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DOI:
10.1523/JNEUROSCI.1059-18.2018

Document Version
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Citation for published version (APA):
Trébuchet, G., Cattenoz, P. B., Zsámboki, J., Mazaud, D., Siekhaus, D. E., Fanto, M., & Giangrande, A. (2019). The Repo Homeodomain Transcription Factor Suppresses Hematopoiesis in Drosophila and Preserves the Glial Fate. *Journal of Neuroscience*, *39*(2), 238-255. https://doi.org/10.1523/JNEUROSCI.1059-18.2018

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Download date: 14. Jan. 2025

The Repo homeodomain transcription factor suppresses hematopoiesis in *Drosophila* and preserves the glial fate

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Running title: The Repo transcription factor suppresses hematopoiesis

Keywords: *Drosophila*/Repo/Gcm/Glial cells/ hemocytes

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Abstract

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, cells of immune origin that move into the brain during development and become the resident macrophages of the nervous system. Interestingly, glia and hemocytes require the same transcription factor 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 pan-glial direct target of Glide/Gcm ensures 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 understanding the bases of cell specification.

Significance statement

Distinct cell types often require the same pioneer transcription factor, raising the issue of how does 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

Glide/Gcm transcription factor, glia originate from the ectoderm, 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 insulate the central nervous system (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 reshaping the nervous system. Thus, *Drosophila* glia behave like microglia, 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 (Logan and Freeman, 2007; Kurant, 2011). Outside the fly nervous system, hemocytes play a key role in cellular and humoral immunity. They can move very fast 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, 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 (see (Cattenoz and Giangrande, 2014) for a review). Thus, the same transcription factor works in functionally related cells that originate from the neurogenic ectoderm (glia) and from the procephalic mesoderm or 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 (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 embryonic glia and blood differentiation in the correct tissue. We here show that mesodermal cues contribute to prevent glial differentiation in the hemocyte anlagen. The mesodermal transcription factor Twist induces the expression of *miR-1*, which in turn represses the expression of Repo. As a consequence, the gliogenic potential of Gcm is inhibited 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.

Results

The mesoderm-specific transcription factor Twist represses the expression of the Repo pan-glial 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; Bernardoni et al., 1998; Lebestky et al., 2000; Alfonso and Jones, 2002; Cattenoz et al., 2016). 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. Twist (Twi) is an early mesoderm-specific transcription factor and a potent mesodermal determinant (Baylies and Bate, 1996), we therefore asked whether it represses the expression of Repo, the most characterized glial-specific target of Gcm. Repo also represents the only transcription factor expressed exclusively in glia and in all glia (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 or GOF), in the neural territory, as well as embryos that lack Twi expression (Loss Of Function or LOF) or express low levels of Twi.

First, the ectopic expression of Twi in the neurogenic region mediated by the *scabrousGal4* driver (*sca>twi*) (Mlodzik et al., 1990) significantly reduces the number of Repo positive cells in the ventral nerve cord from an average of 29.3 +/-1.1 cells per hemisegment in control to 8.6 +/-1.1 cells in *twi* GOF embryos (n hemisegments=10, n embryos=3, ANOVA $p=8.10^{-11}$) (**Table 1**,

114 **Figure 1 A,B**). Second, since the expression of Gcm in the mesoderm triggers gliogenesis at the
115 expense of muscles (Bernardoni et al., 1998), we performed the same experiment in embryos that
116 carry half a dose of Twi and found that this enhances the gliogenic potential of Gcm in the
117 mesoderm. This data were obtained upon expressing Gcm with the *twistGal4* driver (*twi>gcm*) in
118 *twi/+* heterozygous embryos (**Table 1**, **Figure 1C,D**). Third, although Twi is a major mesodermal
119 determinant that induces severe and early defects when absent (Thisse et al., 1987), it is not
120 absolutely required for the initial determination of the hemocyte fate (Spahn et al., 2014). This
121 allowed us to analyze the few *twi* null embryos that reached relatively late stages and revealed the
122 presence of the Repo protein in cells that express the early hemocyte marker Serpent (Srp) (no cell
123 in control and an average of 8.9 +/-4.3 cells Srp and Repo positive per embryo *twi* LOF, n
124 embryos=5, Wilcoxon (W) p=0.0038) (**Figure 1E,F''**). Unless otherwise specified, low
125 magnifications of all the figures show confocal projections whereas high magnifications of the
126 insets shown single confocal sections, for the sake of simplicity. This explains why the labeling in
127 the insets corresponds partially to that shown in the low magnification panels. Altogether, our
128 results strongly suggest that the lack of Twi allows ectopic Repo expression in the hemocyte
129 anlagen, the PM, hence biasing the gliogenic potential of Gcm in that territory.

130 We then asked whether over-expressing Gcm in its own domain of expression, the PM,
131 leads to the differentiation of supernumerary hemocytes or whether it bypasses the molecular brake
132 imposed by Twi, hence allowing ectopic Repo expression. For this purpose, we crossed a *gcmGal4*
133 driver with a transgenic line expressing the Gal4 inhibitor Gal80 in glial cells, the other territory
134 of Gcm expression (**Table 1**, *gcmGal4, repoGal80* or *gcm(hemo)Gal4)* (Lee and Luo, 1999), so as
135 to confine Gcm overexpression to the PM (**Figure 1G**). *gcm(hemo)>gcm* embryos do display Repo
136 expression in the hemocyte anlagen and this is a dosage dependent phenotype, the stronger the *UAS*
137 *gcm* transgene, the higher the levels of Repo (**Figure 1H**). Moreover, and in line with our

138 hypothesis, co-over-expressing Gcm and Twi (*gcm(hemo)>gcm + twi*) abolishes the induction of
139 Repo expression in the PM (**Figure 1I**).
140 The fact that Gcm over-expression induces Repo expression in the PM could mean that glial
141 differentiation simply requires higher Gcm levels than hematopoiesis. If that were the case,
142 hypomorphic *gcm* mutant embryos should express hemocyte markers in the nervous system. The
143 *gcm*^{34} mutation is an imprecise excision that still expresses the *LacZ* gene carried by the P element
144 located at the *gcm* locus and results in low Gcm levels (Vincent et al., 1996). Neither *gcm*^{34}
145 homozygous nor *gcm*^{34}/*Df(2L)132* transheterozygous animals (the *Df(2L)132* deficiency
146 completely deletes the gene (Kammerer and Giangrande, 2001)) show Srp ectopic expression in
147 the nervous system (**Figure 1J-L**). This excludes mere dosage dependency for the establishment
148 of the glial vs. the blood cell fate and further supports the idea that tissue-specific factors are
149 responsible for it.
150 In sum, the Twist mesodermal factor negatively affects the expression of the pan-glial
151 transcription factor Repo.
152
153 **The micro RNA *miR-1* inhibits Repo expression post-transcriptionally**
154 The microRNA *miR-1* is a direct target of Twi expressed and required in the mesoderm
155 (Biemar et al., 2005; Sokol and Ambros, 2005). We found that *miR-1* has two putative target sites
156 in the *repo 3'UTR* (miRanda: http://www.microrna.org/microrna/home.do) (**Figure 2A**) and
157 therefore explored the possibility that it acts post-transcriptionally on Repo. First, we found that
158 animals lacking *miR-1* display ectopic Repo expression in the PM, similar to the *twi* embryos
159 (**Figure 2B-D**). Second, we asked whether *miR-1* directly acts on the *repo 3'UTR* by co-
160 transfecting S2 *Drosophila* cells with a *miR-1* expression vector and a luciferase reporter carrying
161 either the *repo 3'UTR* or its own *3'UTR* (**Figure 2E**). By measuring the luciferase activity, we

162 found that *miR-1* specifically acts on the *repo 3'UTR* to repress *repo* expression **(Figure 2E-F)**.
163 Third, this negative control is abolished upon mutagenizing the two putative *miR-1* target sites
164 (**Figure 2F**). Thus, *miR-1* inhibits Repo expression post-transcriptionally.
165 In sum, our data indicate that mesoderm-specific cues prevent Gcm from triggering Repo
166 expression in the PM.
167
168 **Repo is sufficient to repress the expression of hemocyte markers in the PM**
169 The tight repression of Repo expression in the hemocyte anlagen suggests that gliogenesis
170 is alternative to hemocyte differentiation. We therefore analyzed the effects of Repo ectopic
171 expression in the PM upon using the *UAS-repo* transgene (Yuasa et al., 2003). *gcm*(*hemo)>repo*
172 (or *repo* GOF) hemocytes are severely affected: many of them aggregate and show altered
173 morphology as well as migratory defects (**Figure 3E,F**). Moreover, they no longer express the late
174 hemocyte marker NimC/P1, which is a scavenger receptor (Kurucz et al., 2007) (**Figure 3C,D**),
175 and the expression of the early hemocyte marker Srp is severely downregulated (**Figure**
176 **3A'',A''',B'',B''').** The hemocytes express Srp at low levels. To quantify this phenotype, we
177 measured the intensity of Srp labeling and found a significant difference between control and *repo*
178 GOF hemocytes (control: 83.4 +/-3.9 arbitrary unit (AU, see materials and methods), *repo* GOF:
179 12.4 +/-1.5 AU; n=50 cells in 3 embryos, ANOVA $p=6.10^{-23}$). Of note, Srp is also expressed in the
180 fat body and yet such expression remains unchanged in *repo* GOF animals (Hoshizaki et al., 1994)
181 (**Figure 3A',B'**), showing that the hemocyte defects are specific and cell autonomous.
182 A more direct evidence for the specific effects of Repo on the *srp* gene was obtained by
183 using a Gal4 plasmid that carries a fragment of the *srp* promoter specific to hemocytes and called
184 *srp(hemo)>* (Bruckner et al., 2004). Co-transfecting S2 *Drosophila* cells with a Repo expression
185 vector and the *srp(hemo)>GFP* plasmid severely reduces the expression of the GFP, and this is a

186 dosage dependent effect (**Figure 3I**). Moreover, *srp(hemo)>repo* embryos display similar features
187 than *gcm*(*hemo)>repo* embryos, with reduced number of hemocytes (**Figure 3J-K''**). Indeed, we
188 found 252.8 +/-27.4 hemocytes in control and 136.8 +/-19.8 in *repo* GOF embryo (n=7 embryos,
189 ANOVA p=0.0028, counted on 30μm stacks of confocal images taken from stage 13 embryos
190 (lateral views)). Of note, the presumptive hemocytes that ectopically express Repo with the
191 *gcm(hemo)>* or with the *srp(hemo)* driver do not express late glial markers (as monitored by the
192 Nazgul antibody (von Hilchen et al., 2010; Ryglewski et al., 2017) (**Figure 3E-F'',J-K'').**

193 The reduction in the number of hemocytes in *repo* GOF is due, at least partially, to enhanced
194 cell death, as shown by the apoptosis marker cleaved death caspase-1 (DCP-1) (Song et al., 1997)
195 (**Figure 3G-H''**, 9.1% +/-1.3 of hemocytes display co-labeling with DCP1 in control vs. 16.1%
196 +/-2.1 in *repo* GOF embryos, n=7 embryos, ANOVA p=0.0150).

197 Altogether, the above data strongly suggest that the expression of the Repo pan-glial factor
198 in the PM is detrimental to hemocyte differentiation and are also in line with the fact that Repo is
199 not sufficient to induce the glial fate when ectopically expressed (Yuasa et al., 2003).

200 Given the ability of Gcm over-expression in the PM to induce Repo ectopic expression, we
201 re-examined that phenotype, in order to understand the relative roles of the two transcription factors
202 in blood and glial development. Interestingly, the over-expression of Gcm in the PM induces both
203 Repo and Nazgul expression in the presumptive hemocytes (von Hilchen et al., 2010) (**Figure 4A-**
204 **D'''**, 21.7%+/-3.4 of Repo positive hemocytes/embryo, n=4 embryos, W p=0.0105 and 53.4% +/-
205 6.4 Nazgul positive hemocytes/embryo, n=3 embryos, W p=0.0318 in *gcm(hemo)>gcm*, compared
206 to 0% in control). Moreover, the cells that express Repo also express the hemocyte marker Srp
207 (**Figure 4E-E'''**) (Rehorn et al., 1996), at levels that are comparable to those found in wild-type
208 embryos (the intensity of Srp labeling in hemocytes from control = 83.4 +/-3.9 AU and from
209 *gcm(hemo)>gcm* = 73.1 +/-6.2 AU, n=50 hemocytes in 3 embryos, ANOVA p=0.22). Thus, Gcm

210 over-expression induces the expression of glial genes without blocking hemocyte differentiation.
211 Since Srp constitutes an early hemocyte gene (Reuter, 1994; Bernardoni et al., 1997; Lebestky et
212 al., 2000), we asked whether late hemocyte markers are also detected in those cells or whether
213 hematopoiesis is blocked at its early stages. The hemocyte-specific scavenger receptor Croquemort
214 (Crq) (Franc et al., 1996; Franc et al., 1999) co-localizes with the pan-glial maker Repo (**Figure
215 4F-F'''**), indicating a mixed glial and hemocyte phenotype. This finding is in accord with the
216 expression/requirement of Gcm in both hemocytes and glia. Of note, we never observed Repo
217 expression in *gcm(hemo)>gcm* hemocytes at larval stages, suggesting that the Repo expressing
218 cells do not survive or that Repo expression is not maintained. Finally, because the *gcmGal4* driver
219 is expressed transiently and early in the hemocyte lineages, we confirmed these data by using
220 additional hemocyte-specific drivers: *srp(hemo)Gal4, hemolectinGal4* and *hemeseGal4* (Bruckner
221 et al., 2004) (**data not shown**).
222

223 **Repo represses the expression of hemocyte markers in glial cells**

224 Given the ability of Repo to inhibit the hemocyte fate in the PM, we asked whether it also
225 represses that fate in glial cells. In the simplest view, the lack of Repo could transform glial cells
226 into hemocytes, as glia represent the resident macrophages of the nervous system. By analyzing
227 the role of Repo first in ectopic glial cells and then in endogenous glia, we found that this
228 transcription factor represses the expression of hemocyte markers.
229 First we found that Gcm expression throughout the neurogenic region (*sca>gcm)* triggers
230 ectopic gliogenesis, whereas the same experiment in *repo* null embryos (*repo* loss-of-function,
231 *repo* LOF) triggers ectopic Srp expression within the nervous system. (**Figure 5A-B'''**). To
232 identify the cells expressing Srp ectopically, we needed a lineage marker that traces the glial cells
233 in wild-type embryos and the presumptive glia in embryos lacking Repo. We hence analyzed

sca>gcm; repo LOF embryos that also carry the *repo-nuclearGFP* (*repo-nGFP*) transgene, which faithfully recapitulates the expression profile of Repo. Since Srp is a nuclear marker, using the nuclear GFP tagging we could show Srp/GFP co-localisation (**Figure 5A-B''')**: 3.5 +/-0.8 cells/hemisegment show Srp/GFP co-localisation in *sca>gcm;repo* LOF,*repo-nGFP* embryos as compared to 0 cells in *sca>gcm;repo-nGFP* embryos (n=6 hemisegments in 3 embryos, W $p=2.10^{-4}$). This indicates that the presumptive glia express Srp, as opposed to the possibilities that *repo-GFP* positive cells phagocyte Srp positive cells (Jones, 2005; Laneve et al., 2012) or that the lack of Repo induces Srp expression non autonomously. Similar results were obtained upon using a second early hemocyte marker, U-shaped (Ush) (**Figure 5C-D'''**): 6.8 +/-0.6 cells/hemisegment show Ush/GFP co-localisation in *sca>gcm;repo* LOF,*repo-nGFP* embryos as compared to 2.8+/-0.7 cells in *sca>gcm;repo-nGFP* embryos (n=10 hemisegments in 3 embryos, ANOVA $p=6.10^{-4}$). Within the neural tissue, we also found Srp or Ush positive cells that are GFP negative (empty arrowheads in **Figure 5B'-B''',D'-D'''**). These cells likely represent hemocytes that have moved into a neural tissue that is no longer properly formed/insulated (Shklyar et al., 2014).

Second, 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) in the *repo* LOF embryos (**Figure 6A-C''**). This does not occur in control animals and is in agreement with the finding that Repo represses the expression of the *srp(hemo)* promoter in S2 cells (**Figure 3I**). Because the GFP of the *srp(hemo)>GFP* line is localized in the membrane and the RFP of the *repo-nRFP* line in the nuclei, we could not formally exclude the possibility that the co-localization indicated the presence of hemocytes within the mutant nervous system and engulfing the presumptive glia. We hence used the anti-Srp antibody and again found expression of the hemocyte marker in presumptive glial cells (*repo* LOF, *repo-nGFP*) (**Figure 6E,F**). In

258 similar assays, we found nuclear co-localization between Ush labeling and GFP (**Figure 6G,H**). In
259 total, 11,6 % of the presumptive glia (GFP positive cells) express Srp (2.2 +/-0.8 cells per
260 hemisegment are double positive GFP/Srp, n=10 hemisegments in 3 embryos, W p= 0.0105) and
261 26 % express Ush ectopically (4.9 +/-0.5 cells per hemisegment are double positive GFP/Ush, n=3
262 hemisegments in 3 embryos, W p= 0.009). This reveals for the first time a hematopoietic potential
263 for *Drosophila* embryonic glial cells.

264 In addition, we analyzed the expression of another hemocyte marker by labelling the *repo*
265 *LOF; repo-nGFP* embryos with the Singed antibody. *singed* (*sn*) codes for a Fascin ortholog that
266 is crucial for hemocyte migration (Zanet et al., 2009) and the antibody strongly labels the
267 embryonic hemocytes (**Figure 6I-L''**). The *repo* LOF embryos show Sn labeling in 6% of the GFP
268 positive cells. (**Figure 6I-L''**). As in the assays performed on ectopic glia, we also found Sn
269 expressing cells that corresponds to hemocytes migrating into the defective nervous system (Sn
270 positive/GFP negative cells, **Figure 6M-N'**).

271 Finally, we asked whether the lack of Repo converts glial cells into mature and functional
272 hemocytes by monitoring the expression of the hemocyte-specific phagocytosis receptor Crq
273 (Franc et al., 1999), but found no ectopic expression of that protein (**Figure 6O,P**), in agreement
274 with the hypothesis that the lack of Repo does not simply reveal a default hemocyte fate. Thus, the
275 lack of the Repo transcription factor triggers the epxression of subsets of hemocyte markers in a
276 fraction of presumptive glia. This could mean that Repo is not sufficient to repress a hemocyte fate
277 in all glial cells or that distinct glial subtypes express different hemocyte markers in the *repo* LOF
278 embryos. To discriminate between the two hypotheses, we followed the approach described by
279 Sklyar *et al.* (Shklyar et al., 2014) and subdivided the ventral nerve cord in two parts along the Z
280 axis: the ventral part mainly contains cortex glial cells, the dorsal part mainly contains axon-
281 associated glial cells (Ito et al., 1995) (**Figure 6D**). The presumptive glia ectopically expressing

282 the hemocyte transcription factors Srp or Ush are only located dorsally and they correspond to the
283 axon-associated glia. This was confirmed by using anti-Fas2, which recognizes the three dorsally
284 located longitudinal axonal fascicles of the ventral cord (Santos et al., 2007) (**Figure 6E,F**) or a
285 second neuronal marker, anti-HRP (**Figure 6G,H**). On the other hand, the cells that express Sn are
286 located at the position of the cortex glia and are mostly located ventrally **(Figure 6I-L'')**. This
287 phenotype matches the observation that cortex glia are more motile in *repo* mutant embryos
288 (Shklyar et al., 2014).

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

291

292 **Repo acts as the guardian of the glial fate**

293 The fact that only a fraction of the presumptive glia expresses any hemocyte marker in *repo*
294 LOF embryos prompted us to ask whether these cells display other defects. Since Gcm represses
295 the neuronal fate and gain of function experiments suggest that Repo contributes to the process
296 (Yuasa et al., 2003), we explored the possibility that glial cells lacking Repo express neuronal
297 features. We indeed found that a fraction of the presumptive glial cells (22 %) express the pan-
298 neuronal marker Elav (Yao and White, 1991; Berger et al., 2007) in *repo* LOF; *repo-nGFP*
299 embryos (**Figure 7A-D''',F**). These cells are scattered throughout the ventral nerve cord (**Figure
300 7B,D**) and do not co-express the hemocyte markers Srp (**Figure 7E-E'''**) or Ush (**data not shown**).

301 We hence ypothesized that the neuronal and the hemocyte transcriptional programs may
302 compete with each other and asked whether hemocyte markers are ectopically expressed in the
303 ventral cord of embryos lacking Elav, a key factor for neuronal differentiation. No mutant
304 phenotype was observed in these embryos **(last column in Figure 7F,G)**. Interestingly, however,
305 *elav;repo* LOF double mutant embryos that also carry the *repo-nGFP* transgene show twice as

306 many cells expressing the Srp hemocyte marker in presumptive glia as compared to those observed
307 in *repo* LOF embryos (23 % vs. 11% (**3rd and 2nd columns, respectively, in Figure 7F,G**). Thus,
308 the glial factor Repo contributes to repress the neuronal as well as the hemocyte fates and the
309 neuronal factor Elav contributes to repress the hemocyte fate.

310 To further our understanding on the role of the Repo transcription factor on the glial fate,
311 we also scored the total number of presumptive glia and assessed their proliferative and cell death
312 profile in *repo* LOF embryos. The number of nuclei expressing the GFP in *repo LOF; repo-nGFP*
313 embryos is 30 % lower compared to that observed in wild-type animals (345.8+/-6.9 per embryo
314 in WT compared to 196.0+/-35.8 in *repo LOF*, n=3 embryos, ANOVA p=0.0383). This is in
315 agreement with a slight reduction in cell division and a slight increase in apoptosis: anti-PH3 (Juan
316 et al., 1998) was used to score for glial cell division: 4.9+/-1.0 dividing cells are present per 6
317 hemisegments in WT embryos compared to 0.3+/-0.3 in *repo LOF* (n=3 embryos, W p=0.0361).
318 Apoptosis was scored using the anti-CM1 antibody that recognizes the activated Caspase-3 (**Figure**
319 **8A-B’’’**). No cells were observed in WT compared to 10.6+/-1.2 dying cells in *repo LOF* (n=3
320 embryos, 6 hemisegments were counted per embryo, W p=0.0318). It is therefore likely that some
321 cells missing the Repo protein no longer acquire/maintain the right identity and eventually die. To
322 make sure that the co-localization between the presumptive glia (nuclear GFP) and the death maker
323 CM1 identifies dying cells (**Figure 8A-B’’’,E**), rather than glial cells that are phagocytosing dead
324 bodies, we compared the results obtained on *repo LOF; repo-nGFP* with those obtained on *repo;*
325 *repo-CD8GFP* embryos, in which the GFP is tagged to the membrane (**Figure 8E**).

326 As expected, in the latter case we did not observe co-localization between the GFP and
327 CM1 (**Figure 8C-C’’’**). Moreover, this data further confirmed the lack of phagocytosis observed
328 in *repo LOF* embryos **(Figure 8D)**, likely due to defective SIMU and Draper expression (Shklyar

329 et al., 2014). Indeed, while in wild-type embryos glial cell membranes completely enwrap apoptotic
330 bodies (**Figure 8C-C’’’**), in *repo* LOF embryos these contacts are no longer established.

331 In sum, Repo acts as a true guardian of the glial fate, in line with the fact that it is the only
332 transcription factor that is expressed in all glia and only in glia.

333

334

335
Discussion

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

346

347
Tissue-specific cues inhibit the gliogenic potential of Gcm in the hematopoietic anlagen

348 The *Drosophila* transcription factor Gcm is expressed and required for the differentiation
349 of glia and blood, which share immune features but also perform specific functions in the immune
350 and nervous systems. These cells originate from different layers, glia from the ectoderm, hemocytes
351 from the mesoderm, and therefore display distinct molecular landscapes. We here show that the

352 mesoderm-specific transcription factor Twi and its target *miR*-1 repress the expression of the pan-
353 glial gene Repo in the hemocyte anlagen. Thus, the mesodermal molecular landscape controls Gcm
354 activity and biases its transcriptional output towards hemocyte differentiation.

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

363

364 **The Repo homeodomain containing factor locks cells in the glial fate**

365 Gcm is expressed and necessary at early stages of glial development, whereas the
366 homeodomain containing Repo protein is stably expressed in the glial cells. The lack of late glial
367 markers observed in *repo* mutant embryos initially suggested a role of Repo in glial terminal
368 differentiation (Xiong et al., 1994; Yuasa et al., 2003). However, the ectopic expression of non-
369 glial markers in those embryos shows that Repo also controls cell plasticity. This shows that
370 homeodomain containing transcription factors can provide the molecular relay from multipotency
371 to a fully differentiated state once the transient expression of pioneer factors extinguishes.

372 The robustness of the glia and hemocyte fates relies on the activity of cell-specific genes:
373 Repo as well as Elav repress the expression of Srp in the nervous system, whereas Twi/*miR-1*
374 repress the expression of Repo in the mesoderm. Moreover, Srp and Gcm co-expression in the

375 mesoderm also repress Repo expression (**data not shown**). These inhibitory interactions ensure
376 that the glial and the hemocyte fates are mutually exclusive.
377 Our data also suggest that glial (Repo) and neuronal (Elav) factors both repress ectopic
378 hematopoiesis in the neural territory while counteracting each other to maintain the glial and the
379 neuronal fates, respectively. This molecular network explains why cells adopt the neuronal default
380 fate in the absence of Gcm whereas they start expressing hemocyte markers in the absence of Repo,
381 and even more so in the absence of both Repo and Elav.
382 Thus, cell-specific pathways and feedback loops allow a single pioneer factor to affect
383 different cell fates. Such molecular checkpoints acting in parallel and in sequence allow the
384 maintenance of a stable fate.
385

386 **Lack of Repo triggers different phenotypes in distinct glial subtypes**

387 The glial cells of the embryonic ventral nerve cord are subdivided into three main subtypes
388 (surface, cortex and axon-associated) based on their morphology, position and function (Ito et al.,
389 1995; Beckervordersandforth et al., 2008). The large and flattened glial cells associated to the
390 surface form the BBB (Auld et al., 1995). Glial cells located in the cortex are star-shaped and
391 intermingled with neuronal bodies, their cytoplasmic projections contacting multiple synapses
392 (Freeman and Doherty, 2006; Freeman, 2015). Cortex glia help clearing the debris induced by
393 neuronal programmed cell death (Freeman et al., 2003; Shklyar et al., 2013; Shklyar et al., 2014)
394 (Kurant et al., 2008). Finally, glial cells associated to the axons enwrap them in a multi-layer sheath
395 promoting the conduction of nerve impulses and a subset of them has also been called astrocyte-
396 like glia (Hidalgo and Booth, 2000; Sepp et al., 2000; Sepp and Auld, 2003; Freeman and Doherty,
397 2006; Freeman, 2015). These glia are known to act as scavengers in response to developmental
398 signals and to trauma, likely due to their proximity to signaling axons. Typically, in the adult brain

399 they phagocyte degenerating axons after brain injury (Doherty et al., 2009) and, after puparium
400 formation, axon-associated glia of the mushroom body control ecdysone-dependent axons pruning
401 (Awasaki and Ito, 2004; Kato et al., 2011; Kato and Hidalgo, 2013; Boulanger and Dura, 2014;
402 Hakim et al., 2014).

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

412 Finally, our data strongly suggest that, although glial cells act as macrophages, they do not
413 have a default hemocyte phenotype, rather, they constitute a very specialized population of
414 scavenger cells. Similarly, vertebrate microglia, cells of immune origin that provide the first
415 response to nervous system challenge, display a molecular signature that is distinct from that of
416 macrophages (Prinz and Priller, 2014).

417

418 **Of flies and vertebrates…**

419 *Drosophila* and vertebrate glial cells share numerous functions controlling neuron
420 homeostasis, recycling neurotransmitters and insulating axons (Freeman and Doherty, 2006),
421 however the transcriptional program triggering the first steps of gliogenesis are not evolutionarily

422 conserved. In *Drosophila*, the Gcm transcription factor constitutes the major regulatory gene and
423 acts as a molecular switch between neuron and glial cells. Although the vertebrate Gcm orthologs
424 seem to maintain some gliogenic potential *in vitro* (Kim et al., 1998; Reifegerste et al., 1999;
425 Buzanska et al., 2001; Iwasaki et al., 2003; Soustelle et al., 2007), they are neither expressed nor
426 required in glia. Moreover, no true glial determinant has been so far identified in vertebrates
427 (Hitoshi et al., 2011). Even more strikingly, the vertebrate genomes do not contain the coding
428 sequences for Repo (no orthologs found so far), the only fly transcription factor that is specific to
429 all lateral glia and only to glia, a molecular signature that seems shared throughout the Arthropod
430 clade (Wakamatsu, 2004; Boyan et al., 2011; Mysore et al., 2011; Nasu and Hara, 2012).
431 Our findings raise the question of the evolutionary link between vertebrate and *Drosophila*
432 gliogenesis (Hartline, 2011). While the hypothesis of an independent origin of vertebrate and
433 invertebrate glia remains to be tested, the comparative analysis of those glia has tremendously
434 improved our understanding of the bases of nervous system regeneration. *Drosophila* glia indeed
435 constitute an excellent model to investigate the mechanisms governing CNS repair following
436 traumatic injury (Leyssen and Hassan, 2007; Kato et al., 2011). In this contest, and in light of recent
437 data showing that mature astrocytes and oligodendrocytes can be reprogrammed into functional
438 neurons to promote CNS regeneration (Heinrich et al., 2010; Guo et al., 2014; Su et al., 2014), it
439 will be interesting to study whether the loss of Repo triggers glial cell conversion into neurons in
440 the adult *Drosophila* injured CNS.
441 Finally, sequencing the genome and analyzing the single cell transcriptome of simple
442 organisms has become an important tool to understand the molecular and cellular bases of
443 evolution. Future analyses will establish when Gcm and Repo appear in evolution and where are
444 they expressed/required within/outside the nervous system.
445

Materials and Methods

Fly stocks

Flies were kept at 25 °C. w^{1118} was used as wild-type. *repo-nGFP* was used to drive nuclear GFP expression under the control of the 4.3kb *repo* promoter, which recapitulates the full *repo* expression pattern (Jones, 2005; Laneve et al., 2012). 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) and $elav^{4}$ (Bloomington Center) are null alleles.

The *UAS/Gal4* system was used for cell-specific manipulation of gene expression. *srp(hemo)Gal4* triggers expression in hemocytes (Bruckner et al., 2004), *scaGal4* throughout the neurogenic region (Bloomington stock Center), *twiGal4* (Baylies and Bate, 1996) throughout the mesoderm and *gcmGal4* (Soustelle and Giangrande, 2007) combined to *repo-Gal80* (gift of B. Altenhein) 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* (Bloomington stock Center), *UAS-GFP* (Bloomington stock Center); *UAS-repo* (Yuasa et al., 2003); *UAS-twi* (Baylies and Bate, 1996); *UAS-gcm(F18A)* **(Figure 5)** (weak Gcm over-expression), *UAS-gcm(RS1)* **(Figure 1C,D,I)** or *UAS-gcm(M24A)* **(Figure 1H, Figure 4)** (medium Gcm over-expression) (Bernardoni et al., 1998). The combination of *UAS-gcm(M24A)* and *UAS-gcm(F18A)* provided a strong Gcm over-expression **(Figure 1H)**.

469 **Immunohistochemistry**

470 Embryo collections were done on plates containing agar, apple juice and yeast.
471 Dechorionated embryos were fixed in 4% formaldehyde in PBS for 20 min, permeabilized with
472 0.3 % Triton-x100 in PBS (PTX), blocked by 0.5% Blocking Reagent (Roche) in PTX for 1 h and
473 labeled overnight at 4 °C with the following antibodies: rabbit (rb) anti-Repo (1/10), mouse (m)
474 anti-Repo (1/10), m anti-Singed (1/50) and rat anti-Elav (1/200) (DHSB); guinea pig (gp) anti-
475 Repo (1/1000) and gp anti-Nazgul, (1/200) (gift of B. Altenhein) (von Hilchen et al., 2010); mouse
476 (m) anti-Ush (1/1000) (Cubadda et al., 1997); rb anti-Srp (1/1000) (gift of R. Reuter) (Sam et al.,
477 1996; Petersen et al., 1999); m anti-P1 (1/10) (gift of E. Kurucz) (Kurucz et al., 2007); rb anti-Crq
478 (1/500) (gift of J.L Dimarcq and J. Hoffmann) (Franc et al., 1996); m anti-Fas2 (1/100) (gift of
479 C.S. Goodman) (Grenningloh et al., 1991); rb anti-HRP (1/500) and rb anti-β-Gal (1/500) (Cappel)
480 and chicken anti-GFP (1/1000) (Abcam); m anti-β-Gal (1/200) (Sigma); rat anti-RFP (1/100)
481 (chromotek); rb anti-DCP-1 (1/50) (Cell Signaling Technology).

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

485 Srp signal intensity was measured on confocal images acquired with hybrid detector in
486 photon counting mode. The mean gray value measurement tool from Fiji was used to estimate the
487 intensity of the signal (in Arbitrary Unit, AU) from 50 hemocytes in at least 3 embryos (Schindelin
488 et al., 2012).

489

490 **Co-transfection, Western blot and luciferase assays**

491 *Drosophila* S2 cells were grown in Schneider medium (Fisher Scientific) complemented
492 with 10% heat inactivated Fetal Calf Serum and 0.5% Penicillin/Streptomycin. 6 x 10^6 cells were

493 cultured in six well culture dish 12 h prior transfection. 5 µg of total plasmid mix were transfected
494 using the Effectene Kit (Qiagen) according to manufacturer's instructions. The *psrp(hemo)Gal4*
495 plasmid provided a *srp* transcriptional reporter (Bruckner et al., 2004) upon co-transfection with
496 the *pUAS-GFP* plasmid. The *pPac5C-repo* plasmid was used to induce Repo expression (Yuasa et
497 al., 2003) and *pPac5C-lacZ* as a transfection control. The *pPac5C* plasmid was used to equilibrate
498 the amount of transfected DNA. Cells were harvested 24 h after transfection in Tris-HCl 25 mM
499 pH 7.9, 400 mM KCl, 10 % glycerol and total proteins were extracted by three freezing-thawing
500 steps. Protein expression was detected from protein lysate according to standard Western blot
501 procedure. The following primary antibodies were used: m anti-β-Gal (1/2500, Sigma), rb anti-
502 GFP (1/5000, Molecular Probes), m anti-Repo (1/20, DHSB). m anti-HRP and rb anti-HRP
503 (1/5000, Jackson ImmunoResearch) were used as secondary antibodies.

504 For the luciferase assay, *Drosophila* S2 cells were cultured in a 24-well plate, in the same
505 conditions as previously described. Plasmid transfections were carried out using Effectene
506 (Qiagen) following manufacturer's instructions. *pMTGal4-GFP, pUAST-Luciferase-Luciferase*
507 *3'UTR, pUAST-Luciferase-Repo 3'UTR*, *pUAST-Luciferase-Repo 3'UTR ΔmiR-1* and *pTK-Renilla*
508 were all used at 20 ng/mL and *pTub-miR-1* was used at 50 ng/mL. The cells were cultured 2 days
509 prior induction with 500 µM of copper sulphate. The luciferase assay was done 18h after induction,
510 using the Dual-Glo Luciferase assay kit (Promega) according to manufacturer's instructions. Three
511 independent transfections were averaged with standard deviation. Statistical significance was
512 calculated with Graphpad Prism software using t-test.

513

514 **RNA extraction, reverse transcription and qPCR**

515 Total RNA was purified from stage 5-11 embryos by TriReagent (MRC). 1 µg of purified
516 RNA was reverse transcribed by SuperScript II reverse transcriptase (Invitrogen) using oligodT

517 primers (5 µM). mRNAs were analyzed by qPCR using Sybr Green (Roche) Master Mix, the
518 thermocycler Roche LightCycler480 and the following oligonucleotides:
519 *repo* forward : 5' AAGCAGCAGCAAGAAGAAGG 3'
520 *repo* reverse : 5' ATACGGAGCACGTTCAAAGG 3'
521 *actin5C* forward : 5' GCAGCAACTTCTTCGTCACA 3'
522 *actin5C* reverse : 5' CTTAGCTCAGCCTCGCCACT 3'
523 For each gene, the mRNA levels were automatically calculated (LightCycler480 Software,
524 release 1.5.0) by calibration to gene-specific standard curves generated on input cDNAs. Collected
525 values, derived from three amplification reactions, each performed in three independent
526 experiments, were normalized to *actin5C* mRNA amounts.
527
528 **Statistics**
529 All the experiments were performed in at least three biological replicates. Statistical
530 relevance was assigned by calculating means, standard errors. Whenever the data showed normal
531 distribution **(Figure 1H,I, 3I)**, they were analyzed by the ANOVA test, whenever they did not
532 **(Figure 7F,G)** by Kruskal-Wallis (KW) and Wilcoxon (W) tests. * = $p < 0.05$; ** = $p < 0.01$; ***
533 = $p < 0.001$.
534
535
536 ## *Author contribution*
537 GT and AG designed the experiments. GT, PC and DM did the experiments. GT, PC, JZ,
538 DM, MF and AG analyzed the data. GT, PC and AG finalized the manuscript.

Acknowledgements

We thank B. Altenhein, K. Brückner, M. Crozatier, L. Waltzer, M. Logan, E. Kurant, R. Reuter, E. Kurucz, J.L Dimarcq, J. Hoffmann, C. Goodman, the DHSB and the Bloomington Stock Center for reagents and flies. We thank all the lab members for comments on the manuscript, C. Diebold, C. Delaporte, M. Pezze, the fly, imaging and antibody facilities for technical assistance and D. Dembele for help with statistics. We thank Alison Brewer for help with Luciferase assays. This work was supported by INSERM, CNRS, UDS, Ligue Régionale contre le Cancer, Hôpital de Strasbourg, ARC and ANR grants. P. Cattenoz was funded by the ANR and by the ARSEP, G Trebuchet by governmental and by ARC fellowships. This work was also supported by grants from the Ataxia UK (2491) and the NC3R (NC/L000199/1) awarded to M.F. The IGBMC was also supported by a French state fund through the ANR labex.

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748

749

750 ***Table 1***

751 **Drivers used to target the neurogenic region, the mesoderm and the procephalic mesoderm.**

752 The 1st column indicates the genotype, the 2nd column indicates the region expressing the driver

753 (embryo at stage 8, lateral view, anterior to the left) and in a cross-section of the embryo (dorsal to

754 the top) and the 3rd column indicates the region targeted.

755

756

757 ***Figure legends***

758 **Figure 1: Twi negatively regulates Repo expression.**

759 **A-D**) Confocal projections of embryos stage 14 *scaGal4* or *sca>* (Control, **A**), *sca>twi* (*twi* GOF,

760 **B**), *twi>gcm* (*gcm* GOF, **C**) and *twi>gcm;twi-/+* (*gcm* GOF, *twist* het, D) immunolabeled for the

761 glial marker Repo (blue). Ventral view. Unless otherwise specified, all scale bars represent 100 µm

762 and anterior is to the left. (**E-F''**) Confocal projections of wild-type (**E**) and *twi-/-* (*twi* LOF, **F**)

763 embryos labeled for the Repo glial marker (blue) and for the hemocyte Srp marker (red). Lateral

764 view. (**F'**) and (**F''**) represent a single section of the inset indicated in (**F**), they show Srp labeling

765 only and co-labeling with Repo, respectively. The white arrowheads indicate cells expressing Srp

766 and Repo. (**G**) Confocal projections of *gcmGal4,repoGal80/+;UAS-CD8GFP* (*gcm(hemo)>GFP*)

767 embryos labeled for Repo (blue) and GFP (green). Lateral view (upper panel) and ventral view

768 (lower panel). The region defined by the dashed line indicates the Central Nervous System (CNS).

769 Note that GFP expression is excluded from glia. (**H,I**) Relative quantification of *repo* mRNA by

770 qPCR from stage 5-11 embryos of the following genotypes: *gcm(hemo)>* (Control) and

771 *gcm(hemo)> gcm* GOF (Weak, Medium and Strong *gcm* GOF) in (**H**); *gcm(hemo)>* (Control),

772 *gcm(hemo)>medium gcm* (*Med. gcm* GOF) and *gcm(hemo)>medium gcm + twi* (*Med. gcm* GOF,

773 *twi* GOF) in (**I**). *gcm* levels are relative to *actin* levels, n indicates the number of independent
774 assays, see the Experimental Procedure section for the statistic tests. (**J-L**) Confocal projections of
775 embryonic ventral cords of the following genotypes: gcm^{34}/+ (**J**), gcm^{34}/gcm^{34} (**K**) and
776 gcm^{34}/*Df(2L)132* (**L**). Labeling: β-Gal (green), Srp (red) and the neuronal marker Elav (gray). The
777 gcm^{34} line represents a P element partial excision that retains the LacZ gene, allowing monitoring
778 of *gcm* expression. β-Gal/Srp double positive cells (yellow, asterisks) are located outside the
779 ventral cord (dashed line) and label the circulating hemocytes.
780

781 **Figure 2: *miR-1* prevents Repo expression in the hemocyte lineage.**

782 (**A**) Schematic representation of the *repo* locus in the *Drosophila* genome (dm3). UTRs and coding
783 exons are indicated by plain blue boxes (thin and thick, respectively) and the intron by a blue line.
784 The two putative *miR-1* binding sites in the *repo* 3'UTR are indicated. (**B,C**) Confocal projections
785 of embryos of the following genotypes: wild-type and *miR-1* LOF (-/-), lateral view, stage 14,
786 labeled for Repo (blue) and Srp (red). (**C'**) and (**C''**) represent a single section of the inset indicated
787 in (**C**), they show Srp labeling only and co-labeling with Repo, respectively. (**D**) Number of
788 hemocytes expressing Srp and Repo in wild-type and in *miR-1* mutant embryos (-/+ and -/-). n
789 indicates the number of embryos analyzed for each genotype. (**E**) Schematic representation of the
790 three Luciferase reporter vectors that were used in the co-transfection assays: the top one is the
791 Control vector carrying the Firefly Luciferase coding sequence and the Firefly 3'UTR under the
792 UAS promoter. In the second construct (middle), the 3'UTR has been replaced by the *repo* 3'UTR
793 and in the last construct (bottom), the two *miR-1* binding sites of the *repo* 3'UTR have been
794 mutated. (**F**) Quantification of the Luciferase activity in extracts from S2 cells co-transfected with
795 *pTub-miR-1*, *pTK-Renilla* and either *pUAST-Luciferase-Luciferase-3'UTR* (*Firefly* 3'UTR, gray),

796 *pUAST-Luciferase-Repo-3'UTR* (*repo* 3'UTR, green) or *pUAST-Luciferase-Repo-3'UTRΔmiR-1*
797 (*repo* 3'UTR *ΔmiR-1*, red), the values are normalized with the Renilla activity.
798
799 **Figure 3: Repo can repress hemocyte differentiation.**
800 **(A-H'')** Embryos *gcm(hemo)>CD8GFP* (Control) or *gcm(hemo)>CD8GFP,repo* (*repo* GOF).
801 **(A,B)** represent confocal projections of embryos labeled for GFP (green), Srp (red) and Repo
802 (blue), dorsal view, stage 16, the empty arrowheads indicate the Srp positive GFP negative cells of
803 the fat body. **(A')** and **(B')** show the Srp signal alone. (**A'',A''',B'',B'''**) show single sections of
804 the insets indicated in (**A,B**), the arrowheads indicate the hemocytes (GFP/Srp double positive
805 cells). Note that Repo is expressed in GFP positive cells in *repo* GOF (**B''**) and that the levels of
806 Srp upon Repo overexpression (**B'''**) are much lower compared to those observed in the wild-type
807 embryo (**A'''**). (**C,D**) represent confocal projections of embryos labeled for the hemocyte marker
808 P1 (red), dorsal view, stage 14. (**E-F**) represent confocal projections of embryos labeled for GFP
809 (green) and the glial marker Nazgul (red), lateral view, stage 14, (**E',F'**) show the Nazgul signal
810 alone. (**G,H**) represent single confocal sections of embryos labeled for DAPI (blue), CD8GFP
811 (green) and the apoptotic marker DCP-1 (gray). (**G',G'',H',H''**) show the insets indicated in
812 (**G,H**), the arrowheads indicate cells double positive for CD8GFP and DCP-1. (**I**) Western blot on
813 protein extracts from S2 cells co-transfected with *psrp(hemo)Gal4, pUAST-GFP* and increasing
814 amounts of *pPac5C-repo* (0 to 3 μg). *pPac5C-lacZ* was used as a transfection control. The
815 histogram represents GFP/β-Gal relative quantification. The amounts of transfected Repo were also
816 verified. n indicates the number of co-transfection assays. (**J-M**) Embryos *srp(hemo)>RFP*
817 (Control) or *srp(hemo)>RFP,repo* (*repo* GOF), lateral view, stage 14. (**J,K**) represent confocal
818 projections of embryos labeled for RFP (red) and Repo (green). (**J',K'**) show the Srp signal alone

from (**J,K**). (**J'',K''**) represent single sections of the insets indicated in (**J,K**). (**L,M**) represent confocal projections of embryos labeled for Nazgul (red).

Figure 4: 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'**). **(A-B')** represent confocal projections of embryos labeled for GFP (green) and Repo (blue), lateral view, stage 14. **(B'',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'',D''')** represent single confocal sections of the inset indicated in **(D')**, arrowheads indicate ectopic glial labeling in hemocytes over-expressing 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, those that also express Repo by arrowheads. Note that Repo ectopic expression does not affect Srp or Crq expression. Scale bars in (**E,F**): 50 μm.

Figure 5: 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 line indicates the ventral nerve cord (VNC) **(A-D)**. **(B'-B''', D'-D''')** represent single sections of the insets indicated in **(B, D)**, they show nGFP labeling only, Srp or Ush labeling only and co-labeling

843 Srp or Ush with nGFP, respectively. White arrowheads indicate nGFP/Srp **(B'-B''')** or nGFP/Ush
844 **(D'-D''')** double positive cells, empty arrowheads indicate Srp or Ush positive and nGFP negative
845 cells in *gcm* GOF *repo* LOF embryos. These are hemocytes recruited to the VNC that is not
846 properly insulated due to the mutant background (Shklyar et al., 2014).
847
848 **Figure 6: Repo is required to repress hemocyte transcription factors in developing glia.**
849 (**A,B**) Confocal projections of embryos *srp(hemo)>CD8GFP/repo-nRFP* (Control, **A**) and
850 *srp(hemo)>CD8GFP/repo-nRFP;repo-/-* (*repo* LOF, **B**) labeled for GFP (green) and RFP (red),
851 ventral view. (**B'-B''', C-C''**) show single sections of the insets indicated in (**B**). Note that the
852 single sections were acquired at different focal planes in the VNC. The arrows indicate GFP/RFP
853 double positive cells. (**D**) Schematic representation of a transversal section of the VNC from a
854 mature embryo. Glial cell subtypes are defined according to their localization: surface pale (blue),
855 cortex (red) and axon-associated glia (green) (Ito et al., 1995; Beckervordersandforth et al., 2008).
856 (**E-P**) Embryos of the following genotypes: *repo-nGFP* (Control) and *repo-/-;repo-nGFP* (*repo*
857 LOF). The analyses were performed upon subdividing the VNC in a ventral and in a dorsal part,
858 according to the schematic shown in (**D**), the position of the section along the dorso/ventral axis of
859 the VNC is indicated on the left side of the panels. Scale bar in (**E-P**): 50 μm. Stage 15 embryos
860 are labeled for GFP (green), Fas2 (gray) and Srp (red) (**E,F**); GFP (green), HRP (gray) and Ush
861 (red) (**G,H**). Stage 14 embryos are labeled for GFP (green) and Sn (red) (**I-L''**), (**L',L''**) show
862 single sections of the inset indicated in (**L**). (**M,N**) Confocal projections of the whole VNC labeled
863 for GFP (green), Sn (red) and Elav (gray), the dash line indicates the position of the z-axis
864 reconstitution of the VNC presented in (**M',N'**). Note the presence of Sn positive/GFP negative
865 cells within the VNC in *repo* LOF embryo; these are hemocytes recruited to the VNC following
866 the loss of *repo* (Shklyar et al., 2014). (**O,P**) Embryos labeled for GFP (green) and Crq (red).

867

868 **Figure 7: Repo represses both hemocyte and neuronal differentiation**

869 (**A-D'''**) Embryos of the following genotypes: *repo-nGFP* (Control) and *repo-/-;repo-nGFP* (*repo*
870 LOF), ventral view, stage 15. The ventral and the dorsal parts of the VNC were analyzed separately.
871 Labeling: GFP (green) and Elav (gray). (**B'-B''',D'-D'''**) show single sections of the insets
872 indicated in (**B, D**). Arrowheads indicate ectopic GFP/Elav double positive cells. (**E-E'''**) Dorsal
873 part of a *repo-/-;repo-nGFP* (*repo* LOF) embryo labeled for Srp (red), Elav (gray) and GFP (green),
874 the channels are presented individually in (**E'**), (**E''**) and (**E'''**), respectively. White arrowheads
875 indicate GFP/Elav double positive cells and empty arrowheads indicate GFP/Srp double positive
876 cells. Scale bars in (**A,E**): 50µm. (**F,G**) Graphs showing the number and the percentage of
877 GFP/Elav double positive cells (**F**) or GFP/Srp double positive cells (**G**) per hemisegment in
878 Control, *repo* LOF, *repo* LOF *elav* LOF double mutant and *elav* LOF embryos. n indicates the
879 number of hemisegments counted in 3 embryos.

880

881 **Figure 8: *repo -/-* glia undergo apoptosis.**

882 **(A-D)** Embryos of the following genotypes: *repo-nGFP* (Control, **A**) and *repo-/-;repo-nGFP* (*repo*
883 LOF, **B**) express nuclear GFP. *repo-CD8GFP* (Control, **C**) and *repo-/-;repo-CD8GFP* (*repo* LOF,
884 **D**) express GFP at the membrane, ventral view, stage 15. Labeling: GFP (green) and the apoptotic
885 marker CM1 (red). (**B'-B''',C'-C'''**) show single sections of the insets indicated in (**B, C**).
886 Arrowheads in **(B'-B''')** indicate glial cells undergoing apoptosis (co-localisation of nuclear GFP
887 and CM1), whereas arrowheads in (**C'-C'''**) indicate glial cells enwrapping apoptotic bodies
888 (CD8GFP surrounding CM1 labeled bodies). **(E)** Schematic representation of the GFP/CM1 co-
889 labelling in apoptotic cells expressing nuclear GFP and in phagocytic cells expressing GFP at the
890 membrane.

Table 1

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(hemoGal4)* *(srp(hemo)>)*		Procephalic mesoderm

Table 1
Drivers used to target the neurogenic region, the mesoderm and the procephalic mesoderm. The 1st column indicates the genotype, the 2nd 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 3rd column indicates the region targeted.

Figure 1

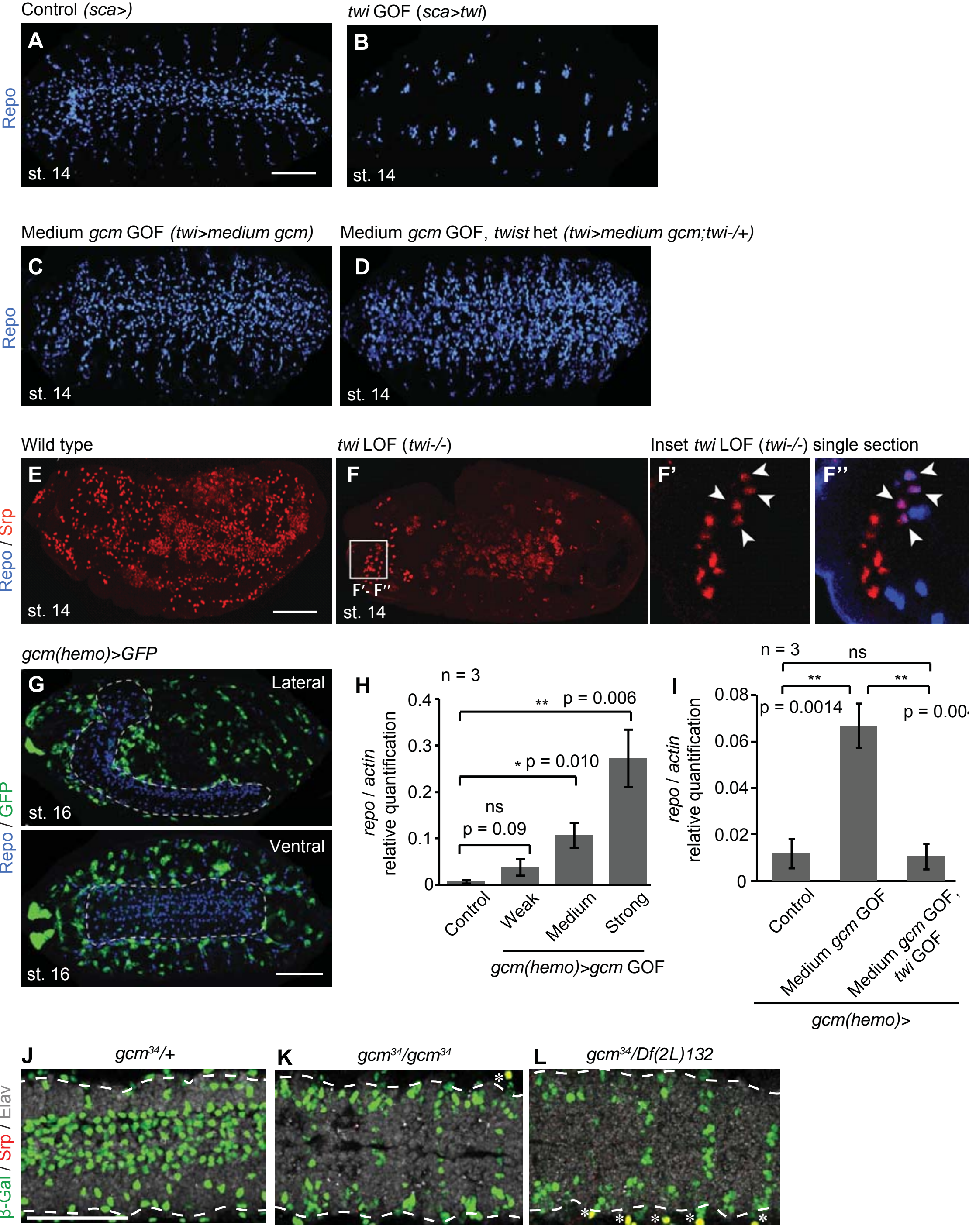
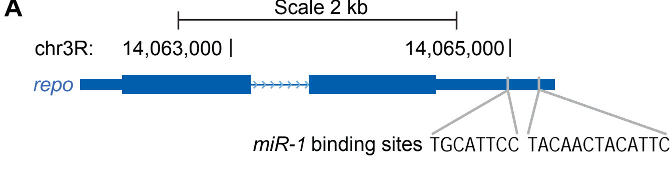


Figure 2



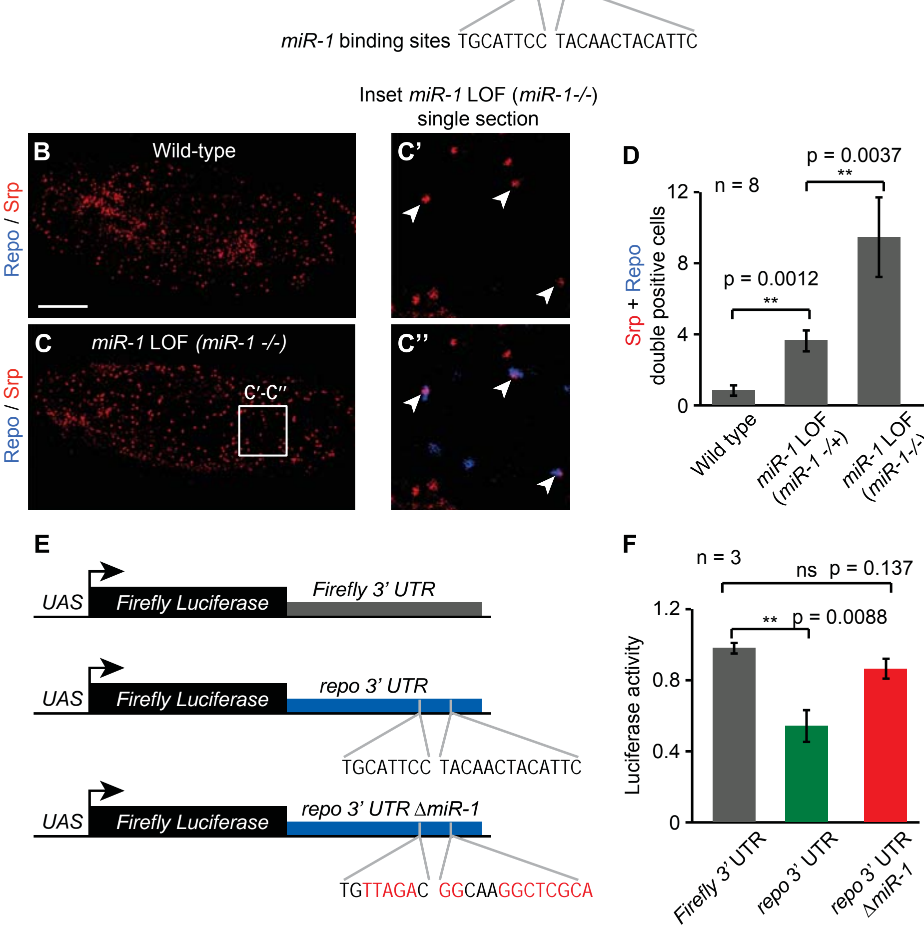


Figure 3

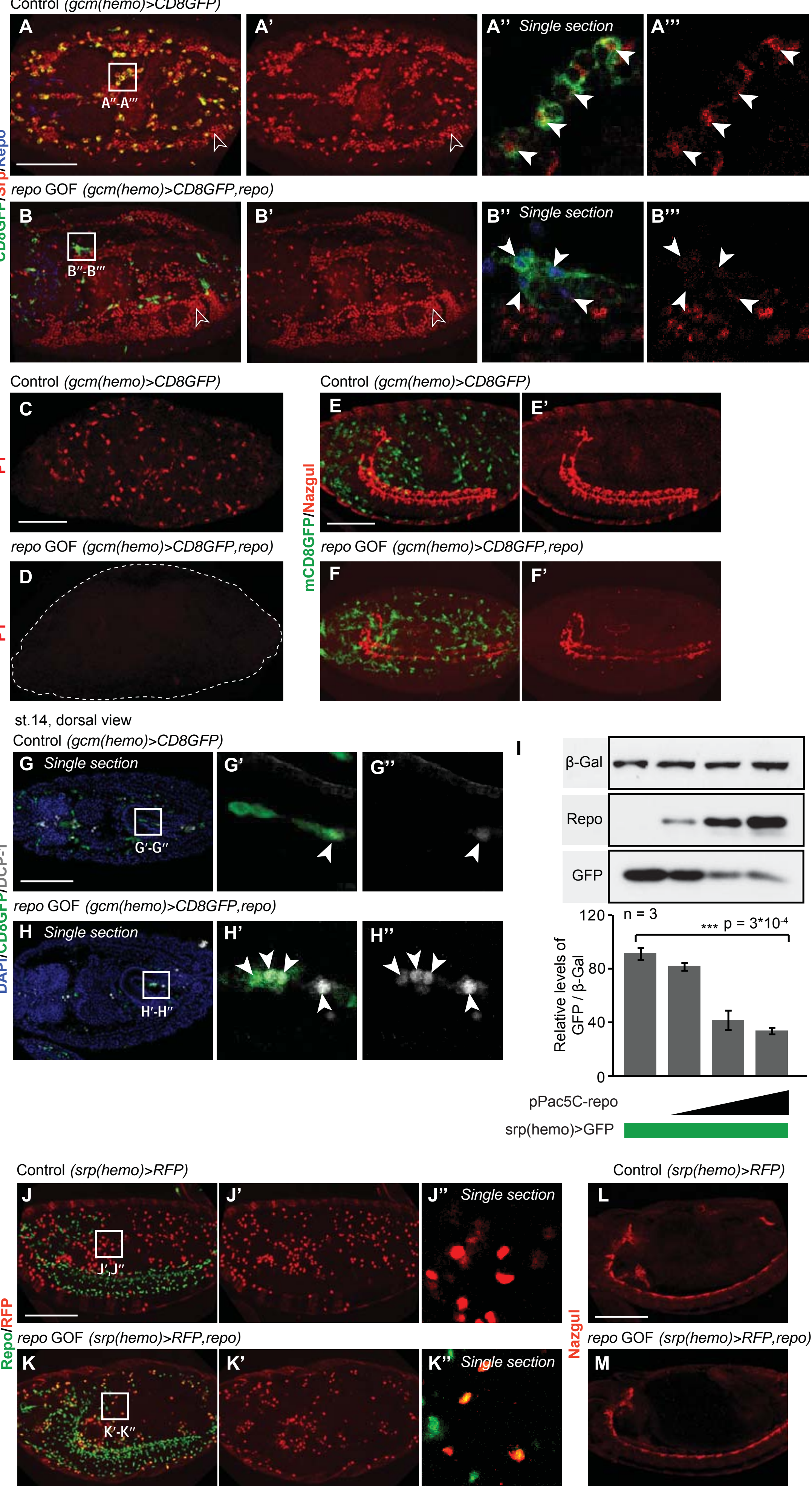


Figure 4

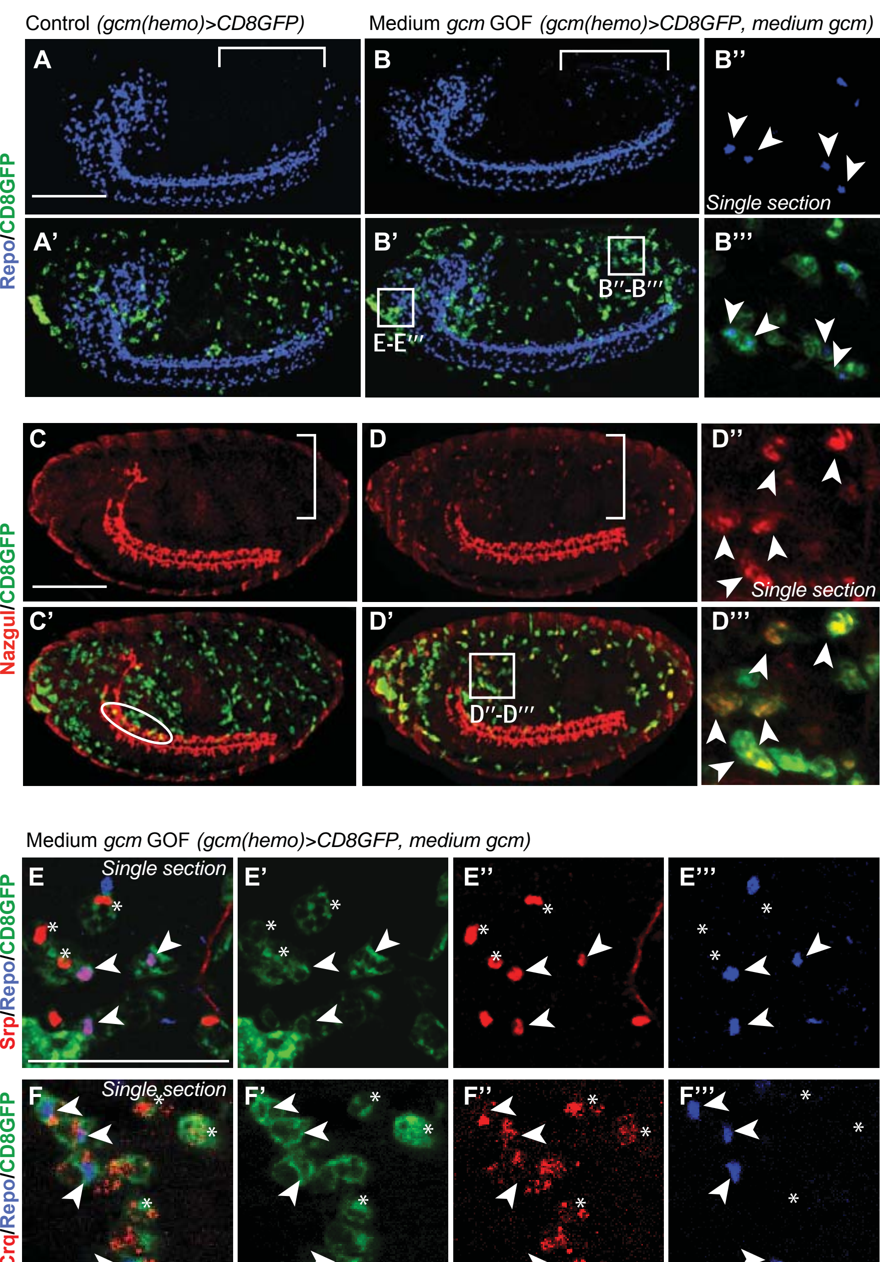


Figure 5

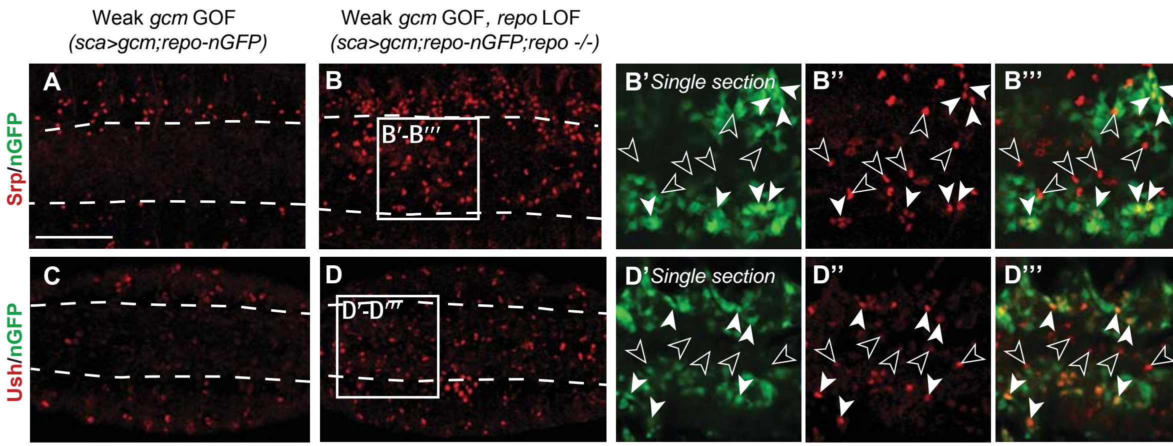


Figure 6

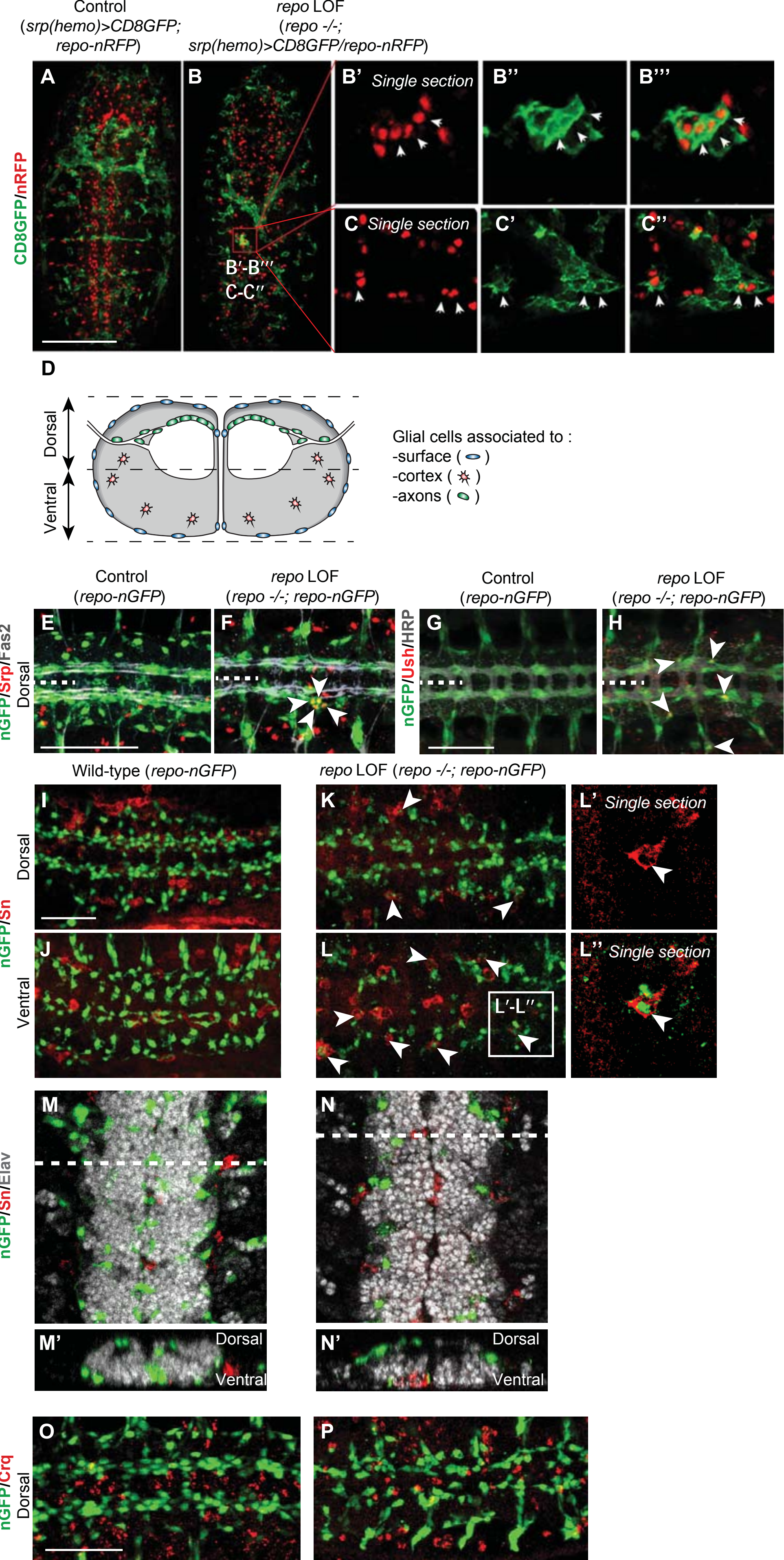


Figure 7

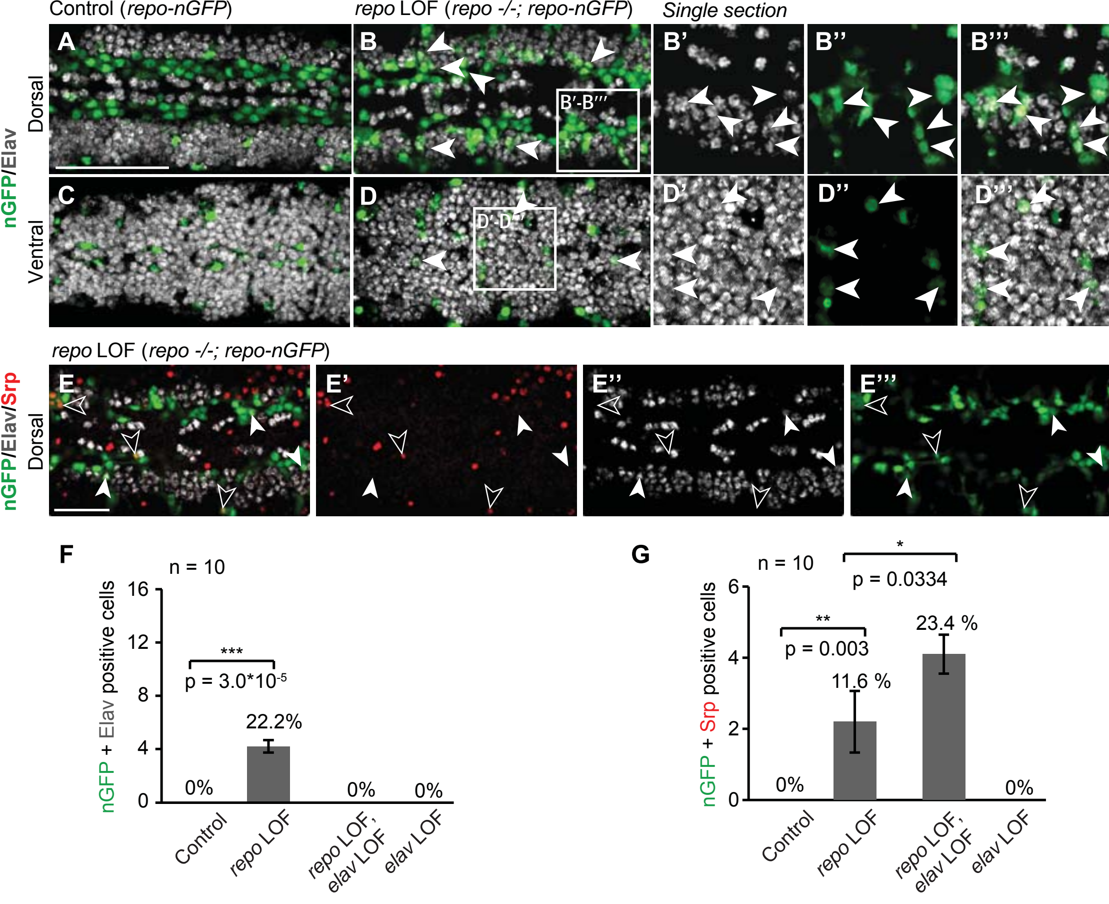


Figure 8

Control (*repo-nGFP*)

repo LOF (*repo -/-; repo-nGFP*)

nGFP/CM1

A B B' B'' B''' B'-B'''

Control (*repo-CD8GFP*)

repo LOF (*repo -/-; repo-CD8GFP*)

CD8GFP/CM1

C C' C'' C''' C'-C''' D

E

	CM1	GFP	GFP/CM1
Apototic cell *repo-nGFP*			
Phagocytic cell *repo-CD8GFP*			