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Developmental regulation of glial cell phagocytic function during *Drosophila* embryogenesis

Boris Shklyar [1], Yael Sellman [1], Jeny Shklover, Ketty Mishnaevski, Flonia Levy-Adam, Estee Kurant *

Department of Genetics and Developmental Biology, The Rappaport Family Institute for Research in the Medical Sciences, Faculty of Medicine, Technion – Israel Institute of Technology, Haifa 31096, Israel

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ABSTRACT

The proper removal of superfluous neurons through apoptosis and subsequent phagocytosis is essential for normal development of the central nervous system (CNS). During *Drosophila* embryogenesis, a large number of apoptotic neurons are efficiently engulfed and degraded by phagocytic glia. Here we demonstrate that glial proficiency to phagocytose relies on expression of phagocytic receptors for apoptotic cells, SIMU and DRPR. Moreover, we reveal that the phagocytic ability of embryonic glia is established as part of a developmental program responsible for glial cell fate determination and is not triggered by apoptosis per se. Explicitly, we provide evidence for a critical role of the major regulators of glial identity, *gcm* and *repo*, in controlling glial phagocytic function through regulation of SIMU and DRPR specific expression. Taken together, our study uncovers molecular mechanisms essential for establishment of embryonic glia as primary phagocytes during CNS development.

Introduction

Efficient recognition and elimination of apoptotic cells through phagocytosis is crucial for normal development of multicellular organisms. Apoptotic cell clearance is accomplished by two types of phagocytes: 'professional' macrophages and immature dendritic cells and 'non-professional' tissue-resident neighboring cells, whose role is critical during development (Elliott and Ravichandran, 2008; Henson and Hume, 2006; Scheib et al., 2012). Phagocytes must recognize apoptotic particles with high level of specificity in order to specifically remove apoptotic cells but not living normal cells. This very precise recognition is achieved through transmembrane phagocytic receptors or secreted bridging molecules, which recognize 'eat me' signals on apoptotic surfaces (Kinchen and Ravichandran, 2007, 2008a, 2008b; Lauber et al., 2004; Ravichandran, 2011; Ravichandran and Lorenz, 2007; Stuart and Ezekowitz, 2005).

In mammals, a large number of transmembrane receptors and soluble bridging molecules have been shown to play a role in recognition and engulfment of apoptotic particles (Hanayama et al., 2002; Miyanishi et al., 2007; Park et al., 2007, 2008, 2009). Importantly, most of these are exclusively expressed in phagocytic cell populations. However, the molecular mechanisms controlling their specific expression remain elusive. Several phagocytic receptors for apoptotic cells are also known in *Drosophila*. These receptors show highly specific expression in phagocytic cell populations during embryogenesis. For example, the CD36 homolog Croquemort (CRQ) is expressed mostly in professional phagocytes, the macrophages (Franc et al., 1999). Two other receptors, Draper (DRPR) and Six Microns Under (SIMU), are expressed both in macrophages and in glia, the non-professional phagocytes of the central nervous system (CNS) (Freeman et al., 2003; Kurant et al., 2008).

During late embryogenesis a large number of neurons die through apoptosis and embryonic glia function as the main phagocytes in the CNS, which efficiently remove them (Freeman et al., 2003; Kurant et al., 2008). Although different functions of glia have recently received great attention (Allen and Barres, 2005; Barker and Ullian, 2010; Barres, 2008; Chotard and Salecker, 2004; Edenfeld et al., 2005; Farina et al., 2007; Freeman, 2006; Freeman and Doherty, 2006; Halassa and Haydon, 2010; Kurant, 2011; Logan and Freeman, 2007; Pfrieger, 2010; Vilhardt, 2005), mechanisms responsible for preparing glia to be potent phagocytic cells remain poorly understood.

We have previously shown that SIMU, which is required for recognition and engulfment of apoptotic neurons by glial cells, is expressed exclusively during stages of developmental apoptosis in *Drosophila*: mid-to-late embryogenesis, pupae and early adult (Kurant et al., 2008). How this specific expression, which entirely correlates with developmentally programmed cell death, is regulated

* Correspondence to: Department of Genetics and Developmental Biology, The Rappaport Family Institute for Research in the Medical Sciences, Faculty of Medicine, Technion, Efron Street, P.O. Box 9697, Haifa 31096, Israel. Fax: +972 4 8295403.
E-mail address: kurante@tx.technion.ac.il (E. Kurant).
[1] These authors equally contributed to this study.

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remains unclear. In the work presented here, we demonstrate that the apoptotic process itself does not affect expression levels of phagocytic receptors SIMU and DRPR in glia but this specific expression is part of a developmental program responsible for glial cell determination. Specifically, we show that a master regulator of glial cell fate, *glial cells missing* (*gcm*), which is expressed in precursors of macrophages and early developing glia (Bernardoni et al., 1997; Hosoya et al., 1995; Jones et al., 1995), induces the expression of SIMU and DRPR in glia, but not in macrophages. We also demonstrate that a key regulator of lateral glia development, *reversed polarity* (*repo*) (Halter et al., 1995; Xiong et al., 1994), is required for DRPR but not SIMU expression in glia. Moreover, we provide evidence that GCM directly regulates *simu* expression in glia through its specific binding sites, although *drpr* glial expression requires *repo*. Altogether, our study uncovers molecular mechanisms responsible for establishment of glial phagocytic ability during development.

Results

Developmental apoptosis during embryogenesis does not affect expression of the phagocytic receptors SIMU and DRPR

The strong correlation between SIMU expression and developmental apoptosis (Kurant et al., 2008) prompted us to test whether apoptosis itself promotes expression of phagocytic receptors during embryonic development. In order to address this question, we first examined the level of SIMU and DRPR receptors in embryos lacking developmental apoptosis. A specific deletion of a genomic region containing three pro-apoptotic genes *reaper*, *grim* and *hid* (*H99*) completely abrogates caspase activation in the embryo, resulting in lack of developmental apoptosis (White et al., 1994). We monitored caspase activation in the embryonic CNS by staining with an anti-cleaved caspase 3 antibody (CM1), which specifically labels apoptotic particles in wild type embryo (Fig. 1A) and shows no reactivity in *H99* embryos (Fig. 1B). In *H99* mutant embryos we tested protein expression using anti-SIMU (Fig. 1C, D, G, and H) and anti-DRPR (Fig. 1E–H) antibodies and detected SIMU and DRPR proteins on glial membranes similarly to wild type staining which has been described previously (Kurant et al., 2008, Fig. 1G and H), indicating that lack of apoptosis does not affect protein levels of phagocytic receptors. We also did not notice any change in GFP expression of the *simu-cytGFP* reporter, which contains a 2 kb region upstream of *simu* translation start site fused to cytoplasmic GFP, which completely recapitulates SIMU embryonic expression (Kurant et al., 2008, Fig. 1I and J).

Moreover, we measured the levels of *simu* and *drpr* transcripts by qRT-PCR analysis. We detected similar levels of *simu* cDNA in the mutant embryos compared to wild type embryos (Fig. 1K), suggesting that lack of developmental apoptosis does not affect *simu* transcriptional levels. Similarly, comparable levels of *drpr* cDNA were detected in *H99* and wild type embryos (Fig. 1K), demonstrating that *drpr* transcript levels are not affected by lack of apoptosis as well.

To explore, reciprocally, whether elevated apoptosis stimulates expression of phagocytic receptors during embryogenesis, we examined *simu* and *drpr* expression in embryos with high levels of apoptosis. To induce excess apoptosis we used *elavGal4*::*hid* transgenic embryos, which show upregulated neuronal apoptosis. Apoptotic rates were assessed by staining with the CM1 antibody (Fig. 2A and B). When we tested protein expression of SIMU (Fig. 2A′, A″, B′, and B″) and DRPR (Fig. 2E and F) in the *elavGal4*::*hid* embryos we observed an abnormal shape of glial cells labeled with anti-SIMU (Fig. 2B″) and anti-DRPR (Fig. 2F) antibodies. This glial shape is a result of increased glial engulfment of higher amounts of apoptotic particles in the *elavGal4*::*hid* embryos (Fig. 2B) as compared to wild type (Fig. 2A). However, similar levels of SIMU and DRPR proteins were detected (Fig. 2A″, B″, E, and F), suggesting that increased apoptosis does not affect protein levels of phagocytic receptors. Moreover, since *elavGal4* is transiently expressed in embryonic glia (Berger et al., 2007), in order to test whether the transient expression of HID in glia affected glial cell number, we quantified REPO-labeled glial cells in entire *elavGal4*::*hid* embryonic CNS (Fig. 2D) and compared it to wild type controls (Fig. 2C). No significant difference in glial cell number was detected between *elavGal4*::*hid* and control embryos (Fig. 2G), demonstrating no glial death in these embryos.

In addition, qRT-PCR analysis of *elavGal4*::*hid* and control embryos showed that increased apoptosis did not affect the transcriptional levels of *simu* and *drpr* during late embryogenesis (Fig. 2H). Since the expression levels of *simu* and *drpr* are relatively high in normal embryos, it may be difficult to detect an increase in their expression at this developmental stage. We, therefore, induced ectopic apoptosis during larval stages when normally no apoptosis takes place (Fig. 2I). In wild type larvae *simu* expression levels are undetectable at this stage (Kurant et al., 2008, Fig. 2K), whereas *drpr* expression is lower, as compared to embryogenesis (Flybase expression data, Figs. S1 and 2I′).

To bypass HID-induced neuronal apoptosis during embryogenesis, which is lethal, we conditionally expressed *hid* in larval neurons (*elavGal4*::*hid*) using the temperature-sensitive Gal80 repressor (*tubGal80^{ts}*). At 18 °C *tubGal80^{ts}* is expressed in all embryonic tissues and prevents *elavGal4* from induction of *hid* neuronal expression. At 29 °C Gal80 is inactivated and *elavGal4* is derepressed leading to expression of *hid* specifically in larval neurons. We placed the progeny *elavGal4*::*hid*; *tubGal80* at 18 °C until the 2nd instar larvae stage and then shifted them to 29 °C for 24 h. Dissected larval brains of the *tubGal80^{ts}*; *elavGal4*::*hid* third instar larvae were stained with the CM1 antibody to confirm induction of apoptosis (Fig. 2J and J″), and with specific antibodies for SIMU and DRPR in order to evaluate their protein expression levels. No detectable expression of SIMU was found in these brains (Fig. 2L) and no difference in DRPR expression was obtained as compared to wild type (Fig. 2I′, I″, J′, and J″), suggesting that elevated apoptosis did not affect SIMU and DRPR expression in larval brains. Moreover, the progeny *elavGal4*::*hid*; *tubGal80* and control *elavGal4*; *tubGal80* were subjected to qRT-PCR analysis following incubation at 29 °C as described above. We found no increase in *drpr* expression compared to wild type (Fig. 2H) and no detectable *simu* expression in the *elavGal4*::*hid; tubGal80* larvae. These results indicate that induced apoptosis does not influence *simu* and *drpr* expression in larval stages, similarly as during embryogenesis. Altogether, these data suggest that expression of phagocytic receptors (in glia and macrophages) is not affected by levels of apoptosis, raising the possibility that their expression is part of the developmental program responsible for phagocytic cell fate determination.

glial cells missing (gcm) differentially affects expression of SIMU and DRPR in haemocytes and glia

gcm is expressed in precursors of 'professional' macrophages, haemocytes, and early lateral glia (Bernardoni et al., 1997; Hosoya et al., 1995; Jones et al., 1995), making it a promising candidate for regulation of phagocytic ability of two phagocytic cell populations, macrophages and glia. In addition, two GCM putative binding sites have been identified (Genomatix software) within a 2 kb region upstream of *simu* translation start site, which completely recapitulates SIMU embryonic expression (Kurant et al., 2008, Fig. 3A).

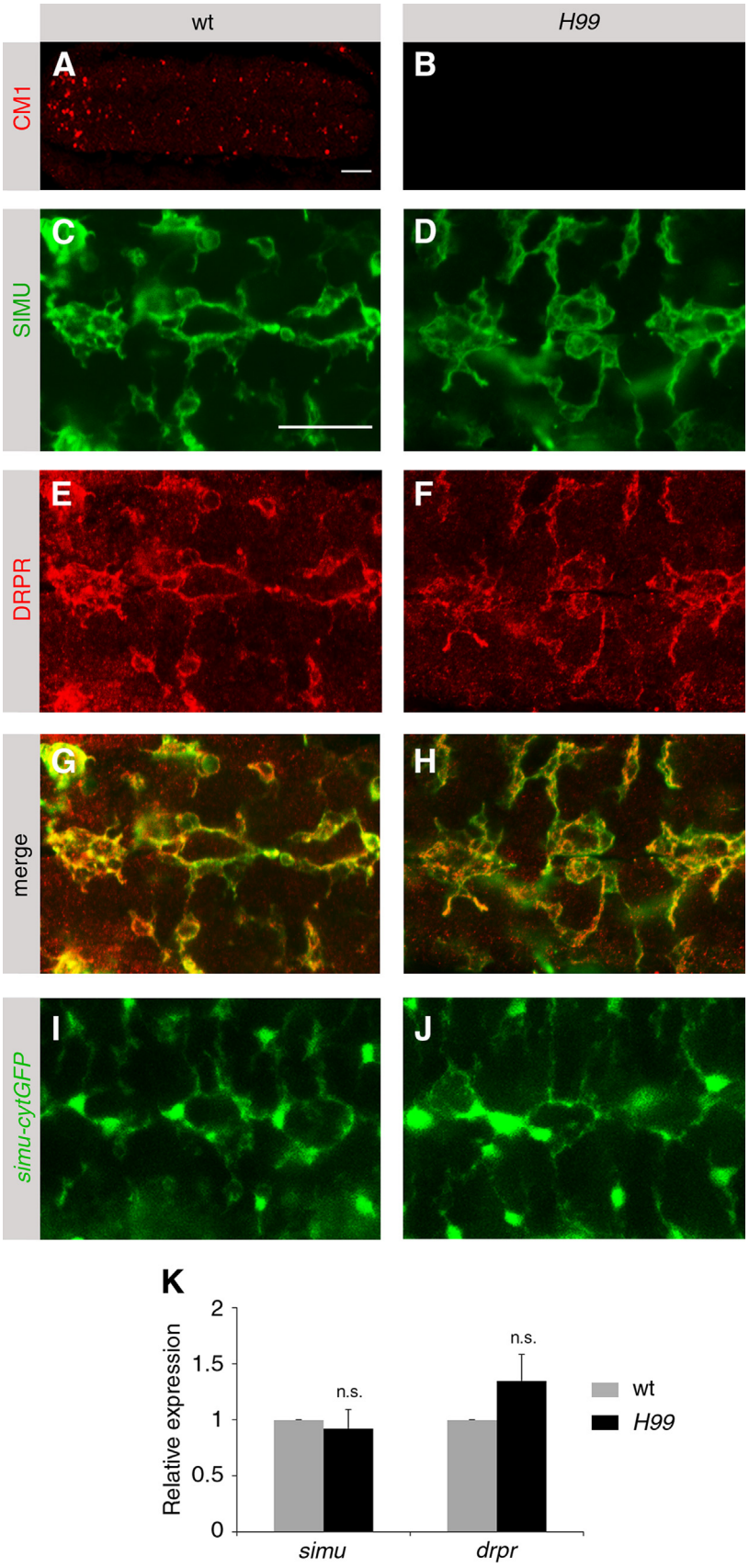

Fig. 1. SIMU and DRPR are expressed on glial membranes in embryos lacking apoptosis. (A–J) Projections from confocal stacks of the CNS at embryonic stage 16, ventral view. (A, B) Apoptotic particles are labeled with CM1 (red). Bar, 20 μm. (A) Wild type; (B) *H99* mutant embryo, in which no CM1 staining is evident. (C, D, G, H) SIMU is localized on glial membranes of wild type embryo (C, G) and *H99* mutant embryo alike (D, H) as detected with anti-SIMU antibody. (E, F, G, H) DRPR is localized on glial membranes of wild type embryo (E, G) and *H99* mutant embryo (F, H) as detected with anti-DRPR antibody. (I, J) *simu-cytGFP* reporter is expressed in wild type embryo (I) and *H99* mutant embryo (J). (K) qRT-PCR for *simu* or *drpr* on wild type and *H99* embryos; bars represent mean ± SEM. No significant changes (n.s., $p > 0.05$) in levels of *simu* or *drpr* expression are detected based on Student's *t*-test statistical analysis.

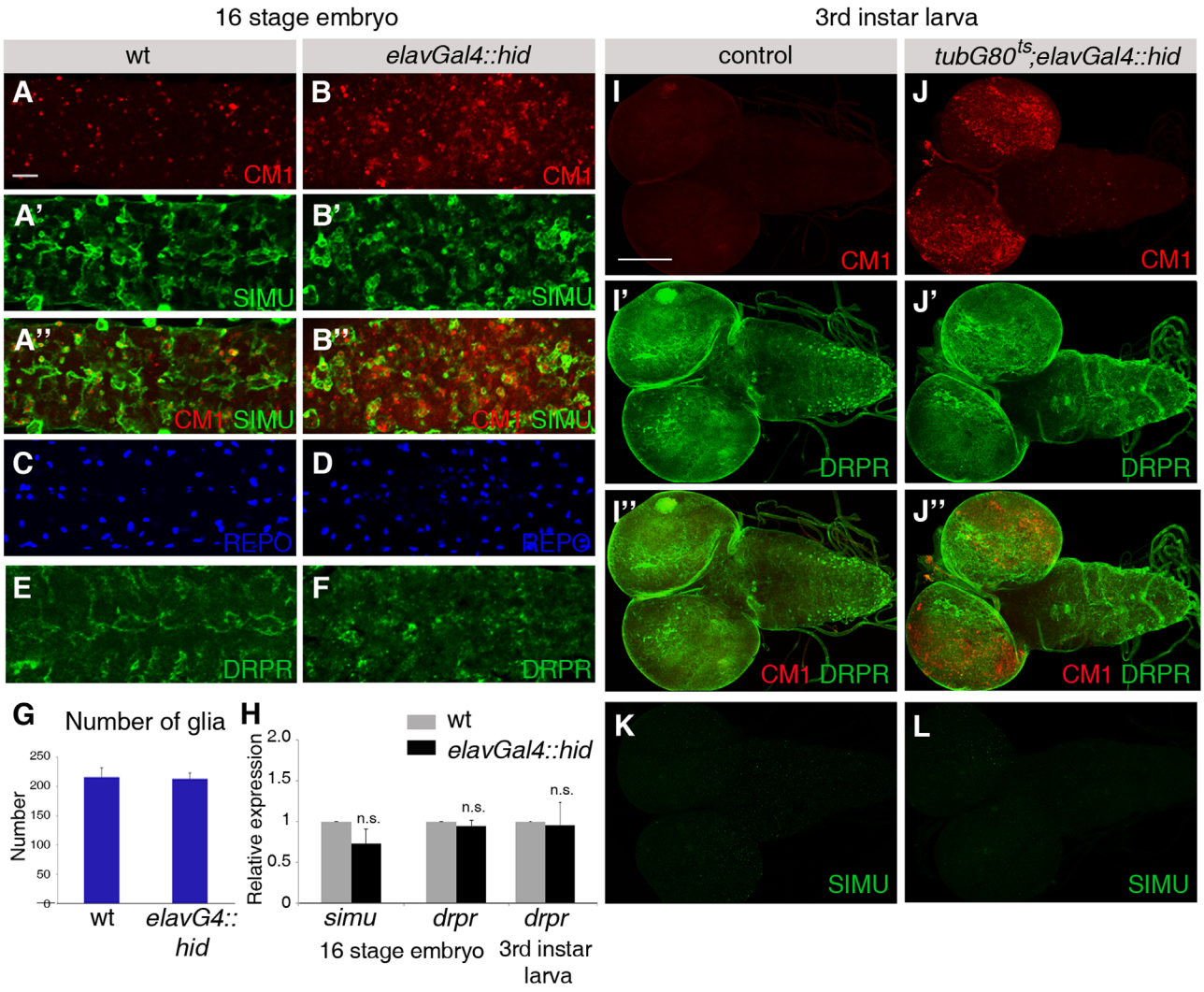

Fig. 2. SIMU and DRPR expression is unaffected in embryos and larvae with elevated apoptosis. (A–F) Projections from confocal stacks of the CNS at embryonic stage 16, ventral view. Apoptotic particles are labeled with CM1 (red). Bar, 20 μm. (A–A″, C, E) wild type; (B–B″, D, F) *elavGal4*::*hid* embryo. (A′, A″, B′, B″) SIMU staining with anti-SIMU in green. (C, D) staining of glial nuclei with anti-REPO in blue. (E, F) DRPR staining with anti-DRPR in green. (G) Quantification of glial cell number in wild type and *elavGal4*::*hid* CNS. Columns represent mean total number of REPO-positive nuclei within confocal stacks of the entire CNS ± SEM, $n=7$. n.s. (not significant) $p>0.05$, as determined by one-way ANOVA. (H) qRT-PCR for *simu* or *drpr* on wild type and *elavGal4*::*hid* embryos and qRT-PCR for *drpr* on wild type and *elavGal4*::*hid* 3rd instar larvae; bars represent mean ± SEM. No significant changes (n.s., $p>0.05$) in levels of *simu* and *drpr* expression are detected based on Student's *t*-test analysis. Levels of *simu* expression in *tubGal80*^{ts}; *elavGal4*::*hid* 3rd instar larvae were under detection limits of the method. (I–L) Projections from confocal stacks of the 3rd instar larval brains. Apoptotic particles are labeled with CM1 (red). Bar, 100 μm. (I–I″, K) wild type; (J–J″, L) *tubGal80*^{ts}; *elavGal4*::*hid* brain shows increased CM1 staining. (I′, I″, J′, J″) anti-DRPR in green. (K, L) anti-SIMU in green.

gcm and its homolog, *gcm2*, which is expressed in a very similar pattern to *gcm* but at lower levels, share regulatory sequences and have partially redundant function (Alfonso and Jones, 2002; Kammerer and Giangrande, 2001). Deletion of both *gcm* and *gcm2* (*Df(2L)Exel7042*) results in the elimination of all lateral glial cells (Alfonso and Jones, 2002), and in about 60% reduction in the number of macrophages (Bernardoni et al., 1997). To test the role of *gcm* in regulation of *simu* and *drpr* expression, we examined embryos lacking the *gcm* function. In the mutant *Df(2L)Exel7042* embryos we did not detect SIMU and DRPR protein expression in presumptive glia using specific antibodies against SIMU (Fig. 3D and D″) and DRPR (Fig. 3F and F″). However, we observed that SIMU and DRPR were still expressed in macrophages labeled with the anti CRQ antibody (Fig. 3B′, B″, C′, C″, D′, D″, E′, E″, F′, and F″). These results indicate that expression of SIMU and DRPR in macrophages does not depend on *gcm*, suggesting that *gcm* affects SIMU and DRPR expression specifically in glia. In addition, we found a very high amount of apoptotic particles in the *gcm* mutant (Fig. 3H′ and H″) compared to wild type (Fig. 3G′ and G″), indicating an increase in cell death and/or reduction in clearance of apoptotic cells.

reversed polarity (repo) is required for glial expression of DRPR but not SIMU

In *gcm* mutant embryos, the undifferentiated glial cells change their fate and turn into neurons (Hosoya et al., 1995; Jones et al., 1995; Mao et al., 2012), leading us to evaluate *simu* and *drpr* expression and glial phagocytic ability in mutants for genes acting downstream to *gcm* specifically in glial cell determination. *repo* is a master regulator of lateral glia, which is expressed exclusively in lateral glia during all stages of fly development (Halter et al., 1995; Xiong et al., 1994). To investigate whether *repo* is involved in regulation of SIMU and DRPR expression, the pattern of these proteins was examined in *repo* mutant embryos (*repo*^{03702}). To specifically label glial cells we used *repoGal4*::*cytGFP* transgenic embryos, where all lateral glial cells express

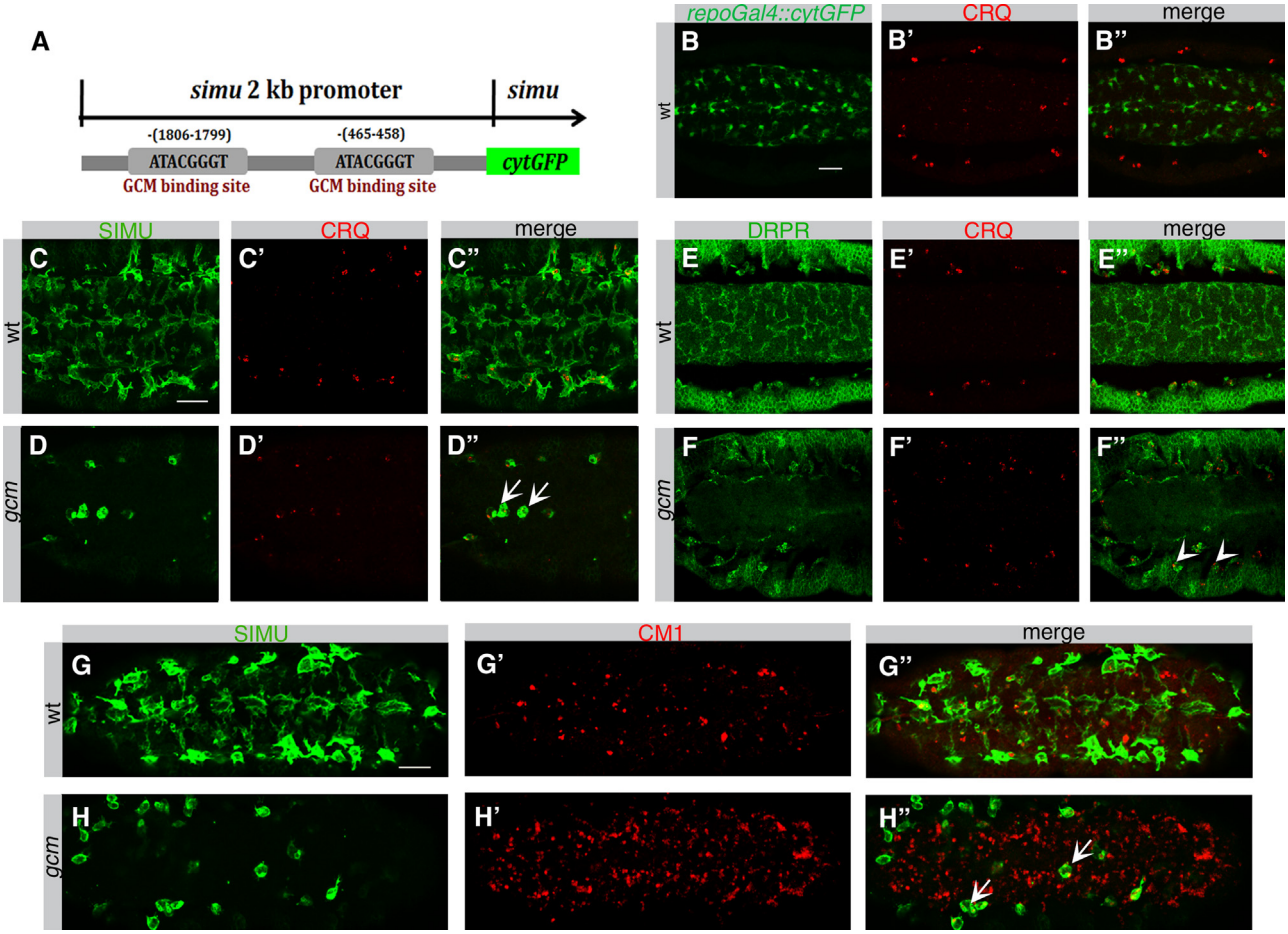

Fig. 3. GCM differentially affects SIMU and DRPR expression in embryonic glia and hemocytes. (A) Schematic illustration of *simu* promoter region with two putative GCM-binding sites depicted. (B–H″) Projections from confocal stacks of the CNS at embryonic stage 16, ventral view. Bar, 20 μm. (B–B″) Wild type embryo. Macrophages are labeled with anti-CRQ (red) and glia are labeled with *repoGal4*::*cytGFP* (green). (C–D″) Wild type and *gcm* mutant embryos are stained with anti-CRQ (red) and anti-SIMU (green). Note that SIMU is present in mutant macrophages (arrows). (E–F″) Wild type and *gcm* mutant embryos are stained with anti-CRQ (red) and anti-DRPR (green). Note that DRPR is present in mutant macrophages (arrowheads). (G–H″) Wild type and *gcm* mutant embryos are stained with CM1 (red) and anti-SIMU (green). Note the increased number and volume of apoptotic particles in the mutant.

cytoplasmic GFP and their shape and behavior are well visualized. In wild type CNS, GFP-positive glia are stereotypically organized and express SIMU and DRPR in high levels on their membranes (Fig. 4A, A″, C, C″, E, and E″). However, in *repo* mutant embryos, the GFP-positive glial cells appear different and their organization is impaired (Fig. 4B, B″, D, D″, F, and F″). When we examined expression of SIMU and DRPR in *repo* mutants we found that SIMU was expressed on certain glial membranes (45 ± 6.2% of mutant glia express SIMU, $n=7$, Fig. 4D′ and D″) whereas DRPR was absent in all glial cells (Fig. 4F′ and F″). Importantly, in the wild type glia, SIMU is not expressed in all lateral glia but only in a subset of glial cells (63 ± 1.5%, $n=7$, Fig. 4C′ and C″), whose number seems to be reduced in the *repo* mutant (see below). In contrast, SIMU and DRPR are normally expressed in macrophages (Fig. 4D′, D″, F′, and F″).

repo is required for the phagocytic function of glia

Given that in *repo* mutant embryos glial cells appear abnormal and do not express DRPR on their membranes, we tested glial phagocytosis of apoptotic cells in these embryos. In order to evaluate if there are more apoptotic particles in the CNS, which would result from an impaired phagocytosis, we measured the volume of apoptotic particles labeled with the CM1 antibody. We observed a much higher volume of CM1-positive apoptotic particles in the *repo* mutant CNS compared to wild type (Fig. 5A′, B′, and D). Moreover, we found that 33 ± 6.6% of the apoptotic particles in *repo* mutants are not engulfed by phagocytes ($n=7$), glia or macrophages, compared to 8% in wild type (Kurant et al., 2008). In addition to impaired phagocytosis, the increase in the volume of apoptotic particles may result from elevated cell death. We took two approaches to distinguish between these two possibilities. The first approach was to test whether mutant glia die by apoptosis. We measured the volume of GFP-positive glial cells labeled by *repoGal4*::*cytGFP* in mutant embryos (Fig. 5B and B‴) and compared it to the wild type embryos (Fig. 5A and A‴). We found that the volume of glial cells in mutant embryos is half the normal glial volume (Fig. 5C), suggesting that the amount of glial cells may be reduced.

To quantify the number of glial cells, we marked glial nuclei with *repoGal4*::*nucGFP* (Fig. 5G, G″, H, and H″) and counted the total number of GFP+ nuclei. The same number of GFP-positive nuclei in *repo* mutants and wild type embryos were detected (Fig. 5H‴), indicating that the same number of glial cells are formed in the *repo* mutant as in the wild type. Furthermore, we performed TUNEL analysis to test whether a portion of these nuclei were apoptotic (Fig. 5H and H″). We found minimal colocalization of nuclear GFP and TUNEL labeling in *repo* mutants (4.3 ± 0.6 nuclei), which is non-significant from wild type (3 ± 0.4, $P > 0.05$ as determined by one-way ANOVA), suggesting that most glia do not die. Therefore, we propose that the 50 percent reduction in glial volume is a consequence of abnormal differentiation of certain glial cells resulting in their smaller volume (Fig. 5B) and leading to the decreased overall volume of glial cells.

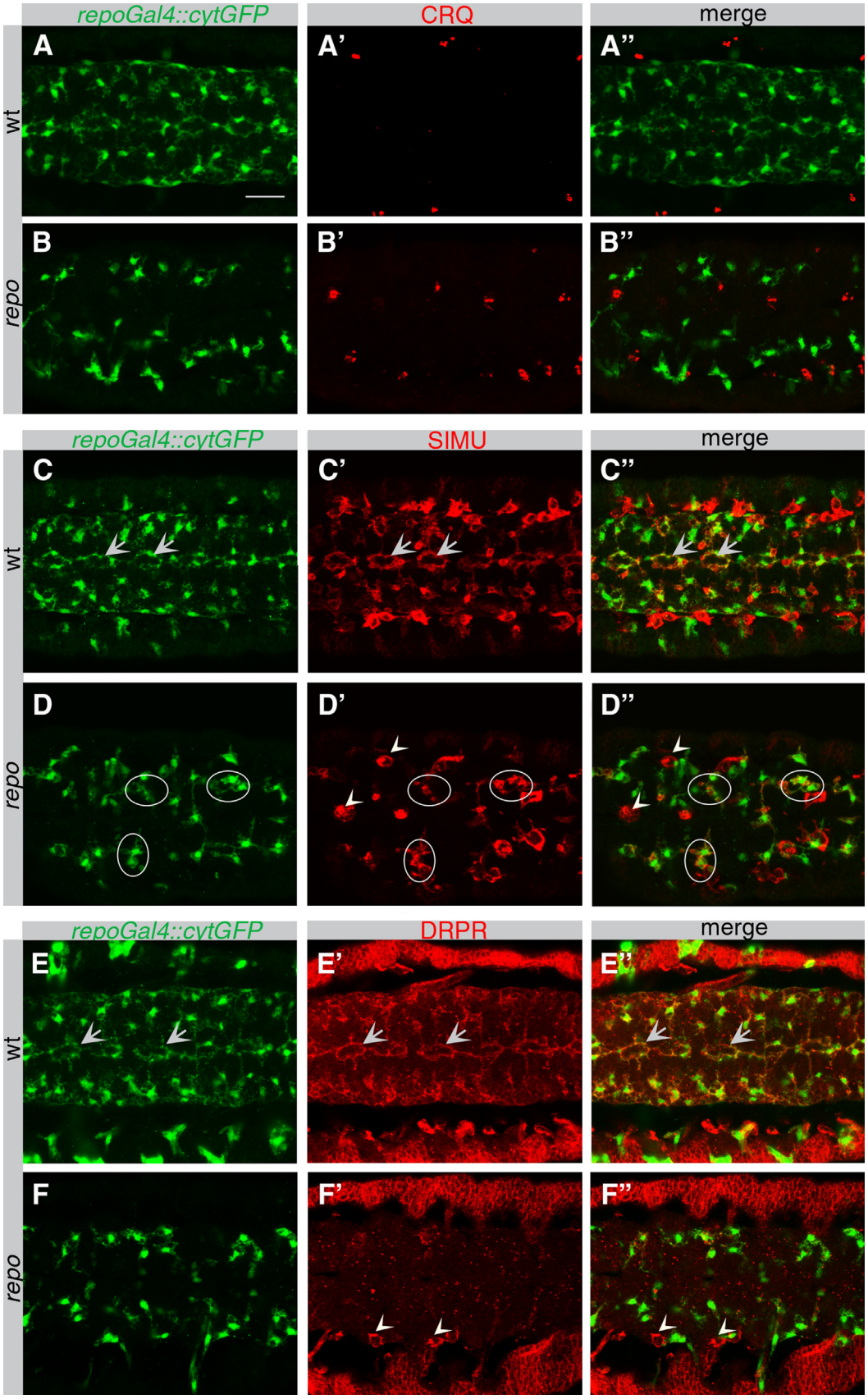

Fig. 4. REPO is required for DRPR expression, but is dispensable for SIMU expression in embryonic glia. Projections from confocal stacks of the CNS at embryonic stage 16, ventral view. Glia are labeled with *repoGal4*::*cytGFP* (green). Bar, 20 μm. (A–A″) Wild type and (B–B″) *repo* mutant embryos are stained with anti-CRQ (red). (C–C″) Wild type and (D–D″) *repo* mutant embryos are stained with anti-SIMU (red). $63 \pm 1.5\%$ ($n=7$) of glial cells express SIMU on their membranes in wild type embryo (C′–C″, arrows) whereas $45 \pm 6.2\%$ ($n=7$) of glial cells express SIMU on their membranes in the mutant (D–D″, ellipses); this is a significant difference with p value < 0.001, as determined by one-way ANOVA. SIMU is normally expressed in macrophages (D″, arrowheads). (E–E″) Wild type and (F–F″) *repo* mutant embryos are stained with anti-DRPR (red). Many glial cells express DRPR on their membranes in wild type embryo (E″, arrows) but no glial cells express DRPR in *repo* mutant embryo (F″). DRPR is normally expressed in macrophages of the mutant (F″, arrowheads).

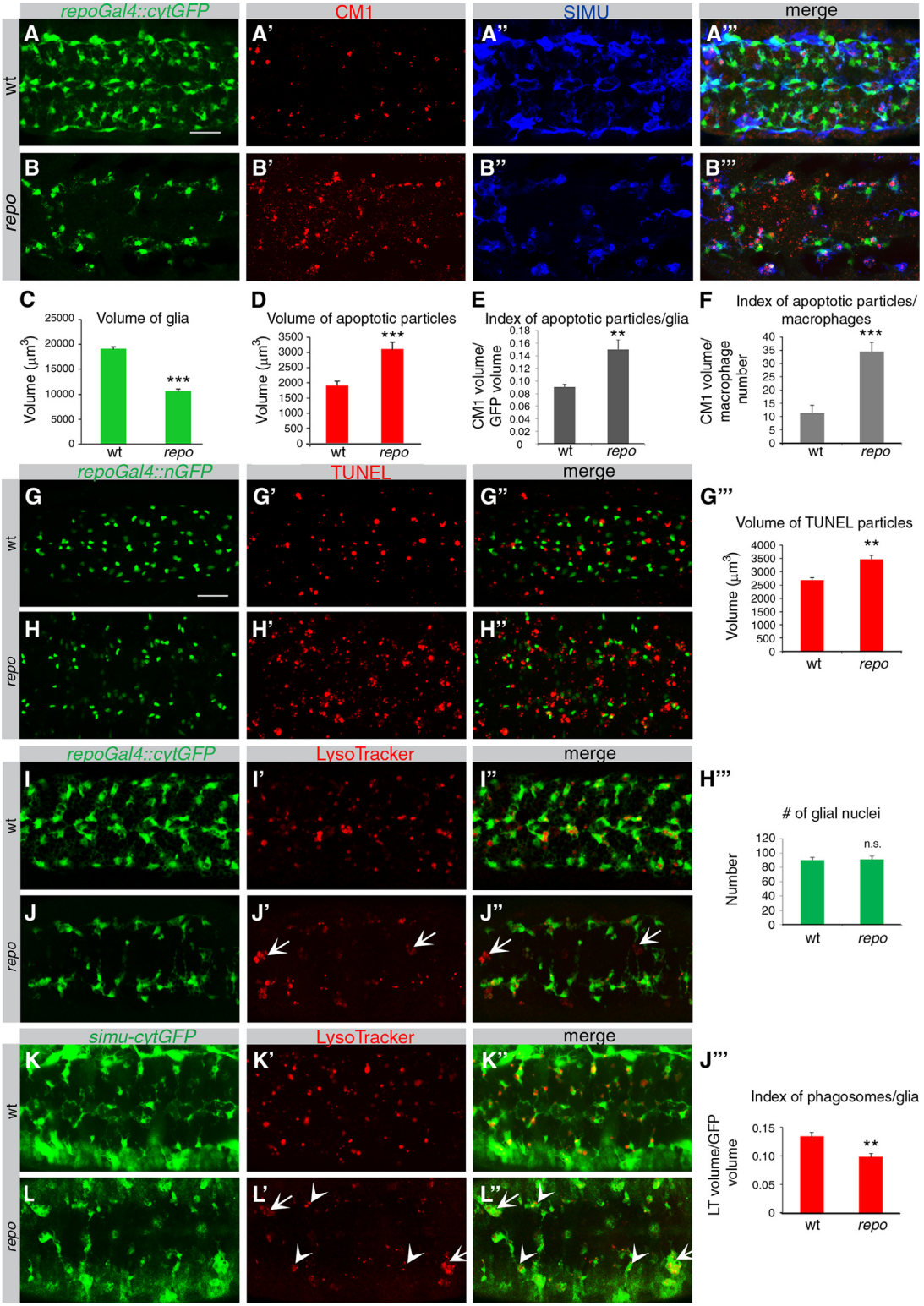

Fig. 5. REPO is required for differentiation of embryonic glia and their phagocytic function. (A–B‴, G–G″, H–H″, I–J″, K–L″) Projections from confocal stacks of the CNS at embryonic stage 16, ventral view. Bar, 20 μm. (A–B‴) Glia are labeled with *repoGal4*::*cytGFP* (green). (A–A″) Wild type and (B–B‴) *repo* mutant embryos are stained with CM1 (red) and anti-SIMU (blue). (C) Quantification of glial volume. Columns represent mean total volume of GFP-positive cells within confocal stacks of the CNS, ± SEM, $n=7$. (D) Quantification of phagocytosis phenotype. Columns represent mean total volume of apoptotic particles within confocal stacks of the CNS, ± SEM, $n=7$. (E) Index of apoptotic particles per glia (volume/volume). Columns represent ratio ± SEM, $n=7$. (F) Index of apoptotic particles per macrophages (volume/number). Columns represent ratio ± SEM, $n=7$. (G–H″) Glial nuclei are labeled with *repoGal4*::*nucGFP* (green) and TUNEL is in red. (G–G″) Wild type and (H–H″) *repo* mutant embryos. (G‴) Columns represent mean total volume of TUNEL-positive particles within confocal stacks of the CNS, ± SEM, $n=7$. (H‴) Quantification of glial number. Columns represent mean of GFP-positive nuclei within confocal stacks of the CNS, ± SEM, $n=7$. (I–J″) Live imaging of *repoGal4*::*cytGFP* labeled glia following LT injections. (I–I″) Wild type and (J–J″) *repo* mutant. Arrows indicate large masses of LT in GFP-negative macrophages. (J‴) Ratio of LT positive area and glial volume. Columns represent mean ratio within confocal stacks of the CNS, ± SEM, $n=7$–10. (C–F, G‴, H‴, J‴) Asterisks indicate statistical significance versus wild type, as determined by one-way ANOVA, $^{***}p<0.001$, $^{**}p<0.01$, n.s. (not significant) $p>0.05$. (K–L″) Glia and macrophages are labeled by *simu-cytGFP* marker and phagosomes and lysosomes are marked by LT. (L′, L″) Note phagosomes in macrophages (arrows) and in mutant glia (arrowheads).

Our second approach was to evaluate phagocytosis by glial cells. To do this we used LysoTracker (LT) to label phagosomes (Fig. 5I′, I″, J′, and J″) and calculated the ratio between the volume of LT-positive area and the GFP-positive glia. This ratio was significantly lower in the CNS of *repo* mutants as compared to wild type (Fig. 5J″), demonstrating an impaired phagocytic ability of *repo* mutant glia. In addition, we calculated the ratio between the volume of engulfed CM1 particles and GFP-positive glia. This ratio was significantly higher in the *repo* mutant than in wild type (Fig. 5E), indicating that apoptotic particles are overloaded inside the phagocytic glia, which resembles the *drpr* mutant phenotype (Kurant et al., 2008). Altogether, our data show that the increase in apoptotic cell volume in *repo* mutants likely results from impaired glial phagocytosis.

We also detected large masses of LT in cells which were not labeled with *repoGal4*::*cytGFP* (Fig. 5J′ and J″), presumably macrophages. Indeed, when we used the *simu-cytGFP* marker, which specifically labels macrophages and glia during late embryogenesis (Kurant et al., 2008), we noticed that the large masses of LT were located inside the macrophages (Fig. 5L′ and L″), indicating their higher phagocytic activity in *repo* mutants than in wild type, probably by doing the 'glial' job in clearance of apoptotic cells in the CNS. To evaluate phagocytosis by macrophages, we counted the index of apoptotic particles per macrophage and found that it was significantly higher in *repo* mutants than in wild type (Fig. 5F), suggesting that macrophages perform more phagocytosis in the mutant. In the wild type condition, at late embryonic stages, macrophages are unable to enter the CNS because of a physical barrier formed by surface glia (Kurant et al., 2008, Fig. S4A–C″). Interestingly, in *repo* mutants macrophages are found inside the CNS (Fig. S4D–F″).

repo mutant glia behave in a 'macrophage-like' fashion

To monitor the in vivo dynamics of apoptotic cell clearance by (GFP-positive) glial cells, we used an early marker for apoptotic cells, Annexin V, which labels specifically Phosphatidylserine (PS) on apoptotic cell surfaces (van den Eijnde et al., 1998; Shklyar et al., 2013b). In the wild type embryonic CNS most of apoptotic particles are engulfed by glia and co-localize with Annexin V inside the phagocytes (Fig. 6A–A″ and C). In addition, phagocytic glia are barely motile and constantly send their protrusions to engulf apoptotic particles (Kurant et al., 2008; Shklyar et al., 2013a, movie S2, Fig. 6A–A″ and C). However, *repo* mutant glia are abnormally located inside the CNS and lack appropriate contacts with each other (Fig. 6B, B″, D, and E). Remarkably, some of the mutant glial cells exhibit increased motility resulting in 'macrophage-like' movement and are able to engulf multiple apoptotic particles (Movie S3, Fig. 6D, E, and F–F‴), two 'macrophage-like' behaviors. Importantly, this unusual performance of specific glial cells correlates with expression of SIMU on their surfaces. We found that all glial cells that contain multiple apoptotic particles express SIMU (Fig. 6F–F‴), suggesting that SIMU is a prerequisite for upregulated engulfment.

Supplementary material related to this article can be found online at http://dx.doi.org/10.1016/j.ydbio.2014.07.005.

SIMU and DRPR expression rescues repo phagocytosis phenotype

To investigate whether impaired glial phagocytic ability in the *repo* mutant is a result of decreased expression of the receptors SIMU and DRPR (less or no glial expression, respectively), we performed rescue experiments. We expressed SIMU or DRPR or both proteins specifically in *repo* mutant glial cells using the *repoGal4* driver, which results in strong expression of both receptors on glial membranes (Fig. 7D, D″, E, E″, F and F″). In all cases, the morphology and localization of glia appeared abnormal, similarly to the *repo* mutant glia (Fig. 7B and B″). Nevertheless, when *simu* was expressed in additional glial cells, they were overloaded with apoptotic particles (Fig. 7D″). This phenotype strongly resembles the *drpr* single mutant phenotype, where excess apoptotic particles accumulate inside glia and macrophages due to the abnormal degradation of the particles inside the phagocytes (Kurant et al., 2008). Since in our analysis of phagocytosis phenotype we measure the volume of all apoptotic cells in the CNS (inside and outside glia), the volume of apoptotic cells is barely affected in the *simu* rescue embryos and stays close to the volume in the *repo* mutant (Fig. 7G). However, we detected a significantly higher percentage of apoptotic particles inside phagocytes in *repo* mutant expressing *simu* in additional glial cells (*repoGal4*::*simu, repo*^{03702}) than in *repo* mutant alone (Fig. 7H). These results suggest that *simu* expression in glia rescues engulfment defects. When we similarly expressed *drpr* in additional glial cells, we did not rescue the mutant phenotype (Fig. 7G). Since *drpr* is mostly involved in degradation (Kurant et al., 2008), its expression in the mutant glia cannot overcome defects in engulfment, resulting in excessive apoptotic particles outside glial cells (Fig. 7E″); thus, rescue of phagocytosis is not observed. Strikingly, when we expressed both the SIMU and DRPR receptors specifically in glia, we fully rescued the mutant phenotype (Fig. 7F–G), indicating that *simu* is sufficient for engulfment of apoptotic cells by glial cells and *drpr* is sufficient for their degradation. Altogether, our data show that impaired phagocytosis in the *repo* mutant background is a consequence of abnormal expression of SIMU and DRPR phagocytic receptors.

GCM is sufficient to induce SIMU ectopic expression

Given that GCM and REPO are required for phagocytic function of glia, particularly by regulating expression of phagocytic receptors SIMU and DRPR, we tested whether these factors are also sufficient for the receptor expression. We tested this question in third instar larvae, a stage in which developmental apoptosis does not occur. Normally, GCM and SIMU are not expressed at this stage (Kurant et al., 2008, Figs. 8A′ and S1), while DRPR is expressed at relatively low levels (Figs. 8C′ and S1). We ectopically expressed *gcm* in larval glia using *repoGal4* driver, which is active during larval stages, and examined SIMU and DRPR expression by immunohistochemistry. Ectopic expression of GCM led to high levels of SIMU expression in larval CNS glia (Fig. 8B′, B‴, and b), as compared to wild type controls in which SIMU was undetectable (Fig. 8A′, A‴, and a). Importantly, larval brains ectopically expressing GCM exhibited abnormal shape with much longer ventral nerve cord (VNC) (Fig. 8B‴ and b) compared to wild type brains (Fig. 8A‴ and a). In contrast, DRPR levels were unaltered in *repoGal4*::*gcm* larval brains (Fig.8C′, C‴, c, D′, D‴, and d). Interestingly, there was no increase in REPO expression in *repoGal4*::*gcm* larval brains (Fig. 8B″ and D″) compared to control (Fig. 8A″ and C″) supporting our data obtained in *repo* mutant embryos, where REPO is required for DRPR expression. Thus, GCM is both necessary and sufficient to drive SIMU expression but it is insufficient for upregulating DRPR expression during third instar larvae.

GCM regulates simu expression directly through its binding sites in the simu promoter

As mentioned above, the 2 kb promoter region of *simu*, which recapitulates *simu* expression during embryogenesis, contains two GCM putative binding sites (Fig. 3A). To test whether GCM regulates *simu* expression in glia directly through these sites, we generated transgenic flies containing the *simu-cytGFP* reporter construct lacking the GCM sites (2 *kbΔgcm-cytGFP*). Two putative GCM binding sites (Figs. 3A and 9C) were specifically removed

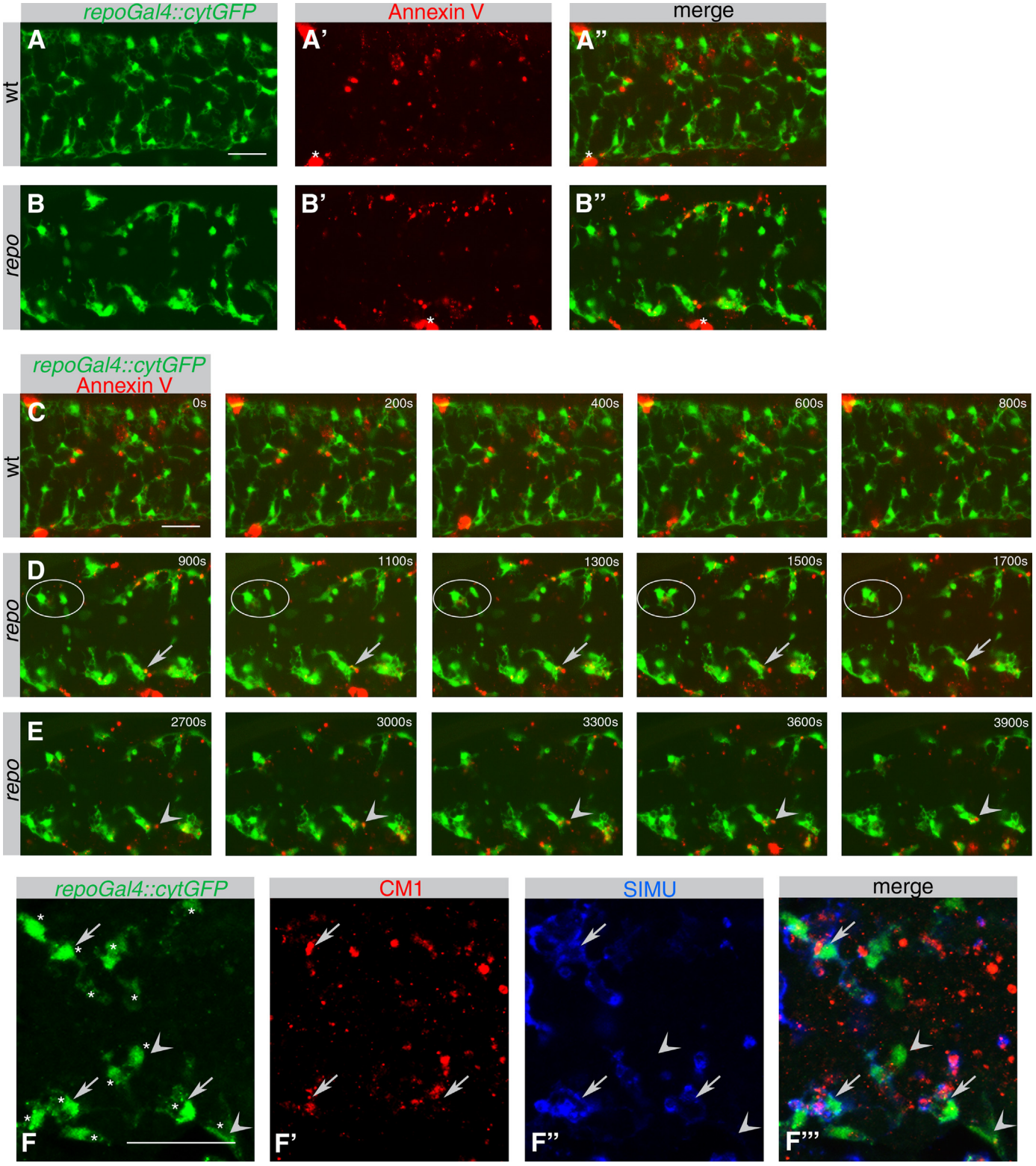

Fig. 6. Dynamic analysis of glial phagocytosis of apoptotic particles in the embryonic CNS. (A–F‴) Embryonic glia are labeled with *repoGal4::cytGFP*. Bar, 20 μm. (A–E) Time-lapse recordings of phagocytosis in stage 16 embryos. PS exposure is labeled by the fluorescent Annexin V (red); (A–A″, C) Wild type embryo. (B–B″, D–F‴) *repo* mutant embryo. (C–E) Selected frames are shown (movies are available in the Supplemental data). In wild type (A–A″, C) most of PS positive particles are inside the glial cells. Some are outside the CNS (asterisks). (B–B″, D, E) *repo* mutant glia exhibit abnormal shape and localization inside the CNS. Some glial cells become more motile and show macrophage-like movement (D, ellipse). Some glial cells are super capable of engulfment (D, arrows, E, arrowheads). (F–F‴) Staining with CM1 and SIMU shows glial cells expressing SIMU with many apoptotic particles inside (arrows), arrowheads point to glia, which do not express SIMU. (F) All GFP-positive glia are labeled with asterisks.

from 2 kb *simu* promoter region using overlapping PCR. The same landing site (86Fb) was used for generation of transgenic flies containing the 2 kb control and the modified 2 *kbΔgcm-cytGFP* construct. We stained the 2 *kbΔgcm-cytGFP* transgenic embryos with the anti-SIMU antibody to follow SIMU protein expression (Fig. 9A′ and B′) and the anti-CRQ antibody to label macrophages

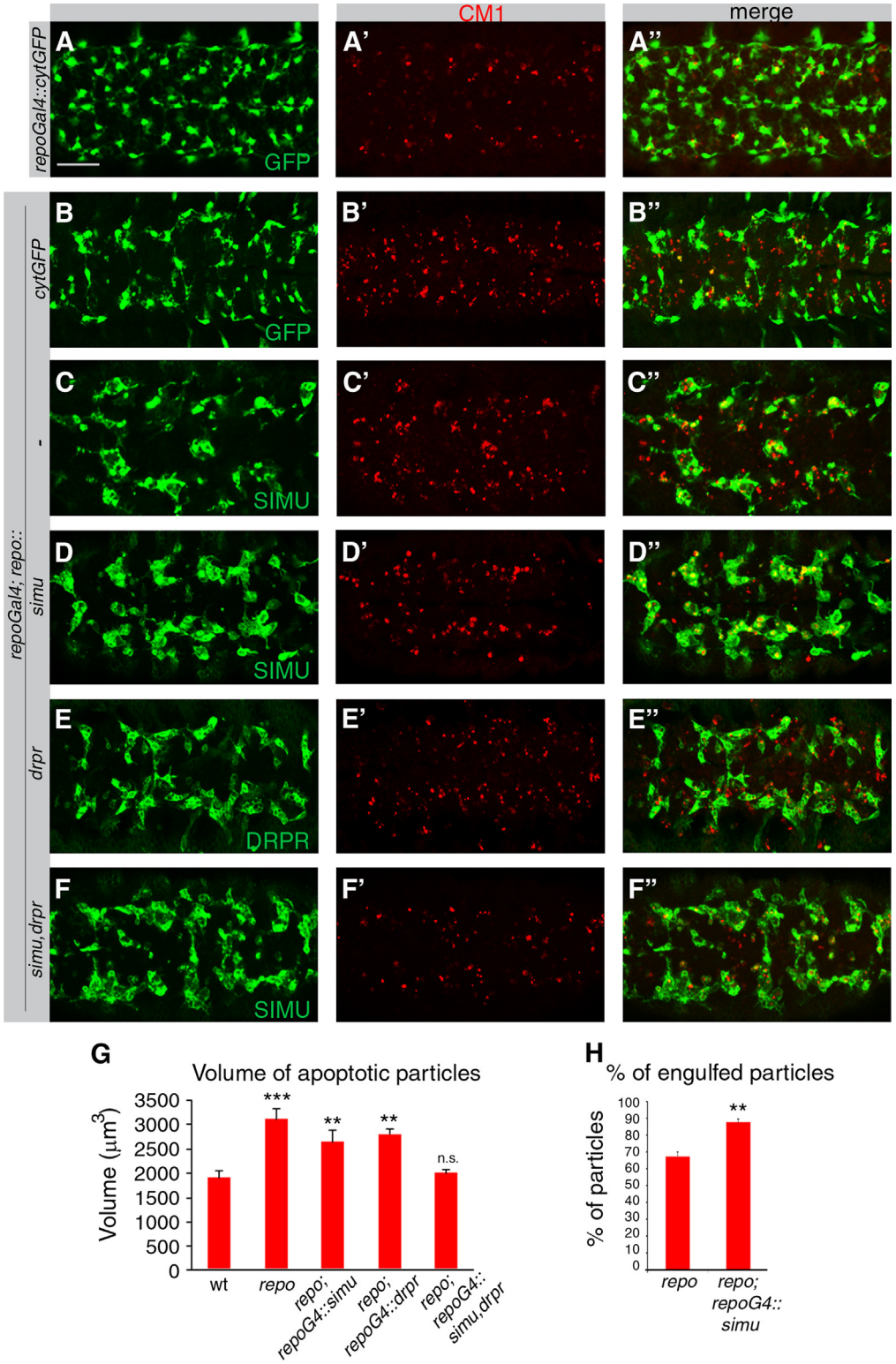

Fig. 7. SIMU and DRPR expression in glia rescues *repo* mutant phenotype. (A–F″) Projections from confocal stacks of the CNS at embryonic stage 16, ventral view; apoptotic cells are in red (CM1) and glia in green (*repoGal4*::*cytGFP*). Bar, 20 μm. In wild type embryo (A–A″) apoptotic particles are mostly inside GFP-positive glia. In *repo* mutant embryo (B–C″) many apoptotic particles are outside GFP-positive glia (B″) or SIMU-labeled glia and macrophages (C″). In rescue experiments (D–F″) SIMU and DRPR expression is detected in glial cells (*repoGal4* driver) and macrophages with anti-SIMU (D, D″, F, F″) and anti-DRPR (E, E″). (F–G) Complete rescue of the *repo* null phenotype with SIMU and DRPR glial specific expression. No rescue is detected with only SIMU (D–D″, G) or only DRPR (E–E″, G) proteins expressed in glial cells. (G) Quantification of phenotypic rescue of *repo* null mutants by the different transgenes. Columns represent mean total volume of apoptotic particles within confocal stacks of the CNS, ± SEM, $n=7$. (H) Percent of engulfed apoptotic particles in *repo* mutant and *repo* mutant expressing *simu* with *repoGal4* driver where substantial rescue of engulfment is detected. Columns represent mean percent of apoptotic particles inside SIMU-labeled cells within confocal stacks of the CNS, ± SEM, $n=7$. (G, H) Asterisks indicate statistical significance versus wild type, as determined by one-way ANOVA, $^{***}p<0.001$, $^{**}p<0.01$, n.s. (not significant) $p>0.05$.

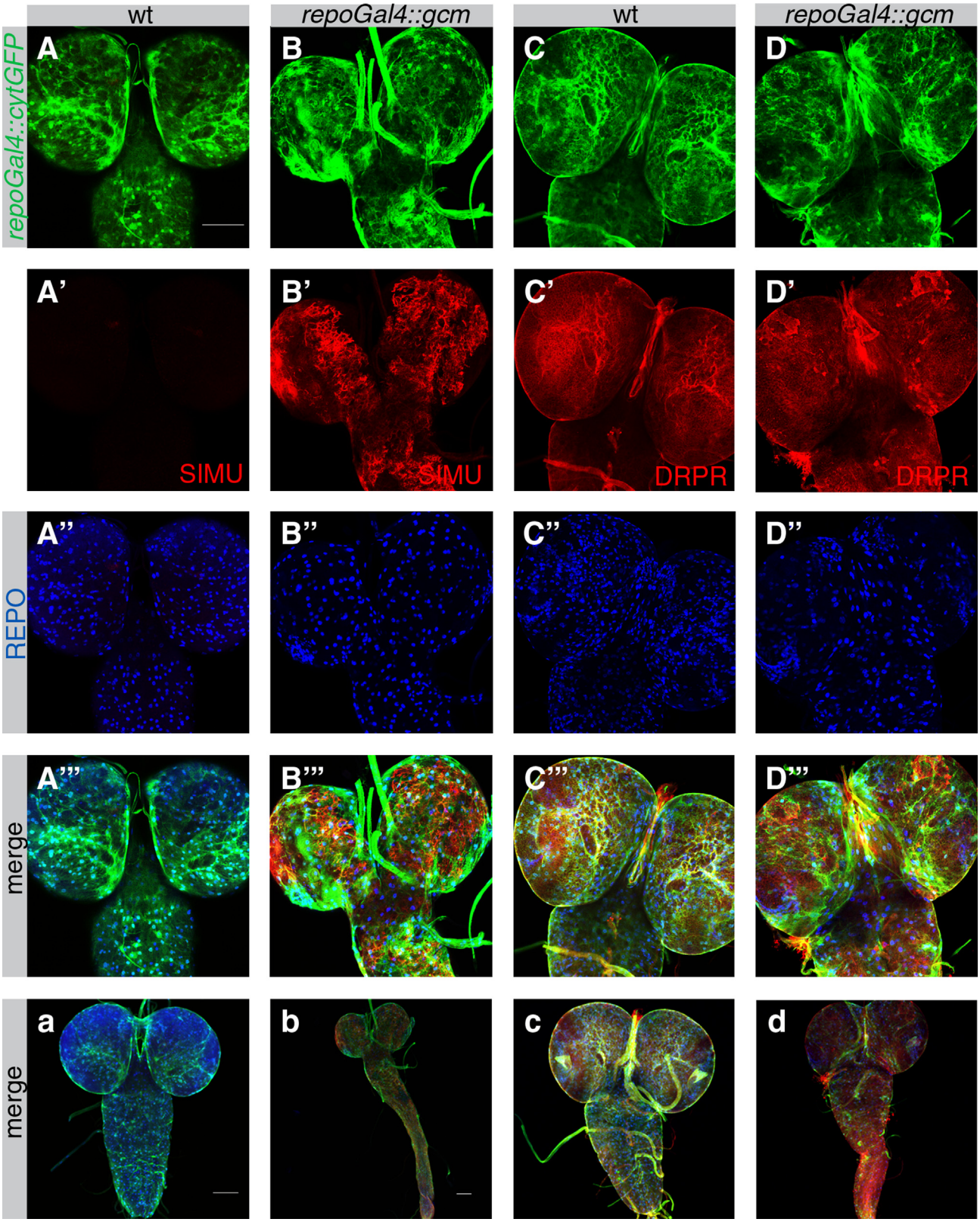

Fig. 8. GCM is sufficient to drive SIMU expression in larval glia but it is dispensable for DRPR expression. (A–D‴) Projections from confocal stacks of the 3rd instar larval brain lobes stained with anti-REPO (blue, A″, A‴, B″, B‴, C″, C‴, D″, D‴), glia are labeled with *repoGal4::cytGFP* (green, A, A‴, B, B‴), anti-SIMU (A′, A‴, B′, and B‴) and anti-DRPR (C′, C‴, D′, and D‴). (a–d) Lower magnification of larval brains (A, B, C, D) showing abnormal shape of *repoGal4::gcm* brains (b, d) compared to wild type brains (a, c). Bar, 100 μm.

(Fig. 9A″ and B″). No GFP expression was found in glia whereas in macrophages GFP appeared normal (Fig. 9B and B‴). These data demonstrate that the two GCM binding sites in the 2 kb promoter are responsible for *simu* expression in embryonic glia and are dispensable for *simu* expression in macrophages. This suggests that GCM directly regulates *simu* expression in embryonic glia.

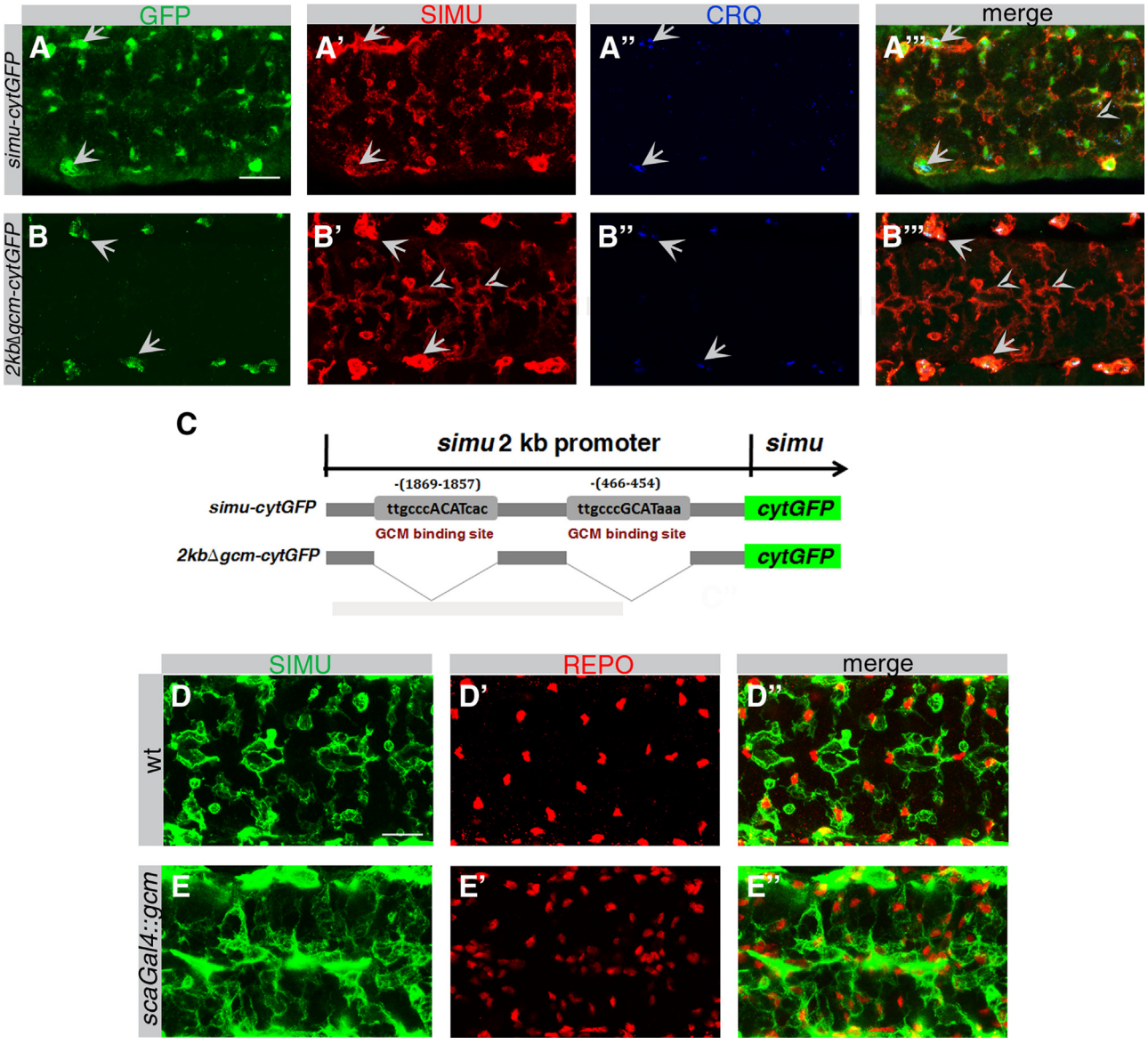

Fig. 9. GCM regulates SIMU glial expression directly through its binding sites. (A–B‴, D–E″) Projections from confocal stacks of the CNS at embryonic stage 16, ventral view; Bar, 20 μm. (A–A‴) *simu-cytGFP* reporter (GFP); (B–B‴) 2 *kbΔgcm-cytGFP* reporter (GFP). SIMU is in red (A′, A‴, B′, B‴) and macrophages are in blue (CRQ, A″, A‴, B″, B‴). (C) Schematic representation of *simu-cytGFP* and 2 *kbΔgcm-cytGFP* reporters. (D–E″) SIMU is in green and REPO is in red in wild type (D–D″) and *scaGal4*::*gcm* (E–E″) embryos.

Moreover, we ectopically expressed *gcm* in wild type embryos using the *scaGal4* neuroectoderm driver and tested SIMU expression. We found strong ectopic SIMU expression in *scaGal4*::*gcm* embryos (Fig. 9E and E″) suggesting that GCM is sufficient to drive SIMU expression in neurons.

Discussion

Efficient clearance of apoptotic neurons is crucial for normal CNS development. Recently, vertebrate microglia have been shown to play a critical role in normal brain development through clearance of unneeded neurons and remodeling synapses (Cunningham et al., 2013; Nandi et al., 2012; Sierra et al., 2013; Sierra et al., 2010; Tremblay et al., 2011). However, the molecular mechanisms responsible for the formation of microglia as potent phagocytes during development remain elusive. Unlike vertebrates, the *Drosophila* nervous system does not contain microglia and functionally homologous neuroectoderm-derived glia function as the main phagocytes during CNS development. We and others have previously shown that *Drosophila* phagocytic glia efficiently clear apoptotic neurons during embryonic development and that two transmembrane receptors SIMU and DRPR are required for this function (Freeman et al., 2003; Kurant et al., 2008; Logan et al., 2012; MacDonald et al., 2006; Shklyar et al., 2013a). However, how this glial phagocytic ability is established during development and whether apoptosis is involved in this process was unknown. In this study we showed that the phagocytic receptors SIMU and DRPR are expressed on membranes of embryonic phagocytes, 'professional' macrophages and 'non-professional' glia, independently of developmental apoptosis itself. We found that this specific expression is differentially regulated in macrophages and glia and showed that it is part of the glial fate determination program. Moreover, we found that GCM is required for both SIMU and DRPR expression whereas REPO is dispensable for SIMU expression and is essential for DRPR expression in glia.

Using *Drosophila* embryonic CNS as a model, we examined whether lack of or increase in apoptosis can influence expression of phagocytic receptors, which are required for proper removal of apoptotic cells during development. Surprisingly, we found that the rate of apoptosis has no effect on expression levels of phagocytic receptors, suggesting that phagocytes are ready to recognize and remove apoptotic particles as part of their correct differentiation. As we have previously shown, engulfment of apoptotic cells occurs rapidly (Kurant et al., 2008; Shklyar et al., 2013a) and there is likely not enough time to up-regulate transcription or translation of phagocytic receptors. Therefore, they have to be placed on phagocytic membranes ahead of the uptake itself. However, posttranslational modification of DRPR in response to apoptosis cannot be ruled out. On the other hand, during normal development in the absence of apoptosis, the expression of SIMU is below detection, and during apoptotic waves its expression is extremely high (Fig. S3). Since SIMU does not undergo any post-translational modifications (Kurant et al., 2008), it seems to be tightly regulated transcriptionally in order to be present only when needed. However, DRPR activity can be modulated by posttranslational modifications (Ziegenfuss et al., 2008); hence it might be less dependent on the levels of transcription, which barely change during development (Fig. S1). Interestingly, recent studies on glial engulfment of injured axons in adult *Drosophila* brain showed that levels of DRPR were elevated as a response to the injury (Macdonald et al., 2013) suggesting that different molecular pathways regulate DRPR levels in the developing and adult CNS.

Both SIMU and DRPR are glial phagocytic receptors (Freeman et al., 2003; Kurant et al., 2008; Logan et al., 2012; MacDonald et al., 2006; Shklyar et al., 2013a) though their expression pattern is different. DRPR is expressed during all stages of fly development and has been shown to play a role in many different processes mediated by glia. In most of these processes it is required for the degradation of engulfed material inside phagocytic glia (Kurant et al., 2008; Logan et al., 2012; MacDonald et al., 2006). However, SIMU is expressed exclusively during developmental apoptosis and it is required specifically for the recognition and engulfment of apoptotic particles (Kurant et al., 2008; Shklyar et al., 2013a). In search for regulators of SIMU and DRPR expression, we first focused on GCM, whose expression correlates with that of SIMU and DRPR during embryonic development in macrophages and glia. Moreover, *drpr* was found in the genetic screen for GCM-regulated genes expressed during embryogenesis (Freeman et al., 2003). Recently, it has been shown that the GCM protein is still present in glia at late embryonic stages (Laneve et al., 2013) when *simu* and *drpr* are highly expressed. Indeed, we found that GCM is responsible for SIMU and DRPR expression though only in glia (Fig. 10) and not in macrophages, suggesting that distinct developmental programs function in different phagocytic cells. Moreover, our experiments show that ectopic expression of GCM in larval glia induces SIMU expression (Fig. 8B′, B‴, and b) while DRPR as well as REPO levels remain unchanged (Fig. 8D′, D‴, and d). Furthermore, we demonstrated that GCM controls SIMU expression in glia directly, through its binding sites in the *simu* promoter (Fig. 9A–C). In addition, since GCM is expressed in all lateral glia, we propose that *gcm*-regulated expression of SIMU is repressed in a subset of lateral glia, which does not normally express SIMU. It will be intriguing to further investigate this suggestion and explore the mechanism, which restricts SIMU expression to the specific glial cells. The situation is different for DRPR, where GCM acts through its downstream target REPO, which is required and sufficient for DRPR embryonic expression (Fig. 10), since in *repo* mutant embryos there is no DRPR in glia even in the presence of GCM (Fig. 4) and ectopic expression of *repo* with the neuroectoderm driver *scaGal4* leads to ectopic expression of DRPR in neurons (Fig. S5).

repo and *drpr* are required for diverse glial functions, apart from apoptotic cell clearance, and are expressed during all stages of development. However, *simu* expression does not depend on *repo* (Fig. 10) and *simu*-expressing *repo* mutant glia are capable of engulfing apoptotic particles very efficiently. Strikingly, some *repo* mutant cells resemble macrophages in their increased motility and uptake capacity resulting in engulfment of multiple apoptotic cells. We found that these cells express SIMU on their surfaces. This 'macrophage-like' behavior could be a transitional state of developing glia, and only when glia further differentiate, they make proper cell-cell contacts, stop moving and become imbedded in their determined place. An important part of this glial differentiation is to be able not only to recognize and engulf apoptotic particles, but also to degrade them intracellularly, which necessitates REPO regulation of DRPR expression. An intriguing idea would be to test DRPR's role as an inhibitory receptor, preventing glial membrane extension, analogous to the role of DRPR's vertebrate homologs MEGF10/MEGF11 in tiling of retinal neurons (Kay et al., 2012).

Our rescue experiments show that when additional glial cells express *simu*, the engulfment defects of *repo* mutant glia are substantially rescued, which is reflected by much a higher percentage of engulfed apoptotic particles compared to *repo* mutant (Fig. 7H). Nonetheless, *simu* expressing mutant glia are unable to degrade apoptotic particles, resulting in accumulation of particles trapped inside the phagocytes (Fig. 7). Moreover, DRPR expression alone is unable to rescue phagocytosis because DRPR is not sufficient for engulfment. Normally, not all lateral glia perform phagocytosis. Here we provide evidence that if SIMU and DRPR are expressed in additional glial cells, they become completely functional phagocytes suggesting that intracellular phagocytic machinery responsible for cytoskeleton rearrangement and phagolysosome formation (Ravichandran and Lorenz, 2007) is already established in these cells.

Taken together, our data show that the ability of embryonic glia to properly remove and degrade apoptotic cells is built up by distinct steps during differentiation. Initial steps governing formation of intracellular phagocytic machinery seem to be common in macrophages and glia while later steps, which involve SIMU and DRPR receptors expression, are specifically determined during glial cell fate determination. In summary, using the *Drosophila* embryonic CNS as a model, our study uncovers molecular mechanisms essential for establishment of embryonic glia as primary phagocytes during CNS development. Given the critical role of glial phagocytosis in development and maintenance of the CNS we believe that our data may open new directions in understanding glial cell biology of higher organisms.

Conclusions

Removal of apoptotic cells is crucial for normal development of multicellular organisms, especially in the developing CNS where many neurons die through apoptosis. These apoptotic neurons are efficiently cleared by glia, which are primary phagocytes in the CNS. It is not clear how these highly phagocytic cells are established during development. Using *Drosophila* embryonic CNS as a model we show that glial phagocytic ability relies on expression of phagocytic receptors SIMU and DRPR, which are key factors involved in distinct steps of apoptotic cell clearance, engulfment and degradation. We provide evidence that SIMU and DRPR are sufficient to make each glial cell phagocytic. However, their embryonic expression is not affected by apoptosis but strongly depends on master regulators of glial fate determination, GCM and REPO, which differentially control SIMU and DRPR. GCM directly regulates *simu* expression whereas *drpr* requires REPO function. These data indicate that the glial developmental program generates competence in the glial cells to act as primary phagocytes in the CNS.

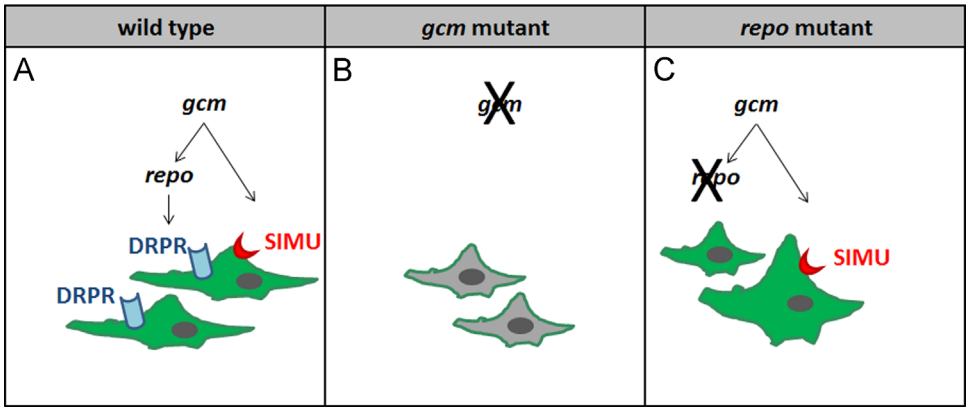

Fig. 10. Schematic representation of developmental regulation of SIMU and DRPR expression in embryonic glia. (A) Wild type. (B) In *gcm* mutant no glia are formed and no SIMU and DRPR expression is found in cells, which turn from glia to neurons. (C) *repo* mutant glia appear in abnormal shapes. SIMU is expressed on some glial cells, which are often bigger than wild type glia and show macrophage-like behavior.

Materials and methods

Fly strains and constructs

The following fly strains were used in this work: *repoGal4* (B. Jones), *UAScytGFP* (# 1521; Bloomington), *repo*^{03702}/*TM3* (# 11604; Bloomington), *elavGal4* (O. Schuldiner), *UAShid* (E. Arama), *Df (3L) H99* (H. Steller), *simu-cytGFP* (Kurant et al., 2008), *Df(2L)Exel7042* (#7812; Bloomington), *UASnGFP* (# 4775; Bloomington), *UASrepo* and *UASdrpr* (M. Freeman), *UASgcm* (#5446; Bloomington), *UASsimu* (Shklyar et al., 2013a), *tubGal80*^{ts} (#7019; Bloomington), *scaGal4* (A. Salzberg). We placed progeny *elavGal4::hid*; *tubGal80* at 18 °C until the 2nd instar larval stage and then shifted them to 29 °C for 24 h.

Reporter constructs were generated by cloning the 2 kb or 2 *kb*Δ*gcm* upstream regulatory region of *simu* into the pattB vector containing a cytoplasmic GFP coding sequence. These transgenes were inserted into the attP86 site on chromosome 3R using the QC31 system (Venken et al., 2006). All strains were raised at 25 °C. w1118 flies were used as wild type control.

qRT-PCR

RNA was isolated from tissue samples (embryos or larvae) using RNazol®RT (MRC) and a hand homogenizer. Following DNase treatment RNA was used as a template for cDNA synthesis (M-MLV RT and RNasin: Promega). Gene expression was evaluated using TaqMan gene expression assays (Applied Biosystems) specific for *simu* (*nimC4*) – Dm01791334_s1, *drpr* – Dm01832226_g1 or *RPII140* – Dm02134593_g1 (as an internal control). The PCR reactions were set up following the manufacturer's instructions. The assays were performed in triplicate with RNA samples from at least three different isolations. Relative gene expression was quantitated using the comparative method (ΔΔCT). Graphs represent the fold change of target gene expression in mutant compared to control samples. The Student's *t*-test was used for statistical analysis. $P < 0.05$ was considered to indicate a statistically significant difference.

Immunohistochemistry and live imaging

For immunohistochemistry embryos were fixed and stained according to standard procedures. Guinea pig anti-SIMU (Shklyar et al., 2013a) and rabbit anti-DRPR (M. Freeman) were used at a 1:5000 and 1:500 concentrations respectively. Rabbit anti-activated caspase 3 (CM1, BD) and mouse anti-GFP (Roche) were used at 1:100 concentration. Rabbit anti-CRQ antibody (1:500) was a gift from N. Franc. Mouse anti-Repo antibody (Hybridoma bank) was used at 1:5 concentration. Fluorescent secondary antibodies (Cy3/Jackson ImmunoResearch; Alexa Fluor 488/Molecular Probes) were used at 1:200 dilutions. All confocal images were acquired on a confocal microscope Zeiss LSM 700 using an EC Plan-Neofluar 40 × /1.30 Oil DIC M27 or a Plan-Apochromat 20 × / 0.8 M27 lens. 75% Glycerol solution was used as the imaging medium. Image analysis was performed using Zeiss LSM 700 and Imaris (Bitplane) software. To quantitate the volume of apoptotic particles or glial cells, confocal stacks (5 sections; total 7.5 μm) were acquired from the neural cortex of stage 16 ventral nerve cords. For quantification of glial cell number in entire CNS (Fig. 2C and D) 10 sections, total 15 μm were acquired.

Live imaging was carried out by dechorionating embryos (stage 15), mounting them under Halocarbon oil, injecting 2%-3% egg volume Alexa fluor 555 conjugated Annexin V (Molecular Probes) or LysoTracker (Molecular Probes) as described in (Shklyar et al., 2013b). Recording started 30 min following injection.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2014.07.005.

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