© 2016. Published by The Company of Biologists Ltd | Development (2016) 143, 180 doi:10.1242/dev.133835

CORRECTION

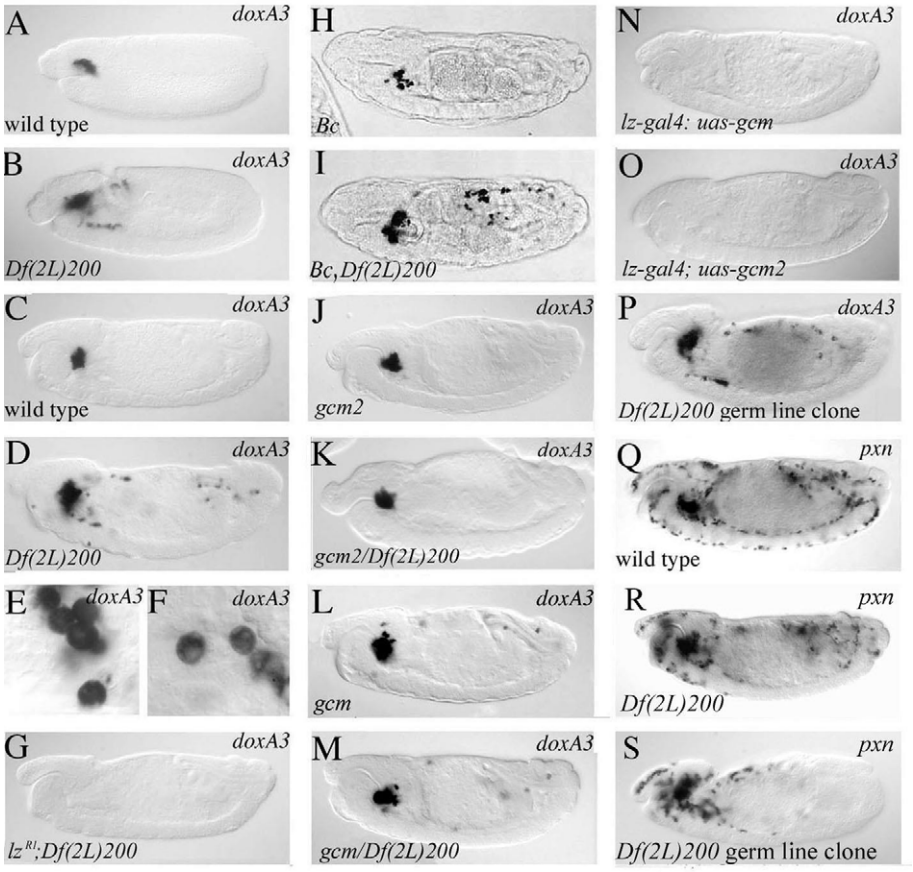
Resolving embryonic blood cell fate choice in *Drosophila*: interplay of GCM and RUNX factors

Laetitia Bataillé, Benoit Augé, Géraldine Ferjoux, Marc Haenlin and Lucas Waltzer

There was an error published in Development **132**, 4635-4644.

In Fig. 2, the incorrect image was shown in panel K. Owing to an error during figure assembly, Fig. 2K is a duplication of the wild-type embryo image shown in Fig. 5A. The corrected Fig. 2 appears below.

This error does not affect the conclusions of the paper. The authors apologise to readers for this mistake.



Resolving embryonic blood cell fate choice in *Drosophila*: interplay of GCM and RUNX factors

Laetitia Bataillé, Benoit Augé, Géraldine Ferjoux, Marc Haenlin* and Lucas Waltzer

Centre de Biologie du Développement, CNRS UMR 5547, 118 route de Narbonne, 31062 Toulouse, France
*Author for correspondence (e-mail: haenlin@cict.fr)

Accepted 9 August 2005

Development 132, 4635-4644
Published by The Company of Biologists 2005
doi:10.1242/dev.02034

Summary

The differentiation of *Drosophila* embryonic blood cell progenitors (prohemocytes) into plasmatocytes or crystal cells is controlled by lineage-specific transcription factors. The related proteins Glial cells missing (GCM) and GCM2 control plasmatocyte development, whereas the RUNX factor Lozenge (LZ) is required for crystal cell differentiation. We have investigated the segregation process that leads to the formation of these two cell types, and the interplay between LZ and GCM/GCM2. We show that, surprisingly, *gcm* is initially expressed in all prohemocytes but is rapidly downregulated in the anterior-most row of prohemocytes, which then initiates *lz* expression. However, the *lz*+ progenitors constitute a mixed-lineage population whose fate depends on the relative levels of LZ and GCM/GCM2. Notably, we demonstrate that GCM/GCM2 play a key role in controlling the size of the crystal cell population by inhibiting *lz* activation and maintenance. Furthermore, we show that prohemocytes are bipotent progenitors, and that downregulation of *gcm/gcm2* is required for *lz*-induced crystal cell formation. These results provide new insight into the mechanisms controlling *Drosophila* hematopoiesis and establish the basis for an original model for the resolution of the choice of blood cell fate.

Key words: RUNX, GCM, Hematopoiesis, Cell fate choice, *Drosophila*

Introduction

Understanding how cell lineages are established remains a central task in developmental biology and particularly in the field of hematopoiesis. Strikingly, it has become clear over the past few years that several aspects of hematopoiesis have been conserved between *Drosophila* and man (Evans et al., 2003). Thus, *Drosophila* may provide a valuable model system with which to gain insight into the mechanisms of blood cell lineage segregation in vivo. As in vertebrates, hematopoiesis in the fruit fly occurs in two waves: blood cell progenitors arise from the head mesoderm in the early embryo and from a specialised organ, the lymph gland, in the larva (Holz et al., 2003). These progenitors (prohemocytes) give rise to three terminally differentiated cell types (collectively called hemocytes), related to the vertebrate myeloid lineages: plasmatocytes, crystal cells and lamellocytes (Meister, 2004). Approximately 95% of hemocytes are plasmatocytes, which function as macrophages, engulfing apoptotic cells and small pathogens such as bacteria (Sears et al., 2003; Tepass et al., 1994). Crystal cells constitute a smaller population of blood cells (about 5%) that participate in melanisation, an insect-specific process involved in wound healing and the encapsulation of foreign invaders (Rizki et al., 1980). Finally, the lamellocytes encapsulate foreign bodies too large to be engulfed by macrophages (Crozatier et al., 2004). They are only generated during larval hematopoiesis, under specific conditions such as parasitisation of the larvae by wasp eggs.

Despite the evolutionary distance between *Drosophila* and vertebrates, many of the molecular pathways governing hematopoiesis have been conserved (Evans et al., 2003). In particular, transcription factors of the GATA, FOG and RUNX families, which regulate several steps of hematopoiesis in vertebrates, also control *Drosophila* hematopoiesis (Fossett et al., 2001; Lebestky et al., 2000; Rehorn et al., 1996). In the embryo, all prohemocytes express the GATA transcription factor Serpent (SRP), which is required for blood cell precursor specification and maintenance (Rehorn et al., 1996). From this pool of prohemocytes, two populations of hematopoietic cells will emerge, plasmatocytes and crystal cells (Lebestky et al., 2000). SRP participates in their differentiation and remains expressed in the differentiated blood cells (Fossett et al., 2003; Waltzer et al., 2002). Furthermore, key lineage-specific factors are required for the differentiation into these two cell types. On the one hand, the RUNX transcription factor Lozenge (LZ) forms a functional complex with SRP to induce crystal cell formation (Waltzer et al., 2003), and in a *lz* mutant no crystal cells appear (Lebestky et al., 2000). On the other hand, the two related transcription factors Glial cells missing (GCM) and GCM2 (also known as Glide and Glide2) are jointly required for plasmatocyte differentiation (Alfonso and Jones, 2002; Bernardoni et al., 1997; Kammerer and Giangrande, 2001). In the absence of both *gcm* and *gcm2* (*gcm/gcm2*), plasmatocytes do not differentiate normally and their number is strongly reduced, whereas crystal cell formation appears unaffected (Alfonso and Jones, 2002). In addition, enforced expression

of GCM in crystal cells converts them into plasmatocytes (Lebestky et al., 2000). By contrast, ectopic expression of LZ in plasmatocytes induces crystal cell marker expression but does not repress plasmatocyte cell fate (Waltzer et al., 2003).

It is proposed that, for their differentiation, crystal cells must express *lz* but not *gcm*/*gcm2,* whereas plasmatocytes have to express *gcm*/*gcm2* but not *lz* (Lebestky et al., 2000). Interestingly, *gcm*/*gcm2* expression is detected from stage 5 in the hematopoietic primordium (Alfonso and Jones, 2002; Bernardoni et al., 1997), whereas the onset of *lz* expression is only detected later (at stage 10) (Lebestky et al., 2000). These observations raise several questions: (1) do the crystal cell and the plasmatocytes precursors emerge from the same pool of prohemocytes; (2) when do the two populations segregate; and (3) what is the relationship between *lz* and *gcm*/*gcm2* during blood cell lineage choice?

To address these questions, we have undertaken an analysis of the mechanism of segregation of the two embryonic blood cell lineages. We find that *gcm* is expressed early on in all prohemocytes but is rapidly downregulated in the anterior-most cells of the hematopoietic primordium, which initiate *lz* expression by stage 7. Our results suggest that the coordinated repression of *gcm* and activation of *lz* in the anterior row of prohemocytes is a key step in the regulation of blood cell lineage choice. In the absence of both *gcm* and *gcm2*, we observe a striking increase in the number of crystal cells, indicating that *gcm*/*gcm2* actually regulates crystal cell development. We further show that *gcm*/*gcm2* inhibits crystal cell formation in two steps: first by regulating the number of cells that initiate *lz* expression, and second by interfering with *lz* maintenance in these cells. Furthermore, contrary to what has been reported during larval hematopoiesis (Duvic et al., 2002; Lebestky et al., 2003), we demonstrate that Notch signalling is neither sufficient nor required for crystal cell formation. Finally, our results indicate that prohemocytes are bipotent progenitors, and that the interplay between *gcm*/*gmc2* and *lz* expression dictates the cell fate choice.

Materials and methods

Fly stocks

Most *Drosophila melanogaster* lines were obtained from the Bloomington Drosophila Stock Center. The following strains were kindly provided by different laboratories*: uas-gcm, uas-gcm2, gcm-lacZ (gcm^{rA87}), Df(2L)200* and *Df(2L)Gcm2* (B. Jones, University, MS, USA); *uas-lz* and *lz^{R1}* (U. Banerjee, Los Angeles, CA, USA); *stg^{2}* (M. Crozatier, Toulouse, France); *N^{55e11} FRT10.1* (A. Martinez-Arias, Cambridge, UK). *uas-srp, srp-gal4* and *pg33* have been previously described by Waltzer et al. (Waltzer et al., 2002). To generate *gcm*- and *gcm2*-deficient germ-line clones, males carrying *hs-flp; FRT40A ovo^{D1}/CyO* were crossed to *Df(2L)200 FRT40A/CyO* females. Larvae from this cross were heat shocked daily for 1 hour at 37°C for 3 days, and the emerging *Df(2L)200 FRT40A/FRT40A ovo^{D1}* adult females were crossed to *Df(2L)200/CyO (twi-lacZ)* males. *Notch* germ-line clone mutants were generated according to a similar protocol, using the *N^{55e11}FRT10.1* and the *ovo^{D1}FRT10.1; hs-flp* stocks.

Unless specified, crosses and embryo collections were performed at 25°C. To induce transient expression of GCM, *hs-gal4; uas-gcm* embryos were collected at 18°C for 3 hours, aged at 18°C for 6, 9 or 12 hours, heat shocked twice at 37°C for 20 minutes, and aged at 18°C for 16, 13 or 10 hours, respectively, before being processed for analysis.

Plasmids and transgenesis

The 1.5 kb upstream regulatory region of *lz* (nucleotides 234118 to 235562 on the genomic scaffold AE003446) was cloned into pCasper-hs43-lacZ to generate pLZ-lacZ. The corresponding P{lz-lacZ} transgenic lines were generated by standard P-element-mediated transformation into *w^{1118}* flies.

In situ hybridization and antibody staining

The in situ hybridization technique and probes used have been previously described by Waltzer et al. (Waltzer et al., 2003).

For double fluorescent staining, the following antibodies were used: rabbit anti-Serpent antibody (1/500) (Reuter, 1994), rabbit anti-β-GAL antibody (1/500; Cappel Pharmaceutical), goat anti-rabbit Alexa Fluor 488 (1/400; Molecular Probes), sheep anti-DIG antibody (1/500; Roche), donkey anti-sheep antibody Alexa Fluor 488 (1/400; Molecular Probes) and/or anti-fluorescein AP (1/1000; Roche), revealed with Fast Red substrate (Vector).

Results

As a first step to elucidate the mechanisms underlying embryonic blood cell fate choice, we compared the expression patterns of the key transcription factors *srp*, *gcm* and *lz* from the earliest stage of hematopoiesis, i.e. from stage 5, when *srp* expression defines the hematopoietic anlage in the mesoderm (Rehorn et al., 1996).

gcm is initially expressed in all prohemocytes but is rapidly downregulated in crystal cell precursors

GCM and GCM2 are co-expressed transcription factors that act redundantly to induce plasmatocyte differentiation (Alfonso and Jones, 2002) and that have been suggested to be plasmatocyte specific (Alfonso and Jones, 2002; Lebestky et al., 2000). We investigated whether *gcm* is expressed only in a sub-population of prohemocytes that will give rise to plasmatocytes or in all prohemocytes, including the prospective crystal cells. As shown Fig. 1, at stage 5 (2 hours and 10 minutes to 2 hours and 50 minutes after egg laying; AEL), *gcm* and *srp* transcripts co-localise in all of the cells that constitute the hematopoietic primordium (Fig. 1A). *gcm2* is also expressed in the hematopoietic anlage from stage 5, but at a much lower level than *gcm* (Alfonso and Jones, 2002), therefore precluding the precise analysis of its expression domain by fluorescent in situ hybridization. Interestingly, at stage 6 (2 hours and 50 minutes to 3 hours AEL), *gcm* transcripts are no longer detected in the anterior-most row of *srp*-expressing cells (Fig. 1B). We extended this observation by comparing the localisation of the *gcm* transcripts with that of the SRP protein (Fig. 1C). Thus, although *gcm* is initially expressed in all prohemocytes, its expression is subsequently downregulated in a sub-population of prohemocytes.

To confirm that *gcm* is also expressed in the crystal cell precursors, we analysed the expression of the *P{lacZ}* insertion in *gcm* (*gcm^{rA87}*), previously shown to recapitulate *gcm* expression (Bernardoni et al., 1997). Taking advantage of the long half-life of β-gal, we observed that almost all of the crystal cells (*DoxA3*-positive cells) co-expressed β-gal by stage 11 (Fig. 1K). However, β-gal staining diminished progressively in the crystal cell population at later stages (Fig. 1L,M). For comparison, SRP (Lebestky et al., 2000) or *srp-gal4/uas-lacZ* expression is maintained in crystal cells throughout embryogenesis (Fig. 1N). Hence, although *gcm* is

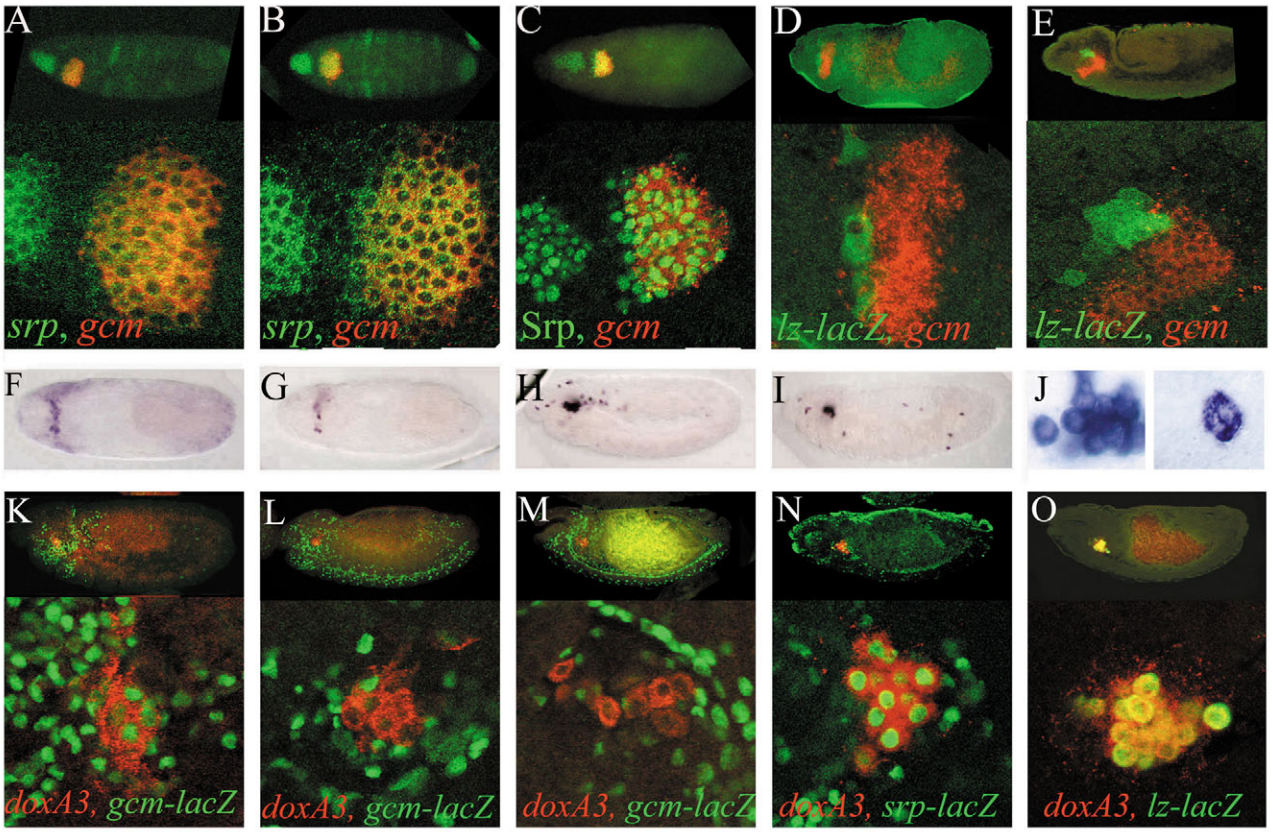


Fig. 1. The downregulation of *gcm* precedes the induction of *lz* in the anterior-most row of prohemocytes. (A,B) *gcm* (red) and *srp* (green) transcription in stage 5 (A) or stage 6 (B) embryos. (C) *gcm* transcript (red) and SRP protein (green) expression in stage 6 embryo. (D,E) *lz-lacZ* embryo processed to reveal *gcm* transcript (red) and β-gal protein (green) at stage 7 (D) and at stage 10 (E). (F-J) *lacZ* expression in *lz-lacZ* (F) or *lz-gal4/uas-gal4; uas-gal4/+; uas-gal4/uas-lacZ* (G-J) embryos at stage 7 (F,G, ventral views), stage 11 (H, side views) or stage 14 (I, side views). (J) High magnification views of *lacZ*-expressing cells localised in the crystal cell cluster (left panel) or far from the cluster (right panels) in a stage 14 *lz-gal4/uas-gal4; uas-gal4/+; uas-gal4/uas-lacZ* embryo. (K-M) Side views of *gcm-lacZ* embryos processed to reveal *DoxA3* mRNA (red) and β-gal protein (green) at stage 11 (K), 14 (L) or 15 (M). (N,L) Side views of *DoxA3* mRNA (red) and β-gal protein (green) in *srp-Gal4;uas-lacZ* (N) or *lz-lacZ* (O) embryos at stage 15. Contrary to *lz-lacZ* or *srp-lacZ*, *gcm-lacZ* expression is progressively lost in the crystal cells during embryogenesis: 26 out of the 28 *DoxA3*$^{+}$ cells are *gcm-lacZ*$^{+}$ at stage 11 (K), against 9 out of 27 at stage 14 (L) and 0 out of 27 at stage 15 (M). *lz-lacZ* codes for a cytoplasmic β-gal whereas *gcm-lacZ* and *uas-lacZ* code for a nuclear β-gal.

initially expressed in all prohemocytes, it is rapidly repressed in the crystal cell precursors. Thus, the transcriptional downregulation of *gcm* in the presumptive crystal cell progenitors (and its maintenance in the remaining prohemocytes) is the earliest known manifestation of a blood cell-lineage choice.

lz expression is induced by stage 7 in the anterior subpopulation of prohemocytes that has downregulated *gcm* expression

lz activity is required for crystal cell formation (Lebestky et al., 2000). Using direct in situ hybridization and immunostaining, it was shown that *lz* is weakly expressed in the crystal cell precursors from stage 10 (4 hours and 20 minutes to 5 hours and 20 minutes AEL). However, our results with *gcm* suggest that the initial blood cell lineage choice takes place at around stage 6, one hour earlier than the reported *lz* expression. Because low levels of expression might hinder *lz* detection during the early stages of hematopoiesis, we re-assessed the onset of *lz* expression by different means. First, we made use of the *lz-gal4* enhancer-trap line that recapitulates *lz* expression (Lebestky et al., 2000). By generating an auto-amplification loop with three copies of *uas-gal4* transgenes (Hassan et al., 2000), we observed *lacZ* expression as early as stage 7 (3 hours to 3 hours and 10 minutes AEL; Fig. 1G). In parallel, we generated transgenic lines containing different regulatory regions of *lz* cloned upstream of a *lacZ* reporter gene. Similarly, we found that a 1.5 kb-long *lz*-upstream region drove *lacZ* expression from stage 7 in a row of prohemocytes and, subsequently, in differentiated crystal cells (Fig. 1F,O). Remarkably, the *lz-lacZ*-expressing cells are not homogeneously distributed among the prohemocyte population but comprise the anterior-most row of cells in which *gcm* transcripts are no longer detected (Fig. 1D,E). It thus appears that *lz* expression is induced by stage 7 in a specific domain of the hematopoietic anlage that corresponds to the domain where *gcm* expression is lost.

Two fates for *lozenge*-expressing cells: crystal cells and plasmatocytes

Crystal cell precursors differentiate into two bilaterally symmetrical groups of cells that remain localised around the

Table 1. The fate of cells that initiate *lz* expression

	Wild type (*n*=26)		*gcm*$^{N7-4}$ (*n=15*)		*Df(2L)200* (*n*=14)	
	Clustered	Scattered	Clustered	Scattered	Clustered	Scattered
lz$^+$ cells (*lz-gal4;uas-lacZ*$^+$)	**27** (±4.4)	**17.2** (±5.6)	**53.8** (±5.6)	**42.5** (±7.8)	**53.2** (±6.0)	**43.4** (±10.3)
% Clustered	61%		56%		55%	
% Scattered		39%		44%		45%
doxA3$^+$ cells	**27** (±4.4)	**1.7** (±1.3)	**53.8** (±5.6)	**9.6** (±3.4)	**53.2** (±6.0)	**39.9** (±11.8)
% of differentiation *(doxA3*$^+$*)/(lz*$^+$*)*	100%	10%	100%	23%	100%	92%

proventriculus (Lebestky et al., 2000). In the course of our experiments with the *uas-gal4* amplification system, we noticed that some *lz-gal4/uas-lacZ*-positive cells were localized outside the crystal cell clusters (Fig. 1H,I) and did not express *DoxA3* (Fig. 3E), suggesting that not all the cells that initiate *lz* expression differentiate into crystal cells. This enticed us to investigate the fate of all of the cells that initiate *lz* expression (so-called *lz*$^+$ progenitors). As shown in Table 1, by stage 14, 60% of the marked cells are clustered along the proventriculus and co-express the crystal cell marker *DoxA3*. The remaining 40% are scattered throughout the embryo, lack the expression of crystal cell-specific markers and morphologically resemble macrophages (Fig. 1J). Therefore, only a fraction of the *lz*$^+$ progenitors differentiates into crystal cells, while the rest become plasmatocytes. These data, together with the observation that *gcm* is initially expressed in all prohemocytes and is required for plasmatocyte differentiation (Alfonso and Jones, 2002; Kammerer and Giangrande, 2001), led us to re-assess the role of *gcm/gcm2* during blood cell lineage choice.

Crystal cell development is repressed by GCM and GCM2

To address the possible role of *gcm* and *gcm2* during crystal cell development, we first analysed the phenotype of embryos carrying a deficiency that removes both genes *(Df(2L)200)* (Alfonso and Jones, 2002). In *Df(2L)200* mutant embryos, we observed a striking increase in the size of the crystal cell clusters and the presence of numerous ectopic *DoxA3*-expressing cells scattered throughout the embryo (Fig. 2B,D compare with 2A,C). Similar results were obtained when we analysed the expression of other crystal cell markers, such as *CG5579* and *CG8193*. These results were confirmed with a smaller deficiency (*Df(2L)Exel7042*) that also removes *gcm* and *gcm2* (data not shown). Morphological observation strongly suggested that these *DoxA3*$^+$ cells are crystal cells (Fig. 2F), despite the ectopic localisation of some of them. Furthermore, these cells efficiently melanised in a *Bc* mutant context and were not observed in a *lz* mutant background (Fig. 2G,I). Thus, in sharp contrast to previous reports (Alfonso and Jones, 2002; Lebestky et

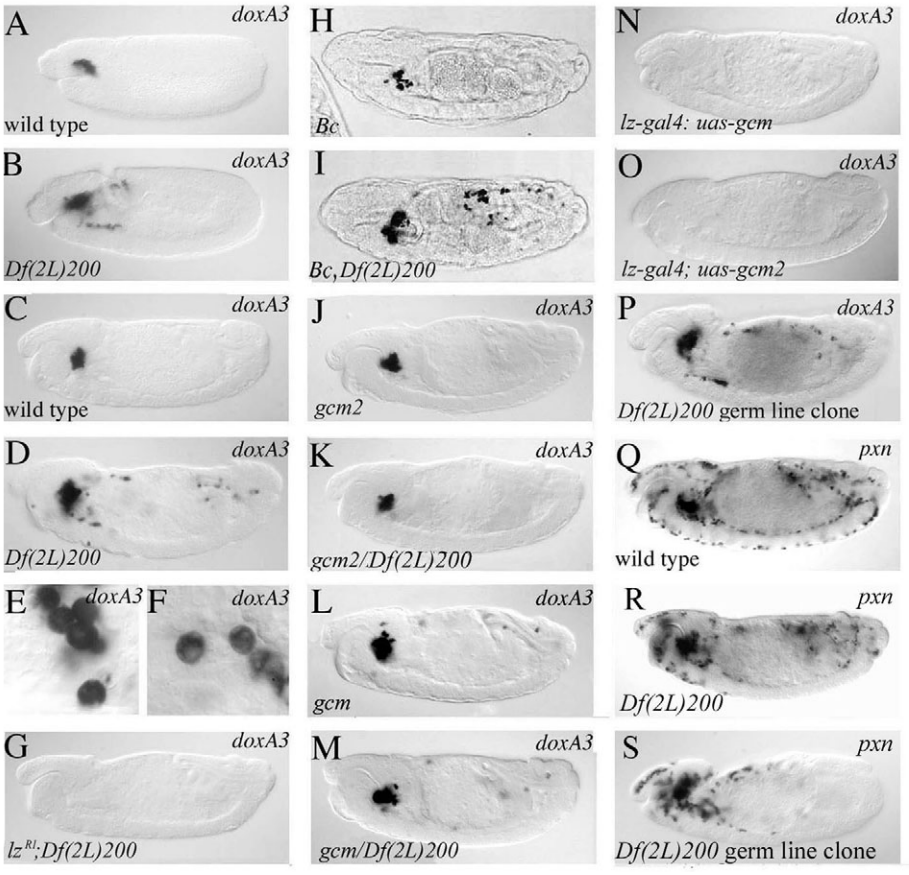


Fig. 2. GCM and GCM2 inhibit crystal cell formation. (A-D) Side views of *DoxA3* expression in stage 11 (A,B), or 15 (C,D) embryos. (A,C) Wild-type embryos; (B,D) *Df(2L)200* embryos. (E,F) Higher magnification views of *DoxA3*-expressing cells located in the crystal cell cluster in a wild-type embryo (E), or located ectopically in a *Df(2L)200* embryo (F). (G) Side views of *DoxA3* expression in a *lz*^{R1};*Df(2L)200* mutant embryo at stage 15. (H,I) Side views of stage 17 embryos carrying the *Bc*^{1} mutation that induces spontaneous melanisation of the crystal cells. (H) *Bc*^{1}, (I) *Bc*^{1},*Df(2L)200*. (J-P) Side views of *DoxA3* expression in stage 15 embryos. (Q-S) Side views of *Pxn* expression in stage 15 embryos. Genotypes as indicated in the lower part of each panel.

al., 2000), our data indicate that *gcm* and *gcm2* normally inhibit crystal cell development.

To ensure that the above phenotypes were due to the lack of *gcm* and/or *gcm2* but not to another gene deleted by the deficiencies, we also examined the phenotypes of *gcm* or *gcm2* single mutants. Lack of *gcm* resulted in a marked increase in the number of crystal cells (Fig. 2L, Table 1), whereas lack of *gcm2* alone did not significantly affect their development (Fig. 2J). The phenotypes were not enhanced when a *gcm* or *gcm2* mutation was combined with *Df(2L)200* (Fig. 2K,M). Nonetheless, the strongest phenotype was observed when both *gcm* and *gcm2* were deleted (Fig. 2D, Table 1). These results indicate that *gcm* is primarily responsible for inhibiting crystal cell development, and that its lack of function can be partially compensated for by *gcm2*. To verify that *gcm2* is also able to inhibit crystal cell fate, we overexpressed *gcm2* in crystal cell precursors. Similar to what was shown for GCM (Lebestky et al., 2000) (Fig. 2N), *lz-gal4*-driven expression of GCM2 inhibited crystal cell formation (Fig. 2O). Thus, both *gcm* and *gcm2* are capable of inhibiting crystal cell formation.

Comparison between the expression patterns of the crystal cell-specific marker *DoxA3* and the panhemocyte marker *peroxidasin* (*Pxn*) (Nelson et al., 1994) indicated that most hemocytes do not acquire the crystal cell fate in *gcm/gcm2* mutant embryos (Fig. 2D, compared with 2R). Thus, in a *Df(2L)200* mutant embryo, it was shown previously that there are approximately 250 PXN-labeled cells (Alfonso and Jones, 2002), whereas we observed about 90 crystal cells, and 40% of them were mislocated (Table 1). Because some *gcm* activity is maternally contributed (Bernardoni et al., 1997), we wondered whether this might explain why only some prohemocytes adopt a crystal cell fate or some crystal cells migrate. However, even in embryos derived from *Df(2L)200* germline clones, ectopic crystal cells were still present (Fig. 2P) and most hemocytes did not differentiate as crystal cells (Fig. 2S). Thus, although *gcm* and *gcm2* inhibit crystal cell development, their absence is not sufficient to cause a complete switch in the fate of the hematopoietic precursors from plasmatocytes to crystal cells.

GCM and GCM2 inhibit crystal cell formation by a two-step process

Next, we tried to understand how *gcm* and *gcm2* control crystal cell fate. As downregulation of *gcm* in the anterior row of prohemocytes shortly precedes *lz* activation, we investigated whether *lz* activation was modified in the absence of *gcm* and *gcm2*. On monitoring *lz-lacZ* expression in stage 7 embryos (i.e. before proliferation resumes), we observed more lz^+ progenitors in the *Df(2L)200* mutant embryos than in wild type (Fig. 3B). Interestingly, all the lz^+ progenitors remained localised to the anterior of the hematopoietic domain, indicating that *lz* induction is still spatially restricted. Thus, in the absence of *gcm/gcm2* there is an increase in the size of the prohemocyte subpopulation that initiates *lz* expression.

We next analysed the fate of the lz^+ progenitors. In stage 14 embryos, we observed a large increase in the number of *lz-gal4/uas-lacZ*-expressing cells in the absence of *gcm/gcm2* (Table 1, Fig. 3D). Double labelling indicated that almost all of the *lz-gal4/uas-lacZ*-expressing cells, even those scattered throughout the embryo, co-expressed the crystal cell marker *DoxA3* (Table 1, Fig. 3F). This is in marked contrast with the wild-type situation, where only the *lz-gal4/uas-lacZ* cells located around the proventriculus express *DoxA3* (Table 1, Fig. 3E). Thus, in the absence of *gcm/gcm2,* all the lz^+ progenitors, both those that remain near the proventriculus and those that migrate to distant positions, differentiate into crystal cells.

To ensure that the increase in crystal cells was directly related to a rise in the number of lz^+ progenitors and not to a modification of the proliferation program, we determined the number of crystal cells formed in the absence of cell proliferation. Accordingly, we introduced the *string* (Drosophila *cdc25*) mutation, which blocks all post-blastodermal cell divisions (Edgar and O'Farrell, 1990), and monitored the number of crystal cells formed in wild-type or in *Df(2L)200* mutant embryos. Even in a *string* mutant context, lack of *gcm/gcm2* induced a twofold increase in the number of crystal cells (Fig. 3, compare G with H).

In summary, the absence of *gcm/gcm2* results in an increase in the number of lz^+ progenitors and allows all of them to

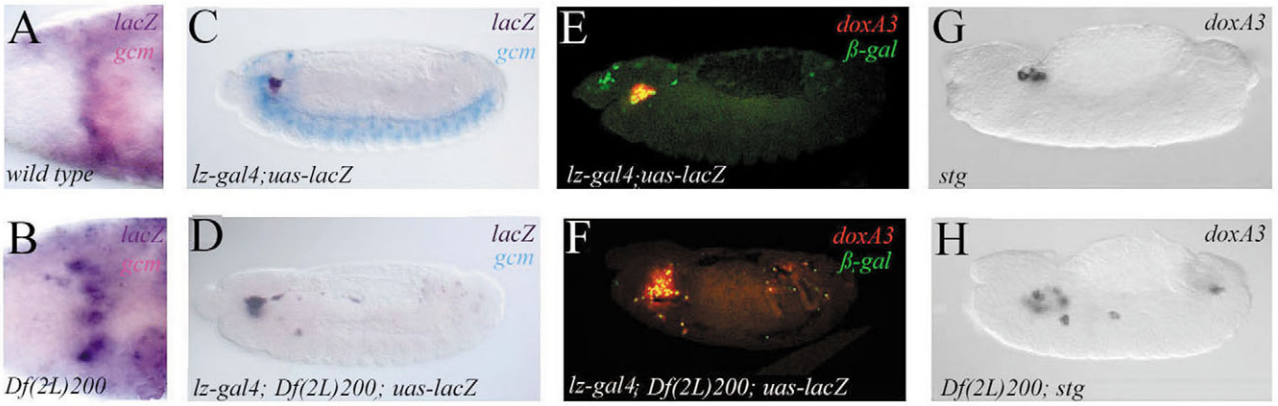


Fig. 3. GCM/GCM2 regulates both the number of lz^+ progenitors and their subsequent differentiation. (A,B) *gcm/gcm2* restricts *lz* activation in the prohemocytes. Ventral views of *lacZ* (dark purple) and *gcm* (red) transcripts in stage 7 *lz-lacZ* (A) and *Df(2L)200;lz-lacZ* (B) embryos. (C-F) *gcm/gcm2* inhibits the differentiation of the lz^+ progenitors into crystal cells. Side views of stage 13 *lz-gal;uas-lacZ* (C,E) and *lz-gal4;Df(2L)200;uas-lacZ* (D,F) embryos showing *lacZ* (dark purple) and *gcm* (blue) transcripts (C,D), or β-gal (green) and *DoxA3* (red) expression (E,F). (G,H) The lack of *gcm/gcm2* induces an increase in the number of crystal cells even in the absence of cell proliferation. Side view of *DoxA3* expression in stg^2 (G) and *Df(2L)200;*stg^2 (H) stage 13 embryos.

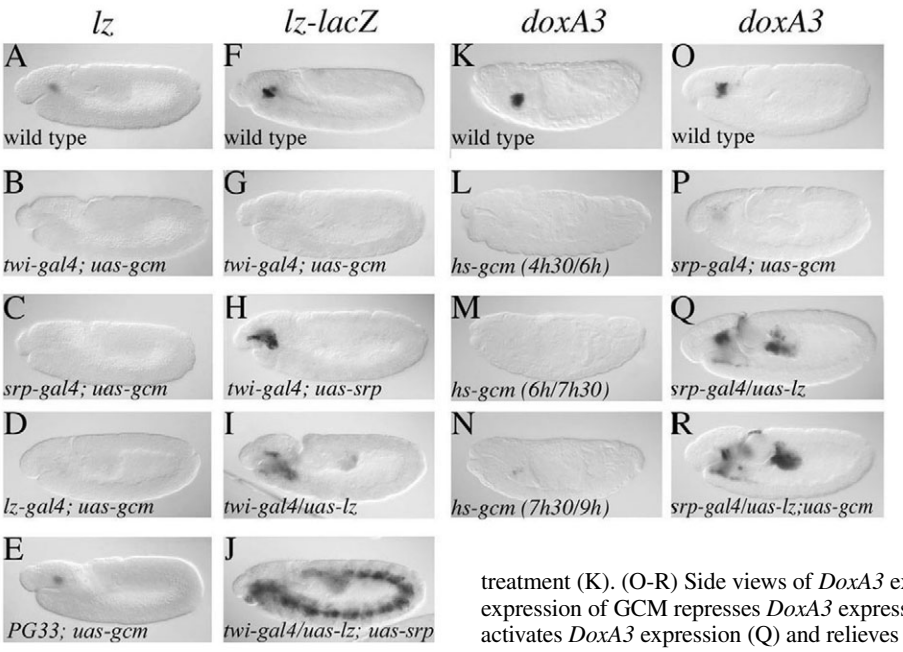


Fig. 4. GCM inhibits *lz* induction and maintenance but not LZ activity. (A-E) Side views of *lz* expression in stage 11 embryos. (A) Wild type. *lz* expression is repressed by GCM when *gcm* expression is driven in the whole mesoderm (B), the hemocytes (C) or the prospective crystal cells (D), but not when it is overexpressed in the plasmatocytes (E). (F-J) Side views of *lz-lacZ* expression in stage 11 embryos. *lz-lacZ* transcription is repressed upon overexpression of GCM in the mesoderm (G). Pan-mesodermal expression of SRP (H) or LZ (I) induces restricted activation of *lz-lacZ*, whereas co-expression of LZ and SRP (J) induces synergistic activation throughout the mesoderm. (K-N) Side views of *DoxA3* expression in stage 16 embryos. Crystal cell formation is repressed by heat-shock induced transient expression of GCM around stage 10 (L), 11 (M) or 12 (N). No repression was observed in the absence of heat-shock treatment (K). (O-R) Side views of *DoxA3* expression in stage 11 embryos. *srp-gal4*-driven expression of GCM represses *DoxA3* expression (P). *srp-gal4*-driven expression of LZ activates *DoxA3* expression (Q) and relieves GCM-induced repression upon *DoxA3* (R).

differentiate into crystal cells. These data suggest that *gcm* and *gcm2* inhibit crystal cell differentiation by a two-step process. First, they limit the induction of *lz* expression to a subset of prohemocytes, thereby regulating the number of crystal cell precursors. Second, they inhibit the acquisition of the crystal cell fate in 40% of the lz^+ progenitors, which take on a plasmatocyte fate instead.

Activation and maintenance of *lz* are inhibited by GCM and GCM2

The preceding results suggested that *gcm* represses *lz* expression. To test this hypothesis, we performed a gain-of-function analysis using the *uas/gal4* system. Interestingly, GCM (and GCM2, data not shown) repressed *lz* when it was expressed throughout the mesoderm (*twi-gal4*), in the entire hemocyte population (*srp-gal4*) or in the presumptive crystal cells (*lz-gal4*) (Fig. 4B-D). Similar results were obtained when we monitored the more robust *lz-lacZ* expression instead of *lz*. (Fig. 4G). By contrast, *lz* expression was not affected when GCM was expressed under the control of the plasmatocyte-specific driver *pg33* (Fig. 4E) (Waltzer et al., 2003). Thus *gcm* and *gcm2* can repress *lz* expression in a cell-autonomous manner.

All of the cells initiating *lz* expression differentiate into crystal cells in the absence of *gcm*/*gcm2*, whereas 40% of them do not in a wild-type situation. To address the possibility, that *gcm* also inhibits the maintenance of *lz*, we induced *gcm* overexpression at different times during embryogenesis using a *hs-gal4* driver (see Materials and methods for details). Interestingly, transient ectopic expression of GCM around stage 9, 10 or 11 still repressed crystal cell formation (Fig. 4L-N). Therefore *gcm* probably also inhibits the maintenance of *lz* expression.

As *lz* expression must be maintained for crystal cell differentiation (Lebestky et al., 2000), we wondered whether *lz* expression might be auto-activated. Reminiscent of its capacity to induce ectopic crystal cell markers (Waltzer et al., 2003), *twi-gal4*-driven LZ expression activated *lz-lacZ* in the *srp*-expressing domains (Fig. 4I). Indeed, SRP and LZ form a functional complex to induce crystal cell development (Waltzer et al., 2003). Therefore we ascertained whether they also cooperate to regulate *lz* expression. Whereas we observed restricted ectopic activation of *lz-lacZ* by SRP or LZ alone (Fig. 4H,I), *lz-lacZ* was strongly activated throughout the mesoderm when SRP and LZ were co-expressed (Fig. 4J). Thus SRP and LZ cooperate to maintain *lz* expression via a positive-feedback loop.

If *gcm* (and *gcm2*) antagonizes crystal cell development only by repressing *lz* expression, we surmised that we might rescue crystal cell formation by uncoupling *lz* expression from *gcm* regulation. Accordingly, we co-expressed LZ and GCM under the control of the *srp-gal4* driver and monitored crystal cell marker expression. As shown in Fig. 4, LZ induced *DoxA3* expression to a similar extent in the presence or in the absence of overexpressed GCM, indicating that GCM does not inhibit LZ function (compare Fig 4Q with 4R). All together, these data demonstrate that *gcm* and *gcm2* repress crystal cell formation by inhibiting both the induction and the maintenance of *lz* transcription.

Notch signalling is not required for crystal cell formation in the embryo

During larval hematopoiesis, Notch signalling in the lymph gland is required and is sufficient to induce crystal cell formation (Duvic et al., 2002; Lebestky et al., 2003). Furthermore, the number of crystal cells is decreased in a *Notch* mutant embryo, suggesting that Notch signalling might also be required for embryonic crystal cell formation (Lebestky et al., 2003). Since the remaining crystal cells in a *Notch* mutant might reflect Notch maternal contribution (Lebestky et al., 2003), we generated *Notch* mutant germ line clones. We still observed crystal cells, both in embryos derived from *Notch* germline clones (12.8±2.6 crystal cells; n=21) and in *Notch* zygotic mutants (15.9±3.5 crystal cells; n=19; Fig. 5B,C).

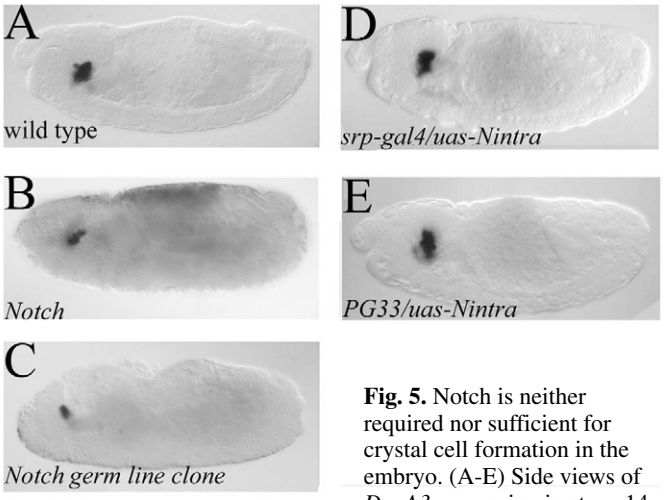


Fig. 5. Notch is neither required nor sufficient for crystal cell formation in the embryo. (A-E) Side views of *DoxA3* expression in stage 14 embryos. (A) Wild type; (B) N^{55e11} zygotic mutant; (C) N^{55e11} germline clone mutant; (D) *srp-gal4;uas-*N^{intra}; (E) *pg33; uas-*N^{intra}.

Therefore, although Notch participates in embryonic crystal cell development, it is clearly not required. To test whether ectopic Notch signalling activates crystal cell formation in the embryo, we expressed an activated form of Notch in all of the prohemocytes using the *srp-gal4* driver or in plasmatocytes using the *pg33* driver. In neither case did we observe more crystal cells (Fig. 5D,E). Therefore, contrary to the previous suggestion (Lebestky et al., 2003), we show that Notch signalling is neither sufficient nor required to induce crystal cell formation in the embryo.

Prohemocytes are biptotential hematopoietic progenitors

In a *gcm/gcm2* mutant background, as in wild type, only a fraction of the prohemocytes develops into crystal cells. In addition, unlike *gcm*, which can convert crystal cells into plasmatocytes (Lebestky et al., 2000), ectopic expression of *lz* induced crystal cell marker expression without repressing plasmatocyte differentiation (Waltzer et al., 2003) (Fig. 6C,G,K). This raised the question: do all prohemocytes have the ability to develop as bona fide crystal cells? To address this issue, we expressed *lz* in all of the prohemocytes in a *gcm/gcm2* mutant background with the *srp-gal4* driver and monitored hemocyte differentiation. Under these conditions, almost all of the hemocytes remained around the proventriculus, judging by the expression of the hemocyte marker *Pxn* (Fig. 6H). Furthermore, all the hemocytes expressed high levels of crystal cell-specific markers and morphologically resembled differentiated crystal cells (Fig. 6D,L). Thus, in the absence of *gcm/gcm2, lz* is capable of inducing differentiation of all the prohemocytes into crystal cells. In conclusion, these results strongly support the hypothesis that prohemocytes are bipotential hematopoietic progenitors that can differentiate either into plasmatocytes or into crystal cells depending on the respective activity states of *gcm/gcm2* and *lz*.

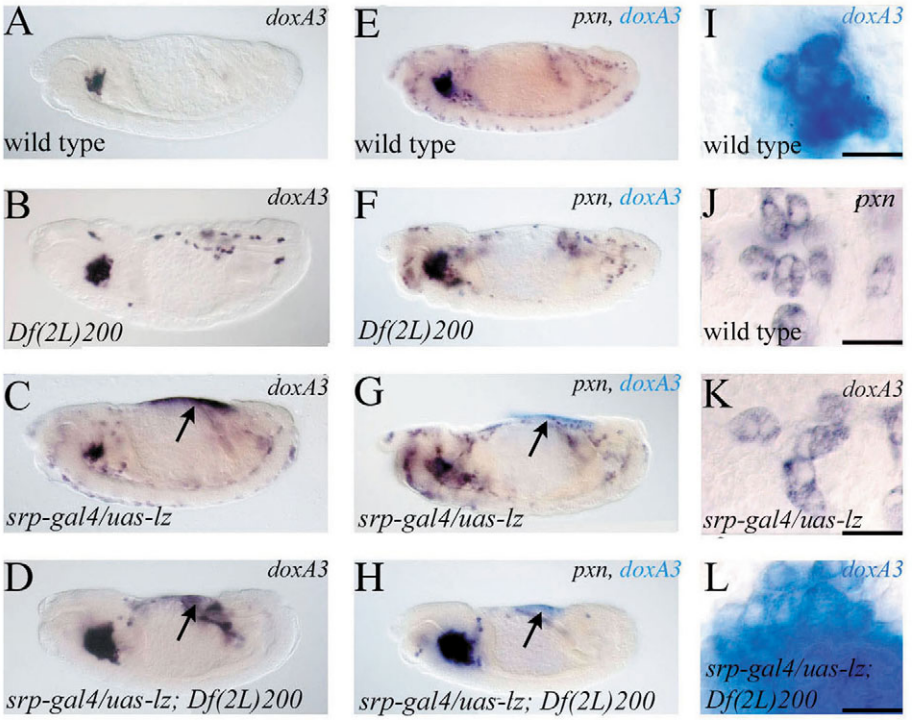


Fig. 6. All the prohemocytes have the capability to develop as crystal cells. (A-H) Side views of stage 14 to 15 embryos processed to reveal *DoxA3* (A-D), or *DoxA3* (blue) and *Pxn* (black) expression (E-H). The arrows in C,D,G and H point to the ectopic activation of *DoxA3* expression in the amnioserosa upon *srp-gal4*-induced expression of *uas-lz* in this tissue. (I-L) Higher magnification views of wild-type crystal cells expressing *DoxA3* (I), wild-type plasmatocytes expressing *Pxn* (J), and *DoxA3*-expressing hemocytes upon *srp*-driven expression of LZ in a wild-type embryo (K) or in a *Df(2L)200* embryo (L). Scale bar in I-L: 12 μm.

Discussion

We have taken advantage of the relatively simple model provided by Drosophila embryonic hematopoiesis to attempt to unravel the mechanisms that underlie the choice of two blood cell fates in vivo. Our data indicate that crystal cells and plasmatocytes develop from a pool of bipotential hematopoietic progenitors. We show that the earliest detectable manifestation of the segregation of the two blood cell lineages occurs in the anterior row of prohemocytes with the downregulation of *gcm* and the induction of *lz*. Furthermore, we demonstrate that the number of *lz*-expressing precursors, and their final differentiation into crystal cells or plasmatocytes, is regulated by *gcm/gcm2* activity, which inhibits *lz* induction and maintenance. Thus, embryonic blood cell lineage segregation is revealed to be a highly dynamic process in which the interplay between the transcription factors *gcm/gcm2* and *lz* plays a crucial role.

gcm/*gcm2* play a pivotal role in the plasmatocyte versus crystal cell developmental decision during embryonic hematopoiesis

It was shown previously that *gcm* and *gcm2* are required for the proper differentiation of plasmatocytes, and GCM and GCM2 were claimed to be plasmatocyte-specific lineage transcription factors that are not involved in crystal cell development (Alfonso and Jones, 2002; Lebestky et al., 2000). By contrast, our results clearly demonstrate that *gcm* and *gcm2* inhibit crystal cell formation. Furthermore, we detected the expression of *gcm* in all of the prohemocytes. including the prospective crystal cell precursors, at stage 5, a result confirmed by tracing *gcm-lacZ* expression into early differentiating crystal cells. Thus, *gcm* and *gcm2* participate in blood cell fate segregation by regulating not only plasmatocyte development but also that of crystal cells.

gcm and *gcm2* have been most intensively studied during neurogenesis, where they are required to promote glial cell development at the expense of neuronal cell fate (Van De Bor and Giangrande, 2002). We show here that they also regulate a binary cell fate choice during hematopoiesis. However, although their expression is restricted to glial precursors during neurogenesis (Alfonso and Jones, 2002; Kammerer and Giangrande, 2001; Vincent et al., 1996), they are initially expressed in all prohemocytes irrespective of their subsequent fate. Furthermore, in the absence of *gcm*/*gcm2*, whereas almost all presumptive glial cells are transformed into neurons (Alfonso and Jones, 2002; Kammerer and Giangrande, 2001; Vincent et al., 1996), only a small proportion of the presumptive plasmatocytes adopts a crystal cell fate. Therefore, the function and mechanism of action of *gcm*/*gcm2* in regulating cell fate choice during neurogenesis and hematopoiesis are different.

gcm and *gcm2* interfere with crystal cell development at different levels

We have deduced that *gcm* and *gcm2* control crystal cell formation by a two-step process. First, *gcm*/*gcm2* determines the number of crystal cell precursors by restricting the initiation of *lz* expression in the prohemocyte population (Fig. 7). In the absence of *gcm*/*gcm2*, we observed more lz^+ progenitors, correlating with a greater number of differentiated crystal cells at later stages. Our data indicate that *gcm* is expressed early in the entire hematopoietic primordium but is rapidly downregulated in the prospective *lz* expression domain. Maintaining GCM or GCM2 expression in the lz^+ progenitors inhibited crystal cell differentiation. Thus, repressing *gcm*/*gcm2* expression in the anterior population of prohemocytes is most probably a prerequisite for the emergence of crystal cells.

Second, *gcm* and *gcm2* regulate the proportion of lz^+ progenitors that ultimately differentiate in crystal cells: whereas 40% of these cells differentiate into plasmatocytes in wild-type embryos, all of them become crystal cells in the absence of *gcm*/*gcm2*. Lebestky et al. already noted that some *lz*-expressing cells differentiate into plasmatocytes and suggested that this might be due to the de novo activation of *gcm* expression in these cells (Lebestky et al., 2000). Our results extend their observations and demonstrate that *gcm* participates in this process, although it is not re-expressed in the lz^+ cells. Our data further suggest that the residual *gcm*

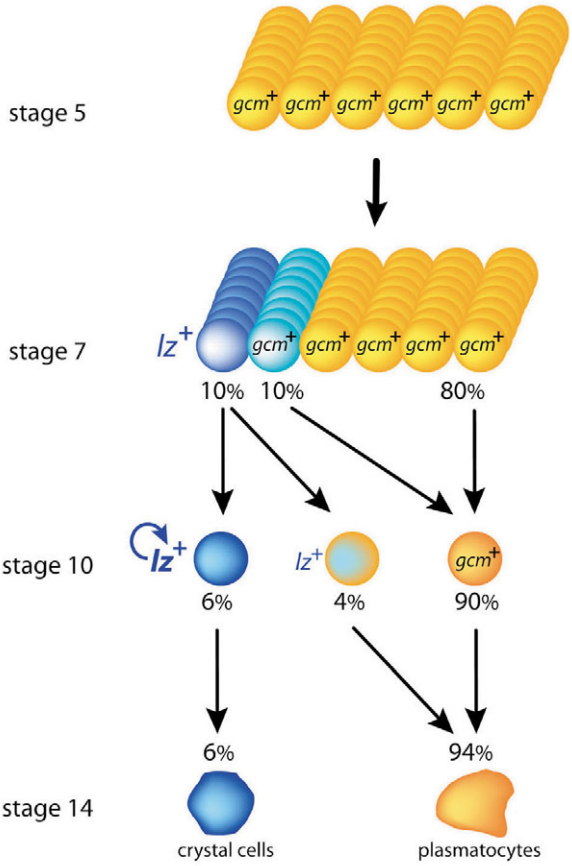


Fig. 7. Schematic representation of blood cell fate resolution during *Drosophila* embryogenesis. Initially all the prohemocytes express *gcm* but not *lz*. Then *gcm* transcription is turned off and *lz* expression activated in the first row of prohemocytes but not in the others that subsequently differentiate into plasmatocytes. 60% of these lz^+ progenitors manage to maintain *lz* expression through an autoactivation loop and differentiate into crystal cells, while in the remaining 40%, the presence of residual GCM interferes with *lz* expression and promotes plasmatocyte differentiation. In the absence of *gcm*/*gcm2*, more prohemocytes (potentially the second row) initiate *lz* expression and all the lz^+ progenitors differentiate into crystal cells.

activity present in the lz^+ progenitors may contribute to the relative plasticity in the fate of these progenitors, allowing some of them to differentiate into plasmatocytes. In summary, we provide compelling evidence that *gcm* and *gcm2* play a key role in regulating cell fate choice in prohemocytes and lz^+ progenitors.

The modulation of *lz* expression controls crystal cell formation

Our study yields new insight into the regulation and mode of action of *lz* during embryonic crystal cell development. Although plasmatocytes migrate through the embryo, crystal cells gather around the proventriculus. Strikingly, we showed that in the absence of *gcm*/*gcm2*, *srp*-driven high-level expression of *lz* induced the transformation of all of the hemocytes to authentic crystal cells that remain clustered. By contrast, when *lz* is expressed under the control of its own promoter, 40% of lz^+ cells migrate through the embryo whether

or not they express *gcm*/*gcm2*. Hence, our data suggest that high levels of *lz* are required for crystal cell clustering and that *lz* induction in prohemocytes is heterogeneous. Below a certain threshold, lz^+ progenitors retain the default migratory behaviour of hemocytes and, in the presence of *gcm*/*gcm2*, can differentiate into plasmatocytes. It is noteworthy that *gcm*/*gcm2* participate in (but are not required for) hemocyte migration (Alfonso and Jones, 2002; Bernardoni et al., 1997). Thus, *lz* and *gcm*/*gcm2* appear to have opposite effects on blood cell migration, with *gcm*/*gcm2* promoting a migratory behaviour that dominates the inhibitory effect of *lz*.

It has been shown that *lz* function is continuously required to promote crystal cell development (Lebestky et al., 2000). Here, we have identified an enhancer of *lz* that is transactivated by the SRP/LZ complex. This observation suggests that, once initiated, *lz* expression can be maintained by a positive auto-regulatory feedback loop, thereby providing a simple mechanism to stabilise crystal cell lineage commitment. This enhancer contains several RUNX-binding sites and we are currently investigating the role of these sites in *lz* autoregulation. Interestingly, the three mammalian homologues of the RUNX factor LZ contain several conserved RUNX-binding sites in their promoters (Otto et al., 2003). Furthermore, RUNX2 maintains its own expression through an auto-activation loop in differentiated osteoblasts (Ducy et al., 1999), whereas RUNX3 inhibits *RUNX1* expression in B lymphocytes (Spender et al., 2005). Thus, auto- or cross-regulation might be a common feature of the *RUNX* family. In addition, we showed that GCM/GCM2 repress *lz* expression. However, no consensus GCM-binding sites are present in the *lz* crystal cell-specific enhancer. Interestingly, it was recently shown that zebrafish *gcmb* is expressed in macrophages (Hanaoka et al., 2004). Yet, the putative functions of the two *gcm* homologues and their possible interplays with RUNX factors have not been investigated during vertebrate hematopoiesis.

Triggering blood cell fate choice

Because *gcm* is expressed early in the entire hematopoietic anlage, it is tempting to speculate that prohemocytes are primed to differentiate into plasmatocytes (i.e. macrophages). Thus, it appears likely that *Drosophila* blood cells progenitors are not 'naïve'. Similarly, mammalian stem and progenitor blood cells express low levels of lineage-affiliated genes and it has been suggested that they are primed for differentiation (Graf, 2002). Furthermore, from an evolutionary perspective, macrophages are certainly the oldest and most pervasive blood cell type (Lichanska and Hume, 2000), and it is remarkable that another hematopoietic cell type may have evolved from this lineage through the use of a conserved RUNX transcription factor.

Acquisition of crystal cell fate involves both the repression of the primary fate (i.e. repression of *gcm*) and the activation of *lz*. Our data show that these two steps are coordinated in space and time. Nonetheless, the induction of *lz* is not the mere consequence of relieving *gcm*/*gcm2*-mediated repression of *lz* but requires an active and localised process. How *gcm* transcription is repressed and *lz* activated in the anterior row of prohemocytes is currently unknown. In the lymph gland, Notch/Serrate signalling is necessary and sufficient to induce crystal cell formation by activating *lz* expression (Duvic et al., 2002; Lebestky et al., 2003). However our results demonstrate that, contrary to the situation in larvae, Notch is not required for crystal cell formation in the embryo. In this respect, it is interesting to note that neither *gcm* nor *gcm2* is expressed in the lymph gland (B.A., unpublished). Hence, the process that segregates crystal cells from plasmatocytes relies on different mechanisms in the embryo and in the larval lymph gland. Similarly, in vertebrates, primitive and definitive hematopoiesis may also depend on partially distinct programs (Shepard and Zon, 2000). For instance, in mouse, the transcription factor PU.1 plays an essential role in the emergence of definitive macrophages but does not seem to be required for the formation of primitive macrophages in the yolk sac (Lichanska et al., 1999).

The coincident repression of *gcm* and activation of *lz* between stages 6 and 7 in a row of prohemocytes is remarkable, as it suggests that the head mesoderm is delicately patterned at this early stage of development. The hematopoietic primordium is located in the posterior head region, whose patterning involves several genes including *buttonhead*, *empty spiracles*, *orthodenticle* and *collier* (Crozatier et al., 1999). However, mutations of these genes do not specifically suppress crystal cell or plasmatocyte development (L.B., unpublished). Further work will thus be required to understand the coordination permitting the silencing of *gcm* and the activation *lz* that triggers the choice of one fate at the expense of the other.

Resolving blood cell fate choice

It was shown that *gcm* can induce the differentiation of all of the prohemocytes into plasmatocytes (Lebestky et al., 2000). The data presented here demonstrate that, in the absence of *gcm*/*gcm2*, *lz* can transform all of the hemocytes to crystal cells. Thus, *Drosophila* prohemocytes are bipotent progenitors. However, the incapacity of *lz* to repress *gcm* (and thereby plasmatocyte fate) implies that the resolution of cell fate choice does not rely on reciprocal antagonism between two 'lineage-specific' transcription factors like between GATA1 and PU.1 during myeloid/erythroid cell fate choice in vertebrates (Galloway et al., 2005; Graf, 2002; Rhodes et al., 2005). Instead, we propose that *Drosophila* embryonic blood cell fate segregation is a process that can be divided into two consecutive phases (Fig. 7). A local cue triggers the process by downregulating *gcm* and activating *lz* in the anterior population of prohemocytes, whereas *gcm* expression is maintained in the remaining cells, which differentiate into plasmatocytes. Then, in the lz^+ progenitors, the relative levels of LZ and GCM will dictate lineage choice. If the ratio of LZ to GCM is high enough to overcome GCM-mediated repression of *lz* expression, LZ can elicit its autoregulatory activation loop and the progenitor will differentiate into a crystal cell. If not, GCM inhibits *lz* autoactivation and the progenitor differentiates into a plasmatocyte. Such a mechanism of segregation could provide some plasticity, because the size of a population may be regulated at different times by physiological cues influencing either the initiation event or the feed-back loop required for its development.

In conclusion, our data shed light on the transition in vivo from bipotent hematopoietic progenitors to lineage-restricted precursors. Interestingly, the embryonic *Drosophila* cell fate choice occurs though an original mechanism distinct from that observed during larval hematopoiesis. Moreover, this process

does not seem to involve reciprocal negative regulation between two 'lineage-specific' transcription factors. Hence, the mechanisms leading to the resolution of hematopoietic lineages in vivo appears to be more complex and diverse than expected.

We are grateful to J. Smith and members of the CBD for critically reading the manuscript, and to M. Tauzin for her help. We thank B. Ronsin and the IFR109 imaging platform. We also thank U. Banerjee, R. Reuter, A Giangrande and B. Jones for reagents and fly stocks. This work was supported by grants from the CNRS and ARC. G.F. is supported by a post-doctoral fellowship from the ARC.

References

Alfonso, T. B. and Jones, B. W. (2002). *gcm2* promotes glial cell differentiation and is required with *glial cells missing* for macrophage development in *Drosophila*. *Dev. Biol.* **248**, 369-383.

Bernardoni, R., Vivancos, B. and Giangrande, A. (1997). *glide/gcm* is expressed and required in the scavenger cell lineage. *Dev. Biol.* **191**, 118-130.

Crozatier, M., Valle, D., Dubois, L., Ibnsouda, S. and Vincent, A. (1999). Head versus trunk patterning in the Drosophila embryo; *collier* requirement for formation of the intercalary segment. *Development* **126**, 4385-4394.

Crozatier, M., Ubeda, J. M., Vincent, A. and Meister, M. (2004). Cellular immune response to parasitization in Drosophila requires the EBF orthologue *collier*. *PLoS Biol.* **2**, E196.

Ducy, P., Starbuck, M., Priemel, M., Shen, J., Pinero, G., Geoffroy, V., Amling, M. and Karsenty, G. (1999). A Cbfa1-dependent genetic pathway controls bone formation beyond embryonic development. *Genes Dev.* **13**, 1025-1036.

Duvic, B., Hoffmann, J. A., Meister, M. and Royet, J. (2002). Notch signaling controls lineage specification during Drosophila larval hematopoiesis. *Curr. Biol.* **12**, 1923-1927.

Edgar, B. A. and O'Farrell, P. H. (1990). The three postblastoderm cell cycles of Drosophila embryogenesis are regulated in G2 by string. *Cell* **62**, 469-480.

Evans, C. J., Hartenstein, V. and Banerjee, U. (2003). Thicker than blood: conserved mechanisms in Drosophila and vertebrate hematopoiesis. *Dev. Cell* **5**, 673-690.

Fossett, N., Tevosian, S. G., Gajewski, K., Zhang, Q., Orkin, S. H. and Schulz, R. A. (2001). The Friend of GATA proteins U-shaped, FOG-1, and FOG-2 function as negative regulators of blood, heart, and eye development in Drosophila. *Proc. Natl. Acad. Sci. USA* **98**, 7342-7347.

Fossett, N., Hyman, K., Gajewski, K., Orkin, S. H. and Schulz, R. A. (2003). Combinatorial interactions of Serpent, Lozenge, and U-shaped regulate crystal cell lineage commitment during Drosophila hematopoiesis. *Proc. Natl. Acad. Sci. USA* **100**, 11451-11456.

Galloway, J. L., Wingert, R. A., Thisse, C., Thisse, B. and Zon, L. I. (2005). Loss of Gata1 but not Gata2 converts erythropoiesis to myelopoiesis in zebrafish embryos. *Dev. Cell* **8**, 109-116.

Graf, T. (2002). Differentiation plasticity of hematopoietic cells. *Blood* **99**, 3089-3101.

Hanaoka, R., Ohmori, Y., Uyemura, K., Hosoya, T., Hotta, Y., Shirao, T. and Okamoto, H. (2004). Zebrafish gcmb is required for pharyngeal cartilage formation. *Mech. Dev.* **121**, 1235-1247.

Hassan, B. A., Bermingham, N. A., He, Y., Sun, Y., Jan, Y. N., Zoghbi, H. Y. and Bellen, H. J. (2000). *atonal* regulates neurite arborization but does not act as a proneural gene in the Drosophila brain. *Neuron* **25**, 549-561.

Holz, A., Bossinger, B., Strasser, T., Janning, W. and Klapper, R. (2003). The two origins of hemocytes in Drosophila. *Development* **130**, 4955-4962.

Kammerer, M. and Giangrande, A. (2001). Glide2, a second glial promoting factor in *Drosophila melanogaster*. *EMBO J.* **20**, 4664-4673.

Lebestky, T., Chang, T., Hartenstein, V. and Banerjee, U. (2000). Specification of Drosophila hematopoietic lineage by conserved transcription factors. *Science* **288**, 146-149.

Lebestky, T., Jung, S. H. and Banerjee, U. (2003). A Serrate-expressing signaling center controls Drosophila hematopoiesis. *Genes Dev.* **17**, 348-353.

Lichanska, A. M. and Hume, D. A. (2000). Origins and functions of phagocytes in the embryo. *Exp. Hematol.* **28**, 601-611.

Lichanska, A. M., Browne, C. M., Henkel, G. W., Murphy, K. M., Ostrowski, M. C., McKercher, S. R., Maki, R. A. and Hume, D. A. (1999). Differentiation of the mononuclear phagocyte system during mouse embryogenesis: the role of transcription factor PU.1. *Blood* **94**, 127-138.

Meister, M. (2004). Blood cells of Drosophila: cell lineages and role in host defence. *Curr. Opin. Immunol.* **16**, 10-15.

Nelson, R. E., Fessler, L. I., Takagi, Y., Blumberg, B., Keene, D. R., Olson, P. F., Parker, C. G. and Fessler, J. H. (1994). Peroxidasin: a novel enzyme-matrix protein of Drosophila development. *EMBO J.* **13**, 3438-3447.

Otto, F., Lubbert, M. and Stock, M. (2003). Upstream and downstream targets of RUNX proteins. *J. Cell Biochem.* **89**, 9-18.

Rehorn, K. P., Thelen, H., Michelson, A. M. and Reuter, R. (1996). A molecular aspect of hematopoiesis and endoderm development common to vertebrates and Drosophila. *Development* **122**, 4023-4031.

Reuter, R. (1994). The gene *serpent* has homeotic properties and specifies endoderm versus ectoderm within the Drosophila gut. *Development* **120**, 1123-1135.

Rhodes, J., Hagen, A., Hsu, K., Deng, M., Liu, T. X., Look, A. T. and Kanki, J. P. (2005). Interplay of Pu.1 and Gata1 determines myelo-erythroid progenitor cell fate in zebrafish. *Dev. Cell* **8**, 97-108.

Rizki, T. M., Rizki, R. M. and Grell, E. (1980). A mutant affecting the crystal cells in *Drosophila melanogaster*. *Wilhelm Roux's Arch. Dev. Biol.* **188**, 91-99.

Sears, H. C., Kennedy, C. J. and Garrity, P. A. (2003). Macrophage-mediated corpse engulfment is required for normal Drosophila CNS morphogenesis. *Development* **130**, 3557-3565.

Shepard, J. L. and Zon, L. I. (2000). Developmental derivation of embryonic and adult macrophages. *Curr. Opin. Hematol.* **7**, 3-8.

Spender, L. C., Whiteman, H. J., Karstegl, C. E. and Farrell, P. J. (2005). Transcriptional cross-regulation of RUNX1 by RUNX3 in human B cells. *Oncogene* **24**, 1873-1881.

Tepass, U., Fessler, L. I., Aziz, A. and Hartenstein, V. (1994). Embryonic origin of hemocytes and their relationship to cell death in Drosophila. *Development* **120**, 1829-1837.

Van De Bor, V. and Giangrande, A. (2002). glide/gcm: at the crossroads between neurons and glia. *Curr. Opin. Genet. Dev.* **12**, 465-472.

Vincent, S., Vonesch, J. L. and Giangrande, A. (1996). Glide directs glial fate commitment and cell fate switch between neurones and glia. *Development* **122**, 131-139.

Waltzer, L., Bataille, L., Peyrefitte, S. and Haenlin, M. (2002). Two isoforms of Serpent containing either one or two GATA zinc fingers have different roles in Drosophila hematopoiesis. *EMBO J.* **21**, 5477-5486.

Waltzer, L., Ferjoux, G., Bataille, L. and Haenlin, M. (2003). Cooperation between the GATA and RUNX factors Serpent and Lozenge during Drosophila hematopoiesis. *EMBO J.* **22**, 6516-6525.