to the luciferase reporter gene. The reporter gene for the GAL4 fusion proteins contains five GAL4-binding sites upstream of the *hsp70* minimal promoter fused to the luciferase gene. *loco* promoter-luciferase reporter genes AEE-luc and AES-*luc* were constructed by cloning 1.4 kb or 0.7 kb *loco* promoter fragment (extending to 6 bp upstream of the *loco-c1* translational initiation codon) into PGV-B luciferase vector (Toyo Ink). In AEE*-*luc* reporter gene, two CAATTA motifs were each changed to CAGTTA.

S2 cells cultured in a 60 mm diameter dish were transfected with 500 ng of luciferase reporter, 300 ng of effector vector and 50 ng of *Renilla* reference reporter (pACT-Rluc) using Lipofectin (GIBCO BRL) or Effectene (QIAGEN). We used the empty vector (pACT) to adjust the total amount of the effector plasmid to 300 ng, except in the AES-*luc* reporter assay, where the total was 600 ng. Luciferase activity 48 hours after transfection was normalized to values obtained with the *Renilla* reference reporter. Transfections were carried out at least three times.

Fly stocks

The following mutant alleles were used. $repo^{4e19}$ (Xiong et al., 1994), $rk2^{64}$ ($repo^{64}$) (Campbell et al., 1994), $pnt^{\Delta 88}$ (Klämbt, 1993). ttk^{1e11} mutation causes a specific loss of the TTK69 isoform (Xiong and Montell, 1993; Lai and Li, 1999) and ttk^{B330} is a strong hypomorph (Salzberg et al., 1994; Giesen et al., 1997). Homozygous mutant embryos were identified using the *TM3 [Ubx-lacZ]* balancer or by examining their phenotypes. The enhancer-trap strains pnt^{rM254} (Klämbt, 1993), ttk^{0219} (Lai et al., 1996) and $loco^{rC56}$ (Granderath et al., 1999) were used to detect the expression of *pointed*, *ttk* and *loco*, respectively. The Ftz HDS reporter (2X21F) (Nelson and Laughon, 1993) and the M84 strain (Klämbt and Goodman, 1991) were used as glial markers. Ectopic expression was achieved using GAL4 enhancer-trap insertions into *scabrous* (Kramer et al., 1995) and *engrailed* (a gift from Andrea Brand) loci. The UAS-*repo* construct was made by cloning the full-length *repo* cDNA into the pUAST vector (Brand and Perrimon, 1993). The UAS-*gcm*, UAS-*pntp1* and UAS-*ttk69* strains have been described (Hosoya et al., 1995; Klaes et al., 1994; Giesen et al., 1997).

Immunohistochemistry

Anti-REPO antiserum was obtained after immunizing rats with bacterially expressed REPO (amino acids 25-156)-GST fusion protein. Immunohistochemistry was performed as described by Patel (Patel, 1994) with minor modifications. The following primary antibodies were used: mouse anti-BP102 [obtained from Developmental Studies Hybridoma Bank (DSHB)] at 1:4, mouse anti-ELAV at 1:4 (obtained from DSHB), anti-REPO rat antiserum at 1:1000, rat anti-TTK69 at 1:200 (gift from A. Travers) and rabbit anti-β-Galactosidase polyclonal antibody (Cappel) at 1:1000. The following secondary antibodies were used: HRP-conjugated goat anti-mouse, anti-rat, or anti-rabbit IgG (Jackson ImmunoResearch) at 1:1000, and FITC- conjugated donkey anti-rat, Cy3-conjugated donkey anti-rabbit (Jackson ImmunoResearch) at 1:800. Immunofluorescence was visualized with a FLUOVIEW laser-scanning microscope (Olympus). Embryos were staged according to Campos-Ortega and Hartenstein (Campos-Ortega and Hartenstein, 1997).

In situ hybridization

Whole-mount in situ hybridization was conducted using digoxigenin-

labeled RNA probes, essentially as described in Lehmann and Tautz (Lehmann and Tautz, 1994). Full-length *loco-cl* cDNA was obtained by RT-PCR amplification and was used as a template.

RESULTS

REPO is a transcriptional activator

Based on the observation that a *lacZ* reporter gene with a CAATTA homeodomain consensus binding site (*ftz* HDS reporter) was specifically expressed in glial cells, Nelson and Laughon (Nelson and Laughon, 1993) had predicted the presence of a homeodomain transcriptional activator in glial cells. The homeodomain protein REPO is a good candidate for such an activator, because it binds to the CAATTA motif in vitro (Halter et al., 1995), and its expression is confined to glial cells (Campbell et al., 1994; Xiong et al., 1994; Halter et al., 1995). To test whether REPO indeed functions as a transcriptional activator, we tested the transcriptional regulatory activity of REPO in culture cells.

We constructed a luciferase reporter gene with two REPO-binding sites (CAATTA-luc) and tested its transcriptional activity in the *Drosophila* S2 cell line. Co-transfection with a REPO-expressing plasmid (pACT-repo) caused a sevenfold increase in luciferase activity compared with co-transfection with the vector alone (pACT) (Fig. 1A). REPO lacking most of its homeodomain, but retaining a putative nuclear localization signal located from amino acid 1 to 4 of the homeobox (pACT-repoΔbox), was unable to activate transcription of the reporter gene (Fig. 1A), despite being localized to the nucleus (data not shown). Furthermore, transcriptional activation by REPO was dependent on the presence of the CAATTA motif in the reporter gene; a single base substitution in this motif resulted in a complete loss of REPO-dependent transcription (Fig. 1A). We conclude that REPO is a transcriptional activator that can act through the CAATTA motif.

To identify the transcriptional activation domain of REPO, fusion proteins of various segments of REPO with the DNA-binding domain of GAL4 were expressed in S2 cells, and their transcriptional activation activity was assayed by measuring the enzymatic activities of the UAS-luciferase reporter gene. Fusion of the full-length REPO to the GAL4 DNA-binding domain caused a 2.4-fold activation of the reporter gene compared with the GAL4 DNA-binding domain alone. We identified two non-overlapping segments of REPO (amino acids 1-219, 452-612) that had significant levels of transactivation activity. Deletion of either segment retained the transcriptional activation activity present in the full-length fusion, suggesting that REPO may contain regions that inhibit the function of its activation domains. Indeed, deleting the N-terminal 124 amino acids caused an increase in activation that was more than 20-fold, indicating that a strong inhibitory domain is present in the N terminus. The presence of multiple functional domains suggests that REPO may employ different mechanisms of transcriptional regulation depending on the cellular context.

REPO functions as a transcriptional activator in glial cells

To test whether REPO activates transcription through CAATTA sites in vivo, we examined the expression pattern of

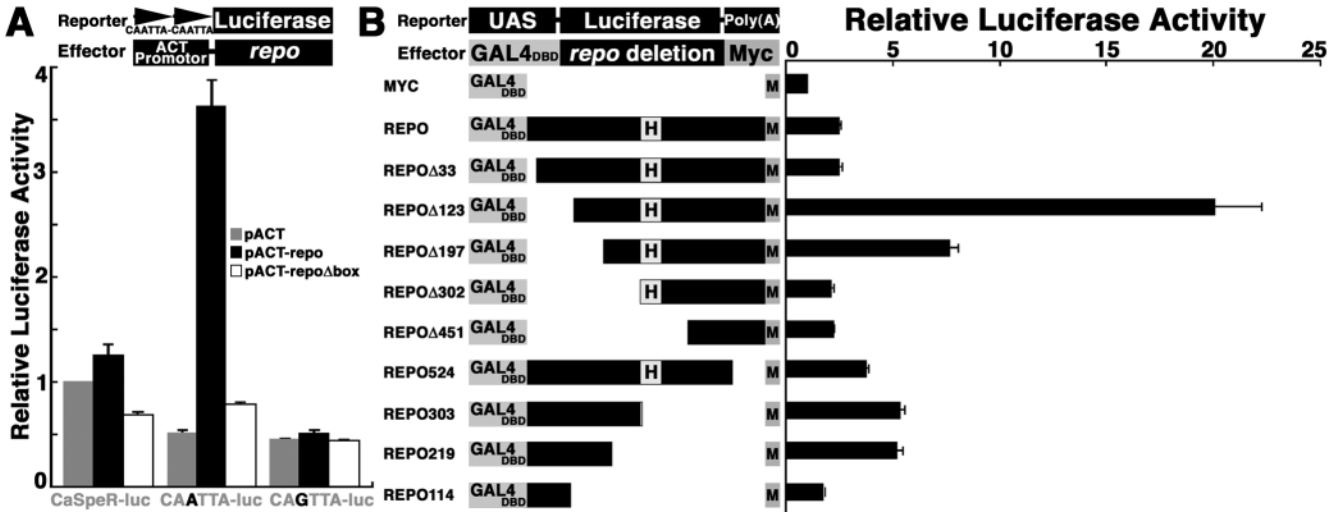

Fig. 1. REPO is a transcriptional activator. (A) REPO activates transcription through a homeodomain-binding site. The effector constructs express REPO (pACT-repo) or REPO lacking its homeodomain (REPOΔbox, pACT-repoΔbox), driven by the *Drosophila Act5C* promoter. The luciferase reporter contains two copies of CAATTA (CAATTA-luc) or CAGTTA (CAGTTA-luc) motifs placed upstream of the *hsp70* minimal promoter. The CaSpeR-luc reporter has a luciferase gene with the *hsp70* minimal promoter alone. Three luciferase reporter plasmids were each transfected into S2 cells with pACT-repo (black column), pACT-repoΔbox (white column) or vector (pACT) alone (gray column). The amount of luciferase activity generated by the co-transfection of CaSpeR-luc and pACT was defined as 1. (B) REPO contains multiple transcriptional activation domains. Various regions of REPO were fused to the GAL4 DNA-binding domain (DBD) and tagged with three copies of the Myc epitope at their C terminus. The reporter was the luciferase gene placed downstream of the *hsp70* TATA and five copies of UAS (GAL4-binding sites). The luciferase activity obtained with each effector is presented as the relative value compared with the value obtained with GAL4-DBD alone. The expression of the GAL4 fusion proteins was confirmed by western blot analysis of whole-cell extracts using a monoclonal antibody directed against the Myc-tag. H, homeodomain; M, Myc tag.

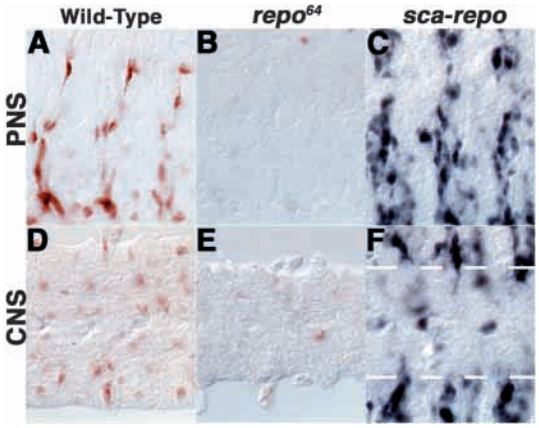

Fig. 2. REPO activates the expression of the *ftz* HDS reporter gene in glial cells. (A,D) Wild-type embryos; (B,E) *repo* mutant embryos; (C,F) ectopic expression of *repo* in *scabrous*-GAL4/UAS-*repo*. (A-C) Stage 16 embryonic PNS preparations. (A) In wild-type embryos, expression of the *ftz* HDS reporter gene is detected in glial cells of the peripheral nervous system, including the support cells of the bipolar dendritic neuron, peripheral glia, ligament cells of the chordotonal organ and exit glia. (B) In the *repo* mutant, the expression of the *ftz* HDS reporter gene in the PNS was absent or dramatically reduced. (C) In response to the ectopic expression of REPO, many cells in the dorsal epidermis expressed the *ftz* HDS reporter. (D-F) Dissected stage 16 embryonic CNS preparations. (D) In the wild-type CNS, the *ftz* HDS reporter gene is expressed in a subset of longitudinal glia, the A glia, B glia and intersegmental nerve root glia. (E) In the *repo* mutant, the expression of the *ftz* HDS reporter gene in the CNS glia was greatly reduced, as it was in the PNS. (F) Ectopic expression of REPO did not induce ectopic expression of the *ftz* HDS reporter gene within the CNS, although many cells showed ectopic expression in the periphery. Anterior is leftwards.

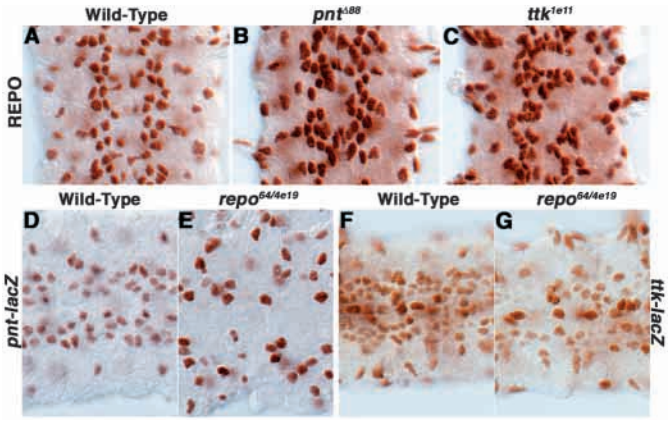

Fig. 3. *repo*, *pointed* and *ttk* are expressed independent of each other. (A-C) The expression of *repo* was demonstrated using a REPO antibody. (A) In wild-type embryos, REPO is expressed in all CNS glia except the midline glia. REPO-positive glial cells were still present in $pnt^{\Delta 88}$ (B) or ttk^{1e11} (C) mutant embryos. (D) A *pointed* enhancer-trap strain (pnt^{rM254}) expressed β-galactosidase in CNS glia that expressed REPO. (E) pnt^{rM254} was expressed in CNS glia in *repo* mutant embryos. (F) A *ttk* enhancer-trap strain (ttk^{0219}) expressed β-galactosidase in all CNS glia, in wild-type (F) as well as *repo* (G) mutant embryos. Anterior is upwards in A-C and leftwards in D-G.

the *ftz* HDS *lacZ* reporter gene in the *repo* mutant background. This reporter gene (2x21F) carries two copies of a 21 mer containing the CAATTA motif, and is expressed in all glial cells in the PNS and a subset of CNS glia (Nelson and Laughon, 1993) (Fig. 2A,D). The glial expression of the *ftz* HDS reporter gene in the PNS overlaps precisely with REPO-expressing cells. The loss of *repo* function abolished *lacZ* expression in both the PNS and CNS glia (Fig. 2B,E), even though glial cells are still present in stage 16 *repo* mutant embryos (Halter et al., 1995). Expression of the *ftz* HDS reporter gene in the antenno-maxillary complex and posterior spiracles, where REPO is not expressed, was unaffected in *repo* mutants (data not shown). These results indicate that REPO acts through the CAATTA site to drive transcription in glial cells.

To address whether REPO is sufficient to activate transcription of the *ftz* HDS reporter gene, we expressed REPO ectopically in the presumptive ventral neurogenic region and the dorsal epidermis. Such embryos expressed *lacZ* in non-glial cells within the dorsal epidermis, adjacent to the PNS (Fig. 2C). Thus, REPO is sufficient for the expression of the *ftz* HDS reporter gene in specific cellular contexts.

Although ectopic REPO induced the appearance of many non-glial *lacZ*-expressing cells in the dorsal epidermis, cells within the CNS did not respond to ectopic REPO. In fact, even in the wild-type background, not all REPO-positive glia in the CNS expressed the *ftz* HDS reporter. This suggests that the mechanism by which REPO regulates transcription may be different in the CNS from the one for peripheral glia. One possible scenario is that the functions of REPO in the CNS requires cooperation with one or more other factors, and that these interactions preclude REPO from acting through the CAATTA motif. TTK69 and PNTP1 are good candidates for such co-factors, because *ttk* and *pointed* are both required for the development of CNS glial cells (Klaes et al., 1994; Giesen et al., 1997). Although *repo*, *ttk* and *pointed* are expressed in overlapping subsets of CNS glial cells, their expression is mutually independent; REPO continued to be expressed in the *ttk* or *pointed* mutant background, and *lacZ* expression levels in enhancer-trap lines of *ttk* or *pointed* were unaffected in *repo* mutant embryos (Fig. 3). Moreover, ectopic expression of REPO in the entire neuroectoderm did not increase the expression of *pointed* P1 mRNA or TTK69, nor did ectopic expression of either TTK69 or PNTP1 affect REPO expression (data not shown). All three genes are most probably regulated independently, downstream of the glial determinant GCM (Hosoya et al., 1995; Jones et al., 1995; Giesen et al., 1997).

REPO cooperates with TTK69 to induce expression of the glial marker M84 in the CNS

To study how REPO controls its target genes in the CNS, we analyzed the regulation of a glial enhancer trap marker M84 (Klämbt and Goodman, 1991), whose expression requires *repo* function (Halter et al., 1995) (Fig. 4B). The M84 strain labels small subsets of glial cells in the CNS (Ito et al., 1995). All M84-expressing glia also expressed REPO and TTK69, but some were negative for PNTP1 expression (data not shown). Although M84 was expressed normally in *pointed* mutants, in *ttk* mutant embryos a prominent decrease in the level of *lacZ* expression was seen in M84-positive glial cells (Fig. 4C,D).

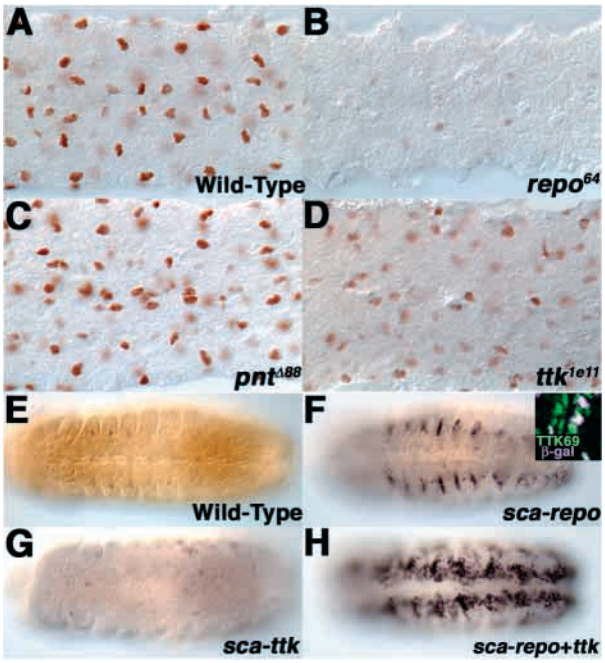

Fig. 4. Cooperative action of REPO and TTK69 activates the glial expression of the M84 marker. (A-D) CNS of stage 16 embryos carrying the M84 glial marker. (A) In wild-type embryos, M84 β-galactosidase expression could be detected in the subperineurial glia and channel glia. (B) In *repo* mutant embryos, M84 expression was absent or dramatically reduced. (C) *pointed* mutant embryos expressed the M84 marker at normal levels, although the arrangement of the glial cells was irregular. (D) In *ttk* mutant embryos, the expression level of M84 was lower than in wild type. (E-H) Ventral views of stage 12 embryos carrying the M84 glial marker. In wild-type embryos, few cells expressed the M84 marker at stage 13 (E). Ectopic expression of REPO induced additional cells to express the M84 marker (F), whereas TTK69 had no effect (G). Co-expression of REPO and TTK69 had a synergistic effect on the activation of the M84 marker (H). (F, inset) Supernumerary M84-positive cells induced by the ectopic expression of REPO also expressed endogenous TTK69. The ectopically expressed M84 marker is shown in magenta and TTK69 is shown in green. An overlay of both colors appears as white. The majority of ectopic M84-positive cells were located in the epidermis, which normally do not express this marker. The number of M84-positive epidermal cells per hemisegment were 10.2±5.8 for REPO misexpression and 32.6±9.7 for co-expression of REPO and TTK69 (n=23). Anterior is leftwards.

Thus, M84 is a suitable glial marker to examine the cooperation between REPO and TTK69.

We examined the effect of the ectopic expression of REPO and TTK69 on M84 expression. The expression of REPO in the neuroectoderm resulted in a precocious expression of the M84 marker in the CNS. In normal embryos, the majority of M84-positive cells appear only in stage 13 among REPO-positive cells that probably differentiate into subperineurial glia and channel glia (data not shown) (Halter et al., 1995; Ito et al., 1995). When REPO was expressed ectopically throughout the entire neuroectoderm, M84-positive cells appeared at stage 12 at the lateral edge of the CNS (Fig. 4F) and could be observed at stage 16 in cells that do not normally express REPO (data not shown). Many of the cells that expressed *lacZ* ectopically and precociously were located in the epidermis. Such cells were also positive for TTK69 (Fig. 4F, inset), suggesting that TTK69 is a prerequisite for REPO to induce expression of the glial marker M84. Although the forced expression of TTK69 alone had no effect on the expression of M84, the co-expression of REPO and TTK69 caused a greater than threefold increase in the number of M84-expressing cells, compared with the expression of REPO alone (Fig. 4G,H). Thus REPO cooperates with TTK69 to induce expression of the glial marker M84.

Expression of *loco-c1* depends on both REPO and PNTP1

Although results presented above suggest a synergistic action of REPO and TTK69 in transcriptional activation, we cannot determine whether REPO and TTK69 act cooperatively on the same target gene, because the gene responsible for M84 is not known. We thus studied the regulation of the gene *loco*, which encodes a member of the family of Regulator of G-protein Signaling (Granderath et al., 1999; Granderath et al., 2000). The *loco* function is required for glial morphogenesis, and *loco-c1*, an isoform of *loco*, is expressed specifically in REPO-positive glial cells, which also express PNTP1 (Granderath et al., 1999; Granderath et al., 2000). The expression of a *loco* reporter gene carrying a glial enhancer element of *loco* (Rrk; Fig. 5D) requires PNTP1 function, as well as an Ets-binding site located within this glial enhancer element (Granderath et al., 2000). Although this *loco*-reporter gene is expressed normally in *repo* mutants (Granderath et al., 2000), we found that in stage 16 *repo* mutant embryos *loco-c1* mRNA was reduced to undetectable levels (Fig. 5B,C), whereas such embryos exhibit robust expression of a *pnt-lacZ* reporter gene in glial cells (Fig. 3E). It is thus likely that proper expression of *loco* depends on both *pointed* and *repo* function.

To test whether REPO acts directly on the *loco* promoter, we focused on a 0.7 kb *loco* promoter fragment (AEE), which partially overlaps with the glial enhancer fragment (Fig. 5D). AEE contains two CAATTA motifs, one of which is located outside the glial enhancer element (Rrk) used by Granderath et al. (Granderath et al., 2000). We constructed luciferase reporter genes that carry either wild type AEE or AEE with single base changes in the CAATTA motifs (AEE-*luc* and AEE*-*luc*, Fig. 5D), and introduced them into S2 cells. Co-transfection of the AEE-*luc* reporter with a REPO expression plasmid caused a 45-fold increase in luciferase activity compared with co-transfection with the vector alone, whereas single base changes in the CAATTA motifs caused a fivefold drop in REPO-dependent activation (Fig. 5E). These results suggest that REPO directly regulates *loco* *trans*cription through the CAATTA motif.

As *loco* expression requires both *repo* and *pointed*, we studied the effects of co-expressing REPO and PNTP1. The *loco* genomic fragment in AEE-*luc* reporter was extended 0.7 kb in the 5′ direction, to include the Ets-binding site through which PNTP1 acts (AES-*luc*, Fig. 5D). In S2 cells transfected with a plasmid that directs REPO expression, a fivefold increase in this reporter gene expression was achieved, compared with transfection with the vector alone (Fig. 5F). Although the expression of PNTP1 had little effect on the reporter gene, co-expression of PNTP1 with REPO resulted in a significantly higher level of reporter expression than the expression of REPO alone (Fig. 5F). Taken together with the

previous work (Granderath et al., 2000), these results show that both PNTP1 and REPO acts through their respective binding sites in the *loco* promoter to activate transcription.

Synergistic effect of REPO and PNTP1 was also observed in vivo. While ectopic expression of either PNTP1 or REPO in the neuroectoderm caused only a minor increase in the number of cells that expressed a *loco* enhancer-trap strain (rC56), co-expression of REPO and PNTP1 in the neuroectoderm caused a dramatic increase in the response (Fig. 5I-K). When misexpression was directed using the *engrailed*-GAL4 driver, co-expression of REPO and PNTP1 caused a fivefold increase in the number of rC56-expressing cells compared with the expression of REPO or PNTP1 alone (Fig. 5L). Such a synergistic effect was not observed between REPO and TTK69, or between PNTP1 and TTK69 (data not shown). We conclude that the glial expression of *loco* is regulated by the cooperation of REPO and PNTP1.

REPO also suppresses neuronal characteristics

In addition to promoting glial differentiation, the glial determinant GCM also inhibits neuronal differentiation (Hosoya et al., 1995; Jones et al., 1995). This function is probably mediated by glial transcription factors that operate downstream of GCM. Because the results presented above established that REPO cooperates with TTK69 and PNTP1 to direct the expression of glial-specific genes in the CNS, we tested whether these proteins also function to inhibit neuronal differentiation.

Ectopic expression of GCM throughout the neuroectoderm has a profound effect on neuronal differentiation; the number of cells that express the neuron-specific marker ELAV is reduced to 5-15% of that in normal embryos (Hosoya et al., 1995). In stage 13 embryos, ectopic expression of REPO or TTK69 alone caused, respectively, little or a modest reduction in the number of ELAV-positive cells (Fig. 6A,C,D). When these two proteins were co-expressed, however, neuronal differentiation was severely blocked, surpassing the inhibition achieved by the ectopic expression of GCM (Fig. 6B,E). Thus, REPO cooperates with TTK69 not only to promote glial development, but also to inhibit neuronal differentiation.

To address whether *repo* activity is indeed necessary for the inhibition of neuronal differentiation, we took advantage of embryos that ectopically express GCM, in which REPO, TTK69 and PNTP1 are all misexpressed. In these embryos, the number of ELAV-positive cells was greatly reduced, and only short stretches of axons could be recognized by staining with an antibody that labels all CNS axons (Fig. 7B,F). The introduction of a *pointed* mutation into this genotype had little effect on the number of ELAV-positive cells or the axonal phenotype, indicating that *pointed* is not essential for inhibiting neuronal differentiation in this genetic background (Fig. 7D,H). However, removal of *repo* function caused a striking effect on the GCM-misexpression phenotype; ELAV-positive cells increased significantly in number, and they grew long axons that made bundles reminiscent of longitudinal or commissural tracts (Fig. 7C,G). The effect of the *repo* mutation was much more pronounced than the removal of TTK (Fig.

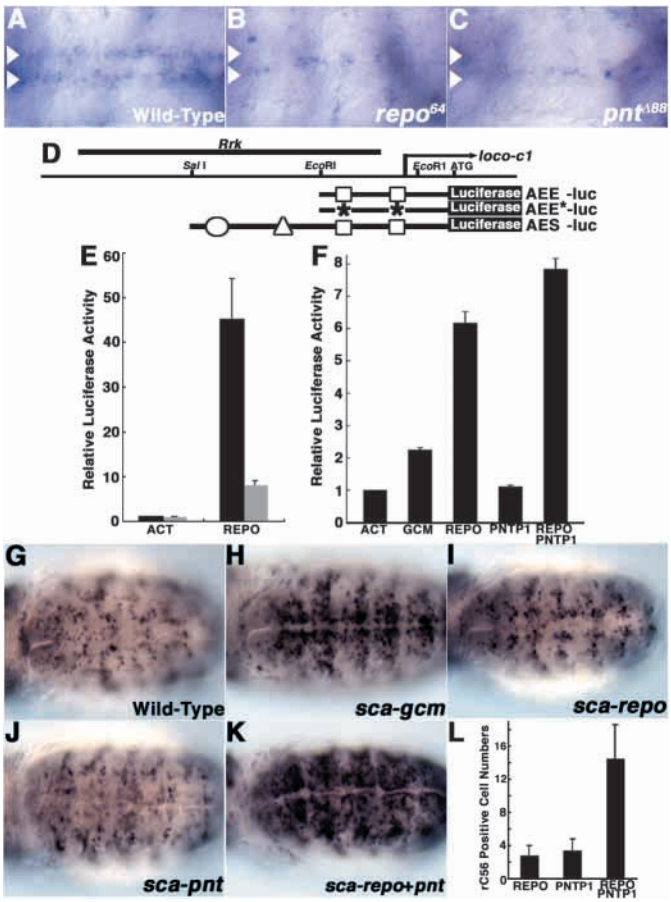

Fig. 5. REPO and PNTP1 cooperate to activate the transcription of *loco*. (A-C) Whole-mount in situ hybridization of stage 16 embryos using the *loco-c1* probe. Ventral views. Wild-type embryos (A) express *loco-c1* mRNA in two rows of longitudinal glial cells (arrowheads). *loco-c1* mRNA expression in longitudinal glia was undetectable in *repo* mutant embryos (B) or in *pointed* mutant embryos (C). Anterior is leftwards. (D) Structure of the *loco-c1* promoter-luciferase reporter genes used for transfection assays. AEE-*luc* carries a 0.7 kb *loco* promoter fragment, which contain two CAATTA motifs (square). Single base changes (asterisk) were introduced in both motifs in AEE*-*luc* reporter. AES-*luc* has a 1.4 kb *loco* fragment, which includes an Ets-binding site (triangle) and a GCM-binding site (oval), both identified by Granderath et al. (Granderath et al., 2000). The glial enhancer fragment (Rrk) used by Granderath et al. (Granderath et al., 2000) is shown above the map. The exact position of the transcriptional start site is not known. (E) REPO activates transcription through the CAATTA motif in the *loco-c1* promoter. S2 cells were transfected with AEE-*luc* (black) or AEE*-*luc* (gray) reporter and REPO-expressing plasmid (REPO) or the empty vector (ACT). Luciferase activity obtained after the transfection of the effector constructs was normalized to the activity of the AEE-*luc* reporter co-transfected with the empty vector. (F) REPO and PNTP1 cooperates on the expression of the *loco* promoter. S2 cells were co-transfected with the AES-*luc* reporter gene and effector constructs that expressed GCM (column 2), REPO (column 3), PNTP1 (column 4) or REPO and PNTP1 (column 5). Luciferase activity was normalized to the value obtained with the empty vector (column 1). (G-L) REPO and PNTP1 has synergistic effects on *loco* expression. (G-K) *lacZ* expression of rC56, an enhancer trap insertion into the *loco* locus. The following transgenes were misexpressed in the entire neuroectoderm using the *scabrous*-GAL4 strain: (G) none; (H) GCM; (I) REPO; (J) PNTP1; (K) REPO and PNTP1. (L) Number of rC56-positive cells upon misexpression using the *engrailed*-GAL4 driver. The number of rC56-positive cells in the *engrailed*-positive region in each segment were scored (n=15).

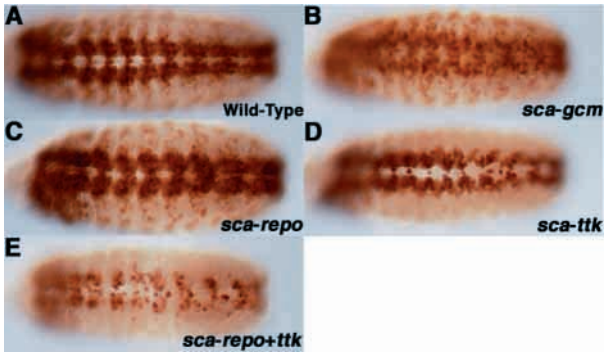

Fig. 6. REPO and TTK69 cooperate to suppress neuronal development. The expression of the neuron-specific protein ELAV in stage 13 embryos. (A) Wild type. (B-E) Ectopic expression using the *scabrous* GAL4 driver. (B) Ectopic expression of GCM reduced the number of ELAV-expressing cells. Ectopic expression of REPO (C) or TTK69 (D) caused, respectively, little or a modest reduction in the number of ELAV-expressing cells. When REPO and TTK69 were co-expressed, they synergistically reduced the number of ELAV-positive cells (E). Anterior is leftwards.

7I,J), which has been regarded as the major factor in repressing neuronal differentiation in glial cells. As the expression of the glial marker M84 was still absent (Fig. 7C), the rescue of neuronal differentiation was achieved without normal glial function, most probably through an autonomous effect on presumptive neurons. We conclude that REPO is an essential factor for mediating the function of GCM, both to promote glial development and to inhibit neuronal differentiation.

DISCUSSION

REPO is the major factor for glial differentiation

Although glial specification by GCM is well established, how the characteristics of individual glial cells are determined is poorly understood. GCM expression is confined to the early stage of glial development, suggesting that GCM itself does not participate in the terminal differentiation of glia. Moreover, GCM also directs blood cell development; GCM is expressed in macrophage precursors and ectopic expression of GCM in crystal cell precursors causes the transformation of crystal cells to macrophages (Bernardoni et al., 1997; Lebestky et al., 2000). These results clearly show that the expression of GCM does not always lead to the determination and terminal differentiation of glia. In glial cells, GCM induces the expression of three transcription factors, REPO, TTK69, and PNTP1 (Hosoya et al., 1995; Jones et al., 1995; Giesen et al., 1997), and the loss of these proteins causes abnormal glial development, although GCM expression remains normal (Halter et al., 1995; Giesen et al., 1997) (data not shown). Although *gcm* can direct *repo* expression in various contexts (Akiyama-Oda et al., 1998; Bernardoni et al., 1998), *repo* is not expressed endogenously in blood cells, but is confined to GCM-positive glial cells, lasting even after *gcm* expression has ceased. In *repo* mutant embryos, the migration, survival and terminal differentiation of glial cells are abnormal (Campbell et al., 1994; Xiong et al., 1994; Halter et al., 1995). In this paper, we showed that the homeodomain protein REPO activates gene expression in glia, and also demonstrated that REPO mediates the suppression of neuronal differentiation (Fig. 8). These results suggest that REPO is the major factor that is necessary for glial development.

Target specificity of REPO is controlled by a combination of cooperating factors

Identifying the target genes of homeodomain proteins has been difficult, partly because most homeodomain proteins bind similar DNA sequences in vitro. REPO has been shown to bind the CAATTA motif in vitro (Halter et al., 1995), and we showed that REPO is necessary and sufficient to activate the transcription of the *ftz* HDS reporter gene that carries the CAATTA motif in its promoter. In cultured *Drosophila* cells, the ability of REPO to activate the CAATTA-*luc* reporter gene was dependent on the presence of the REPO homeodomain and the CAATTA motif in the reporter gene.

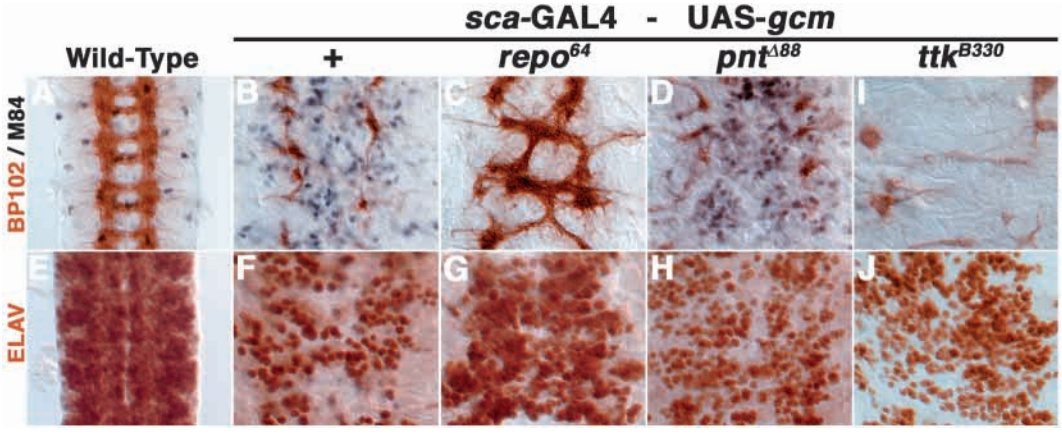

Fig. 7. REPO is necessary for the inhibition of neuronal differentiation by GCM. (A-D,I) The axonal scaffold was labeled using mAb BP102 (brown). (A-D) Glial cells were labeled by β-galactosidase expression from the glial marker M84 (black). (E-H,J) All neurons in the CNS were labeled using the ELAV antibody. (A,E) Wild type. (B,F) Ectopic expression of GCM expression using the *scabrous GAL4* driver. Ectopic expression of GCM in the *repo* mutant background (C,G), *pointed* mutant background (D,H) or *ttk* mutant background (I,J). Ectopic expression of GCM caused a reduction in the number of ELAV-positive neurons and axonal extension, as well as an increased number of cells that expressed the M84 marker (B,F). Removal of *repo* function resulted in a dramatic restoration of ELAV-positive cells and axonal development (C,G). The effect of removing *pointed* (D,H) or *ttk* (I,J) function was, respectively, undetectable or minor (compare with B,F). Note that the *ttk* mutant (I,J) is labeled for axons (I; mAB BP102) and neurons (J; ELAV); this animal did not carry the glial marker M84 (I,J). All embryos were stage 15. Anterior is upwards.

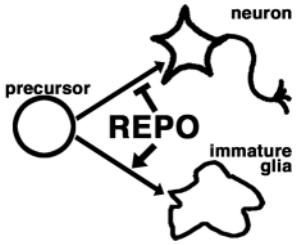

Fig. 8. Model of glial development. Glial cell development involves two processes that both depend on REPO: the activation of glial differentiation and the inhibition of neuronal differentiation.

These results strongly suggest that REPO activates the transcription of the *ftz* HDS reporter gene by directly binding to the CAATTA motif in vivo.

Despite the glia-specific expression of the *ftz* HDS reporter gene, the CAATTA motif is not a specific target site of REPO. FTZ and EN bind the CAATTA motif (Desplan et al., 1988; Schier and Gehring, 1992), which also resembles the consensus binding sequence for ANTP and UBX (Müller et al., 1988; Ekker et al., 1992). Why do other homeodomain proteins fail to drive the *ftz* HDS reporter gene in vivo? Recent results show that homeodomain proteins require co-factors to activate the transcription of their target genes. Co-factors, such as EXD and FTZF1, are also DNA-binding proteins that require specific binding sites in the target gene (Chan et al., 1994; van Dijk and Murre, 1994; Guichet et al., 1997; Yu et al., 1997). Homeodomain proteins other than REPO may be incapable of activating the *ftz* HDS reporter gene because it does not have binding sites for their co-factors.

The behavior of the *ftz* HDS reporter gene suggests that the requirement for co-factors may also apply to REPO. Although the ectopic expression of REPO induced the ectopic expression of the *ftz* HDS reporter gene in the periphery, it did not affect the expression pattern in the CNS. Thus, REPO cannot be the single factor responsible for the activation of the *ftz* HDS reporter gene. Indeed, the expression pattern of the *ftz* HDS reporter gene is altered by changing nucleotides outside the CAATTA motif (Nelson and Laughon, 1993), indicating that *ftz* HDS contains binding sites for factors other than REPO. The simplest interpretation is that such factors are present in the periphery, but not in the CNS.

Using additional target genes of REPO, we provided further evidence that the transcriptional regulation by REPO involves co-factors. Although the enhancer-trap line M84 and the *loco* gene were both dependent on REPO function, and could be expressed precociously and ectopically upon mis-expression of REPO, much stronger responses were obtained when REPO was co-expressed with TTK69 or PNTP. Endogenous expression of M84 and the *loco* gene occurs in cells that co-express REPO and TTK69 or PNTP1, respectively. TTK69 and PNTP1 are thus good candidates for REPO co-factors. Together with an earlier study (Granderath et al., 2000) our results show that REPO and PNTP1 cooperate on *loco* expression through their binding sites in the *loco* promoter. Likewise the synergism between REPO and TTK69 may also occur on the promoter of their target genes.

Our conclusion that the expression of M84 and *loco* are achieved by a cooperation of REPO and TTK69/PNTP1 does not rule out the possibility that these genes are also direct targets of GCM. In fact, reporter genes driven by *loco* enhancer elements are expressed normally in stage 14 *repo* mutant embryos, indicating that other factor(s) activate their transcription at the onset of gliogenesis (Campbell et al., 1994; Granderath et al., 2000). Because the *loco* enhancer element contains GCM-binding sites, GCM can directly regulate *loco* (Granderath et al., 2000). However, as the expression of GCM in glia is transient, transcription initiated by GCM must be sustained by other factors. REPO and PNTP1 are the best candidates for factors that maintain *loco* expression throughout glial development and functioning.

The synergistic effect of REPO and TTK69 on M84 marker expression suggests a positive role of TTK69 on glial differentiation. As the major function of TTK69 has been thought to be the inhibition of neuronal differentiation through transcriptional repression (Brown et al., 1991; Read et al., 1992; Xiong and Montell, 1993; Giesen et al., 1997), the positive action of TTK69 on glial gene expression could be an indirect effect through repressing transcription of a repressor for M84 expression. However, TTK69 can activate transcription in yeast cells (Yu et al., 1999), suggesting that TTK69 may also promote transcription, depending on the cellular context. Recent studies also implicate a role of TTK69 in cell proliferation, through controlling the expression of regulators of the cell cycle (Badenhorst, 2001; Baonza et al., 2002). Badenhorst (Badenhorst, 2001) has shown that overexpression of TTK69 results in the inhibition of glial development, accompanied by the repression of the S-phase cyclin and glial proliferation. As we observe an increase in the number of cells that express M84 glial marker upon co-expression of TTK69 and REPO, our result cannot be accounted for by the ability of TTK69 to inhibit glial cell cycle. Whereas ectopic expression of TTK69 reduces the expression of the endogenous *repo* gene (Badenhorst, 2001), our misexpression paradigm provides exogenous REPO through the GAL4/UAS control. Thus the existence of REPO might modify the activity of TTK69, so that it plays a positive role on glial development.

Repression of neuronal differentiation during glial development

Glial fate determination involves not only the promotion of glial differentiation but also the suppression of neuronal properties. Because ectopic GCM can induce neurogenesis in certain contexts (Akiyama-Oda et al., 1998; Van de Bor et al., 2002), it is unlikely that GCM directly represses neuronal differentiation. TTK69 has been proposed to inhibit neuronal differentiation, mainly because of its loss-of-function phenotype in the sensory organ (Giesen et al., 1997). Here, we have shown that the co-expression of REPO and TTK69 has a potent neuron-suppressing activity, and further demonstrated that the *repo* mutant permits neuronal differentiation even when GCM is overexpressed. This strongly suggests that REPO functions not only to activate the transcription of glial genes, but also to prevent the neuronal differentiation of presumptive glial cells (Fig. 8).

If glia and neuron represent two mutually exclusive cell states that must be chosen between early in development, it is somewhat strange that suppression of neuronal development should be carried out by proteins that are expressed throughout

glial differentiation. The existence of continuous suppression of neuronal properties in glia suggests that cells within the nervous system may retain the potential to become neurons or glia throughout their cellular history. This idea is supported by the observation that GCM is able to transform post-mitotic neurons into glia (Jones et al., 1995). Conversely, in the vertebrate nervous system, glial cells (astrocytes and oligodendrocyte-precursor) can respond to environmental signals and function as neural stem cells, generating neurons (Doetsch et al., 1999; Kondo and Raff, 2000). The role of REPO and TTK69 may be to suppress the ability of glia to respond to cues that would cause them to change into neurons or neural precursors.

We thank C. Klämbt, A. Travers, C. Goodman, C. Montell, Y. Akiyama-Oda, A. Laughon, A. Brand, S. Hayashi and the Developmental Studies Hybridoma Bank, University of Iowa, for fly strains, DNA clones, and antibodies. We also thank all the members of the Okano, Hiromi, Hotta, and Hayashi laboratories for helpful discussions. We acknowledge A. Graham for critically reading the manuscript. This work was supported by grants from the Ministry of Education, Science, Sports and Culture of Japan, and by the Core Research for Evolutional Science and Technology Corporation. Y.Y. was supported by the Japan Society for the Promotion of Science.

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