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Development/Plasticity/Repair

The Repo Homeodomain Transcription Factor Suppresses Hematopoiesis in *Drosophila* and Preserves the Glial Fate

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Despite their different origins, *Drosophila* glia and hemocytes are related cell populations that provide an immune function. *Drosophila* hemocytes patrol the body cavity and act as macrophages outside the nervous system, whereas glia originate from the neuroepithelium and provide the scavenger population of the nervous system. *Drosophila* glia are hence the functional orthologs of vertebrate microglia, even though the latter are cells of immune origin that subsequently move into the brain during development. Interestingly, the *Drosophila* immune cells within (glia) and outside (hemocytes) the nervous system require the same transcription factor glial cells deficient/glial cells missing (Glide/Gcm) for their development. This raises the issue of how do glia specifically differentiate in the nervous system, and hemocytes in the procephalic mesoderm. The Repo homeodomain transcription factor and panglial direct target of Glide/Gcm is known to ensure glial terminal differentiation. Here we show that Repo also takes center stage in the process that discriminates between glia and hemocytes. First, Repo expression is repressed in the hemocyte anlagen by mesoderm-specific factors. Second, Repo ectopic activation in the procephalic mesoderm is sufficient to repress the expression of hemocyte-specific genes. Third, the lack of Repo triggers the expression of hemocyte markers in glia. Thus, a complex network of tissue-specific cues biases the potential of Glide/Gcm. These data allow us to revise the concept of fate determinants and help us to understand the bases of cell specification. Both sexes were analyzed.

Key words: *Drosophila*; Gcm; glia; glide; hemocytes; repo

Significance Statement

Distinct cell types often require the same pioneer transcription factor, raising the issue of how one factor triggers different fates. In *Drosophila*, glia and hemocytes provide a scavenger activity within and outside the nervous system, respectively. While they both require the glial cells deficient/glial cells missing (Glide/Gcm) transcription factor, glia originate from the ectoderm, and hemocytes from the mesoderm. Here we show that tissue-specific factors inhibit the gliogenic potential of Glide/Gcm in the mesoderm by repressing the expression of the homeodomain protein Repo, a major glial-specific target of Glide/Gcm. Repo expression in turn inhibits the expression of hemocyte-specific genes in the nervous system. These cell-specific networks secure the establishment of the glial fate only in the nervous system and allow cell diversification.

Introduction

In the *Drosophila* embryo, lateral glial cells (called glia throughout the text, for the sake of simplicity) constitute the second major population of the nervous system and are necessary for neuronal development, function, and survival. Typically, they

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insulate the CNS upon forming the blood–brain barrier (BBB) and regulate neurotransmitter recycling, axon guidance, or neural proliferation (Trébuchet and Giangrande, 2012). During development and upon injury, *Drosophila* glia also act as scavenger cells and help in reshaping the nervous system. Thus, *Drosophila* glia behave like microglia (Logan and Freeman, 2007; Kurant, 2011), vertebrate immune cells of mesodermal origin that move from the yolk sac into the brain during development and provide the resident macrophages of the CNS (Ginhoux et al., 2010). Outside the fly nervous system, hemocytes play a key role in cellular and humoral immunity. They can move rapidly to patrol the organism and respond to a variety of challenges. The most represented subtype of hemocytes, called plasmatocytes, phagocyte microbes and sculpt tissues by clearing apoptotic cells during development (Meister and Lagueux, 2003).

In addition to sharing the immune function, glia and hemocytes express the same transcription factor, the atypical zinc finger protein Glial cells deficient/Glial cells missing [Glide/Gcm (called "Gcm" throughout the text); Mao et al., 2012; Cattenoz and Giangrande, 2013] at early stages of their development. Gcm is necessary and sufficient to induce gliogenesis and is required for hemocyte differentiation (for review, see Cattenoz and Giangrande, 2015). Thus, the same transcription factor works in functionally related cells that originate from the neurogenic ectoderm (glia) and from the procephalic mesoderm (PM; hemocytes). In the nervous system, Gcm induces the expression of the reverse polarity (Repo) homeodomain containing transcription factor in all the glial cells. Repo is necessary for the execution of the glial differentiation program (Yuasa et al., 2003), and embryos lacking Repo do not express late markers (Halter et al., 1995), including the scavenger receptor Draper (Drpr; Shklyar et al., 2014). As a consequence, *repo* mutant glial cells are not functional and have defective phagocytic activity (Shklyar et al., 2014).

The shared molecular pathway and role of glia and hemocytes call for a cell-specific mechanism triggering the differentiation of embryonic glia and blood in the correct tissue. We here show that mesodermal cues contribute to prevent glial differentiation in the hemocyte anlagen. The mesodermal transcription factor Twist (Twi) induces the expression of *miR-1*, which in turn represses Repo post-transcriptionally. This inhibits the gliogenic potential of Gcm in the PM (Xiong et al., 1994; Halter et al., 1995; Yuasa et al., 2003), showing that the potential of a fate determinant relies on the cell-specific transcriptional landscape. The negative regulation of Repo in the hemocyte anlagen is crucial as Repo represses the hemocyte fate: when expressed in the hemocyte anlagen, it inhibits the expression of hemocyte-specific genes, and the lack of Repo induces the expression of early hemocyte markers in the nervous system. Thus, Repo constitutes a major element in the pathway that discriminates between related but distinct scavenger fates.

Altogether, our work dissects the complex network that allows a single pioneer factor to affect different cell fates.

Materials and Methods

Fly stocks. Flies were kept at 25°C. w^{1118} was used as wild-type (WT). *repo-nGFP* and *repo-nRFP* lines were generated to drive nuclear GFP or RFP expression under the control of the 4.3kb *repo* promoter, which recapitulates the full *repo* expression pattern (Lee and Jones, 2005; Laneve et al., 2013). gcm^{34} (Bernardoni et al., 1999) was used as a *gcm* hypomorphic allele carrying a *lacZ* insertion. The *Df(2L)132* (Kammerer and Giangrande, 2001) deletes the entire *gcm* locus and was used as a null allele. $repo^{52}$, $repo^{84}$ (Xiong et al., 1994; Halter et al., 1995), twi^{1} (Castanon et al., 2001; *twi-/CyO_act5cGFP*), $miR\text{-}1^{KO}$ [catalog #58879, Bloomington Drosophila Stock Center (BDSC); RRID:BDSC_58879], and $elav^{4}$ (catalog #541, BDSC; RRID:BDSC_541) are null alleles.

The *UAS/Gal4* system was used for cell-specific manipulation of gene expression. *srp(hemo)Gal4* triggers expression in hemocytes (Brückner et al., 2004), *scaGal4* throughout the neurogenic region (Budnik et al., 1996), *twiGal4* (Baylies and Bate, 1996) throughout the mesoderm, and *gcmGal4* (Soustelle and Giangrande, 2007) combined with *repoGal80* (a gift from B. Altenhein, University of Cologne - Zoological Institute) throughout the hemocyte anlagen. Finally, *repoGal4* was used to drive gene expression in glial cells (Lee and Jones, 2005).

The following transgenes were also used: *UAS-CD8GFP* (targeting GFP expression to the membrane), *UAS-RFP* (BDSC), *UAS-GFP* (BDSC); *UASrepo* (Yuasa et al., 2003); *UAS-twi* (Baylies and Bate, 1996), *srp(hemo)RFP* (Gyoergy et al., 2018); *UAS-gcm(F18A)* (see Figs. 2*G*,*G*′, Fig. 7; weak Gcm overexpression); and *UAS-gcm(RS1)* (Fig. 1*C–E*) or *UAS-gcm(M24A)* (Fig. 2*G–H*; and see Fig. 6; medium Gcm overexpression; Bernardoni et al., 1998). The combination of *UAS-gcm(M24A)* and *UAS-gcm(F18A)* provided a strong Gcm overexpression (Fig. 2*G*,*G*′).

Immunohistochemistry. Embryo collections were performed on plates containing agar, apple juice, and yeast. Dechorionated embryos were fixed in 4% formaldehyde in PBS for 20 min, permeabilized with 0.3% Triton X-100 in PBS (PTX), blocked by 0.5% Blocking Reagent (Roche) in PTX for 1 h and labeled overnight at 4°C with the following antibodies: rabbit (rb) anti-Repo (1 of 10), mouse (m) anti-Repo (1 of 10; anti-Repo; catalog #8D12, DSHB; RRID:AB_528448), m anti-Singed (Sn; 1 of 50; catalog #sn 7C, DSHB; RRID:AB_528239), and rat anti-Elav (1 of 200; catalog #Rat-Elav-7E8A10 anti-elav, DSHB; RRID:AB_528218); guinea pig (gp) anti-Repo (1 of 1000) and gp anti-Nazgul (1 of 200; a gift from B. Altenhein, University of Cologne - Zoological Institute; von Hilchen et al., 2010); mouse (m) anti-Ush (1 of 1000; Cubadda et al., 1997); rb anti-Srp (Serpent; 1 of 1000; a gift from R. Reuter, Institute for Cell biology - University of Tübingen; Sam et al., 1996; Petersen et al., 1999); m anti-P1 (1 of 10; a gift from E. Kurucz, Institute of Genetics, Szeged, Hungary; Kurucz et al., 2007a); rb anti-Croquemort (Crq; 1 of 500; a gift from J.L Dimarcq and J. Hoffmann, Institut de Biologie Moléculaire et Cellulaire, Strasbourg; Franc et al., 1996); m anti-Fas2 (1 of 100; a gift from C.S. Goodman, Howard Hughes Medical Institute, University of California; Grenningloh et al., 1991); rb anti-HRP (1 of 500) and rb anti-β-Gal (1 of 500; Cappel); and chicken anti-GFP (1 of 1000; catalog #ab92456, Abcam; RRID:AB_10561923); m anti-β-Gal (1 of 200; Sigma-Aldrich); rat anti-RFP (1 of 100; catalog #5f8100, ChromoTek; RRID:AB_2336064); and rb anti-DCP-1 (death caspase-1; 1 of 50; catalog #9578, Cell Signaling Technology; RRID:AB_2721060).

The secondary antibodies were FITC-, Cy3, or Cy5 conjugated (1 of 400; Jackson ImmunoResearch). Images were taken with the SP2 or the SP5 confocal microscopes (Leica) and processed using Fiji (Schindelin et al., 2012).

Srp signal intensity was measured on confocal images acquired with hybrid detector in photon-counting mode. The mean gray value measurement tool from Fiji (Fiji; RRID:SCR_002285) was used to estimate the intensity of the signal (in A.U.) from 50 hemocytes in at least three embryos (Schindelin et al., 2012).

Cotransfection, Western blot, and luciferase assays. Drosophila S2 cells were grown in Schneider medium (Thermo Fisher Scientific) complemented with 10% heat-inactivated fetal calf serum and 0.5% penicillin/streptomycin. A total of 6×10^6 cells were cultured in six-well culture dish 12 h prior to transfection. Five micrograms of total plasmid mix were transfected using the Effectene Kit (Qiagen) according to manufacturer instructions. The *psrp(hemo)Gal4* plasmid provided an *srp* transcriptional reporter (Brückner et al., 2004) upon cotransfection with the *pUAS-GFP* plasmid. The *pPac5C-repo* plasmid was used to induce Repo expression (Yuasa et al., 2003), and *pPac5C-lacZ* as a transfection control. The *pPac5C* plasmid was used to equilibrate the amount of transfected DNA. Cells were harvested 24 h after transfection in Tris-HCl 25 mM pH 7.9, 400 mM KCl, and 10% glycerol, and total proteins were extracted by three freezing–thawing steps. Protein expression was detected from protein lysate according to standard Western blot procedures. The following primary antibodies were used: m anti-β-Gal (1 of 2500; Sigma-Aldrich), rb anti-GFP (1 of 5000; Invitrogen), and m anti-

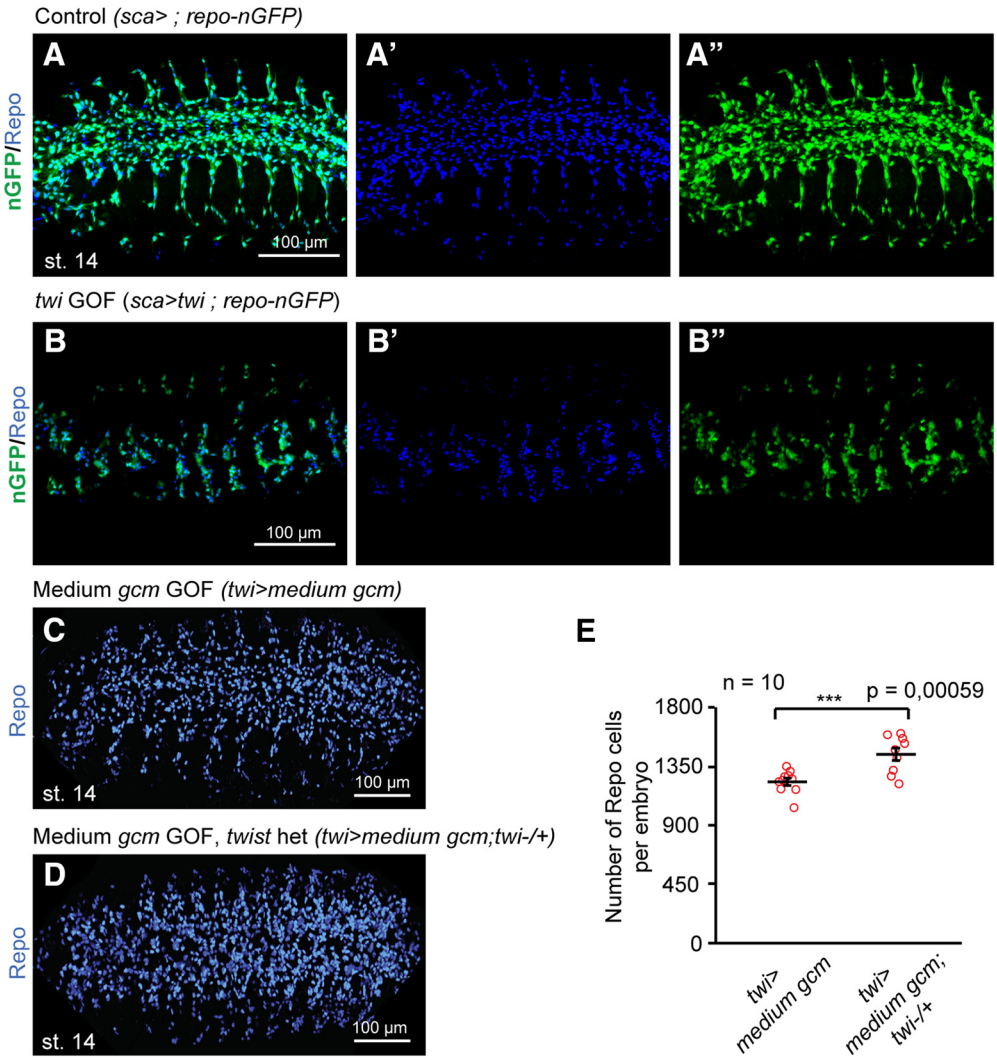

Figure 1. Twi negatively regulates Repo expression in the nervous system. ***A–D***, Confocal projections of stage 14 *sca>;repo-nGFP* (Control; ***A–A"***) and *sca>twi; repo-nGFP* (*twi* GOF; ***B–B"***) embryos immunolabeled for the glial marker Repo (blue) and the glial reporter *repo-nGFP* (green). Ventral view. Here and in all figures, anterior is to the left. ***C***, ***D***, Confocal projections of embryos stage 14 *twi>gcm* (*gcm* GOF; ***C***) and *twi>gcm;twi-/+* (*gcm* GOF, *twist* het; ***D***) immunolabeled for Repo (blue). ***E***, Quantification of the number of Repo-positive cells per embryo in *twi>medium gcm* and *twi>medium gcm;twi-/+*; $n = 10$ embryos, the mean ± SEM is represented on the chart and the *p* value was calculated by ANOVA. Scale bar, 100 µm. $^{***}p < 0.001$.

Repo [1 of 20; Developmental Studies Hybridoma Bank at the University of Iowa (DHSB)]. m anti-HRP and rb anti-HRP (1 of 5000; Jackson ImmunoResearch) were used as secondary antibodies.

For the luciferase assay, *Drosophila* S2 cells were cultured in a 24-well plate, in the same conditions as previously described. Plasmid transfections were performed using Effectene (Qiagen) following manufacturer instructions. *pMTGal4-GFP*, *pUAST-Luciferase-Luciferase 3′UTR*, *pUAST-Luciferase-Repo 3′UTR*, *pUAST-Luciferase-Repo 3′UTR ΔmiR-1*, and *pTK-Renilla* were all used at 20 ng/ml; and *pTub-miR-1* was used at 50 ng/ml. The cells were cultured 2 d prior to induction with 500 µM copper sulfate. The luciferase assay was performed 18 h after induction, using the Dual-Glo Luciferase assay kit (Promega) according to manufacturer instructions. Three independent transfections were averaged with SD. Statistical significance was calculated with GraphPad Prism software (GraphPad Software; RRID:SCR_002798) using a *t* test.

RNA extraction, reverse transcription, and quantitative PCR. Total RNA was purified from stage 5–11 embryos using Tri Reagent (MRC). One microgram of purified RNA was reverse transcribed by SuperScript II reverse transcriptase (Invitrogen) using oligodT primers (5 µM). mRNAs were analyzed by quantitative PCR (qPCR) using Sybr Green (Roche) Master Mix, the thermocycler LightCycler480 (Roche) and the following oligonucleotides: *repo*: forward, 5′ AAGCAGCAGCAAGAAGAAGG 3′; reverse, 5′ ATACGGAGCACGTTCAAAGG 3′; and *actin5C*: forward, 5′ GCAGCAACTTCTTCGTCACA 3′; reverse, 5′ CTTAGCTCAGCCT CGCCACT 3′, For each gene, the mRNA levels were automatically calculated (LightCycler480 Software, release 1.5.0) by calibration to gene-specific standard curves generated on input cDNAs. Collected values, derived from three amplification reactions, each performed in three independent experiments, were normalized to *actin5C* mRNA amounts.

Statistics. All the experiments were performed in at least three biological replicates. Statistical relevance was assigned by calculating means and SEs. Whenever the data showed normal distribution (Figs. 1*E*, 2*G–H*, 3*F*, 4*G′*), they were analyzed by ANOVA; whenever they did not, (see Fig. 10*F*,*G*) they were analyzed by Kruskal–Wallis and Wilcoxon (W) tests ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). The data analysis for this article were generated using the Real Statistics Resource Pack software [release 5.4; Charles Zaiontz (www.realstatistics.com)].

Results

The mesoderm-specific transcription factor Twist represses the expression of the Repo panglial protein

The Gcm transcription factor is expressed in the glial as well as in the hemocyte lineages, where it controls the expression of glial and hemocyte genes, respectively (Jones et al., 1995; Bernardoni et al., 1997, 1998; Lebestky et al., 2000; Alfonso and Jones, 2002;

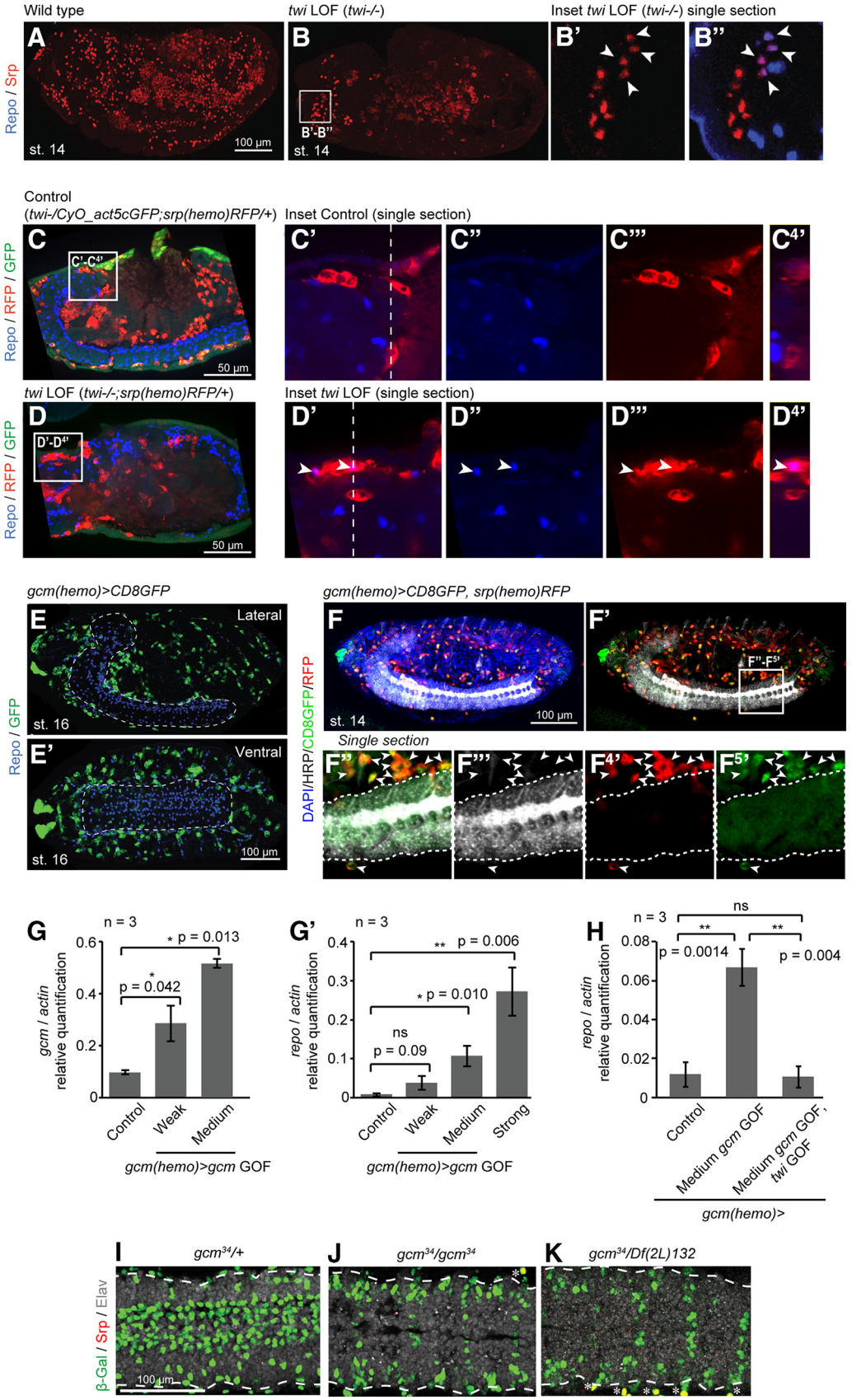

Figure 2. Twi negatively regulates Repo expression in the hemocytes. ***A–B″***, Confocal projections of wild-type (***A***) and *twi*$^{-/-}$ (*twi* LOF; ***B–B″***) embryos labeled for the glial marker Repo (blue) and for the hemocyte marker Srp (red). Lateral view. ***B′*** and ***B″*** represent a single section of the inset indicated in ***B***, they show Srp labeling only and colabeling with Repo, respectively. The white arrowheads indicate cells coexpressing Srp and Repo. Scale bar, 100 μm. ***C–D$^{4'}$***, Confocal projections of control (*twi-/CyO_act5cGFP;srp(hemo)RFP/+*; ***C–C$^{4'}$***) and *twi LOF* (*twi*$^{-/-}$*;srp(hemo)RFP*; ***D–D$^{4'}$***) embryos, labeled for GFP (green), Repo (blue), and the hemocyte reporter *srp(hemo)RFP* (red; Gyoergy et al., 2018). ***C′–C$^{4'}$*** and ***D′–D$^{4'}$*** show single sections (*Figure legend continues.*)

Cattenoz et al., 2016; Bazzi et al., 2018). Since glia differentiate from the ectoderm, and hemocytes from the PM, we hypothesized that tissue-specific factors regulate the expression of the Gcm targets in a cell-specific manner. Since Twi is an early mesoderm-specific transcription factor, we asked whether it represses the expression of Repo, the most characterized glial-specific target of Gcm that is expressed in all glia. The Repo also represents the only transcription factor that is exclusively expressed in glia (Campbell et al., 1994; Xiong et al., 1994; Halter et al., 1995).

To show that Twi inhibits Repo expression *in vivo*, we analyzed embryos in which we induced Twist expression ectopically [gain of function (GOF)], in the neural territory, as well as embryos that lack Twi expression [loss of function (LOF)] or express low levels of Twi.

The ectopic expression of Twi in the neurogenic region mediated by the *scabrousGal4* driver (*sca>twi*; Budnik et al., 1996) significantly reduces the number of Repo-positive cells in the ventral nerve cord (VNC) from an average of 923 ± 62 cells in control to 458 ± 38 cells in *twi* GOF embryos (embryos, $n = 5$; ANOVA, $p = 0.000114$; Table 1, Fig. 1*A*,*B*″). In addition to the Repo antibody, we used the *repo-nuclearGFP* (*repo-nGFP*) transgene to follow the glial cells produced in *sca>twi* embryos (Fig. 1*A*,*B*″). The *repo-nGFP* transgene faithfully recapitulates the expression profile of the Repo protein (Lee and Jones, 2005; Laneve et al., 2013), as we observed coexpression of *repo-nGFP* and Repo in >95% of the glial cells ($n = 3$ embryos). Using the transgene allowed us to show that all the cells that succeed in expressing the *repo* gene also express the Repo protein and hence maintain the glial identity. The decrease in the number of Repo-positive cells suggests that Twi represses Repo expression; however, we could not exclude that the forced expression of Twi in the neurogenic region affects the ability of the neuroblasts to produce glia. If that were the case, the effect of Twi on Repo would only be indirect. Hence, we complemented the GOF data by analyzing embryos that lack Twi expression (analyzing *twi* LOF) partially or completely. Since the expression of Gcm in the mesoderm triggers gliogenesis at the expense of muscles (Bernardoni et al., 1998), we performed the same experiment in embryos that carry half a dose of Twi, which is a potent muscle determinant (Baylies and Bate, 1996) and found that this significantly enhances the gliogenic potential of Gcm in the mesoderm. These data were obtained upon expressing Gcm with the *twistGal4* driver (*twi>gcm*) in *twi*/+ heterozygous embryos (Table 1, Fig. 1*C*–*E*).

Finally, we analyzed the Repo expression in embryos that completely lack Twi. Although the absence of Twi induces severe and early defects (Thisse et al., 1987), it is not absolutely required for the initial determination of the hemocyte fate (Spahn et al., 2014). This allowed us to analyze the few *twi*-null embryos that reached relatively late stages and revealed the presence of the Repo protein in cells that express the early hemocyte marker Srp (no cell in control, and an average of 8.9 ± 4.3 cells Srp and Repo positive per embryo *twi* LOF; $n = 5$ embryos; W test, $p = 0.0038$; Fig. 2*A*,*B*″). In addition to hemocytes, Srp is also expressed in the fat body. Thus, to confirm that the cells coexpressing Srp and Repo in *twi* LOF are hemocytes, we combined the *srp(hemo)RFP* construct that induces RFP expression specifically in embryonic hemocytes (Gyoergy et al., 2018) with the *twi* mutation (Fig. 2*C*–*D*$^{4\prime}$). Similar to the *twi* LOF embryos, the *twi LOF;srp(hemo)RFP* embryos display hemocytes expressing Repo, with a 40-fold increase of double-labeled (Repo/RFP) cells compared with those observed in the control embryos (0.1 ± 0.06% RFP-/Repo-positive cells in Control embryos *twi-/+;srp(hemo)RFP* compared with 4.0 ± 0.9% RFP-/Repo-positive cells in *twi* LOF embryos $twi^{-/-}$*;srp(hemo)RFP*; $n = 4$ embryos; ANOVA, $p = 0.014$).

Altogether, our results strongly suggest that the lack of Twi allows ectopic Repo expression in the hemocyte anlagen, the PM, hence biasing the gliogenic potential of Gcm in that territory.

We then asked whether overexpressing Gcm in its own domain of expression, the PM, leads to the differentiation of supernumerary hemocytes or whether it bypasses the molecular brake imposed by Twi and allows ectopic Repo expression. For this purpose, we crossed a *gcmGal4* driver with a transgenic line expressing the Gal4 inhibitor Gal80 (Lee and Luo, 1999) in glial cells, the other main territory of Gcm expression [Table 1, *gcmGal4*, *repoGal80*, or *gcm(hemo)Gal4*; Fig. 2*E*,*E*′], so as to confine Gcm overexpression to the hemocytes (Fig. 2*F*–*F*$^{5\prime}$). *gcm(hemo)>gcm* embryos display Repo expression in the hemocyte anlagen, and this is a dosage-dependent phenotype. Several *UAS-gcm* transgenes were used alone or in combination to generate a gradient of Gcm levels, which allowed us to show a positive correlation between the levels of Gcm and those of Repo, the stronger the *UAS-gcm* transgene, the higher the levels of Repo (Fig. 2*G*,*G*′). Moreover, and in line with our hypothesis, cooverexpressing Gcm and Twi (*gcm(hemo)>gcm + twi*) abolishes the induction of Repo expression in the PM (Fig. 2*H*).

The fact that Gcm overexpression induces Repo expression in the PM could mean that glial differentiation simply requires higher Gcm levels than hematopoiesis. If that were the case, hypomorphic *gcm* mutant embryos should express hemocyte markers in the nervous system. The gcm^{34} mutation is an imprecise excision that still expresses the *LacZ* gene carried by the P element located at the *gcm* locus and results in low Gcm levels (Vincent et al., 1996). Neither gcm^{34} homozygous nor gcm^{34}*/Df(2L)132* transheterozygous animals (the *Df(2L)132* deficiency completely deletes the gene; Kammerer and Giangrande, 2001) show Srp ectopic expression in the nervous system (Fig. 2*I*–*K*). This excludes mere dosage dependency for the establishment of the glial versus the blood cell fate and further supports the idea that tissue-specific factors are responsible for it.

In sum, the Twist mesodermal factor negatively affects the expression of the panglial transcription factor Repo.

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(*Figure legend continued.*) of the insets indicated in ***C*** and ***D***, respectively, displaying Srp and Repo labeling (***C′***, ***D′***), Repo only (***C″***, ***D″***), Srp only (***C‴***, ***D‴***), and in the orthogonal section along the *z*- and *y*-axes of the dashed lines (***C*$^{4\prime}$**, ***D*$^{4\prime}$**) represented in ***C′*** and ***D′***. Note that colocalization between Repo and RFP is observed only in *twi* LOF (white arrowheads). Scale bar, 50 μm. ***E–F*$^{5\prime}$**, Confocal projections of an embryo *gcmGal4,repoGal80/+;UAS-CD8GFP* (*gcm(hemo)>CD8GFP*; ***E***, ***E′***) labeled for Repo (blue) and GFP (green), and of an embryo *gcmGal4,repoGal80/+;UASCD8GFP/srp(hemo)RFP* (*gcm(hemo)>GFP,srp(hemo)RFP*; ***F–F*$^{5\prime}$**) labeled for GFP (green), RFP (red), and HRP (gray). The region defined by the dashed line indicates the CNS. ***F″–F*$^{5\prime}$** show the inset indicated in ***F′*** displaying the labeling for HRP, GFP, and RFP (***F″***), HRP only (***F‴***), RFP only (***F*$^{4\prime}$**), and GFP only (***F*$^{5\prime}$**). The white arrowheads indicate the hemocytes; note that GFP expression is excluded from glia. Scale bar, 100 μm. ***G–H***, Relative quantification of *gcm* (***G***) and *repo* mRNA (***G′***, ***H***) by qPCR from stage 5–11 embryos of the following genotypes: *gcm(hemo)>* (Control) and *gcm(hemo)> gcm* GOF (weak, medium, and strong *gcm* GOF; ***G***, ***G′***), *gcm(hemo)>* (Control), *gcm(hemo)>medium gcm* (*Med. gcm* GOF), and *gcm(hemo)>medium gcm + twi* (*Med. gcm* GOF, *twi* GOF; ***H***). *gcm* and *repo* levels are relative to *actin* levels, *n* indicates the number of independent assays (for the statistical tests, see the section on experimental procedures, means ± SEM are represented on the charts and the p values were calculated by ANOVA). ***I–K***, Confocal projections of embryonic ventral cords of the following genotypes: gcm^{34}/+ (***I***), gcm^{34}/gcm^{34} (***J***), and gcm^{34}*/Df(2L)132* (***K***). Labeling: β-Gal (green), Srp (red), and the neuronal marker Elav (gray). The gcm^{34} line represents a P element partial excision that retains the LacZ gene, allowing monitoring of *gcm* expression. β-Gal/Srp double-positive cells (yellow, asterisks) are located outside the ventral cord (dashed line) and label the circulating hemocytes. Scale bar, 100 μm. *$p < 0.05$, **$p < 0.01$.

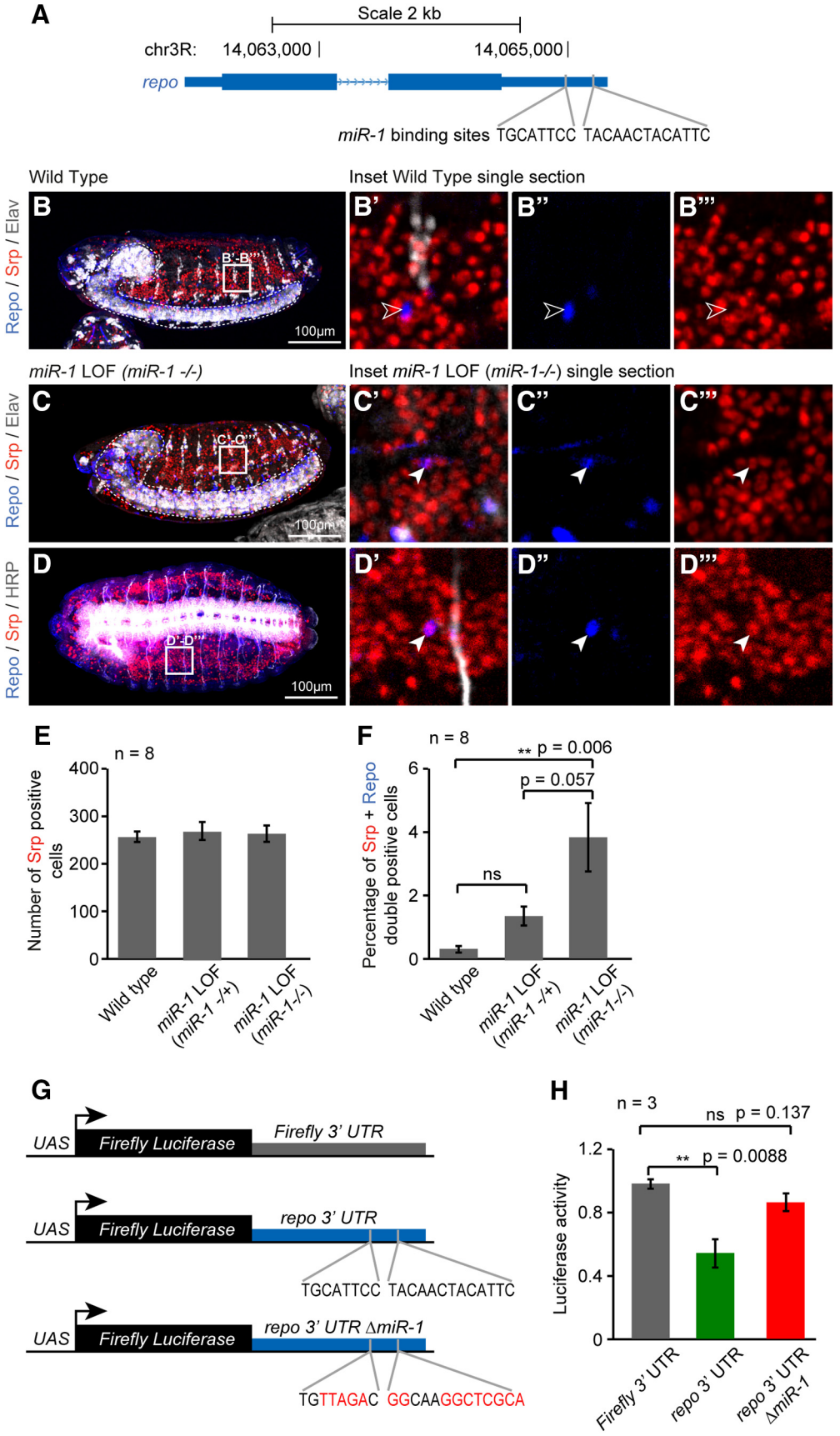

Figure 3. *miR-1* prevents Repo expression in the hemocyte lineage. ***A***, Schematic representation of the *repo* locus in the *Drosophila* genome (dm3). UTRs and coding exons are indicated by plain blue boxes (thin and thick, respectively), and the intron by a blue line. The two putative *miR-1* binding sites in the *repo* 3′ UTR are indicated. ***B–D***, Confocal projections of embryos of the following genotypes: wild-type and *miR-1* LOF (−/−), lateral view, stage 14, labeled for Repo (blue), Srp (red), and Elav (gray; ***B***, ***C***) or HRP (***D***). Note that ***B′–B‴***, ***C′–C‴***, (*Figure legend continues.*)

The microRNA *miR-1* inhibits Repo expression post-transcriptionally

The microRNA *miR-1* is a direct target of Twi expressed and required in the mesoderm (Biemar et al., 2005; Sokol and Ambros, 2005). We found that *miR-1* has two putative target sites in the *repo 3′UTR* (miRanda; http://www.microrna.org/microrna/home.do; Fig. 3*A*) and therefore explored the possibility that it acts post-transcriptionally on Repo. First, we found that animals lacking *miR-1* display ectopic Repo expression in the PM, similar to the *twi* embryos (Fig. 3*B–F*). Second, we asked whether *miR-1* directly acts on the *repo 3′UTR* by cotransfecting S2 *Drosophila* cells with a *miR-1* expression vector and a luciferase reporter carrying either the *repo 3′UTR* or its own *3′UTR* (Fig. 3*G*). By measuring the luciferase activity, we found that *miR-1* specifically acts on the *repo 3′UTR* to repress *repo* expression (Fig. 3*G*,*H*). Third, this negative control is abolished upon mutating the two putative *miR-1* target sites (Fig. 3*H*). Thus, *miR-1* inhibits Repo expression post-transcriptionally.

In sum, our data indicate that mesoderm-specific cues prevent Gcm from triggering Repo expression in the PM.

Repo is sufficient to repress the expression of hemocyte markers in the PM

The tight repression of Repo expression in the hemocyte anlagen suggests that gliogenesis is alternative to hemocyte differentiation. We therefore analyzed the effects of Repo ectopic expression in the PM upon using the *UAS-repo* transgene (Yuasa et al., 2003). *gcm(hemo)>repo* (or *repo* GOF) hemocytes are severely affected: many of them aggregate and show altered morphology as well as migratory defects (Fig. 4*A–D′*). Moreover, they no longer express the late hemocyte marker NimC/P1, which is a scavenger receptor expressed starting from stage 14 (Kurucz et al., 2007a; Fig. 4*E*,*F*), and the expression of the early hemocyte marker Srp is severely downregulated (Fig. 4*A′–C‴*). The hemocytes express Srp at low levels (Fig. 4*A‴*,*C‴*, compare arrowheads). To quantify this phenotype, we measured the intensity of Srp labeling and found a significant difference between control and *repo* GOF hemocytes [control, 83.4 ± 3.9 A.U. (see Materials and Methods); *repo* GOF, 12.4 ± 1.5 A.U.; $n = 50$ cells in three embryos; ANOVA, $p = 6.10^{-23}$]. Of note, Srp is also expressed in the fat body, and yet such expression remains unchanged in *repo* GOF animals (Hoshizaki et al., 1994; Fig. 4*A′*,*C′*), showing that the hemocyte defects are specific and cell autonomous.

More direct evidence for the specific effects of Repo on the *srp* gene was obtained by using a *srp(hemo)Gal4* driver (*srp(hemo)>*) specific to hemocytes (Brückner et al., 2004). Cotransfecting S2 *Drosophila* cells with a Repo expression vector and the *srp (hemo)>GFP* plasmids severely reduces the expression of the GFP, and this is a dosage-dependent effect (Fig. 4*G*,*G′*). We extended the *in vivo* analysis by using the *srp(hemo)>* driver and found phenotypes similar to those observed in *gcm(hemo)>repo* embryos. In line with the aggregation phenotype described above, *srp (hemo)>repo* embryos display reduced levels of the hemocyte marker Sn (also called Fascin; Fig. 5*A–B*$^{5\prime}$), which is expressed from stage 10 onward and is involved in hemocyte migration (Zanet et al., 2009; Gyoergy et al., 2018). Like the *gcm(hemo)>repo* embryos, the *srp(hemo)>repo* embryos also show a reduced number of hemocytes: 252.8 ± 27.4 hemocytes were counted in control and 136.8 ± 19.8 in *repo* GOF embryos [$n = 7$ embryos; ANOVA, $p = 0.0028$; counted on 30 μm stacks of confocal images taken from stage 13 embryos (lateral views); Fig. 5*C–D″*].

Of note, the presumptive hemocytes that ectopically express Repo with the *gcm(hemo)* or with the *srp(hemo)* driver do not express late glial markers (as monitored by the Nazgul antibody; von Hilchen et al., 2010; Ryglewski et al., 2017; Fig. 5*E–H*). The above data strongly suggest that the expression of the Repo panglial factor in the PM is detrimental to hemocyte differentiation, and they are also in line with the fact that Repo is not sufficient to induce the glial fate when ectopically expressed (Yuasa et al., 2003). Finally, there is enhanced hemocyte death in *gcm(hemo)>repo* embryos, as shown by the apoptosis marker cleaved DCP-1 (Song et al., 1997; Fig. 4*H–I″*; 9.1 ± 1.3% of hemocytes display colabeling with DCP-1 in control vs 16.1 ± 2.1% in *repo* GOF embryos, $n = 7$ embryos; ANOVA, $p = 0.0150$). This likely reflects the inability of hemocytes expressing Repo to acquire a stable glial fate.

Given the ability of Gcm overexpression in the PM to induce Repo ectopic expression, we re-examined that phenotype to understand the relative roles of the two transcription factors in blood and glial development. Interestingly, the overexpression of Gcm in the PM induces the expression of both Repo and Nazgul in the presumptive hemocytes (von Hilchen et al., 2010; Fig. 6*A–D‴*, 18.5 ± 1.9% of Repo-positive hemocytes/embryo, $n = 9$ embryos; W test, $p = 0.0061$; and 53.4 ± 6.4% Nazgul-positive hemocytes/embryo, $n = 3$ embryos; W test, $p = 0.0318$ in *gcm(hemo)>gcm*, compared with 0% in control). Moreover, the cells that express Repo also express the hemocyte marker Srp (Fig. 6*E–E‴*; Rehorn et al., 1996) at levels that are comparable to those found in wild-type embryos (the intensity of Srp labeling in hemocytes from control = 83.4 ± 3.9 A.U.; from *gcm(hemo)> gcm* = 73.1 ± 6.2 A.U.; $n = 50$ hemocytes in three embryos; ANOVA, $p = 0.22$). Thus, Gcm overexpression induces the expression of glial genes without blocking hemocyte differentiation. Since Srp constitutes an early hemocyte factor (Reuter, 1994; Bernardoni et al., 1997; Lebestky et al., 2000), we asked whether late hemocyte markers are also detected in those cells or whether hematopoiesis is blocked at its early stages. The hemocyte-specific scavenger receptor Crq (Franc et al., 1996, 1999) colocalizes with the pan-glial maker Repo (Fig. 6*F–F‴*), indicating a mixed glial and hemocyte phenotype. This goes along with the expression/requirement of Gcm in both hemocytes and glia. Of note, we never observed Repo expression in *gcm(hemo)>gcm* hemocytes at larval stages, suggesting that the Repo-expressing cells do not survive or that Repo expression is not maintained. Finally, because the *gcmGal4* driver is expressed transiently and early in the hemocyte lineages, we confirmed these data by using additional hemocyte-specific drivers: *srp(hemo)>*, *hemolectin>* and *hemese>* (Brückner et al., 2004; data not shown).

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(*Figure legend continued.*) and ***D′–D‴*** represent single sections of the insets indicated in (***B***, ***C***, and ***D***), showing Repo, Srp, and Elav or HRP labeling (***B′***, ***C′***, ***D′***), Repo only (***B″***, ***C″***, ***D″***), and Srp only (***B‴***, ***C‴***, ***D‴***). Scale bar, 100 μm. ***E***, ***F***, Number of cells expressing Srp (***E***) and the percentage of Srp cells coexpressing Repo (***F***) in wild-type and in *miR-1* mutant embryos (−/+ and −/−). $n = 8$ embryos analyzed for each genotype. The means ± SEM are represented on the charts and the p values were calculated using ANOVA. ***G***, Schematic representation of the three Luciferase reporter vectors that were used in the cotransfection assays: the top one is the control vector carrying the Firefly Luciferase coding sequence and the Firefly 3′ UTR under the UAS promoter. In the second construct (middle), the 3′ UTR has been replaced by the *repo* 3′ UTR and in the last construct (bottom), the two *miR-1* binding sites of the *repo* 3′ UTR have been mutated. ***H***, Quantification of the Luciferase activity in extracts from S2 cells cotransfected with *pTub-miR-1*, *pTK-Renilla*, and either *pUAST-Luciferase-Luciferase-3′UTR* (*Firefly* 3′ UTR; gray), *pUAST-Luciferase-Repo3′UTR* (*repo* 3′ UTR; green), or *pUAST-Luciferase-Repo-3′UTRΔmiR-1* (*repo* 3′ UTR Δ*miR-1*, red). The values are normalized with the Renilla activity. The means ± standard deviation are represented on the chart and the p values were calculated using student test. $^{**}p < 0.01$.

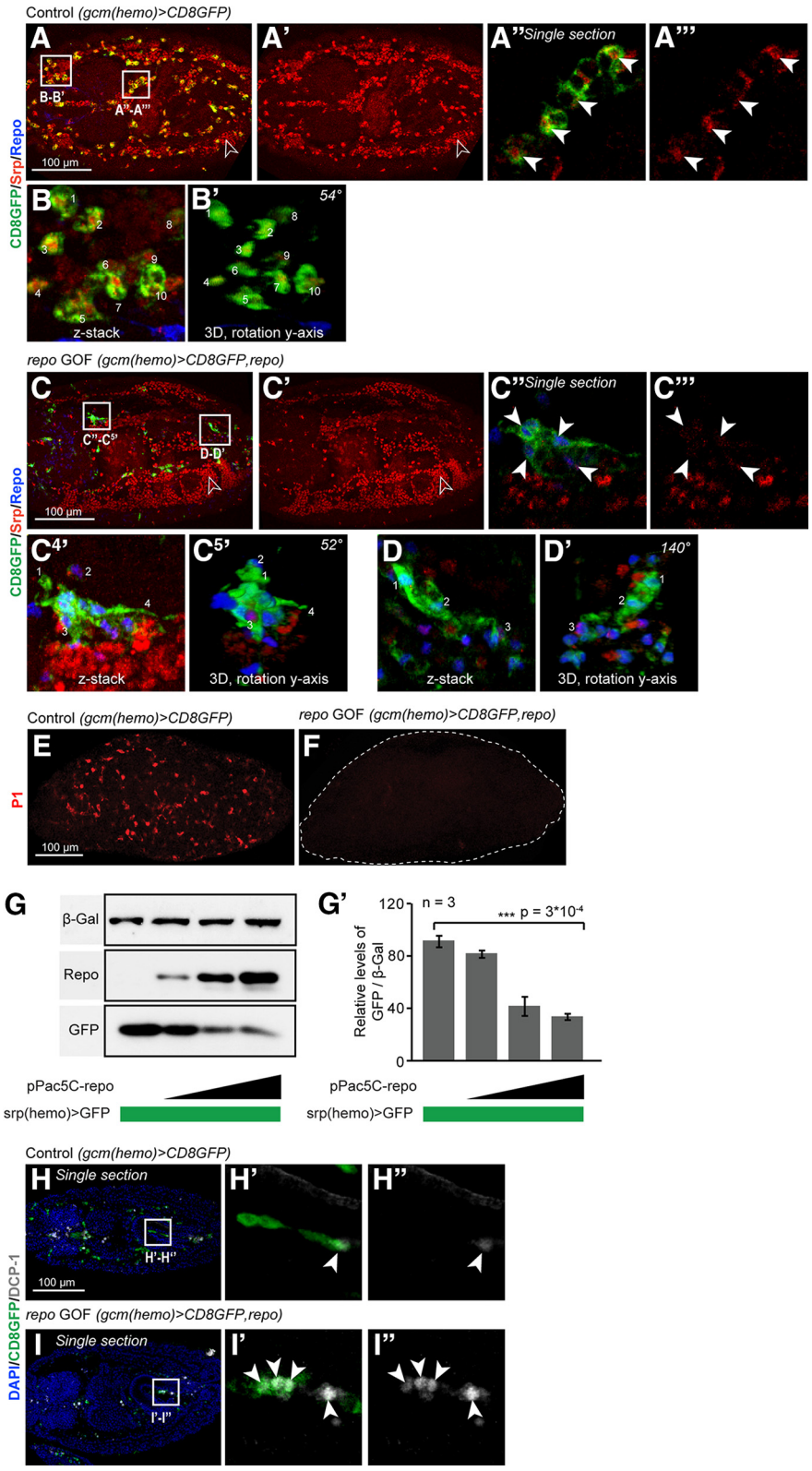


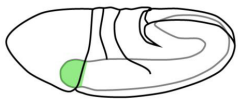
Figure 4. Repo can repress hemocyte differentiation (*gcm(hemo)Gal4* driver). ***A–B′, E, H–H″, C–D′, F, I–I″***, Immunolabeling on *gcm(hemo)>CD8GFP* (Control; ***A–B′, E, H–H″***) or *gcm(hemo)>CD8GFP,repo* (*repo* GOF; ***C–D′, F, I–I″***) embryos. Scale bar, 100 μm. ***A, C***, represent confocal projections of embryos labeled for GFP (green), Srp (red), and Repo (blue), dorsal view, stage 16; the empty arrowheads indicate the Srp-positive GFP-negative cells of the fat body. ***A′*** and ***C′*** show the Srp signal alone. ***A″, A‴, C″***, and ***C‴*** show single sections of the insets indicated in ***A*** and ***C***, the white arrowheads indicate the hemocytes (GFP/Srp double-positive cells). Note that Repo is expressed in GFP-positive cells in *repo* GOF (***C″***) and that the levels of Srp upon Repo overexpression (***C‴***) are much lower compared with those observed in the control embryo (***A‴***). ***B***, ***C4′***, and ***D*** are confocal projections, and ***B′***, ***C5′***, and ***D′*** are 3D reconstructions of the insets indicated in ***A*** and ***C***. The numbers identify the hemocytes in each stack and their localization in the 3D reconstruction after the *y*-axis rotation. Note that in the control, the hemocytes appear as individual cells, whereas in *repo* GOF the hemocytes aggregate. ***E*** and ***F*** represent confocal projections of embryos labeled for the late hemocyte marker P1 (red), dorsal view, stage 14. ***G***, ***G′***, Western blot assay on protein extracts from S2 cells cotransfected with *pSrp(hemo)Gal4*, *pUAST-GFP*, and increasing amounts of *pPac5C-repo* (0–3 μg). *pPac5C-lacZ* was used as a transfection control (***G***). The histogram in ***G′*** represents GFP/β-Gal relative quantification. The amounts of transfected Repo were also verified. *n* indicates the number of cotransfection assays. The means ± SEM are represented on the chart and the p value was calculated using ANOVA. ***H***, ***I***, Single confocal sections of embryos labeled for DAPI (blue), CD8GFP (green), and the apoptotic marker DCP-1 (gray). ***H′, H″, I′, I″***, Insets indicated in ***H*** and ***I***; the arrowheads indicate cells double positive for CD8GFP and DCP-1. ***$p < 0.001$.

Repo represses the expression of hemocyte markers in ectopic glial cells

Given the ability of Repo to inhibit the hemocyte fate in the PM, we asked whether it also represses that fate in glial cells. In the simplest view, the lack of Repo could transform glial cells into hemocytes, as glia represent the resident macrophages of the nervous system. We analyzed the role of Repo first in ectopic glial cells and then in endogenous glia.

Gcm expression throughout the neurogenic region (*sca>gcm*) triggers ectopic gliogenesis, whereas the same experiment in *repo*-null embryos (*repo* LOF) triggers ectopic Srp expression within the nervous system. (Fig. 7*A*,*B‴*). To identify the cells expressing Srp ectopically and to distinguish between hemocytes entering the mutant nervous system (due to the lack of Repo; Shklyar et al., 2014) versus presumptive glial cells expressing hemocyte features in *repo* mutant embryos, we needed a glial lineage marker that traces cells in wild-type and in mutant animals. We hence analyzed *sca>gcm; repo* LOF embryos that also carry the *repo-nGFP* transgene. Since Srp is a nuclear marker, using the nuclear GFP tagging we could show Srp/GFP colocalization as follows (Fig. 7*A–B‴*): 3.5 ± 0.8 cells/hemisegment show Srp/GFP colocalization in *sca>gcm; repo* LOF,*repo-nGFP* embryos compared with 0 cells in *sca>gcm;repo-nGFP* embryos ($n = 6$ hemisegments in three embryos; W test, $p = 2.10^{-4}$). This indicates that presumptive glia express Srp, as opposed to the possibilities that *repo-GFP*-positive cells phagocyte Srp-positive cells (Lee and Jones, 2005; Laneve et al., 2013) or that the lack of Repo induces Srp expression nonautonomously. Similar results were obtained upon using a second early hemocyte marker, Ushaped (Ush; Fig. 7*C–D″*) as follows: 6.8 ± 0.6 cells/

Table 1. Drivers used to target the neurogenic region, the mesoderm and the procephalic mesoderm

Driver	Expression profile in embryo (stage 8, lateral and cross-section	Region
scaGal4 (sca>)		Ventral neurogenic region
twiGal4 (twi>)		Mesoderm Mesectoderm
gcmGal4,repoGal80 (gcm(hemo)>) *srp(hemo)Gal4 (srp(hemo)>)*		Procephalic mesoderm

The 1st column indicates the genotype, the second column indicates the region expressing the driver (embryo at stage 8, lateral view, anterior to the left) and in a cross section of the embryo (dorsal to the top) and the third column indicates the region targeted.

hemisegment show Ush/GFP colocalization in *sca>gcm;repo* LOF,*repo-nGFP* embryos compared with 2.8 ± 0.7 cells in *sca>gcm;repo-nGFP* embryos (n = 10 hemisegments in three embryos; ANOVA, $p = 6.10^{-4}$). Within the neural tissue, we also found Srp- or Ush-positive cells that are GFP negative (Fig. 7*B′–B‴*,*D′*,*D‴*, empty arrowheads). These cells likely represent hemocytes that have moved into a neural tissue that is no longer properly formed/insulated (Shklyar et al., 2014).

Repo represses the expression of hemocyte markers in endogenous glial cells

Next, we found that Repo is sufficient to repress the expression of hemocyte genes in endogenous glia. We introduced the *srp(hemo)>CD8GFP* transgene in *repo* LOF, *repo-nRFP* animals and found GFP expression (hemocyte tracer) in a fraction of RFP-positive cells (glial tracer) that seemed localized dorsally in the ventral cord of the *repo* LOF embryos, in correspondence of the longitudinal connectives (Fig. 8*A–C″*). This does not occur in control animals and is in accord with the finding that Repo represses the expression of the *srp(hemo)* promoter in S2 cells (Fig. 4*G*). Because the GFP of the *srp(hemo)>GFP* line is localized at the membrane and the RFP of the *repo-nRFP* line in the nucleus, we could not formally exclude the possibility that hemocytes invaded the mutant nervous system and engulfed the presumptive glia. We hence used the anti-Srp antibody and analyzed the mutant ventral cord upon subdividing it in two parts along the *z*-axis, following the approach described by Shklyar et al. (2014). The ventral part mainly contains cortex glial cells, and the dorsal part mainly contains neuropil-associated glial cells (Ito et al., 1995) in which astrocyte-like and ensheathing glia can be distinguished (Fig. 8*D*, simplified schematics; for review, see Freeman and Rowitch, 2013; Limmer et al., 2014).

Using anti-Srp and anti-Fas2, which recognizes the three dorsally located longitudinal axonal fascicles of the ventral cord (Santos et al., 2007; Fig. 8*E–F*,*I–J⁴′*), we found expression of the hemocyte marker in presumptive neuropil-associated glial cells (*repo* LOF, *repo-nGFP*; Fig. 8*E–F*). We found that 5.1% of the presumptive glia (GFP-positive cells) express Srp (average of 0.96 ± 0.14 cells/hemisegment are double-positive GFP/Srp of 20 neuropil glia; n = 63 hemisegments in 11 embryos; W test, p = 0.00010). In similar assays using a second neuronal marker, anti-HRP, we found nuclear colocalization between Ush labeling and GFP (Fig. 8*G*,*H*) for 26% of the presumptive glia (4.9 ± 0.5 cells/hemisegment are double-positive GFP/Ush; n = 3 hemisegments in three embryos; W test, p = 0.009). The orthogonal sections of the VNC confirm that the hemocyte markers are expressed in the presumptive glia labeled with *repo-nGFP* in *repo* LOF embryos (Fig. 8*I–J⁴′*).

In the neuropil-associated glia, astrocyte-like and ensheathing glia can be distinguished according to Prospero (Pros) expression, which is present in astrocyte-like glia and is barely detectable in the ensheathing glia (Peco et al., 2016). The colabeling of Srp and Pros indicates that both astrocyte-like glia (Fig. 8*K–K⁴′*, empty arrowheads) and ensheathing glia (Fig. 8*K–K⁴′*, white arrowheads) can express Srp in *repo* LOF embryos.

Since only dorsal cells display colabeling between the glial tracer and the Srp/Ush transcription factors, we asked whether distinct glial subtypes express different hemocyte markers in the *repo* LOF embryos and analyzed the expression of the late hemocyte marker Sn (Fig. 9*A–D″*). The cells that express Sn are located at the position of the cortex glia, mostly in the ventral part (Fig. 9*A–D″*). Since Sn is involved in motility (Zanet et al., 2009), this phenotype matches the observation that cortex glia are more motile in *repo* mutant embryos (Shklyar et al., 2014). As in the assays performed on ectopic glia, we also found Sn-expressing cells that correspond to hemocytes migrating into the defective nervous system (Sn-positive/GFP-negative cells; Fig. 9*E–F′*). The *repo* LOF embryos display Sn labeling in 6% of the GFP-positive cells (Shklyar et al., 2014). Thus, the lack of the Repo transcription factor triggers the expression of subsets of hemocyte markers in a fraction of presumptive glia. These data reveal for the first time a hematopoietic potential for the embryonic glial cells of *Drosophila*.

Finally, we asked whether the lack of Repo converts glial cells into mature and functional hemocytes by monitoring the expression of the hemocyte-specific phagocytosis receptor Crq (Franc et al., 1999), but found no ectopic expression of that protein (Fig. 9*G*,*H*), in agreement with the hypothesis that the lack of Repo does not simply reveal a default hemocyte fate.

In sum, Repo represses the expression of distinct hemocyte markers in specific glial subtypes, hence revealing the complexity of this cell population.

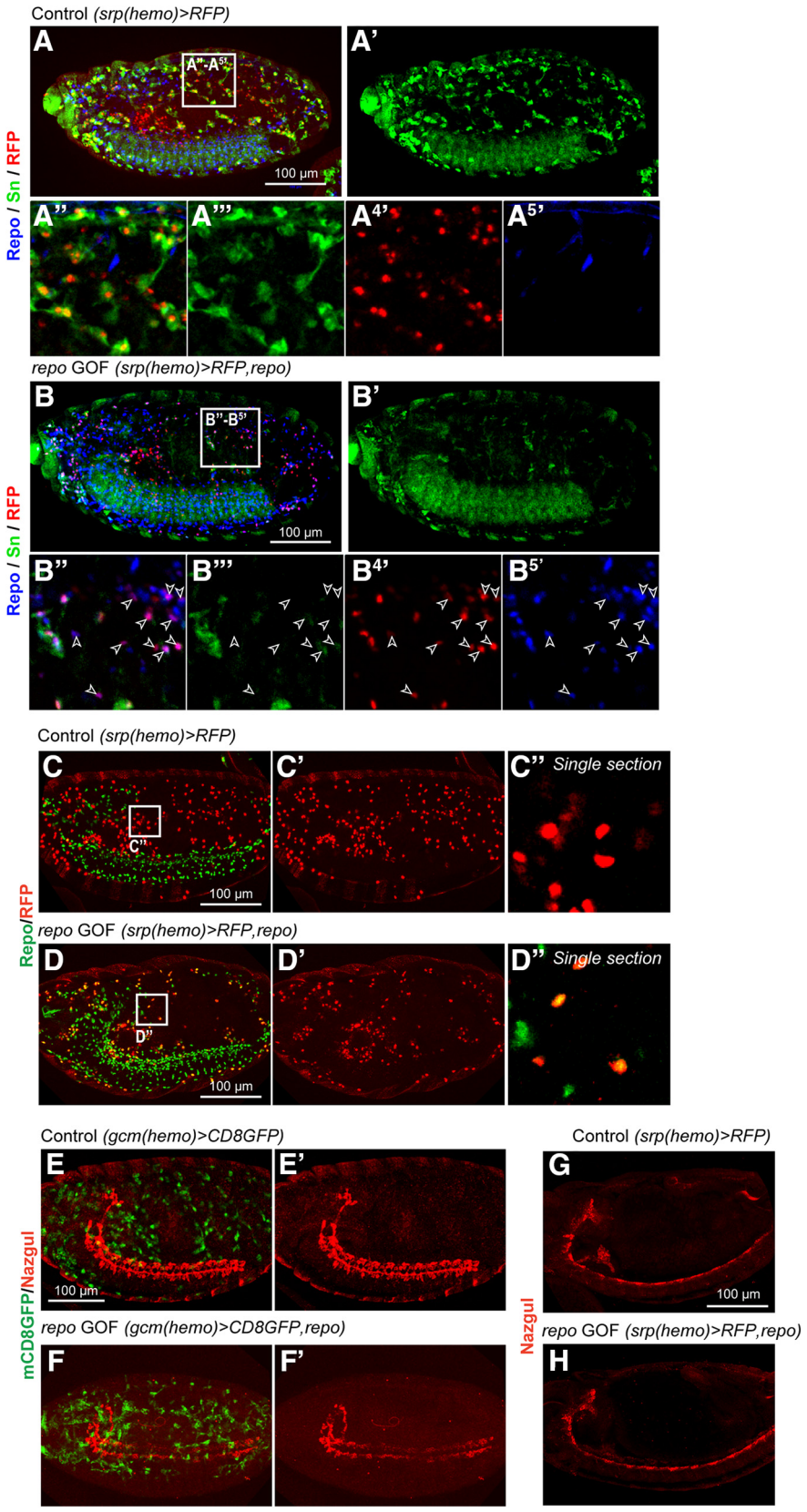


Figure 5. Repo can repress hemocyte differentiation (*srp(hemo)Gal4* driver). ***A–D″***, Immunolabeling on *srp(hemo)>RFP* (Control; ***A–A5′***, ***C–C″***) or *srp(hemo)>RFP,repo* (*repo* GOF; ***B–B5′***, ***D–D″***) embryos. Scale bar, 100 μm. ***A***, ***B***, Confocal projections of embryos labeled for RFP (red), Repo (blue), and the late hemocyte marker Sn (green). ***A′*** and ***B′*** show the Sn signal only, and ***A″–A5′*** and ***B″–B5′*** show the inset indicated in ***A*** and ***B***: from left to right, all channels together, Sn only, RFP only, and Repo only. The empty arrowheads indicate hemocytes expressing RFP and no Sn in *repo* GOF. ***C***, ***D***, Confocal projections of embryos labeled for RFP (red) and Repo (green). ***C′*** and ***D′***) show the RFP signal only, and ***C″*** and ***D″*** represent single sections of the insets indicated in ***C*** and ***D***. ***E–H***, Confocal projections of control and *repo* GOF embryos (using the two different hemocyte-specific drivers) labeled for Nazgul (red): *gcm(hemo)>CD8GFP* (***E***, ***E′***), *gcm(hemo)>CD8GFP,repo* (***F***, ***F′***), *srp(hemo)>RFP* (***G***), and *srp(hemo)>RFP,repo* (***H***). Lateral view, stage 14 (***G′***, ***H′***) show the Nazgul signal alone. Scale bar, 100 μm.

Repo acts as the guardian of the glial fate

The fact that only a fraction of the presumptive glia expresses any hemocyte marker in *repo* LOF embryos prompted us to ask whether these cells display other defects. Since Gcm represses the neuronal fate and gain-of-function experiments suggest that Repo contributes to the process (Yuasa et al., 2003), we explored the possibility that a fraction of the glial cells lacking Repo express neuronal features. Indeed, 22% of the presumptive glial cells express the pan-neuronal marker Elav (Yao and White, 1991; Berger et al., 2007) in *repo* LOF*; repo-nGFP* embryos (Fig. 10*A–D‴*,*F*). These cells are scattered throughout the ventral nerve cord (Fig. 10*B*,*D*) and do not coexpress the hemocyte markers Srp (Fig. 10*E–E‴*) or Ush (data not shown). Thus, the glial factor Repo contributes to repressing the neuronal as well as the hemocytic fates in different glial subtypes. Moreover, removing Elav as well as Repo allows more presumptive glia to express a hemocyte marker, as *elav;repo* LOF double-mutant embryos that also carry the *repo-nGFP* transgene show twice as many cells expressing the Srp hemocyte marker in presumptive glia compared with those observed in *repo* LOF embryos [Fig. 10*F*,*G*, 23% (third column) vs 11% (second column)]. Thus, the neuronal factor Elav is epistatic to Repo and contributes to repression of the hemocyte fate, which may explain why at early embryonic stages Elav is expressed in a considerable number of cells expressing Repo (Berger et al., 2007).

To further our understanding of the role of the Repo transcription factor on the glial fate, we also scored the total number of presumptive glia and assessed their proliferative and cell death profile in *repo* LOF embryos. The number of nuclei expressing the GFP in *repo LOF; repo-nGFP* embryos is 30% lower compared with that observed in wild-type animals (345.8 $\pm$ 6.9 per embryo in WT compared with 196.0 $\pm$ 35.8 in *repo LOF*; $n = 3$ embryos; ANOVA, $p = 0.0383$). This is in agreement with a slight reduction in cell division and a slight increase in apoptosis; anti-PH3 (Juan et al., 1998) was used to

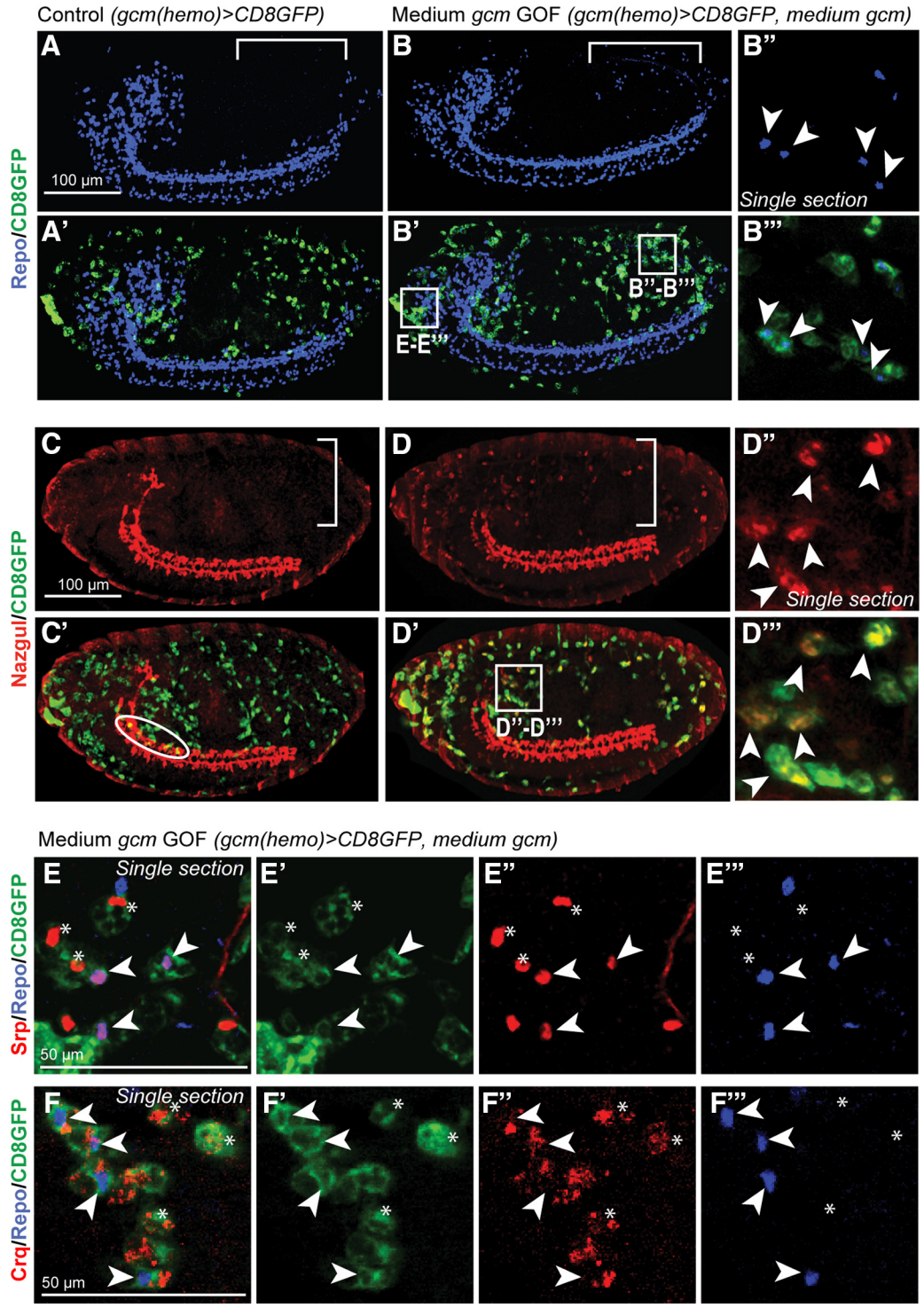


Figure 6. Gcm has a strong gliogenic potential in hemocyte precursors. ***A–D'''***, Embryos *gcm(hemo)>CD8GFP* (Control; ***A***, ***A'***, ***C***, ***C'***) and *gcm(hemo)>CD8GFP,medium gcm* (Medium *gcm* GOF; ***B***, ***B'***, ***D***, ***D'***). Scale bar, 100 μm. ***A–B'*** represent confocal projections of embryos labeled for GFP (green) and Repo (blue), lateral view, stage 14. ***B''*** and ***B'''*** represent single confocal sections of the inset indicated in ***B'***, the arrowheads indicate cells double positive for CD8GFP and Repo. ***C–D'*** represent confocal projections of embryos labeled for GFP (green) and Nazgul (red), lateral view, stage 16. Brackets indicate territories exhibiting hemocytes. Note that the yellow color observed in ***C'*** (oval) is an artifact created by the projection. ***D''*** and ***D'''*** represent single confocal sections of the inset indicated in ***D'***; arrowheads indicate ectopic glial labeling in hemocytes overexpressing Gcm. ***E–F'''***, Single confocal sections of medium *gcm* GOF (*gcm(hemo)>CD8GFP,medium gcm*) embryos labeled for Srp (red), Repo (blue); and GFP (***E–E'''***) and Crq (red), Repo (blue), and GFP (***F–F'''***). Hemocytes are indicated by asterisks, and those that also express Repo by white arrowheads. Note that Repo ectopic expression does not affect Srp or Crq expression. Scale bar, 50 μm. *$p < 0.05$.

score for glial cell division: 4.9 ± 1.0 dividing cells are present per six hemisegments in WT embryos compared with 0.3 ± 0.3 in *repo LOF* embryos ($n = 3$ embryos; W test, $p = 0.0361$). Apoptosis was scored using the anti-CM1 antibody that recognizes the activated Caspase-3 (Fig. 11*A*, *B'''*). No cells were observed in WT embryos compared with 10.6 ± 1.2 dying cells in *repo LOF* embryos ($n = 3$ embryos; six hemisegments were counted per embryo; W test, $p = 0.0318$). It is therefore likely that some cells

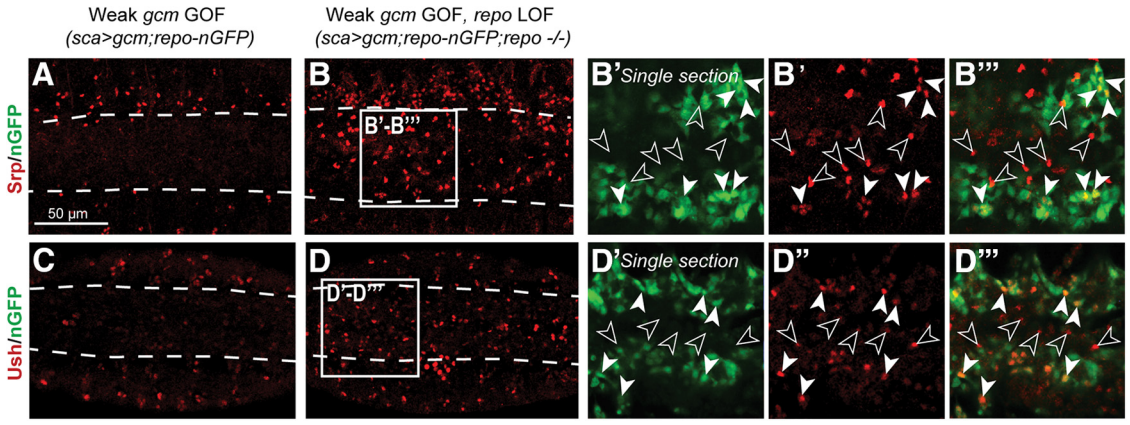


Figure 7. Repo represses the Gcm hematopoietic potential in the neuroectoderm. ***A–D***, Confocal projections of embryos *sca>weak gcm;repo-nGFP* (Weak *gcm* GOF; ***A***, ***C***) and *sca>weak gcm/repo-nGFP;repo* $^{-/-}$ (Weak *gcm* GOF, *repo* LOF; ***B***, ***D***) labeled for GFP (green) and Srp (red; ***A–B‴***) or GFP (green) and Ush (red; ***C–D‴***), ventral view, stage 16. The dashed lines define the VNC (***A–D***). ***B′–B‴***, and ***D′–D‴***) represent single sections of the insets indicated in ***B*** and ***D***; they show nGFP labeling only, Srp or Ush labeling only, and colabeling Srp or Ush with nGFP, respectively. White arrowheads indicate nGFP/Srp (***B′–B‴***) or nGFP/Ush (***D′–D‴***) double-positive cells, empty arrowheads indicate Srp- or Ush-positive and nGFP-negative cells in *gcm* GOF *repo* LOF embryos. These are hemocytes recruited to the VNC that are not properly insulated due to the mutant background (Shklyar et al., 2014). Scale bar, 50 μm.

missing the Repo protein no longer acquire/maintain the right identity and eventually die. To make sure that the colocalization between the presumptive glia (nuclear GFP) and the death maker CM1 identifies dying cells (Fig. 11*A–B‴*,*E*), rather than glial cells that are phagocytosing dead bodies, we compared the results obtained on *repo LOF; repo-nGFP* embryos with those obtained on *repo; repo-CD8GFP* embryos, in which the GFP is tagged to the membrane (Fig. 11*E*).

As expected, in the latter case we did not observe colocalization between the GFP and CM1 (Fig. 11*C–C‴*). Moreover, these data further confirmed the lack of phagocytosis observed in *repo LOF* embryos (Fig. 11*D*), likely due to defective Simu (Six Microns Under) and Drpr expression (Shklyar et al., 2014). Indeed, whereas in wild-type embryos glial cell membranes completely enwrap apoptotic bodies (Fig. 11*C–C‴*), in *repo* LOF embryos these contacts are no longer established.

In sum, the only transcription factor that is expressed in all glia and only in glia, Repo, acts as the true guardian of the glial fate.

Discussion

During development, pioneer transcription factors trigger specific cell fates. More and more data, however, show that these factors act in multiple lineages, raising the question of how each lineage differentiates at the right place. Here we show that a pioneer factor acts in concert with tissue-specific cues to trigger distinct fates in different territories and that this distinction is maintained through reinforcing inhibitory pathways. The *Drosophila* Gcm zinc finger protein promotes hematopoiesis in the procephalic mesoderm, and gliogenesis in the nervous system. The expression of its target and panglial transcription factor Repo is repressed in the hematopoietic anlagen by mesodermal cues. In turn, Repo represses the expression of hemocyte genes. These sequential regulatory steps explain how Gcm induces two functionally related but alternative cell fates in different territories (Fig. 11*F*).

Tissue-specific cues inhibit the gliogenic potential of Gcm in the hematopoietic anlagen

The *Drosophila* transcription factor Gcm is expressed and required for the differentiation of glia and blood, which share immune features but also perform specific functions in the immune and nervous systems. These cells originate from different layers, glia from the ectoderm and hemocytes from the mesoderm, and therefore display distinct molecular landscapes. We show here that the mesoderm-specific transcription factor Twi and its target *miR*-1 repress the expression of the panglial gene Repo in the hemocyte anlagen. Other factors are certainly also involved in the process as the mesodermal coexpression of Gcm and Srp, which is necessary for hemocyte development, also represses Repo expression (data not shown). These tissue-specific cascades induce the mesodermal molecular landscape, which in turn regulates Gcm activity and biases its transcriptional output toward hemocyte differentiation.

The coordinated activity of pioneer and tissue-specific factors allows a limited number of transcription factors to produce the high diversity of cell types present in complex organisms. For example, the vertebrate GATA transcription factors regulate the development of hematopoietic, neural, cardiac, or reproductive tissues (Cantor and Orkin, 2005; Zaytouni et al., 2011; Chlon and Crispino, 2012) and control specific target genes in the different tissues due to the activity of tissue-specific transcription factors that modify the transcriptional output of the GATA factors (Cantor and Orkin, 2005). It will be interesting to see whether in that case as well post transcriptional regulation contributes to the acquisition of cell specificity.

The Repo homeodomain-containing factor locks cells in the glial fate

Gcm is expressed and necessary at early stages of glial development, whereas the homeodomain containing Repo protein is stably expressed in the glial cells. The lack of late glial markers observed in *repo* mutant embryos initially suggested a role of Repo in glial terminal differentiation (Xiong et al., 1994; Yuasa et al., 2003). However, the ectopic expression of non-glial markers in those embryos shows that Repo also controls cell plasticity. This indicates that homeodomain containing transcription factors can provide the molecular relay from multipotency to a fully differentiated state once the transient expression of pioneer factors extinguishes. It is possible that neural-specific factors also help sustain Repo expression in the nervous system, hence providing a favorable landscape for glial differentiation.

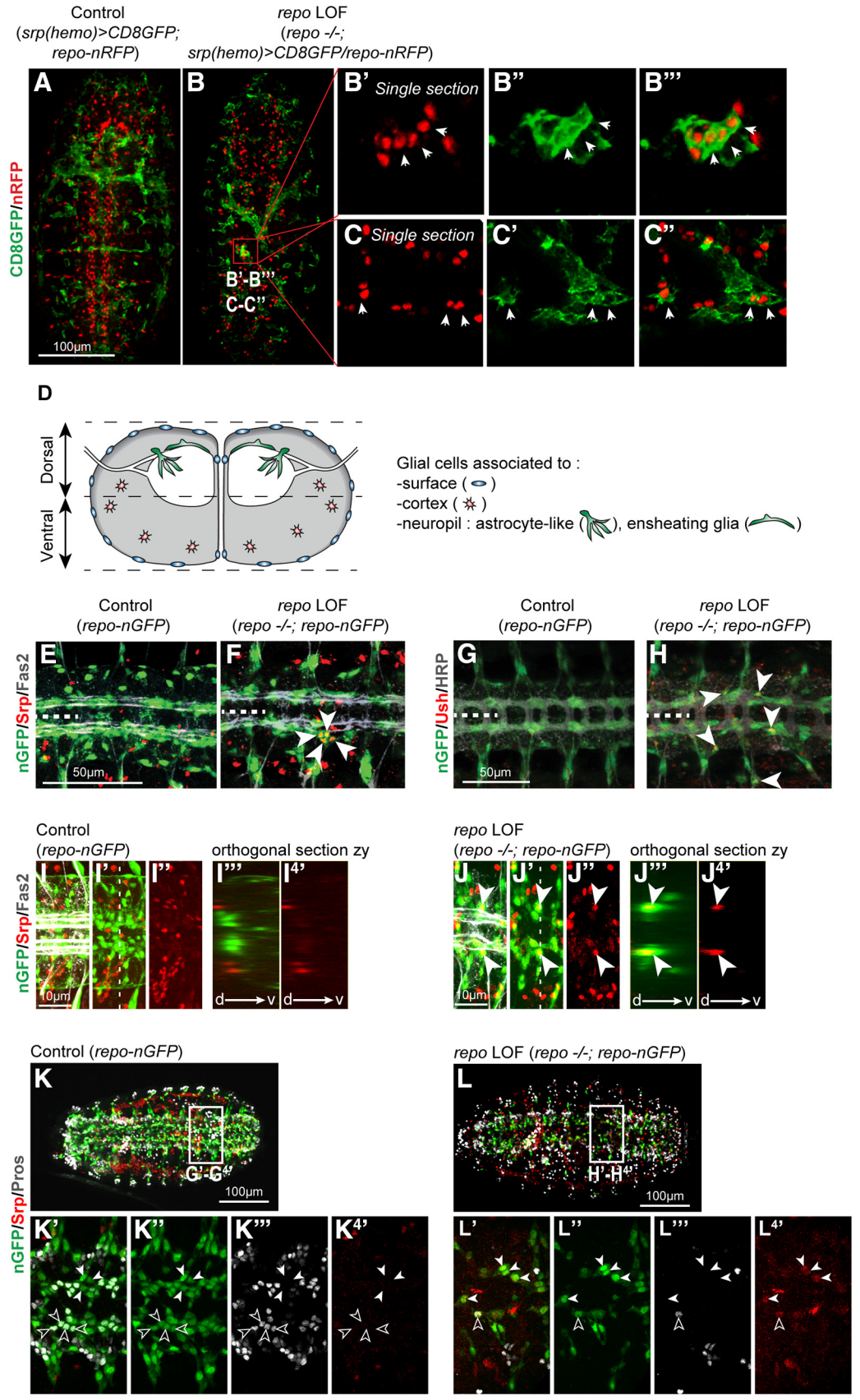


Figure 8. Repo is required to repress hemocyte transcription factors in developing glia. ***A***, ***B***, Confocal projections of embryos *srp(hemo)>CD8GFP/repo-nRFP* (Control; ***A***) and *srp(hemo)>CD8GFP/repo-nRFP;repo* $^{-/-}$ (*repo* LOF; ***B***) labeled for GFP (green) and RFP (red), ventral view. ***B′–B‴***, ***C–C″***, Single sections of the inset indicated in ***B***. Note that the single sections were acquired at different focal planes in the VNC. The arrows indicate GFP/RFP double-positive cells. Scale bar, 100 μm. ***D***, Simplified schematic of a transversal section of the VNC from a mature embryo. Glial cell subtypes are defined according to their localization: surface (pale blue), cortex (red), and neuropil-associated glia (green; Ito et al., 1995; (*Figure legend continues.*)

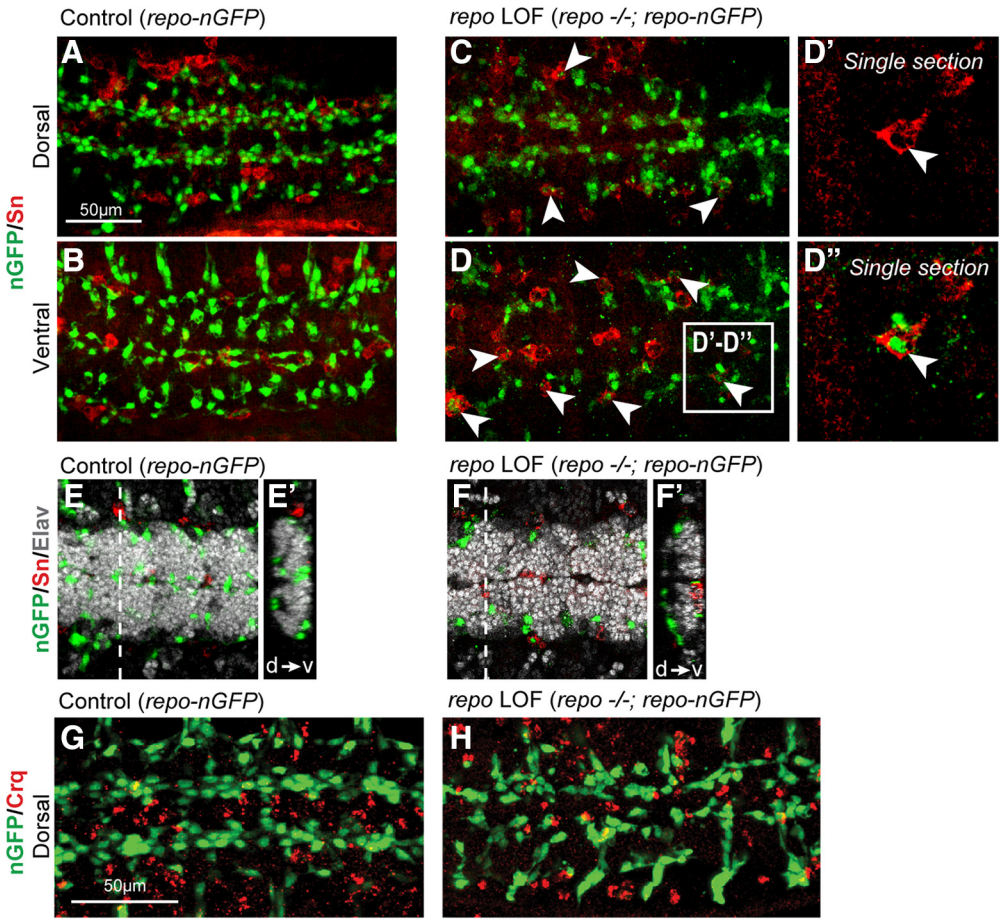


Figure 9. Repo is required to repress the hemocyte marker Sn in developing glia. ***A–H***, Immunolabeling of the VNC of embryos *repo-nGFP* (Control) and *repo* $^{-/-}$*;repo-nGFP* (*repo* LOF). Scale bar, 50 μm. ***A–D″***, Stage 14 embryos labeled for GFP (green) and Sn (red). ***D′*** and ***D″*** show single sections of the inset indicated in ***D***. ***A*** and ***C*** show the dorsal part of the VNC, and ***B*** and ***D*** show the ventral part. ***E***, ***F***, Confocal projections of the whole VNC labeled for GFP (green), Sn (red), and Elav (gray); the dashed line indicates the position of the orthogonal section along the *z*- and *y*-axes of the VNC presented in ***E′*** and ***F′***. Note the presence of Sn-positive/GFP-negative cells within the VNC in *repo* LOF embryo; these are hemocytes recruited to the VNC following the loss of *repo* (Shklyar et al., 2014). ***G***, ***H***, Embryos labeled for GFP (green) and Crq (red).

The robustness of the glial fates relies on the activity of cell-specific genes such as Repo and Elav, which repress the expression of Srp in the nervous system. Our data also suggest that glial (Repo) and neuronal (Elav) factors both repress ectopic hematopoiesis in the neural territory while counteracting each other to maintain the glial and the neuronal fates, respectively. This molecular network explains why cells adopt the neuronal default fate in the absence of Gcm, whereas they start expressing hemocyte markers in the absence of Repo, and even more so in the absence of both Repo and Elav.

Thus, cell-specific pathways and feedback loops allow a single pioneer factor to affect different cell fates. Such molecular checkpoints acting in parallel and in sequence allow the maintenance of a stable fate.

←

(*Figure legend continued.*) Beckervordersandforth et al., 2008). The neuropil-associated glia can be further defined as astrocyte-like and ensheathing glia. ***E–L⁴′***, Immunolabeling of the dorsal part of the VNC of embryos *repo-nGFP* (Control) and *repo* $^{-/-}$*;repo-nGFP* (*repo* LOF). ***E–H***, Confocal projection of stage 15 embryos labeled for GFP (green), Fas2 (gray), and Srp (red; ***E***, ***F***); and GFP (green), HRP (gray), and Ush (red; ***G***, ***H***). Scale bar, 50 μm. The white arrowheads indicate cells coexpressing repo-nGFP and Srp (***E***, ***F***) or *repo-nGFP* and Ush (***G***, ***H***). Scale bar, 50 μm. ***I–J⁴′***, Confocal projections of segments from stage 15 embryos labeled for GFP (green), Fas2 (gray), and Srp (red), acquired with thin section (0.2 μm) to build an orthogonal section. ***I*** and ***J*** show the stack with the three labels, ***I′*** and ***J′*** show the stack with GFP and Srp, and ***I″*** and ***J″*** show the stack with Srp only. ***I‴***, ***I⁴′***, ***J‴***, **and *J⁴′*** show the orthogonal section along the *z*- and *y*-axes of the dashed line in ***I′*** and ***J′*** [d>v, from dorsal (left) to ventral (right)]. The white arrowheads indicate glia expressing Srp in *repo* GOF. Scale bar, 10 μm. ***K***, ***L***, Confocal projection of stage 15 embryos labeled for GFP (green), Pros (gray), and Srp (red). ***K′–K⁴′*** and ***L′–L⁴′*** show the insets indicated in ***K*** and ***L*** with all channels together: GFP only, Pros only, and Srp only, respectively. White arrowheads indicate ensheathing glia (Pros −), and empty arrowheads indicate the astrocyte-like glia (Pros +). Scale bar, 100 μm.

Lack of Repo triggers different phenotypes in distinct glial subtypes

The glial cells of the embryonic ventral nerve cord are subdivided into three main subtypes (surface-associated, cortex-associated, and neuropil-associated) based on their morphology, position, and function (Ito et al., 1995; Beckervordersandforth et al., 2008). The large and flattened glial cells associated with the surface form the BBB (Auld et al., 1995). Glial cells located in the cortex are star shaped and intermingled with neuronal bodies, their cytoplasmic projections contacting multiple synapses (Freeman and Doherty, 2006; Freeman, 2015). Cortex glia help to clear the debris induced by neuronal programmed cell death (Freeman et al., 2003; Shklyar et al., 2013, 2014; Kurant et al., 2008). Finally, glial cells associated with the axons enwrap them in a multilayer sheath promoting the conduction of nerve impulses, and a subset of them has also been called astrocyte-like glia (Hidalgo and Booth,

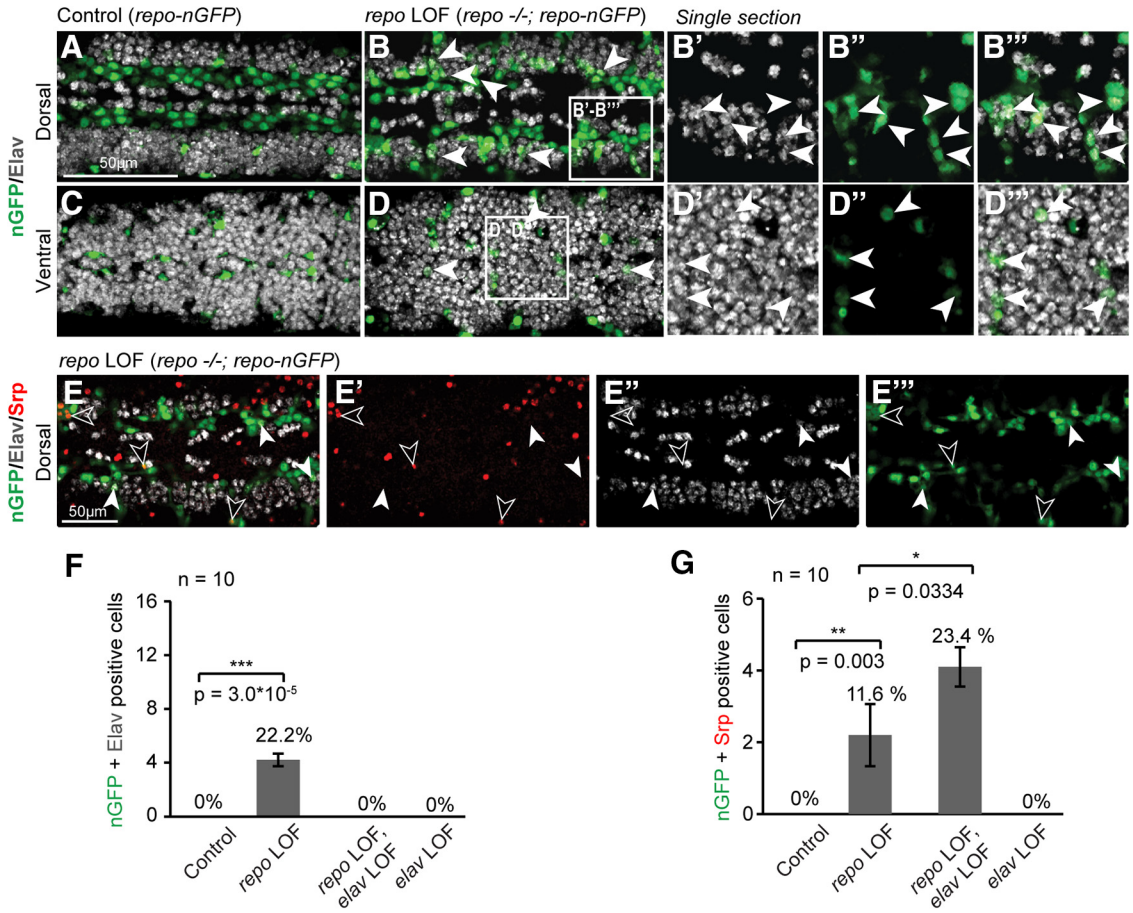


Figure 10. Repo represses both hemocyte and neuronal differentiation. ***A–D'''***, Embryos of the following genotypes: *repo-nGFP* (Control) and *repo* $^{-/-}$*;repo-nGFP* (*repo* LOF), ventral view, stage 15. The ventral and the dorsal parts of the VNC were analyzed separately. Labeling: GFP (green) and Elav (gray). Scale bar, 50 μm. ***B'–B'''*** and ***D'–D'''*** show single sections of the insets indicated in ***B*** and ***D***. Arrowheads indicate ectopic GFP/Elav double-positive cells. ***E–E'''***, Dorsal part of a *repo* $^{-/-}$*;repo-nGFP* (*repo* LOF) embryo labeled for Srp (red), Elav (gray), and GFP (green); the channels are presented individually in ***E'***, ***E''***, and ***E'''***, respectively. White arrowheads indicate GFP/Elav double-positive cells, and empty arrowheads indicate GFP/Srp double-positive cells. Scale bar, 50 μm. ***F***, ***G***, Graphs showing the number and the percentage of GFP/Elav double-positive cells (***F***) or GFP/Srp double-positive cells (***G***) per hemisegment in Control, *repo* LOF, *repo* LOF *elav* LOF double-mutant, and *elav* LOF embryos. *n* indicates the number of hemisegments counted in three embryos. The means ± SEM are represented on the chart and the *p* values were calculated using Krustal-Wallis test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

2000; Sepp et al., 2000; Sepp and Auld, 2003; Freeman and Doherty, 2006; Freeman, 2015). These glia are known to act as scavengers in response to developmental signals and to trauma, likely due to their proximity to signaling axons. Typically, in the adult brain they phagocytose degenerating axons after brain injury (Doherty et al., 2009) and, after puparium formation, axon-associated glia of the mushroom body control ecdysone-dependent axons pruning (Awasaki and Ito, 2004; Kato et al., 2011; Kato and Hidalgo, 2013; Hakim et al., 2014; Boulanger and Dura, 2015).

Repo is expressed in the three cell types, and its lack affects them all (Giesen et al., 1997; Yuasa et al., 2003; Kerr et al., 2014); however, the *repo* mutant phenotypes reveal the underlying diversity of the glial subtypes as, in the absence of Repo, neuropil-associated glia express early hemocyte transcription factors but not Sn, whereas cortex glia express Sn, but not the Srp or Ush transcription factors. Of note, Sn is necessary for cell motility (Adams, 2004; Zanet et al., 2009) and Shklyar et al. (2014) observed that *repo* mutant cortex glia are very motile. In the future, it will be interesting to determine the transcriptional landscape of the different glial subtypes as, for example, cortex glia may be specialized in removing dead cell bodies, whereas neuropil-associated glia may specifically target and remove axons and dendrites.

Although glial cells act as macrophages, they do not have a default hemocyte phenotype, rather, they constitute a very specialized population of scavenger cells. Similarly, vertebrate microglia, cells of immune origin that provide the first response to nervous system challenge, display a molecular signature that is distinct from that of macrophages (Prinz and Priller, 2014). In agreement with the related but distinct function of *Drosophila* glia and hemocytes, their development also presents common and distinct features. The Crq scavenger receptors (Franc et al., 1999) are a potential target of Gcm (Cattenoz et al., 2016) and are expressed only in hemocytes (Shklyar et al., 2014). Another potential Gcm target, Simu (also called Nimrod C4), is a phagocytic receptor necessary in both glia and hemocytes (Kurucz et al., 2007b; Kurant et al., 2008), but Gcm is known to regulate Simu expression in glia, not in hemocytes (Shklyar et al., 2014; Shlyakhover et al., 2018). Finally, the phagocytic receptor Drpr is necessary for phagocytosis by both glia and hemocytes but is not regulated directly by Gcm (Freeman et al., 2003; Shklyar et al., 2014; Shlyakhover et al., 2018). Future experiments will address the point of similarities versus differences existing between glia and hemocytes as well as their specific requirements for the Gcm pathway.

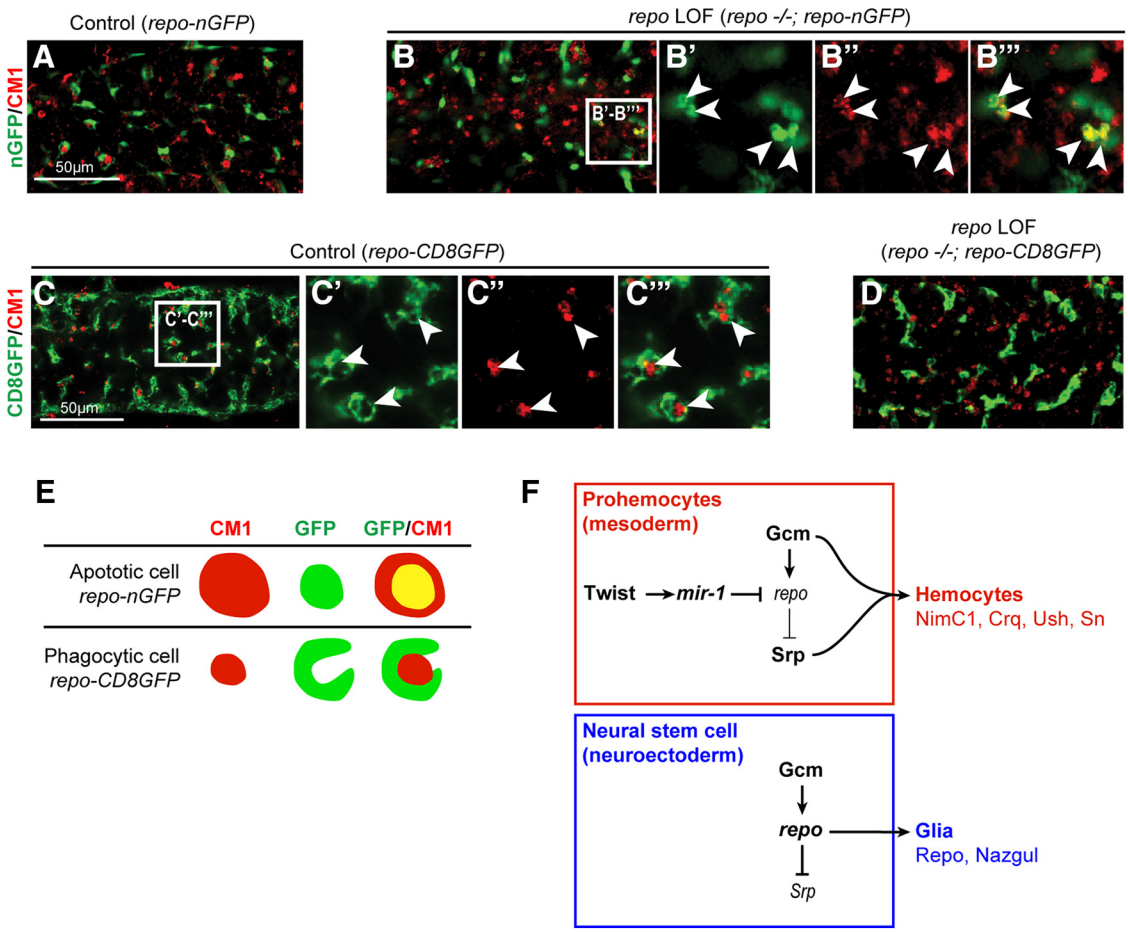


Figure 11. *repo* $^{-/-}$ glia undergo apoptosis. ***A–D***, Embryos of the following genotypes: *repo-nGFP* (Control; ***A***) and *repo* $^{-/-}$*;repo-nGFP* (*repo* LOF; ***B***) express nuclear GFP. *repo-CD8GFP* (Control; ***C***) and *repo* $^{-/-}$*;repo-CD8GFP* (*repo* LOF; ***D***) express GFP at the membrane, ventral view, stage 15. Labeling: GFP (green) and the apoptotic marker CM1 (red). ***B′–B‴*** and ***C′–C‴*** show single sections of the insets indicated in ***B*** and ***C***. Arrowheads in ***B′–B‴*** indicate glial cells undergoing apoptosis (colocalization of nuclear GFP and CM1), whereas arrowheads in ***C′–C‴*** indicate glial cells enwrapping apoptotic bodies (CD8GFP surrounding CM1-labeled bodies). Scale bar, 50 μm. ***E***, Schematic representation of the GFP/CM1 colabeling in apoptotic cells expressing nuclear GFP and in phagocytic cells expressing GFP at the membrane. ***F***, Summary of the interaction between Gcm and its cofactors during hemocyte (top, red) and glia (bottom, blue) differentiation.

Of flies and vertebrates

Drosophila and vertebrate glial cells share numerous functions in controlling neuron homeostasis, recycling neurotransmitters, and insulating axons (Freeman and Doherty, 2006); however, the transcriptional program triggering the first steps of gliogenesis are not evolutionarily conserved. In *Drosophila*, the Gcm transcription factor constitutes the major regulatory gene and acts as a molecular switch between neuron and glial cells. Although the vertebrate Gcm orthologs seem to maintain some gliogenic potential *in vitro* (Kim et al., 1998; Reifegerste et al., 1999; Buzanska et al., 2001; Iwasaki et al., 2003; Soustelle et al., 2007), they are neither expressed nor required in glia. Moreover, no true glial determinant has been so far identified in vertebrates (Hitoshi et al., 2011). Even more strikingly, the vertebrate genomes do not contain the coding sequences for the panglial factor Repo (no orthologs found so far), a molecular signature that seems shared throughout the arthropod clade (Wakamatsu, 2004; Boyan et al., 2011; Mysore et al., 2011; Nasu and Hara, 2012).

Our findings raise the question of the evolutionary link between vertebrate and *Drosophila* gliogenesis. While the hypothesis of an independent origin of vertebrate and invertebrate glia remains to be tested (Hartline, 2011), sequencing the genome and analyzing the single-cell transcriptome of simple organisms will establish when Gcm and Repo appear in evolution and where they are expressed/required within/outside the nervous system.

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