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1	The Repo homeodomain transcription factor suppresses
2	hematopoiesis in Drosophila and preserves the glial fate
3	
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23 Abstract

Despite their different origins, Drosophila glia and hemocytes are related cell populations 24 25 that provide an immune function. Drosophila hemocytes patrol the body cavity and act as 26 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 27 28 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 29 30 hemocytes require the same transcription factor Glide/Gcm for their development. This raises the 31 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 32 33 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 34 in the hemocyte anlagen by mesoderm-specific factors. Second, Repo ectopic activation in the 35 36 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 37 tissue-specific cues biases the potential of Glide/Gcm. These data allow us to revise the concept of 38 39 fate determinants and help us understanding the bases of cell specification.

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42 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.

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54 Introduction

In the *Drosophila* embryo, lateral glial cells (called glia throughout the text, for the sake of 55 56 simplicity) constitute the second major population of the nervous system and are necessary for 57 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 58 guidance or neural proliferation (Trébuchet and Giangrande, 2012). During development and upon 59 injury, Drosophila glia also act as scavenger cells and help reshaping the nervous system. Thus, 60 Drosophila glia behave like microglia, vertebrate immune cells of mesodermal origin that move 61 62 from the volk 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 63 a key role in cellular and humoral immunity. They can move very fast to patrol the organism and 64 65 respond to a variety of challenges. The most represented subtype of hemocytes, called plasmatocytes, phagocyte microbes and sculpt tissues by clearing apoptotic cells during 66 development (Meister and Lagueux, 2003). 67

In addition to sharing the immune function, glia and hemocytes express the same 68 69 transcription factor, the atypical zinc finger protein Glial cells deficient/Glial cells missing 70 (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 71 72 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 73 (glia) and from the procephalic mesoderm or PM (hemocytes). In the nervous system, Gcm induces 74 75 the expression of the Reverse polarity (Repo) homeodomain containing transcription factor in all 76 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 77 the scavenger receptor Draper (Shklyar et al., 2014). As a consequence, repo mutant glial cells are 78 not functional and have defective phagocytic activity (Shklyar et al., 2014). 79

80 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 81 that mesodermal cues contribute to prevent glial differentiation in the hemocyte anlagen. The 82 mesodermal transcription factor Twist induces the expression of miR-1, which in turn represses the 83 expression of Repo. As a consequence, the gliogenic potential of Gcm is inhibited in the PM (Xiong 84 et al., 1994; Halter et al., 1995; Yuasa et al., 2003), showing that the potential of a fate determinant 85 relies on the cell-specific transcriptional landscape. The negative regulation of Repo in the 86 hemocyte anlagen is crucial as Repo represses the hemocyte fate: when expressed in the hemocyte 87 anlagen, it inhibits the expression of hemocyte-specific genes and the lack of Repo induces the 88 expression of early hemocyte markers in the nervous system. Thus, Repo constitutes a major 89 element in the pathway that discriminates between related but distinct scavenger fates. 90

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

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94

95 **Results**

96 The mesoderm-specific transcription factor Twist represses the expression of the Repo pan 97 glial protein

The Gcm transcription factor is expressed in the glial as well as in the hemocyte lineages, 98 where it controls the expression of glial and hemocyte genes, respectively (Jones et al., 1995; 99 Bernardoni et al., 1997; Bernardoni et al., 1998; Lebestky et al., 2000; Alfonso and Jones, 2002; 100 Cattenoz et al., 2016). Since glia differentiate from the ectoderm and hemocytes from the PM, we 101 102 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 103 determinant (Baylies and Bate, 1996), we therefore asked whether it represses the expression of 104 Repo, the most characterized glial-specific target of Gcm. Repo also represents the only 105 transcription factor expressed exclusively in glia and in all glia (Halter et al., 1995). 106

107 To show that Twi inhibits Repo expression *in vivo*, we analyzed embryos in which we 108 induced Twist expression ectopically (<u>Gain Of F</u>unction or GOF), in the neural territory, as well 109 as embryos that lack Twi expression (<u>Loss Of F</u>unction 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**,

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

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

The fact that Gcm over-expression induces Repo expression in the PM could mean that glial 140 differentiation simply requires higher Gcm levels than hematopoiesis. If that were the case, 141 142 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 143 located at the gcm locus and results in low Gcm levels (Vincent et al., 1996). Neither gcm^{34} 144 homozygous nor $gcm^{34}/Df(2L)132$ transheterozygous animals (the Df(2L)132 deficiency 145 completely deletes the gene (Kammerer and Giangrande, 2001)) show Srp ectopic expression in 146 147 the nervous system (Figure 1J-L). This excludes mere dosage dependency for the establishment of the glial vs. the blood cell fate and further supports the idea that tissue-specific factors are 148 149 responsible for it.

In sum, the Twist mesodermal factor negatively affects the expression of the pan-glialtranscription factor Repo.

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153 The micro RNA *miR-1* inhibits Repo expression post-transcriptionally

The microRNA *miR-1* is a direct target of Twi expressed and required in the mesoderm 154 (Biemar et al., 2005; Sokol and Ambros, 2005). We found that *miR-1* has two putative target sites 155 in the repo 3'UTR (miRanda: http://www.microrna.org/microrna/home.do) (Figure 2A) and 156 therefore explored the possibility that it acts post-transcriptionally on Repo. First, we found that 157 158 animals lacking *miR-1* display ectopic Repo expression in the PM, similar to the *twi* embryos (Figure 2B-D). Second, we asked whether *miR-1* directly acts on the *repo 3'UTR* by co-159 transfecting S2 Drosophila cells with a miR-1 expression vector and a luciferase reporter carrying 160 161 either the repo 3'UTR or its own 3'UTR (Figure 2E). By measuring the luciferase activity, we found that *miR-1* specifically acts on the *repo 3'UTR* to repress *repo* expression (Figure 2E-F).
Third, this negative control is abolished upon mutagenizing the two putative *miR-1* target sites
(Figure 2F). Thus, *miR-1* inhibits Repo expression post-transcriptionally.

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

167

168 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 169 is alternative to hemocyte differentiation. We therefore analyzed the effects of Repo ectopic 170 expression in the PM upon using the UAS-repo transgene (Yuasa et al., 2003). gcm(hemo)>repo 171 (or repo GOF) hemocytes are severely affected: many of them aggregate and show altered 172 morphology as well as migratory defects (Figure 3E,F). Moreover, they no longer express the late 173 174 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 3A",A",B",B"). The hemocytes express Srp at low levels. To quantify this phenotype, we 176 measured the intensity of Srp labeling and found a significant difference between control and repo 177 GOF hemocytes (control: 83.4 +/-3.9 arbitrary unit (AU, see materials and methods), repo GOF: 178 12.4 +/-1.5 AU; n=50 cells in 3 embryos, ANOVA p=6.10⁻²³). Of note, Srp is also expressed in the 179 fat body and yet such expression remains unchanged in *repo* GOF animals (Hoshizaki et al., 1994) 180 (Figure 3A',B'), showing that the hemocyte defects are specific and cell autonomous. 181

A more direct evidence for the specific effects of Repo on the *srp* gene was obtained by using a Gal4 plasmid that carries a fragment of the *srp* promoter specific to hemocytes and called *srp(hemo)*> (Bruckner et al., 2004). Co-transfecting S2 *Drosophila* cells with a Repo expression vector and the *srp(hemo)*>*GFP* plasmid severely reduces the expression of the GFP, and this is a

dosage dependent effect (**Figure 3I**). Moreover, srp(hemo) > repo embryos display similar features than gcm(hemo) > repo embryos, with reduced number of hemocytes (**Figure 3J-K''**). Indeed, we found 252.8 +/-27.4 hemocytes in control and 136.8 +/-19.8 in *repo* GOF embryo (n=7 embryos, ANOVA p=0.0028, counted on 30µm stacks of confocal images taken from stage 13 embryos (lateral views)). 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) (**Figure 3E-F'',J-K''**).

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

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

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

over-expression induces the expression of glial genes without blocking hemocyte differentiation. 210 211 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 hematopoiesis is blocked at its early stages. The hemocyte-specific scavenger receptor Croquemort 213 214 (Crq) (Franc et al., 1996; Franc et al., 1999) co-localizes with the pan-glial maker Repo (Figure **4F-F**^{'''}), indicating a mixed glial and hemocyte phenotype. This finding is in accord with the 215 expression/requirement of Gcm in both hemocytes and glia. Of note, we never observed Repo 216 217 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 218 is expressed transiently and early in the hemocyte lineages, we confirmed these data by using 219 220 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

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. By analyzing the role of Repo first in ectopic glial cells and then in endogenous glia, we found that this transcription factor represses the expression of hemocyte markers.

First we found that Gcm expression throughout the neurogenic region (sca>gcm) triggers ectopic gliogenesis, whereas the same experiment in *repo* null embryos (*repo* loss-of-function, *repo* LOF) triggers ectopic Srp expression within the nervous system. (**Figure 5A-B'''**). To identify the cells expressing Srp ectopically, we needed a lineage marker that traces the glial cells 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 234 235 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 +/-0.8 236 cells/hemisegment show Srp/GFP co-localisation in *sca>gcm;repo* LOF.*repo-nGFP* embryos as 237 238 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*-239 GFP positive cells phagocyte Srp positive cells (Jones, 2005; Laneve et al., 2012) or that the lack 240 241 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 242 show Ush/GFP co-localisation in *sca>gcm;repo* LOF.*repo-nGFP* embryos as compared to 2.8+/-243 0.7 cells in *sca>gcm;repo-nGFP* embryos (n=10 hemisegments in 3 embryos, ANOVA p= 6.10^{-4}). 244 Within the neural tissue, we also found Srp or Ush positive cells that are GFP negative (empty 245 arrowheads in Figure 5B'-B''',D'-D'''). These cells likely represent hemocytes that have moved 246 247 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 248 249 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 250 tracer) in the *repo* LOF embryos (Figure 6A-C"). This does not occur in control animals and is in 251 agreement with the finding that Repo represses the expression of the *srp(hemo)* promoter in S2 252 cells (Figure 3I). Because the GFP of the *srp(hemo)*>GFP line is localized in the membrane and 253 the RFP of the *repo-nRFP* line in the nuclei, we could not formally exclude the possibility that the 254 co-localization indicated the presence of hemocytes within the mutant nervous system and 255 engulfing the presumptive glia. We hence used the anti-Srp antibody and again found expression 256 257 of the hemocyte marker in presumptive glial cells (repo LOF, repo-nGFP) (Figure 6E,F). In similar assays, we found nuclear co-localization between Ush labeling and GFP (**Figure 6G,H**). In total, 11,6 % of the presumptive glia (GFP positive cells) express Srp (2.2 +/-0.8 cells per hemisegment are double positive GFP/Srp, n=10 hemisegments in 3 embryos, W p= 0.0105) and 26 % express Ush ectopically (4.9 +/-0.5 cells per hemisegment are double positive GFP/Ush, n=3 hemisegments in 3 embryos, W p= 0.009). This reveals for the first time a hematopoietic potential for *Drosophila* embryonic glial cells.

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

Finally, we asked whether the lack of Repo converts glial cells into mature and functional 271 hemocytes by monitoring the expression of the hemocyte-specific phagocytosis receptor Crq 272 (Franc et al., 1999), but found no ectopic expression of that protein (Figure 60,P), in agreement 273 with the hypothesis that the lack of Repo does not simply reveal a default hemocyte fate. Thus, the 274 lack of the Repo transcription factor triggers the epxression of subsets of hemocyte markers in a 275 276 fraction of presumptive glia. This could mean that Repo is not sufficient to repress a hemocyte fate in all glial cells or that distinct glial subtypes express different hemocyte markers in the repo LOF 277 278 embryos. To discriminate between the two hypotheses, we followed the approach described by Sklvar et al. (Shklvar et al., 2014) and subdivided the ventral nerve cord in two parts along the Z 279 axis: the ventral part mainly contains cortex glial cells, the dorsal part mainly contains axon-280 281 associated glial cells (Ito et al., 1995) (Figure 6D). The presumptive glia ectopically expressing the hemocyte transcription factors Srp or Ush are only located dorsally and they correspond to the axon-associated glia. This was confirmed by using anti-Fas2, which recognizes the three dorsally located longitudinal axonal fascicles of the ventral cord (Santos et al., 2007) (**Figure 6E,F**) or a second neuronal marker, anti-HRP (**Figure 6G,H**). On the other hand, the cells that express Sn are located at the position of the cortex glia and are mostly located ventrally (**Figure 6I-L''**). This phenotype matches the observation that cortex glia are more motile in *repo* mutant embryos (Shklyar et al., 2014).

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

291

292 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* 293 294 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 295 (Yuasa et al., 2003), we explored the possibility that glial cells lacking Repo express neuronal 296 features. We indeed found that a fraction of the presumptive glial cells (22 %) express the pan-297 neuronal marker Elav (Yao and White, 1991; Berger et al., 2007) in repo LOF; repo-nGFP 298 embryos (Figure 7A-D"",F). These cells are scattered throughout the ventral nerve cord (Figure 299 7B,D) and do not co-express the hemocyte markers Srp (Figure 7E-E''') or Ush (data not shown). 300 We hence ypothesized that the neuronal and the hemocyte transcriptional programs may 301 302 compete with each other and asked whether hemocyte markers are ectopically expressed in the ventral cord of embryos lacking Elav, a key factor for neuronal differentiation. No mutant 303 phenotype was observed in these embryos (last column in Figure 7F,G). Interestingly, however, 304 305 *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 as compared to those observed
in *repo* LOF embryos (23 % vs. 11% (**3rd and 2nd columns, respectively, in Figure 7F,G**). Thus,
the glial factor Repo contributes to repress the neuronal as well as the hemocyte fates and the
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, we also scored the total number of presumptive glia and assessed their proliferative and cell death 311 profile in *repo* LOF embryos. The number of nuclei expressing the GFP in *repo* LOF; *repo-nGFP* 312 embryos is 30 % lower compared to that observed in wild-type animals (345.8+/-6.9 per embryo 313 in WT compared to 196.0+/-35.8 in repo LOF, n=3 embryos, ANOVA p=0.0383). This is in 314 agreement with a slight reduction in cell division and a slight increase in apoptosis: anti-PH3 (Juan 315 316 et al., 1998) was used to score for glial cell division: 4.9+/-1.0 dividing cells are present per 6 hemisegments in WT embryos compared to 0.3+/-0.3 in repo LOF (n=3 embryos, W p=0.0361). 317 318 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 embryos, 6 hemisegments were counted per embryo, W p=0.0318). It is therefore likely that some 320 321 cells missing the Repo protein no longer acquire/maintain the right identity and eventually die. To make sure that the co-localization between the presumptive glia (nuclear GFP) and the death maker 322 CM1 identifies dying cells (Figure 8A-B''',E), rather than glial cells that are phagocytosing dead 323 bodies, we compared the results obtained on *repo LOF*; *repo-nGFP* with those obtained on *repo*; 324 *repo-CD8GFP* embryos, in which the GFP is tagged to the membrane (Figure 8E). 325

As expected, in the latter case we did not observe co-localization between the GFP and CM1 (**Figure 8C-C'''**). Moreover, this data further confirmed the lack of phagocytosis observed in *repo LOF* embryos (**Figure 8D**), likely due to defective SIMU and Draper expression (Shklyar et al., 2014). Indeed, while in wild-type embryos glial cell membranes completely enwrap apoptotic
bodies (Figure 8C-C'''), in *repo* LOF embryos these contacts are no longer established.

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

333

334

335 Discussion

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

346

347 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, hemocytes from the mesoderm, and therefore display distinct molecular landscapes. We here show that the mesoderm-specific transcription factor Twi and its target *miR*-1 repress the expression of the panglial gene Repo in the hemocyte anlagen. Thus, the mesodermal molecular landscape controls Gcm activity and biases its transcriptional output towards hemocyte differentiation.

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

363

364 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 nonglial markers in those embryos shows that Repo also controls cell plasticity. This shows 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.

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

mesoderm also repress Repo expression (data not shown). These inhibitory interactions ensure
that the glial and the hemocyte fates are mutually exclusive.

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.

385

386 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 387 (surface, cortex and axon-associated) based on their morphology, position and function (Ito et al., 388 1995; Beckervordersandforth et al., 2008). The large and flattened glial cells associated to the 389 surface form the BBB (Auld et al., 1995). Glial cells located in the cortex are star-shaped and 390 intermingled with neuronal bodies, their cytoplasmic projections contacting multiple synapses 391 (Freeman and Doherty, 2006; Freeman, 2015). Cortex glia help clearing the debris induced by 392 neuronal programmed cell death (Freeman et al., 2003; Shklyar et al., 2013; Shklyar et al., 2014) 393 (Kurant et al., 2008). Finally, glial cells associated to the axons enwrap them in a multi-layer sheath 394 395 promoting the conduction of nerve impulses and a subset of them has also been called astrocytelike glia (Hidalgo and Booth, 2000; Sepp et al., 2000; Sepp and Auld, 2003; Freeman and Doherty, 396 2006; Freeman, 2015). These glia are known to act as scavengers in response to developmental 397 398 signals and to trauma, likely due to their proximity to signaling axons. Typically, in the adult brain

they phagocyte 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; Boulanger and Dura, 2014;
Hakim et al., 2014).

Repo is expressed in the three cell types and its lack affects them all (Giesen et al., 1997; 403 Yuasa et al., 2003; Kerr et al., 2014), however the *repo* mutant phenotypes reveal the underlying 404 diversity of the glial subtypes as, in the absence of Repo, axon-associated glia express early 405 406 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) 407 and Kurant and collaborators (Shklyar et al., 2014) observed that *repo* mutant cortex glia are very 408 motile. In the future, it will be interesting to determine the transcriptional landscape of the different 409 glial subtypes as, for example, cortex glia may be specialized in removing dead cell bodies whereas 410 411 axon-associated glia may specifically target and remove axons and dendrites.

Finally, our data strongly suggest that, 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).

417

418 Of flies and vertebrates...

Drosophila and vertebrate glial cells share numerous functions 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 422 423 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; Buzanska et al., 2001; Iwasaki et al., 2003; Soustelle et al., 2007), they are neither expressed nor 425 426 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 427 sequences for Repo (no orthologs found so far), the only fly transcription factor that is specific to 428 all lateral glia and only to glia, a molecular signature that seems shared throughout the Arthropod 429 clade (Wakamatsu, 2004; Boyan et al., 2011; Mysore et al., 2011; Nasu and Hara, 2012). 430

431 Our findings raise the question of the evolutionary link between vertebrate and Drosophila gliogenesis (Hartline, 2011). While the hypothesis of an independent origin of vertebrate and 432 invertebrate glia remains to be tested, the comparative analysis of those glia has tremendously 433 434 improved our understanding of the bases of nervous system regeneration. Drosophila glia indeed constitute an excellent model to investigate the mechanisms governing CNS repair following 435 traumatic injury (Leyssen and Hassan, 2007; Kato et al., 2011). In this contest, and in light of recent 436 437 data showing that mature astrocytes and oligodendrocytes can be reprogrammed into functional neurons to promote CNS regeneration (Heinrich et al., 2010; Guo et al., 2014; Su et al., 2014), it 438 will be interesting to study whether the loss of Repo triggers glial cell conversion into neurons in 439 the adult Drosophila injured CNS. 440

Finally, sequencing the genome and analyzing the single cell transcriptome of simple organisms has become an important tool to understand the molecular and cellular bases of evolution. Future analyses will establish when Gcm and Repo appear in evolution and where are they expressed/required within/outside the nervous system.

446 Materials and Methods

447 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. *repo⁵², repo⁸⁴* (Xiong et al., 1994; Halter et al., 1995), *twi¹* (Castanon et al., 2001) and *elav⁴* (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
(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).

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467

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

The secondary antibodies were FITC-, Cy3 or Cy5 conjugated (1/400, Jackson). Images were taken with the SP2 or the SP5 Leica confocal microscopes 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 was used to estimate the intensity of the signal (in Arbitrary Unit, AU) from 50 hemocytes in at least 3 embryos (Schindelin 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 10⁶ cells were

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

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

513

514 RNA extraction, reverse transcription and qPCR

Total RNA was purified from stage 5-11 embryos by TriReagent (MRC). 1 μg of purified
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
- 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'
- *actin5C* reverse : 5' CTTAGCTCAGCCTCGCCACT 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.

527

528 Statistics

All the experiments were performed in at least three biological replicates. Statistical relevance was assigned by calculating means, standard errors. Whenever the data showed normal distribution (**Figure 1H,I, 3I**), they were analyzed by the ANOVA test, whenever they did not (**Figure 7F,G**) by Kruskal-Wallis (KW) and Wilcoxon (W) tests. * = p < 0.05; ** = p < 0.01; ***= p < 0.001.

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536 Author contribution

GT and AG designed the experiments. GT, PC and DM did the experiments. GT, PC, JZ,
DM, MF and AG analyzed the data. GT, PC and AG finalized the manuscript.

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- 749

750 *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.

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- 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, **B**), twi > gcm (gcm GOF, **C**) and twi > gcm; twi-/+ (gcm GOF, twist het, **D**) immunolabeled for the 760 glial marker Repo (blue). Ventral view. Unless otherwise specified, all scale bars represent 100 µm 761 762 and anterior is to the left. (E-F") Confocal projections of wild-type (E) and twi-/- (twi LOF, F) embryos labeled for the Repo glial marker (blue) and for the hemocyte Srp marker (red). Lateral 763 view. (F') and (F'') represent a single section of the inset indicated in (F), they show Srp labeling 764 765 only and co-labeling with Repo, respectively. The white arrowheads indicate cells expressing Srp and Repo. (G) Confocal projections of gcmGal4, repoGal80/+; UAS-CD8GFP (gcm(hemo)>GFP) 766 embryos labeled for Repo (blue) and GFP (green). Lateral view (upper panel) and ventral view 767 768 (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, *twi* GOF) in (**I**). *gcm* levels are relative to *actin* levels, n indicates the number of independent assays, see the Experimental Procedure section for the statistic tests. (**J-L**) Confocal projections of embryonic ventral cords of the following genotypes: $gcm^{34}/+$ (**J**), gcm^{34}/gcm^{34} (**K**) and $gcm^{34}/Df(2L)132$ (**L**). 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.

780

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

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

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

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).

821

Figure 4: Gcm has a strong gliogenic potential in hemocyte precursors.

823 (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 824 GFP (green) and Repo (blue), lateral view, stage 14. (B",B"") represent single confocal sections 825 of the inset indicated in (**B**'), the arrowheads indicate cells double positive for CD8GFP and Repo. 826 (C-D') represent confocal projections of embryos labeled for GFP (green) and Nazgul (red), lateral 827 828 view, stage 16. Brackets indicate territories exhibiting hemocytes. Note that the vellow color observed in (C', oval) is an artifact created by the projection. (D'',D''') represent single confocal 829 sections of the inset indicated in (D'), arrowheads indicate ectopic glial labeling in hemocytes over-830 831 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''') 832 and Crq (red), Repo (blue) and GFP (F-F"). Hemocytes are indicated by asterisks, those that also 833 express Repo by arrowheads. Note that Repo ectopic expression does not affect Srp or Crq 834 expression. Scale bars in (E,F): 50 µm. 835

836

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

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 is not 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.

(A,B) Confocal projections of embryos srp(hemo)>CD8GFP/repo-nRFP (Control, A) and 849 srp(hemo)>CD8GFP/repo-nRFP;repo-/- (repo LOF, **B**) labeled for GFP (green) and RFP (red), 850 ventral view. (B'-B''', C-C'') show single sections of the insets indicated in (B). Note that the 851 852 single sections were acquired at different focal planes in the VNC. The arrows indicate GFP/RFP double positive cells. (D) Schematic representation of a transversal section of the VNC from a 853 mature embryo. Glial cell subtypes are defined according to their localization: surface pale (blue), 854 855 cortex (red) and axon-associated glia (green) (Ito et al., 1995; Beckervordersandforth et al., 2008). (E-P) Embryos of the following genotypes: repo-nGFP (Control) and repo-/-; repo-nGFP (repo 856 LOF). The analyses were performed upon subdividing the VNC in a ventral and in a dorsal part, 857 858 according to the schematic shown in (**D**), the position of the section along the dorso/ventral axis of the VNC is indicated on the left side of the panels. Scale bar in (E-P): 50 µm. Stage 15 embryos 859 are labeled for GFP (green), Fas2 (gray) and Srp (red) (E,F); GFP (green), HRP (gray) and Ush 860 (red) (G,H). Stage 14 embryos are labeled for GFP (green) and Sn (red) (I-L''), (L',L'') show 861 single sections of the inset indicated in (L). (M,N) Confocal projections of the whole VNC labeled 862 for GFP (green), Sn (red) and Elav (gray), the dash line indicates the position of the z-axis 863 reconstitution of the VNC presented in (M',N'). Note the presence of Sn positive/GFP negative 864 cells within the VNC in repo LOF embryo; these are hemocytes recruited to the VNC following 865 866 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*
- LOF), ventral view, stage 15. The ventral and the dorsal parts of the VNC were analyzed separately.
- Labeling: GFP (green) and Elav (gray). (**B'-B''',D'-D'''**) show single sections of the insets
- indicated in (**B**, **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 bars in (A,E): 50µm. (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 3 embryos.
- 880

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

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

Table 1



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.









Medium gcm GOF (gcm(hemo)>CD8GFP, medium gcm)











Control (repo-CD8GFP)

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





