A novel role of the glial fate determinant \textit{glial cells missing} in hematopoiesis

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ABSTRACT Glial cell deficient/Glial cells missing (Glide/Gcm) transcription factor is expressed in all glial precursors of the Drosophila embryo. Gcm is necessary and sufficient to induce glial differentiation but also plays a role in other cell types, by interacting with specific factors. To find potential partners of Gcm which trigger these other pathways, we performed a yeast two-hybrid screen and identified \textit{dpias}, a gene involved in post-embryonic hematopoiesis. \textit{dpias} larvae show melanotic tumors due to excess of lamellocytes, a hemocyte lineage that is involved in non-self recognition. We here show that blocking Gcm activity also triggers melanotic tumors and that \textit{gcm} interacts genetically with \textit{dpias}. Moreover, the members of the Janus Kinase (JAK)/Signal Transducer and Activator of Transcription (STAT) pathway, which are known for their role in the vertebrate and invertebrate immune system and are required for \textit{dpias}-dependent tumor formation, act downstream of Gcm. Altogether, this study identifies an unpredicted role of Gcm, dictated by its cofactor \textit{dpias}, allowing Gcm to act in a specific pathway. Together with the recent finding that glia act as scavengers during development and in pathological conditions, our data open new perspectives onto the cellular and molecular pathways involved in non-self recognition within and outside the nervous system.

KEY WORDS: \textit{Drosophila melanogaster, glide/gcm, dpias, JAK/STAT, hematopoiesis}

Introduction

In addition to these transcriptional and post-transcriptional regulatory mechanisms, the activity of Gcm is mediated by cellular context (Miller \textit{et al.}, 1998; Ragone \textit{et al.}, 2003). A cell-specific factor, Huckebein, interacts directly with Gcm and triggers Gcm autoregulation in the thoracic neuroblast lineage \textit{1-1} (NBT1-1). Such protein-protein interaction is necessary to promote glial differentiation and specify the sub-perineural glia fate in that lineage (De Iaco \textit{et al.}, 2006). Finally, while \textit{gcm} is necessary and sufficient to induce embryonic glia, it is clear that this gene is involved in other developmental pathways as well. \textit{gcm} is expressed and required in embryonic hemocytes (Alfonso and Jones, 2002; Bataille \textit{et al.}, 2005; Bernardoni \textit{et al.}, 1998; Kammerer and Giangrande, 2001; Miller \textit{et al.}, 1998; Ragone \textit{et al.}, 2003; Soustelle \textit{et al.}, 2008).

Abbreviations used in this paper: \textit{dpias}, Drosophila protein inhibitor of activated STAT; \textit{gcm}, glial cells missing; JAK, janus kinase; STAT, signal transducer and activator of transcription.

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similar to what is observed in post-embryonic hematopoiesis. This allowed us to reveal an unexpected role of Gcm in the cascade that maintains post-embryonic blood cell homeostasis. In addition, we show that members of the JAK/STAT signaling cascade, which are known for their role in post-embryonic hematopoiesis and rely on dpias, act downstream of gcm. These data allow us to identify a novel Gcm pathway and cofactor acting in such pathway. Finally, our study highlights common features between glia and hemocytes in Drosophila, in line with recent data demonstrating that glia play a scavenger role in physiological and pathological conditions (Awasaki et al., 2006; Freeman et al., 2003; MacDonald et al., 2006). Interestingly, mammalian microglia display highly mobile processes that are constantly screening the nervous system and display a macrophage function (Hanisch and Kettenmann, 2007). Altogether, these data lead to the speculation that invertebrate glia and/or hemocytes may be at the origin of microglia, the scavenger cells of the mammalian nervous system.

The present study was designed to gain insight into the mode of action of the Gcm transcription factor by identifying new cofactors. Gcm is indeed well known to have specific functions dictated by the presence of cell-specific cofactors. Upon performing a yeast two-hybrid screen we identified dpias (Protein Inhibitor of Activated STAT), a protein that controls post-embryonic hematopoiesis. This allowed us to reveal an unexpected role of Gcm and a novel molecular cascade.

**Results**

**Identification of Gcm cofactors by yeast two-hybrid screen**

gcm codes for a transcription factor of 504 amino-acids carrying several motifs including a DNA binding domain (DBD), a nuclear localization signal (NLS), a PEST domain and an activation domain (AD) (Fig.1A). To gain insights into the Gcm mode of action, we performed a yeast two-hybrid screen. To determine the optimal construct for the screen, we removed different domains of Gcm and used the deleted constructs for autoactivation tests in yeasts. Strikingly, we found that a fragment of the C-terminal part of Gcm (aa261-aa421) leads to strong autoactivation, even though the AD is not present in this construct (data not shown). In contrast, we did not detect autoactivation upon using a fragment containing the N-terminal part of Gcm, which includes the DBD, the NLS as well as the PEST domain (aa1-aa261) and thus used this construct for the screen (Fig.1A).

Twelve partners were identified (Fig.1B). Amongst them, Karyopherin-α1 and Pendulin (also called Importin-α1 and Importin-α2, respectively) are known to transport NLS carrying transcription factors from the cytoplasm to the nucleus (Goldfarb et al., 2004). Accordingly, we found that the interaction between Gcm and Karyopherin-α1 or Pendulin needs the NLS domain of Gcm (Fig.1B).

Another candidate identified in our screen is Uba2 (also called Smt3 activating enzyme 2), which requires the PEST domain of Gcm to interact (Fig.1B). uba2 codes for a SUMO-1 conjugation enzyme playing a role in sumoylation, a process that modulates the activity of many proteins (Zhao, 2007). In line with our results, GCMa, the human ortholog of fly Gcm, is also a target of the sumoylation machinery (Chou et al., 2007). In their study, the authors showed that GCMa-mediated transcriptional activation is repressed by sumoylation, due to a decreased DNA binding activity of GCMa, suggesting that this regulation also exists in flies.

Strikingly, one of the candidates identified during the screen for dpias (also called Su(var)2-10 or Zimp), a nuclear protein that is required in hematopoiesis in the Drosophila larva. Because gcm is known to play a role during embryonic hematopoiesis, we focused our attention on this particular candidate.

In order to confirm the interaction between Gcm and dpias, we performed immunoprecipitation experiments from cytoplasmic extracts of S2 cells transiently overexpressing a Flag-tagged version of Gcm and a HA-tagged version of dpias. Then, we determined whether anti-Flag antibody coprecipitates the HA-tagged dpias protein. As shown in Fig.1C (top panel), Flag-Gcm is expressed after transfection of S2 cells and efficiently precipitated. Importantly, HA-dpias is also detected in transfected cells and in the Flag-precipitated proteins (Fig.1C, bottom panel) calling for Gcm – dpias interaction.

**gcm interacts genetically with dpias**

The embryonic hematopoietic anlage produces two types of hemocytes: the crystal cells, which depend on the RUNX factor Lozenge for their differentiation (Lebestky et al., 2000), and the plasmatocytes/macrophages, which are under the control of gcm genes (Alfonso and Jones, 2002; Bernardoni et al., 1997). At post-embryonic stages, however, the production of hemocytes (crystal cells and plasmatocytes/macrophages) resides in a specialized

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Presence of melanotic tumors</th>
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<tbody>
<tr>
<td>dpias1 / dpias1</td>
<td>Embryonic lethal</td>
</tr>
<tr>
<td>gcm26 / gcm26</td>
<td>Embryonic lethal</td>
</tr>
<tr>
<td>gcm-gal4 / gcm-gal4</td>
<td>NO</td>
</tr>
<tr>
<td>gcm-gal4 / gcm26</td>
<td>NO</td>
</tr>
<tr>
<td>dpias1 / dpias1, gcm26</td>
<td>YES L2/L3 (1/200)</td>
</tr>
</tbody>
</table>

The genotypes of analysed larvae are described in the left column. Right column describes the observed phenotypes, note that dpias1, gcm-p and gcm-p homozygous animals die at embryonic stages. Removing one copy of dpias in combination with a gcm-hypomorphic allele (gcm-gal4/dpias1) did not produce any larval melanotic tumor. In contrast, tumors were detected in animal lacking one copy of gcm and dpias in combination with a gcm-hypomorphic allele (gcm-gal4/dpias1, gcm26), indicating that gcm and dpias interact genetically. All experiments were performed at 29°C.
hematopoietic organ, the lymph gland, which produces an additional type of hemocytes, the lamellocytes, in response to parasitism (Sorrentino et al., 2002). Prohemocytes of the lymph gland, the precursors of hemocytes, start hyperproliferating and produce lamellocytes, which aggregate in high number together with circulating hemocytes (Lanot et al., 2001; Meister and Lagueux, 2003). Lamellocytes act in concert with crystal cells and plasmatocytes/macrophages in the cellular immune response by encapsulating pathogens that invade the larval hemolymph. This process, which leads to the formation of melanotic tumors that are clearly identifiable by their black color, also takes place when blood cell homeostasis is altered in mutant conditions. For example, it is known that overactivation of the JAK/STAT signaling activity is sufficient to induce massive lamellocyte differentiation (Harrison et al., 1995; Luo et al., 1995; Zettervall et al., 2004). In Drosophila, dpias owns its name to its role and represents the unique member of the PIAS family, known to act negatively on STAT transcription factors (Betz et al., 2001). Loss of function of dpias gene is known to trigger lamellocyte differentiation and melanotic tumor formation (Betz et al., 2001; Hari et al., 2001).

To show that gcm and dpias interact in vivo, we analyzed animals that loose the two genes simultaneously by constructing a recombinant line carrying a null allele of gcm (gcm26) with dpias1 mutation. Since the dpias1 and the gcm26 homozygous animals die at embryonic stage, we crossed this recombinant line with a weak gcm hypomorphic allele, gcm-gal4 (Soustelle and Giangrande, 2007b). We never found melanotic tumors in original dpias1, gcm 26, and gcm-gal4 lines nor in the recombinant gcm26,dpias1 (see Table 1). In contrast, we did observe melanotic tumors in larvae lacking one copy of dpias and more than one copy of gcm (transheterozygous animals carrying gcm26 in combination with gcm-gal4). These genetic data confirm that gcm and dpias act in the same molecular pathway.

Post-embryonic Gcm loss of function causes larval death and melanotic tumors as observed in dpias mutant

To elucidate the role of Gcm in post-embryonic hematopoiesis we decided to use a time- and tissue-specific mutant allele.

Fig. 1. Gcm and its putative cofactors. (A) Schematic representation of Gcm transcription factor structure. DBD, DNA-binding domain; NLS, nuclear localization signal; PEST, rapid turnover signature; AD, activation domain. Black lines indicate the different baits used in the screen to identify the interaction domain. (B) Table indicating the putative cofactors of Gcm identified in this study, their predicted/demonstrated function, as well as the strength of interaction between baits and putative cofactors (indicated by plus and minus signs). Note that interaction strength is not an absolute value and cannot therefore be used to compare Gcm affinity for different candidates. (1) Flybase: http://flybase.bio.indiana.edu, (2) Escudero et al., 2005, (3) Betz et al., 2001; Hari et al., 2001; Mohr and Boswell, 1999), (4) and (6) (Goldfarb et al., 2004), (5) (Beuchle et al., 2007; Terman et al., 2002), (7) (Brown et al., 2003; Wang et al., 2004), (8) (Hipfner and Cohen, 2003; Hipfner et al., 2004), (9) (Donaghue et al., 2001). (C) Gcm interacts with dpias. Drosophila S2 cells were transiently transfected with expression plasmids encoding Flag-tagged Gcm in combination with HA-tagged dpias. Whole-cell extracts were subjected to immunoprecipitation (IP) with anti-Flag (indicated by IP α-Flag). The samples were subjected to SDS-PAGE, and Western blots were probed by using the anti-Flag (top panel) or the anti-HA (bottom panel) antibody. Input indicates sample of S2 cell extracts prior to immunoprecipitation (Input S2 for untransfected cells, Input S2 transf for transfected S2 cells). S2, S2 transf and SN indicate the product of IP from untransfected S2 cells, transfected S2 cells, and the supernatant from transfected S2 cells, respectively. As seen on top panel, Flag-tagged Gcm is detected as a triplet in transfected S2 cells (Input S2 transf) and efficiently immunoprecipitated (S2 transf). HA-dpias is expressed in transfected S2 cells (Input S2 transf) and immunoprecipitated by anti-Flag (S2 transf). Note the presence of a weak signal in the SN for both Gcm-Flag and dpias-HA.
Expression of a fusion protein containing the Gcm DNA binding domain and the repressor domain of Engrailed (gcm(DN)) provides a dominant negative approach that induces the same phenotypes as those induced by a deficiency eliminating both gcm and its homolog gcm2 (Soustelle et al., 2004). The use of this transgenic construct allows to overcome the embryonic lethality induced by the gcm mutation. Indeed, expression of the gcm(DN) construct can be controlled spatially and temporally by using the TARGET system, which is based on the expression of a temperature-sensitive mutation of the Ga180 protein (Ga180FS), a repressor of Ga14 function (McGuire et al., 2003). Conditional expression of the gcm(DN) construct has already been successfully used to clarify the role of gcm genes in the nervous system and in tendon cells (Soustelle and Giangrande, 2007b; Soustelle et al., 2004; Soustelle et al., 2007).

In a first trial, we used the serpent-gal4 line, which expresses Gal4 in all tissues involved in the innate immune response at post-embryonic stages (Fig. 2A,A'). This includes the lymph gland, the organ producing hemocytes during larval development (Lanot et al., 2001), the fat body, which is known to play a role in the humoral immune response (for reviews, see Cherry and Silverman, 2006; Leclerc and Reichhart, 2004; Lemaire and Hoffmann, 2007), as well as hemocytes (Crozatier et al., 2004). To bypass the embryonic lethality induced by loss of gcm, we expressed the dominant negative construct starting from larval stages (shift at the restrictive temperature, 29°C, during the first instar larval stage or L1), using the line serpent-gal4,tub-gal80D, UAS-gcmDN, UAS-encGFP (srp>gcmDN). Induction of the gcm(DN) construct at these stages leads to the formation of melanotic tumors (Fig. 2C). This phenotype is 100% penetrant (n>200) and all animals die at the pupal stage. Importantly, mutant animals do not show such phenotype at permissive temperature (18°C) and are perfectly viable and fertile, due to the fact that Ga180 is active and represses Gal4 activity. We also noticed that mutant animals display a delay in development after the LIII stage as well as a disintegration of the fat body (data not shown), as it had been previously observed in dpias mutant larvae (Betz et al., 2001; Hari et al., 2001), suggesting that gcm and dpias act in concert during post-embryonic hematopoiesis. Importantly, the observed delay in development occurs during the LIII/pupariation transition, after the appearance of melanotic tumors, indicating that the formation of melanotic tumors is not due to development delay. Finally, we used a mutant construct, gcmN7-4DN, which carries a mutation abolishing DNA binding (Miller et al., 1998; Soustelle et al., 2004; Vincent et al., 1996), serpent-gal4,tub-gal80D, UAS-gcmN7-4DN, UAS-encGFP larvae expressing the gcmN7-4DN construct do not show any phenotype (Fig. 2D), confirming the specificity of the defects observed in srp>gcmDN larvae.

Previous studies have shown that the melanotic tumor phenotype is associated with extensive lamellocyte differentiation (Harrison et al., 1995; Luo et al., 1995). To demonstrate that this is also the case in animals lacking Gcm activity in immune tissues, we counted the number of hemocytes in third instar larvae (LIII). While control larvae (serpent-gal4, UAS-encGFP and srp>gcmN7-4DN, UAS-encGFP, UAS-gcmN7-4DN) provides a significant difference in lamellocyte number (p<0.001) between srp>gcmDN and all other genotypes. (G) Schematic representation of Drosophila development at 18°C and the phenotypes induced upon shifting srp>gcmDN animals at restrictive temperature (29°C) during different developmental stages. Note that when expression of gcm(DN) construct is induced in L1, large and numerous tumors are found in all LIII larvae. The penetrance decreases to 50% when gcm(DN) is induced in LII and the size and number of tumors are smaller. Expression induced in LIII does not cause any melanotic tumor. Note that gcm(DN) expression prior to the larval stages induces embryonic lethality (cross). Scales bar in (A,A') 50 μm; in (B) 1 mm.
4DN do not contain any lamellocyte (Fig. 2F). srp-gcm2DN animals display a strong increase in lamellocyte production (Fig. 2F). Moreover, the ratio of plasmatocytes as compared to total hemocyte number is significantly decreased in srp-gcm2DN animals (10.5% versus 97% in srp-encGFP larvae, p<0.001), as it had been previously observed in other mutant conditions leading to the production of melanotic tumors (Betz et al., 2001; Hari et al., 2001).

Interestingly, we observed that the melanotic tumor phenotype is less severe (fewer larvae showing fewer tumors) when the expression of the gcm2DN construct is induced after LI and no tumor was observed upon gcm2DN induction at early LIII, suggesting that gcm is required during early larval development for normal hematopoiesis (Fig. 2G).

### Tissue specific requirement of Gcm

In order to determine the tissue-specific requirement of gcm during post-embryonic hematopoiesis, we assessed its expression profile. We first analyzed enhancer trap lines inserted into the regulatory regions of gcm (gcm-gal4 and rA87 lines) and found no expression in serpent-positive tissues (data not shown), as had been described in previous studies (Bataille et al., 2005). Because gcm function is required during early larval development, we performed RT-PCR experiments on cells separated by fluorescent activated cell sorter (FACS) (Fig. 3). For this purpose, we collected serpent-gal4, UAS-GFP animals at LI, dissociated them into single cells and collected two fractions: the serpent-positive cells expressing GFP and the serpent-negative cells, which do not express GFP. These two fractions were then used to perform RT-PCR experiments by using different couples of primers (serpent, gcm, gcm2, lozenge, dpias). This approach was validated by two sets of data: 1) srp-positive cells express serpent, lozenge and dpias (Fig. 3A), which are known to be transcribed in immune tissues, 2) serpent as well as the crystal cell-specific marker lozenge are not detected in serpent-negative cells (Fig. 3B).

As shown on Fig. 3, gcm is present in serpent-negative cells due to its expression in neural tissues (Fig. 3B), but also in serpent-positive cells (Fig. 3A). Interestingly, gcm2 was not detected (Fig. 3), indicating that only gcm is expressed in tissues involved in the innate immune defense.

Because the serpent-gal4 line drives expression in several cell types, we aimed at identifying in which cells Gcm acts by using lines that drive expression in restricted cell populations. First, the Gal4 expression profile was established by crossing the driver with a GFP reporter line and analyzed in LI under a fluorescent microscope.
We analyzed between forty and sixty larvae and never found any tumor, indicating a 100% penetrance of the tumor suppression phenotype. Interestingly, mutants for several components that interact with JAK/STAT display melanotic tumors. Gcm genetically interacts with JAK/STAT signaling pathway.

Gcm genetica...
known for their role in post-embryonic hematopoiesis and rely on dpia\textsubscript{s}, act downstream of gcm.

**Discussion**

During nervous system development of *Drosophila* embryos, gcm acts as a glial determinant, being necessary and sufficient to induce the glial fate (Akiyama-Oda et al., 1998; Bernardoni et al., 1998; Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996), for review (Soustelle and Giangrande, 2007a). Interestingly, gcm also acts as a cell fate determinant in hematopoietic lineages, where it controls the plasmatocyte/crystal cell fate choice (Alfonso and Jones, 2002; Bataille et al., 2005; Bernardoni et al., 1997). Despite the fact that gcm also has a gliogenic role at post-embryonic stages (Chotard et al., 2005; Soustelle and Giangrande, 2007b; Soustelle et al., 2007; Yoshida et al., 2005), its function in larval hematopoiesis has not been elucidated. We here identify dpia\textsubscript{s} as a cofactor of Gcm and show that loss of function for each of these genes triggers the same larval phenotype. In addition, we demonstrate that Gcm acts in the fat body and upstream of the JAK/STAT pathway to maintain blood cell homeostasis at post-embryonic stages. Thus, Gcm activity is able to trigger different pathways and depends on protein-protein interactions.

The identification of putative cofactors constitutes a starting point to better understand Gcm mode of action. Future challenge will be to identify which target genes are specifically activated by Gcm-dpia\textsubscript{s} complex in the fat body and how this molecular cascade acts on JAK/STAT signaling to maintain blood cell homeostasis. Whether dpia\textsubscript{s} also acts in concert with Gcm during nervous system development will also be the purpose of future analyses.

Our study identifies the fat body as a tissue that controls blood homeostasis. Until now, the hematopoietic role of the fat body has been associated to the humoral but not to the cellular response. During bacterial infection, circulating hemocytes signal the presence of pathogens to the fat body, which in turn produces antimicrobial peptides (for reviews, see Cherry and Silverman, 2003; MacDonald, 2006; Freeman, 2006). The scavenger activity displayed by *Drosophila* glia and hemocytes also represents a primary feature of microglia, the immune cells of mammalian Central Nervous System (CNS) (Hanisch and Kettenmann, 2007). Our data open new questions on the cellular and molecular pathways involved in non self-recognition. They also prompt us to speculate about the evolutionary origin of microglia and the possible role of gcm genes orthologs in this cell type. Indeed, despite the observation that murine gmc\textsubscript{e} genes are transcribed in the nervous system (Iwasaki et al., 2003; Kim et al., 1998), it is still unknown in which cell type they expressed. Considering our data, we propose that gcm orthologs may play a role in the microglia in normal or pathological conditions and call for revisiting their role in mammalian nervous system.

**Materials and Methods**

**Yeast two-hybrid assay**

The yeast strain L40 (MATa trp1 leu2 his3 lys2::lexA-HIS3 URA3::lexA-lacZ) (Vojtek et al., 1993), which includes the reporter genes HIS3 and lacZ, was used. Transcription of HIS3 gene can be measured by the ability of the strain to grow in the absence of histidine, which requires the HIS3 gene product. The other reporter gene, lacZ, provides a secondary assay of activation by the bait and activation-tagged proteins interacting with it, as well as quantitative information about the interaction. All transformations were performed using the lithium acetate method (Gietz et al., 1995). Sequence encoding the DNA binding domain (DBD), the nuclear localization signal (NLS) and the protein instability element (PEST) (Val-Tyr-Thr\textsuperscript{265}) of Gcm were PCR amplified using the following forward and reverse oligonucleotide primers:

- 5'-GAATTCGGGTTGCTATGTGTGGGCGTCG-3' and 5'-CTCGAGGGTGTACCATGTCGGCGTCG-3'. The PCR product (Gcm\textsubscript{1-261}) was subcloned in pGEM\textsuperscript{®}-T and digested with EcoRI and XhoI and then inserted in frame with the LexA DBD (LexA\textsuperscript{DBD}Gcm\textsubscript{1-261}) into pBTM116. The resulting fusion protein was used as bait to screen a *Drosophila* embryonic cDNA library (4-18h) made into pASV4 carrying the VP16 activation domain (VP16\textsuperscript{AB}) (Beckstead et al., 2001). Yeast two-hybrid screening followed the method of Le Douarin et al. (Le Douarin et al., 2001). β-Galactosidase assays on transformants of the L40 yeast strain were carried out as in (Seipel et al., 1992). To identify the Gcm domains required for interaction, we made two constructs: LexA\textsuperscript{DBD}Gcm\textsubscript{1-233} and LexA\textsuperscript{DBD}Gcm\textsubscript{1-188}, which lack the PEST or the PEST and NLS domains, respectively, using the following reverse oligonucleotide primers:

- 5'-CTCGAGCCCATCCTTTCTGCGGGTGC-3' and 5'-CTCGAGGAAAGTGCGCGTCTCCG-3'.

**Plasmid constructions**

Two Flag epitope tag (DYKDDDDK) were fused to the C-terminus of Gcm by overlapping PCR using the following forward and reverse oligonucleotide primers:

- 5'-TGCAATGGTGCGGAAATCCAGGCTTT-3' and 5'-CCGGGCTCATTTATCTTCGATCTCGGAATAT-3';

and degenerating axons (Awasaki et al., 2006; Freeman et al., 2003; MacDonald et al., 2006). These observations call for an ancestral, scavenger role, of glia and indicate that these cells could be considered as the neural equivalent of hemocyte populations present outside the nervous system. Furthermore, these two cell populations share other properties such as their capacity to proliferate and migrate (Holz et al., 2003; Soustelle and Giangrande, 2007b). In the future, the characterization of Gcm target genes will help to understand the molecular mechanisms as well as the signaling pathways involved in these common features between glia and hemocytes.

The scavenger activity displayed by *Drosophila* glia and hemocytes also represents a primary feature of microglia, the immune cells of mammalian Central Nervous System (CNS) (Hanisch and Kettenmann, 2007). Our data open new questions on the cellular and molecular pathways involved in non self-recognition. They also prompt us to speculate about the evolutionary origin of microglia and the possible role of gcm genes orthologs in this cell type. Indeed, despite the observation that murine gmc\textsubscript{e} genes are transcribed in the nervous system (Iwasaki et al., 2003; Kim et al., 1998), it is still unknown in which cell type they expressed. Considering our data, we propose that gcm orthologs may play a role in the microglia in normal or pathological conditions and call for revisiting their role in mammalian nervous system.
The wild-type C-terminus of gcm was removed from puAST-gcm upon SacII digestion and the Flag-tagged PCR product digested by SacII was inserted. HA epitope tag (YPYDVPDYA) was fused to the C-terminus of dplias isoform (gift from A. Betz) by overlapping PCR using the following forward and reverse oligonucleotide primers:

5' -GGTACCTCGTAATCTGGAAACTGATTTGATAGCTAGGAGT-3' and 5' -GGTACCTGATGAACTTCTGGAAACTGATTTGATAGCTAGGAGT-3'. The PCR product was digested by NotI and KpnI and cloned into puAST.

**DNA transfections, immunoprecipitations and Western Blot assay**

_Drosophila_ S2 cells were cultured in Schneider cell medium (Gibco BRL/Invitrogen) + 10% fetal calf serum. Transient transfection was performed using effecene (Qiagen), according to the manufacturer’s instructions, using 2 μg of DNA containing the following: 500 ng of reporter DNA (puAST-encGFP), 500 ng of pMET-Gal4 (expresses Gal4 under control of the copper-inducible metallothionine promoter) and 500 ng of each expression vector (puAST-gcmFL and puAST-dpliasFL). Transgenes expression was induced 24 h later by adding copper sulfate at 1 mM. Cells were harvested 48 hours after transfection in cold PBS, pelleted, washed, and resuspended in lysis buffer (400 mM KCl, 20 mM Tris-HCl (pH 7.5), 20% glycerol, 5 mM DTT, 0.4 mM PMSF). After three cycles of freeze-thaw in liquid nitrogen, the resulting cell lysate was diluted four times with the lysis buffer without KCl to give a final concentration of 100 mM KCl and then centrifuged for 5 min at 13000 rpm. The protein concentration was determined by the Bradford assay. Three hundred micromolars of protein extract, adjusted to 1 ml with RIPA buffer (PBS, 0.1% SDS, 0.5% sodium deoxycholate, 0.5% NP-40), were incubated for 2 hours at 4°C with 30 μl of anti-Flag M2 Affinity Gel (Sigma A-1205). The agarose beads were then recovered by centrifugation and washed three times with 1 ml of RIPA buffer. The adsorbed proteins were dissociated by boiling for 5 min in 30 μl of Laemmli buffer, resolved by SDS-polyacrylamide gel electrophoresis and electrotransferred onto a nitrocellulose filter. Blocking, washing, and incubation of the membrane with antibodies were carried out in PBS containing 5% skimmed dry milk, 0.1% Triton X-100. blots were further incubated with horseradish peroxidase-linked rabbit anti-mouse immunoglobulins (Jackson Immunoresearch, 1/5000).

**Hemocyte counting**

Larvae were staged according to procedures described in (Andres and Thummel, 1994). Staged larvae were first washed in PBS, bled onto glass slides and labeled with anti-GFP, anti-peroxidasin (Nelson et al., 1994) and DAPI according to the procedure described in (Asha et al., 2003). Circulating hemocytes were manually counted and classified as either plasmatocytes or lamellocytes, based on their morphology and expression of plasmatocyte marker peroxidaxin (data not shown). Circulating crystal cells were not counted separately. Instead, we counted sessile crystal cells in the last two posterior dorsal segments of third-instar larvae (Duvic et al., 2002). They were visualized by heating the larvae for 10 min at 60°C in a water bath. Total circulating hemocytes as well as crystal cells were counted from at least ten larvae of each genotype.

Data were compared using one-way analysis of variance (ANOVA) followed by Dunnett’s post hoc test to determine statistical differences after multiple comparisons (SPSS, SPSS Inc., Chicago, IL). A probability (p) value of less than 0.05 was considered significant.

**Fly strains and transgenic lines**

Wild-type strain was _Sevelen_. _gcm_ mutant described in (Kammerer and Giangrande, 2001; Vincent et al., 1996) carries a small deletion in the regulatory region of _gcm_ and _dplias_ mutant carries a leucine to methionine change at aminoacid 327 (Hari et al., 2001). Recombinant between _gcm_ and _dplias_ was created and used for genetic experiments. Flies were raised at 25°C on standard medium, except for experiments done with tub-gal80P transgene (Bloomington stock center) and genetic experiments shown in Table 1 (see below). Transgenic line carrying _serpent-gal4_ (srp-gal4) construct was obtained from M. Meister (Crozatier et al., 2004). _pp-gal4_ was obtained from P. Leopold (Colombani et al., 2005). _UAS-gcm_ was used to block _gcm_ function and _UAS-gcm_ line as a control (Soustelle et al., 2004). _UAS-mCD8GFP_ targets GFP to the membrane (Bloomington stock center). _UAS-encGFP_ targets GFP to nucleus and cytoplasm (gift of C. Desplan). A _serpent-gal4,tub-gal80°,UAS-encGFP,UAS-gcm_ line was established and maintained at 18°C. _UASRNAi_ stocks (os, upd3, dome, hop and stat92E) were obtained from the Vienna Drosophila RNAi Center (VDRC). _serrate, hemoclot, domels_gal4 (all gifts from U. Banerjee), lozenge, hemose and collagen-gal4 (all three from the Bloomington stock center) were used for targeted expression of the _Gcm_ promoter. _A87a_ and _gcm-gal4_ enhancer trap lines carry a P-element inserted into the _gcm_ promoter (Jones et al., 1995; Vincent et al., 1996). _hop_ mutant was obtained from Bloomington Stock center. Larvae were analyzed using the Leica Macro-FluoTM.

**Preparation and separation of larval cells**

First instar larvae of the _serpent-Gal4, UAS-encGFP_ genotype were dissociated in S2 medium (Schnier's insect medium (Gibco BRL) supplemented with 10% fetal calf serum and 3 mM EDTA) using a homogenizer by gentle movements. Cells were washed two times in S2 medium, pelleted at 1000 rpm for 10 min at 4°C, resuspended in 3 ml of S2 medium and stored on ice. Cells were separated using fluorescence-activated cell sorting (FACS; FACSDiVa, Becton Dickinson) and populations analyzed for GFP expression by flow cytometry to verify sample purity. Samples showing purity lower than 90% were discarded.

**RT-PCR experiments**

RNA from 106 separated cells was prepared using Trizol reagent (Gibco BRL) according to the manufacturer’s instructions. The RNA pellet was dried and dissolved in RNA-free water, then quantified by NanoDrop ND-1000 spectrophotometer. Reverse transcription reactions were performed using the Prototscript First Strand cDNA Synthesis kit (New England Biolabs) using 1 μg total RNA in a 50 μl reaction volume with random primers. To avoid false positive results due to amplification of contaminating genomic DNA in the cDNA preparation, we used primers spanning exon-exon junctions. Primers for polymerase chain reaction (PCR) were as follows:

**gcm**

5'-GAAGCAGCAGGGCAAACAGT-3' (forward) and 5'-ATTCCTGGGCAAACATTAGC-3' (reverse);

**gcm2**

5'-TGCCCGAAAGATCGCTCATT-3' (forward) and 5'-TTGCATGGTAGATGGGATAG-3' (reverse);

**dplias**

5'-ACATTGCGCAAGCTCACCAG-3' (forward) and 5'-GCCTGGTGCGGCAACCAG-3' (reverse);

**lozenge**

5'-TGCCAGGTCTACAAGCCGAA-3' (forward) and 5'-CTAGAGCTCTTGAAGTTAGGG-3' (reverse);

**serpent**

5'-AATGCGATGCGGCTGTAACA-3' (forward) and 5'-AGGACGACACACAGCTATG-3' (reverse). PCR conditions were as follows: denaturation at 95°C for 45 sec, annealing at 65°C for 1 min, polymerization at 72°C for 1 min (35 cycles), and terminal extension at 72°C for 10 min.

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