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A novel role of the glial fate determinant *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 *dpias*, a gene involved in post-embryonic hematopoiesis. *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 *gcm* interacts genetically with 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 *dpias*-dependent tumor formation, act downstream of Gcm. Altogether, this study identifies an unpredicted role of Gcm, dictated by its cofactor 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: *Drosophila melanogaster, glide/gcm, dpias, JAK/STAT, hematopoiesis*

Introduction

Glial cell deficient/glial cells missing (glide/gcm), referred to throughout the text as *gcm*, codes for a transcription factor that is expressed in embryonic glial precursors (Hosoya *et al.*, 1995; Jones *et al.*, 1995; Vincent *et al.*, 1996). Lack of *gcm* leads to loss of most lateral glia, which transform into neurons, while ectopic *gcm* pan-neural expression leads to differentiation of supernumerary glia at the expense of neurons (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). While *gcm2*, the homolog of *gcm*, is also expressed in embryonic glial precursors, its mutation does not induce detectable glial defects, likely due to its very low levels of expression (Alfonso and Jones, 2002; Kammerer and Giangrande, 2001). Expression of the *gcm* gene as well as RNA and protein processing are tightly regulated (Akiyama *et al.*, 1996; Akiyama-Oda *et al.*, 1999; Bernardoni *et al.*, 1999; Jones, 2005; Kammerer and Giangrande, 2001; Miller *et al.*, 1998; Ragone *et al.*, 2003; Soustelle *et al.*, 2008).

In addition to these transcriptional and post-transcriptional regulatory mechanisms, the activity of Gcm is mediated by cellular context (Miller *et al.*, 1998; Ragone *et al.*, 2003). A cell-specific factor, Huckebein, interacts directly with Gcm and triggers Gcm autoregulation in the thoracic neuroblast lineage 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 *et al.*, 2006). Finally, while *gcm* is necessary and sufficient to induce embryonic glia, it is clear that this gene is involved in other developmental pathways as well. *gcm* is expressed and required in embryonic hemocytes (Alfonso and Jones, 2002; Bataille *et al.*, 2005; Bernardoni *et al.*, 1997) and tendon cells (Soustelle *et al.*, 2004); it is also necessary in specific neuronal and glial lineages of the post-embryonic nervous system

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

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(Chotard *et al.*, 2005; Soustelle and Giangrande, 2007b; Soustelle *et al.*, 2007; Yoshida *et al.*, 2005). Importantly, *gcm*-dependent differentiation of these cell types relies on the activation of mutually exclusive molecular pathways (for review (Soustelle and Giangrande, 2007a).

All these data indicate that Gcm activates distinct target genes upon interaction with cell/tissue-specific cofactors to induce distinct differentiation programs.

We here identify several potential partners of Gcm by a yeast two-hybrid approach. Surprisingly, one such partner is coded by *dpias*, a gene required in post-embryonic hematopoiesis, a process that has not been so far described as being Gcm-dependent. We also show that blocking Gcm function in tissues of the post-embryonic immune system triggers a melanotic tumor phenotype, similar to what is observed in *dpias* mutant larvae (Betz *et al.*, 2001; Hari *et al.*, 2001). Altogether, this study identifies *dpias* as a cofactor 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 twohybrid screen we identified dpias (Protein Inhibitor of Activated STAT), a protein that controls post-embryonic hematopoiesis. This allowed us to reveal an upredicted 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 codes 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 anlagen 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 1

GENETIC INTERACTION BETWEEN *GCM* AND *DPIAS*

Genotype	Presence of melanotic tumors
$dpias^{1}$ / $dpias^{1}$	Embryonic lethal
gcm^{26} / gcm^{26}	Embryonic lethal
gcm-gal4 / gcm-gal4	NO
gcm-gal4 / gcm^{26}	NO
gcm-gal4 / $dpias^{1}$	NO
$dpias^{1}$, gcm^{26} / $dpias^{1}$, gcm^{26}	Embryonic lethal
gcm-gal4 / $dpias^{1}$, gcm^{26}	YES L2/L3 (1/200)

The genotypes of analysed larvae are described in the left column. Right column describes the observed phenotypes, note that $dpias^{1}$, gcm^{26} and $dpias^{1}$, gcm^{26} homozygous animals die at embryonic stages. Removing one copy of *dpias* in combination with a *gcm* hypomorphic allele (*gcm-gal4/*$dpias^{1}$) 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/*$dpias^{1}$,gcm^{26}), indicating that *gcm* and *dpias* interact genetically. All experiments were performed at 29°C.

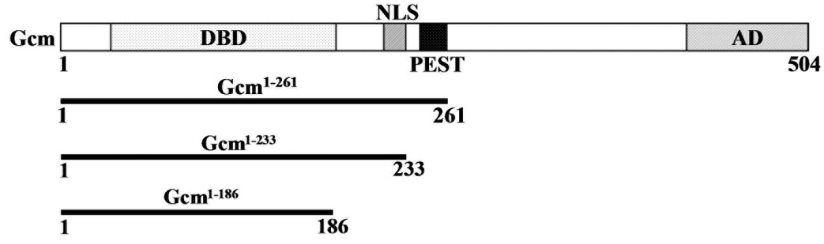
hematopoietic organ, the lymph gland, which produces an additional type of hemocytes, the lamellocytes, in response to parasitism (Sorrentino *et al.*, 2002). Prohemocytes of the lymph gland, the precursors of hemocytes, start hyperproliferating and produce lamellocytes, which aggregate in high number 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* (*gcm*^{26}) with *dpias*^{1} mutation. Since the *dpias*^{1} and the *gcm*^{26} 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 *dpias*^{1}, *gcm*^{26}, and *gcm-gal4* lines nor in the recombinant *gcm*^{26},*dpias*^{1} (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 *gcm*^{26} 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.

A



B

Candidates	Product/Function	Gcm$^{1-261}$	Gcm$^{1-233}$	Gcm$^{1-186}$
CG1244	Predicted transcription factor, unknown function [1]	+++	+++	++
CG2199	Predicted transcription factor, unknown function [1]	+++	+++	+++
CG2926	Predicted transcription factor, unknown function [1]	+++	+	-
CG9715	Predicted transcription factor, unknown function [1]	+++	++	+
Charlatan	Transcription factor, nervous system development [2]	+++	+++	+++
dpias	DNA binding protein, negative regulation of JAK-STAT pathway [3]	+++	+/-	+/-
Karyopherin-α1	Carrier protein, protein import into nucleus [4]	+++	+++	-
Mical	Actin binding protein, axon guidance [5]	+++	+	+/-
Pendulin	Carrier protein, protein import into nucleus [6]	+++	+++	-
Pleiohomeotic like	Transcription factor, transcription regulator activity [7]	+++	+++	+
Slik 1	Receptor signaling protein, regulation of protein localization [8]	+++	-	-
Uba2	Ubiquitin activating enzyme, sumoylation [9]	+++	+/-	+/-

C

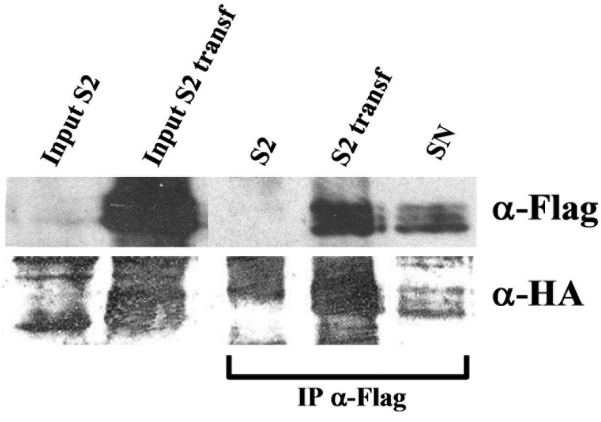


Fig. 1. Gcm and its putative cofactors. (A) *Schematic representation of Gcm transcription factor structure. DBD, DNA-binding domain; NLS, nuclear localization signal; PEST, rapid turnover signature; AD, activation domain. Black lines indicate the different baits used in the screen to identify the interaction 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 putatives 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.*

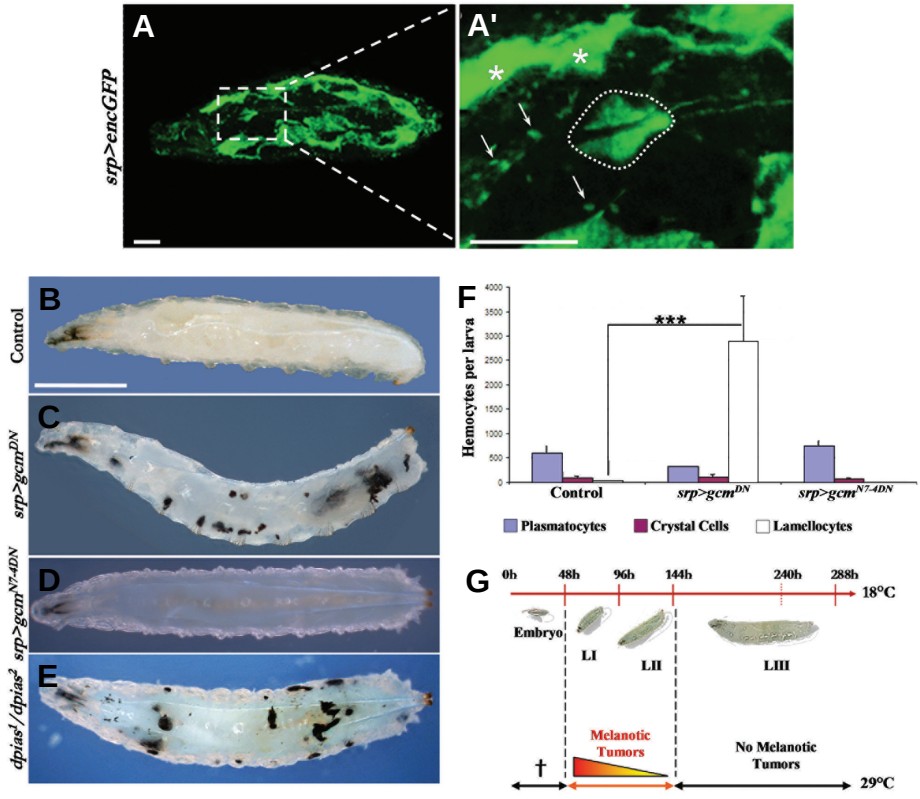


Fig. 2. *gcm* or *dpias* loss of function induces melanotic tumors. (A,A') serpent-gal4,UAS-encGFP (srp>encGFP) *first instar larva.* **(A')** *shows the region delimited in the dashed box in* **(A)**. *GFP is present in the lymph gland (encircled dotted line), in all hemocytes (arrows) and in the fat body (asterisks).* **(B-E)** *Third instar larvae of the following genotypes* srp>encGFP *(control, B),* serpent-gal4,tub-gal80^{ts},UAS-encGFP,UAS-gcm^{DN} (srp>gcm^{DN}, *C),* serpent-gal4,tub-gal80^{ts},UAS-encGFP,UAS-gcm$^{N7\text{-}4DN}$ (srp>gcm$^{N7\text{-}4DN}$, *D) and* dpias^{1}/dpias^{2} **(E)**. *Note that loss of function of* gcm *(C) or* dpias *(E) induces similar melanotic tumor phenotypes.* **(F)** *Hemocytes from at least ten different LIII larvae were examined for each genotype (control,* srp>gcm^{DN} *and* srp>gcm$^{N7\text{-}4DN}$*), and the average number of the three hemocyte subsets are presented. Bars indicate the standard deviation. Note that* gcm *loss of function induces massive differentiation of lamellocytes; ANOVA analysis shows a significant difference in lamellocyte number ($p<0.001$) between* srp>gcm^{DN} *and all other genotypes.* **(G)** *Schematic representation of* Drosophila *development at 18°C and the phenotypes induced upon shifting* srp>gcm^{DN} *animals at restrictive temperature (29°C) during different developmental stages. Note that when expression of* gcm^{DN} *construct is induced in LI, 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 the 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.*

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 Gal80 protein (Gal80^{ts}), a repressor of Gal4 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; Lemaitre 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 LI), using the line *serpent-gal4,tub-gal80^{ts},UAS-gcm^{DN},UAS-encGFP* (*srp>gcm^{DN}*). 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 Gal80 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, *gcm$^{N7\text{-}4DN}$*, which carries a mutation abolishing DNA binding (Miller *et al.*, 1998; Soustelle *et al.*, 2004; Vincent *et al.*, 1996). *serpent-gal4,tub-gal80^{ts},UAS-gcm$^{N7\text{-}4DN}$,UAS-encGFP* larvae expressing the *gcm$^{N7\text{-}4DN}$* construct do not show any phenotype (Fig.2D), confirming the specificity of the defects observed in *srp>gcm^{DN}* 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>gcm$^{N7\text{-}}$*

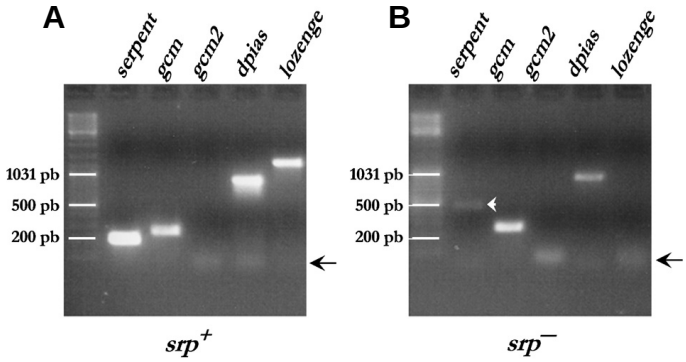


Fig. 3. *gcm* is expressed in cells of the immune system. *RT-PCR experiments on GFP-positive or GFP-negative cells collected from* serpent-gal4,UAS-encGFP *larvae upon Fluorescence-activated cell sorting (FACS). Note that* serpent*-positive cells express* serpent *(expected size: 197 bp),* lozenge *(expected size: 1224 bp),* dpias *(expected size: 839 bp) and* gcm *(expected size: 245 bp)* **(A)** *whereas* serpent*-negative cells only express* dpias *and* gcm **(B)**. gcm2 *is not detected (expected size: 351 bp). Black arrows in (A,B) show the primers. White arrowhead in (B) shows an amplification product issued from* serpent *genomic DNA (expected size: 450 bp).*

^{4DN}) do not contain any lamellocyte (Fig.2F), *srp>gcm^{DN}* 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>gcm^{DN}* 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 *gcm^{DN}* construct is induced after LI and no tumor was observed upon *gcm^{DN}* 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, a stage making *in situ* hybridization experiments difficult, we performed RT-PCR experiments on cells separated by fluorescence 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) *serpent*-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 L1 under a fluorescent

A

Expression domain / Driver	Lymph gland	Hemocytes			Fat body	Other tissues
		Prohemocytes	Crystal cells	Plasmatocytes		
serpent	CZ, MZ, PSC	+	+	+	+	-
gcm	-	-	-	-	-	Nervous system, peritracheal cells
peroxydasin	CZ	+	-	+	-	-
lozenge	CZ	+	+	-	-	-
collagen	-	+	+	+	+	-
serrate	PSC	-	-	-	-	Nervous system, peritracheal cells, gut
hemolectin	-	+	-	+	-	-
domeless	MZ	-	-	-	-	Salivary glands, gut, trachea
hemese	CZ, MZ, PSC (weak)	+	+	+	-	Salivary glands, gut
pumpless	-	-	-	-	+	-

B

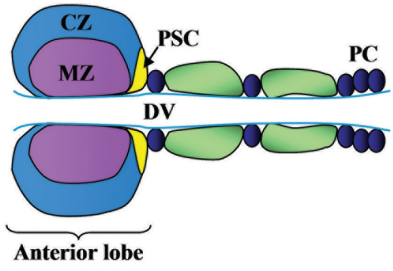
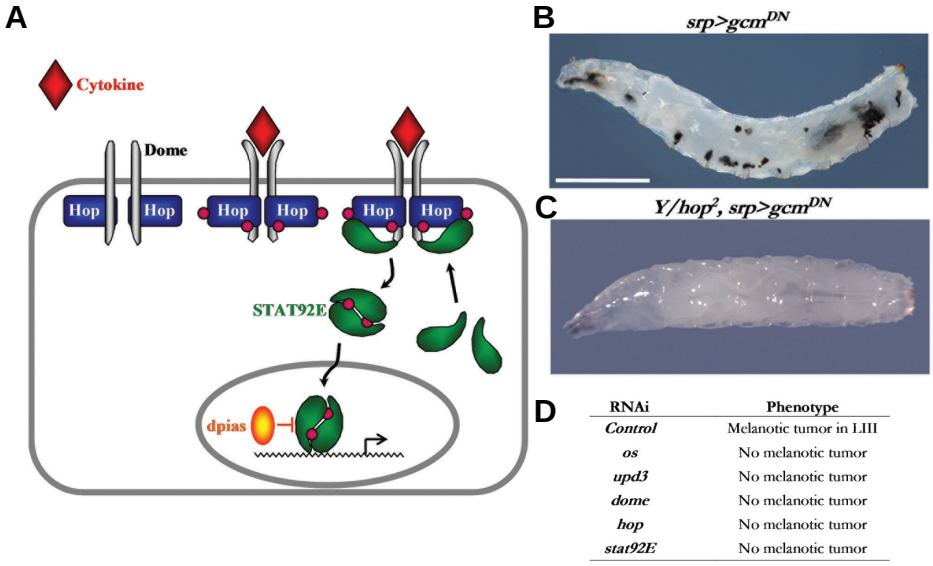


Fig. 4. Expression profile of *gal4* lines used in this study. **(A)** *For each* gal4 *driver, a cross with a UAS-GFP reporter line was performed and analyzed under a macroscope. Columns report the GFP expression profile in the immune system (lymph gland, hemocytes and fat body) and in other tissues in LI larvae. Red rows indicate drivers that induce melanotic tumors upon crosses with the* UAS-gcm^{DN} *line. In the case of the* collagen-gal4 line, *we induced gcm^{DN} expression at LII as induction at LI triggers lethality.* **(B)** *Schematic diagram of third instar lymph gland. The lymph gland flanks the dorsal vessel (DV) and each lobe is separated by pericardial cells (PC). The anterior lobe displays three distinct zones: the cortical zone (CZ), where maturing hemocytes are present; the medullary zone (MZ), which contains prohemocytes; and the posterior signaling center (PSC), which contains a small cluster of signaling cells. Posterior lobes contain immature hemocytes. Note that in LI larvae, only anterior lobes are present (as shown in Fig. 2), posterior lobes appearing at later stages.*



RNAi	Phenotype
Control	Melanotic tumor in LIII
os	No melanotic tumor
upd3	No melanotic tumor
dome	No melanotic tumor
hop	No melanotic tumor
stat92E	No melanotic tumor

Fig. 5. The melanotic tumor phenotype induced by *gcm* loss of function requires the JAK/STAT pathway. (A) *Schematic representation of the JAK/STAT pathway. Following binding of a cytokine (Os or Upd) to its cognate receptor Dome, receptor-associated Hop is activated. STAT92E proteins dimerize upon tyrosine phosphorylation by the Hop kinase and subsequently translocate into the nucleus, where they modulate expression of target genes. dpias interacts with activated STAT92E dimers and inhibits their DNA binding or their transactivating capacity.* **(B,C)** Y/+,serpent-gal4,tub-gal80^{ts},UAS-encGFP,UAS-gcm^{DN} *larva displays melanotic tumors (C) whereas* Y/hop^{2},serpent-gal4,tub-gal80^{ts},UAS-encGFP,UAS-gcm^{DN} *larva does not (C).* **(D)** *To analyze the effects of JAK/STAT pathway down-regulation, we crossed the* serpent-gal4,tub-gal80^{ts},UAS-encGFP,UAS-gcm^{DN} *line with a UAS-RNAi transgenic construct for each member of the JAK/STAT pathway (*os, Upd3, dome, hop, stat92E*). While the control line induces melanotic tumors with 100% penetrance (*serpent-gal4,tub-gal80^{ts},UAS-encGFP,UAS-gcm^{DN}*), tumors are absent in* serpent-gal4,tub-gal80^{ts},UAS-encGFP,UAS-gcm^{DN} *larvae also carrying an RNAi transgenic construct for one member of the JAK/STAT pathway. Note that for each RNAi construct, we analyzed between forty and sixty larvae and never found any tumor, indicating a 100% penetrance of the tumor suppression phenotype.*

macroscope. We crossed *UAS-gcm^{DN}* with *peroxydasin-gal4, lozenge-gal4, serrate-gal4, hemolectin-gal4, domeless-gal4* and *hemese-gal4* drivers, which express Gal4 in specific territories of the immune system (lymph gland, prohemocytes, circulating hemocytes, see Fig.4B). By using these lines, we did not observe any melanotic tumor phenotype (Fig.4A), excluding a role of Gcm in these cell types. In contrast, expression of *gcm^{DN}* in the fat body by using the *collagen-gal4* line, induces the formation of melanotic tumors (Fig.4A), similar to the phenotype induced by the *serpent-gal4* line. We confirmed this phenotype by expressing *gcm^{DN}* specifically in the larval fat body by using the *ppl-gal4* line (data not shown), a fat body specific driver (Colombani *et al.*, 2005).

Altogether, these data strongly suggest that *gcm* is required in the fat body during early larval life and that its misregulation leads to lamellocyte production and melanotic tumor formation.

Gcm genetically interacts with JAK/STAT signaling pathway

dpias was identified as a cofactor for Gcm and melanotic tumors were observed in mutant conditions for both genes. In *Drosophila*, *dpias* is known to play a role in the modulation of the JAK/STAT signal transduction pathway (Betz *et al.*, 2001). This pathway is activated by the binding of an extracellular ligand (Unpaired (Upd) or Outstretched (Os) cytokines) to its receptor (Domeless (Dome)) (Fig.5A) (Arbouzova and Zeidler, 2006). This induces the intracellular recruitment and the phosphorylation of Hopscotch (Hop), the only kinase that is described as being involved in the JAK/STAT pathway in flies (Binari and Perrimon, 1994; Perrimon and Mahowald, 1986). Subsequently, the phosphorylated Hop kinase acts onto cytoplasmic STAT, which translocates to the nucleus and activates its target genes. The role of *dpias* is to bind to STAT and inhibit its function. Interestingly, mutants for several components that activate the JAK/STAT pathway display a melanotic tumor phenotype associated with massive lamellocyte differentiation (Betz *et al.*, 2001; Hari *et al.*, 2001; Harrison *et al.*, 1995; Luo *et al.*, 1995). Moreover, the tumor phenotype induced by the *hop^{Tum-1}* allele, which codes for a constitutively active kinase (Harrison *et al.*, 1995; Luo *et al.*, 1995), is rescued by ectopic expression of *dpias* (Betz *et al.*, 2001).

To determine whether the JAK/STAT pathway is required for lamellocyte production and melanotic tumor formation induced by loss of *gcm*, we performed an epistatic analysis by using the *hop^{2}* null mutation. Contrary to what was observed in *srp>gcm^{DN}* larvae (Fig.5A), none of the *hop^{2}/Y,srp>gcm^{DN}* larvae shows melanotic tumors or lamellocyte production (Fig.5B). Importantly, the two other phenotypes observed in *srp>gcm^{DN}* larvae (fat body disintegration and developmental delay after the LIII stage) are also completely suppressed by the *hop^{2}* mutation. Altogether, these data show that *gcm* acts upstream of *hop* to control post-embryonic hematopoiesis.

To further confirm that the JAK/STAT pathway is required for melanotic tumor formation induced by loss of Gcm, we knocked down five members (*upd3, os, dome, hop, stat92E*) of the JAK/STAT pathway by using transgenic lines carrying RNAi constructs. The down-regulation of each of these genes in *srp>gcm^{DN}* animals (shift at 29°C starting from LI, as above) completely suppresses the melanotic tumor phenotype (Fig.5D), clearly showing that the JAK/STAT pathway acts downstream of *gcm*. Finally, the delay in development as well as the fat body disintegration observed in *srp>gcm^{DN}* larvae are also rescued by the down-regulation of these members of the JAK/STAT pathway.

We identified several potential partners of Gcm, one of them being *dpias*, a gene required in post-embryonic hematopoiesis.

Blocking Gcm function in the fat body triggers a melanotic tumor phenotype, similar to what is observed in *dpias* mutant larvae.

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*.

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 dpias 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-dpias complex in the fat body and how this molecular cascade acts on JAK/STAT signaling to maintain blood cell homeostasis. Whether dpias 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, 2006; Leclerc and Reichhart, 2004; Lemaitre and Hoffmann, 2007). Our study identifies a role in the cellular response as the production of lamellocytes is induced by blocking Gcm activity in the fat body. These data therefore indicate that the fat body is necessary to maintain cellular blood homeostasis. Recent studies have shown that the fat body also plays a crucial role during development by controlling larval growth rates and the final size of adult flies. For example, a Target of Rapamycin (TOR)-mediated nutrient sensor present in the fat body detects nutrient availability and regulates insulin signaling in peripheral tissues (Mirth and Riddiford, 2007). Altogether, these data indicate that the fat body, which is considered as the '*Drosophila* liver', acts as an integrator center for different processes relying on distinct signaling cascades.

Microarray studies have shown that glia- and hemocyte-specific Gcm pathways share common target genes (Altenhein *et al.*, 2006; Freeman *et al.*, 2003). One such example is provided by the transmembrane protein Draper, which is expressed in embryonic glia and in plasmatocytes. Interestingly, glial expression of this macrophage receptor mediates engulfment of apoptotic neurons 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 *gcm* genes are transcribed in the nervous system (Iwasaki *et al.*, 2003; Kim *et al.*, 1998), it is still unknown in which cell type are 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 by 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^{2}-Thr^{263}) of Gcm were PCR amplified using the following forward and reverse oligonucleotide primers:
5'-GAATTCGTTTTGAACGGCATGCCTAT-3' and
5'- CTCGAGGGTGCTATGTGTGGGCGTCG-3'. The PCR product (Gcm^{1-261}) was subcloned in pGEM®-T and digested with EcoRI and XhoI and then inserted in frame with the LexA DBD ($LexA^{DBD}Gcm^{1-261}$) 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 ($VP16^{AD}$) (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: $LexA^{DBD}Gcm^{1-233}$ and $LexA^{DBD}Gcm^{1-186}$, which lack the PEST or the PEST and NLS domains, respectively, using the following reverse oligonucleotide primers:
5'- CTCGAGACCCATCCTTCTGCGCTTGC-3' and
5'- CTCGAGCAAAAGTCGCCTGGCTTCCG-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'-TTGCAATGGTCGCTTGGAAATCCAGGCTT-3' and
5'-CCGCGGTCATTTATCGTCATCGTCTTTGTAGTCTTTATCGTCATCGTCTTTGTAGTCGCAATAGATGGGATCCGTGCTGTTGAC-3'.

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 dpias^{537} isoform (gift from A. Betz) by overlapping PCR using the following forward and reverse oligonucleotide primers:
5'-GCGGCCGCATGGTGCAGATGCTTCGAGT-3' and
5'-GGTACCTCATGCGTAATCTGGAACATCGTATGGGTAAGGACTATC TAGAAGATCAATTACGGAAT-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 effectene (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-gcm^{FLAG} and pUASt-dpias^{HA}). 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 cleared by centrifugation for 5 min at 13000 rpm. The protein concentration was determined by the Bradford assay. Three hundred micrograms 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 and 0.1 % Triton X-100. Mouse anti-Flag M2 antibody (Sigma F3165, 1/5000) and mouse anti-HA antibody (1/2000) were used to detect the Flag-tagged Gcm and the the HA-tagged dpias, respectively. After washing (PBS, 0.1 % Triton X-100) and blocking (PBS, 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 peroxidasin (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^{26}* mutant described in (Kammerer and Giangrande, 2001; Vincent *et al.*, 1996) carries a small deletion in the regulatory region of *gcm*. *dpias^{1}* mutant carries a leucine to methionine change at aminoacid 327 (Hari *et al.*, 2001). Recombinant between *gcm^{26}* and *dpias^{1}* was created and used for genetic experiments. Flies were raised at 25°C on standard medium, except for experiments done with *tub-gal80^{ts}* 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). *ppl-gal4* was obtained from P. Leopold (Colombani *et al.*, 2005). *UAS-gcm^{DN}* was used to block *gcm* function and *UAS-gcm^{N7-4DN}* 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^{ts},UAS-encGFP,UAS-gcm^{DN}* line was established and maintained at 18°C. *UAS-RNAi* stocks (*os*, *upd3*, *dome*, *hop* and *stat92E*) were obtained from the Vienna *Drosophila* RNAi Center (VDRC). *serrate, hemolectin, domeless-gal4* (all gifts from U. Banerjee), *lozenge, hemese* and *collagen-gal4* (all three from the Bloomington stock center) were used for targeted expression of the *Gcm^{DN}* construct. *rA87* and *gcm-gal4* enhancer trap lines carry a P-element inserted into the *gcm* promoter (Jones *et al.*, 1995; Vincent *et al.*, 1996). *hop^{2}* mutant was obtained from Bloomington Stock center. Larvae were analyzed using the Leica Macro-Fluo^{TM}.

Preparation and separation of larval cells

First instar larvae of the *serpent-Gal4, UAS-encGFP* genotype were dissociated in S2 medium (Schneider'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 10^6 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 Protoscript 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'- ATTCCCTGGCCAACATTACG-3' (reverse);
gcm2 5'-TCGCCAAGAAGTCGGTCATT-3' (forward) and
5'-TTGCATGGTAGATGGGATAG-3' (reverse);
dpias 5'-ACATTCGCAACAGCTCCAAG-3' (forward) and
5'-GTCCTGTGCTGGGGACAAAT-3' (reverse);
lozenge 5'-TGCCAGGTCTACAAGCCGAA-3' (forward) and
5'-CTGAGCTCTTGAAGTTAGGG-3' (reverse);
serpent 5'-AATGCATGCGGCCTGTACTA-3' (forward) and
5'-AGGACGACACCAACGTTATG-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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