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Cooperation between the GATA and RUNX factors Serpent and Lozenge during *Drosophila* hematopoiesis

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Members of the *GATA* and *RUNX* families of genes appear to have conserved functions during hematopoiesis from *Drosophila* to mammals. In *Drosophila*, the GATA factor Serpent (Srp) is required in blood cell progenitors for the formation of the two populations of blood cells (plasmatocytes and crystal cells), while the RUNX factor Lozenge (Lz) is specifically required for crystal cell development. Here we investigate the function and the mechanisms of action of Lz during hematopoiesis. Our results indicate that Lz can trigger crystal cell development. Interestingly, we show that Lz function is strictly dependent on the presence of functional Srp and that Srp and Lz cooperate to induce crystal cell differentiation *in vivo*. Furthermore, we show that Srp and Lz directly interact *in vitro* and that this interaction is conserved between *Drosophila* and mammals. Moreover, both Srp and mouse GATA1 synergize with mouse RUNX1 to activate transcription. We propose that interaction and cooperation between GATA and RUNX factors may play an important role in regulating blood cell formation from *Drosophila* to mammals.

Keywords: *Drosophila*/GATA/hematopoiesis/RUNX

Introduction

Hematopoietic development provides an excellent paradigm to address how multipotent cells generate a spectrum of cell types through the combinatorial action of transcription factors (Orkin and Zon, 2002). Recent investigations suggest that *Drosophila* will prove to be a valuable model to study the mechanisms of hematopoietic cell fate choice. Indeed, various aspects of hematopoietic development have been conserved during evolution (Evans and Banerjee, 2003). As in vertebrates, *Drosophila* hematopoiesis occurs in two waves from mesodermally derived progenitors. First, during early embryogenesis, blood cells (hemocytes) originate from the head mesoderm (Tepass *et al*., 1994). A second wave of hematopoiesis occurs during the larval stages in a specialized organ formed from the dorsal mesoderm, the lymph gland (Rizki, 1978). Hemocytes differentiate into two major classes: plasmatocytes and crystal cells (Lebestky *et al*., 2000). Plasmatocytes, which represent 95% of the hemocytes, function as macrophages and play a crucial role in host defense and development (Tepass *et al*., 1994; Franc *et al*., 1996). Crystal cells participate in melanization, an insect-specific process involved in the encapsulation of foreign bodies and in wound healing (Rizki *et al*., 1980). Functionally and morphologically, *Drosophila* blood cells most closely resemble vertebrate monocytes/macrophages and granulocytes.

Strikingly, several transcription factors that are important for hematopoiesis and immunity in vertebrates have been shown to play similar roles in *Drosophila*. First, members of the GATA family are recurrently used during hematopoiesis. For instance, in vertebrates, GATA2 plays a critical role in the proliferation and/or survival of hematopoietic stem cells and controls the production of mast cells (Tsai and Orkin, 1997). Moreover, while GATA2 is downregulated to permit erythrocytic differentiation (Persons *et al*., 1999), GATA1 is necessary for the differentiation of erythrocytes and megakaryocytes (Pevny *et al*., 1991; Fujiwara *et al*., 1996). In *Drosophila*, the only GATA factor known to participate in hematopoiesis is encoded by the gene *serpent* (*srp*) (Rehorn *et al*., 1996) that is alternatively spliced to give rise to different isoforms containing either one (SrpC) or two (SrpNC) GATA zinc fingers (Waltzer *et al*., 2002). Similarly to GATA2, *srp* is expressed in the blood cell precursors and is required for the proliferation and maintenance of this population (Rehorn *et al*., 1996; Sam *et al*., 1996). However, *srp* may also have a function later in hematopoiesis as Srp is still detected in mature plasmatocytes and crystal cells (Lebestky *et al*., 2000). Secondly, members of the friend of GATA (FOG) family are implicated in hematopoietic development in both mammals and *Drosophila*. FOG proteins interact with the N-terminal zinc finger of GATA factors and modulate their activity (Haenlin *et al*., 1997; Tsang *et al*., 1997). In mouse, the interaction between FOG1 and GATA1 is necessary for normal erythroid and megakaryocytic differentiation (Tsang *et al*., 1998). In *Drosophila*, the FOG factor U-shaped (Ush) represses crystal cell fate (Fossett *et al*., 2001) and inhibits the expression of the apoptotic body receptor *croquemort* (*crq*) in plasmatocytes, most likely by interacting with SrpNC (Waltzer *et al*., 2002). Thirdly, factors containing a Runt domain (hereafter collectively called RUNX factors) participate in hematopoiesis in both vertebrates and *Drosophila*. In mouse, RUNX1, also known as acute myeloid leukemia factor 1 (AML1), is required for the emergence of definitive hematopoietic stem cells (Okuda *et al*., 1996; Wang *et al*., 1996). In addition, RUNX1 may promote myeloid progenitor differentiation, as suggested by *in vitro* assays and the frequent chromosomal translocations affecting RUNX1 that are associated with human acute myeloid leukemia (Speck and Gilliland, 2002). In *Drosophila*, Lozenge (Lz), which exhibits 71% identity with RUNX1 in the Runt domain, also plays a role in

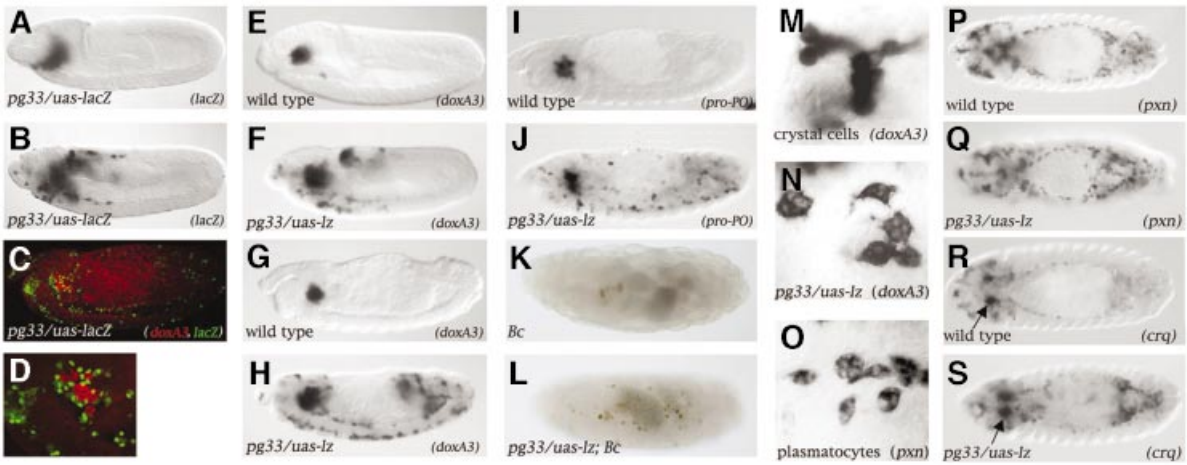

Fig. 1. Lz can induce the crystal-cell-specific genetic program in plasmatocytes. (**A–D**) The Gal4 line *pg33* drives expression in the plasmatocytes. Side views of (A) *pg33/uas-lacZ* stage 9, (B) *pg33/uas-lacZ* stage 11 and (C) *pg33/uas-lacZ* stage 14 embryos processed to reveal *lacZ* expression (A and B) or *doxA3* mRNA (red) and nuclear-LacZ (green) (C). (D) Higher magnification of the head region in (C). (**E–L**) *pg33*-driven expression of Lz induces the ectopic expression of the crystal cell marker *doxA3* and *pro-PO* in plasmatocytes as well as their melanization in a *Bc* mutant context. (E–H) Side views of wild-type (E, G and I) or *pg33/uas-lz* (F, H and J) embryos; *doxA3* expression at stage 11 (E and F) or stage 14 (G and H); *pro-PO* expression at stage 14 (I and J). Side views of *Bc* (K) or *pg33/uas-lz*; *Bc* (L) stage 17 embryos. (**M–S**) Lz does not repress plasmatocyte cell fate. (M) High magnification of the head region around the proventriculus showing wild-type stage 15 crystal cells expressing *doxA3*. (N) High magnification of cells ectopically expressing *doxA3* in a *pg33-gal4/uas-lz* stage 15 embryo (localized ventrally in the trunk). (O) High magnification of wild-type stage 15 plasmatocytes expressing *pxn* (localized ventrally in the trunk). (P–S) Dorsal views of wild-type (P and R) or *pg33/uas-lz* (Q and S) stage 14 embryos: (P and Q) *pxn* expression; (R and S) *crq* expression. The arrows in R and S indicate crystal cells expressing *crq*.

hematopoiesis (Lebestky *et al*., 2000). Indeed, *lz* expression initiates in a small fraction of *srp*-expressing hemocytes in response to Notch signaling and is necessary for crystal cell production (Lebestky *et al*., 2000, 2003). Taken together, the functional conservation of the GATA, FOG and RUNX proteins in *Drosophila* and vertebrate hematopoiesis suggests that aspects of the molecular circuitry that control blood cell development might also be conserved.

Recent genetic studies suggest that *Drosophila* hematopoiesis is organized in a hierarchical cascade of transcription factors controlling progenitor differentiation into the two major cell types: plasmatocytes and crystal cells. Thus *srp* expression in prohemocytes is essential for the expression of the transcription factors *glial cell missing* (*gcm*) and *gcm2* (Bernardoni *et al*., 1997; Alfonso and Jones, 2002), *lz* (Lebestky *et al*., 2000) and *ush* (Fossett *et al*., 2001). Subsequently, while *gcm* and *gcm2* are required for hemocyte differentiation into mature plasmatocytes, *lz* is required for crystal cell determination. Interestingly, this hierarchical cascade is not absolutely linear, as the conserved GATA/FOG interaction provides a feedback loop by which Ush impinges on *srp* function by interacting with SrpNC (Waltzer *et al*., 2002). Whether other players in this cascade interact with each other is not known.

Here we investigate the function and mechanism of action of the RUNX factor Lz during blood cell formation in *Drosophila*. We show that Lz cooperates with Srp to induce crystal cell differentiation *in vivo*. Our data suggest that this cooperation is mediated by the formation of an Srp/Lz complex that synergistically activates transcription. Consistent with this, we find that Srp and Lz interact through their conserved GATA zinc finger and Runt domain, respectively, and that this interaction is conserved through evolution as mouse GATA1 and RUNX1 can interact with each other and with their *Drosophila* counterparts. Finally, we show that GATA and RUNX factors can synergize to activate transcription in cell culture. Together, these results suggest that the interaction and cooperation between GATA and RUNX factors may play an important role during hematopoiesis from *Drosophila* to vertebrates.

Results

lozenge can induce crystal-cell-specific gene expression

The choice between crystal cell and plasmatocyte differentiation appears to be regulated mainly by the antagonistic action of Gcm on Lz. Notably, it was shown that forced expression of *gcm* in crystal cell precursors redirects their fate toward a plasmatocyte destiny (Lebestky *et al*. 2000). To gain further insight into the mechanisms controlling the choice between plasmatocyte and crystal cell formation, we asked whether *lz* is sufficient to impose the crystal cell fate. Normally, Lz expression is activated in a small subset of hemocytes that will give rise to crystal cells but is absent from the vast majority of plasmatocyte-forming hemocytes (Lebestky *et al*., 2000). We used the UAS/GAL4 system to express Lz ectopically in plasmatocytes to see whether they would be converted into crystal cells. The Gal4 driver line *pg33* contains a P(Gal4) insertion upstream of *silver*, a gene with no known function in hematopoiesis (Bourbon *et al*., 2002). This line drives specific expression of a *uas-lacZ* reporter gene in plasmatocytes from stage 9 onward (Figure 1 A–D and Supplementary figure 1 available at *The EMBO Journal* Online). Note that no LacZ expression is detected in crystal cells (Figure 1C and D). To monitor crystal cell formation, we analyzed the expression of an early crystal-cell-specific marker, the monophenol mono-oxygenase

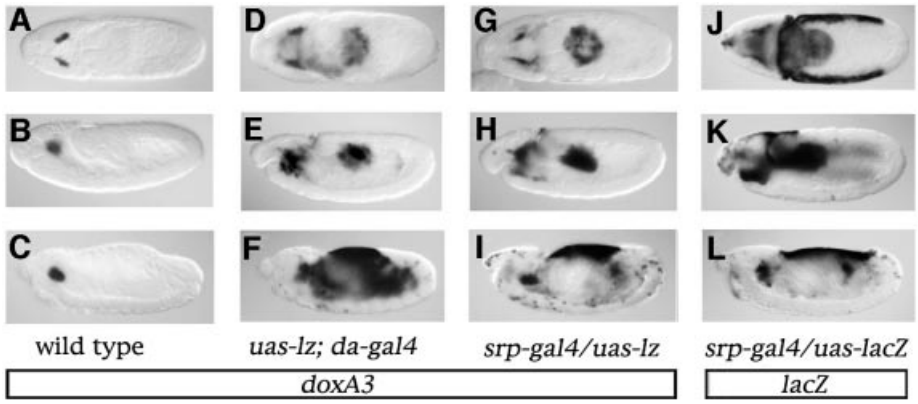

Fig. 2. Lz–mediated activation of crystal-cell-specific genes is restricted to *srp*-expressing cells. (**A–I**) Side views of *doxA3* expression in stage 9 (A, D and G), stage 11 (B, E and H) or stage 14 (C, F and I) embryos: (A–C) wild-type; (D–F) *uas-lz*; *da-gal4*; (G–I) *srp-gal4/uas-lz.* (**J–L**) Side views of *lacZ* expression in *srp-gal4/uas-lacZ* stage 9 (J), stage 11 (K) or stage 14 (L) embryos.

DoxA3 that is expressed as early as stage 9 (see Supplementary figure 1), as well as that of the mature crystal-cell-specific marker prophenoloxidase (Pro-PO), an enzyme of the melanization cascade that can be detected from stage 11 onward (Waltzer *et al*., 2002). Upon *pg33*-driven expression of Lz in plasmatocytes, we observed ectopic expression of *doxA3* in cells that migrated along the normal plasmatocyte migration paths (compare Figure 1F with E and B). At the end of embryogenesis, *doxA3*-expressing cells were scattered throughout the embryo (compare Figure 1H with G). *pg33/uas-lz* induced ectopic *pro-PO* expressing cells along a similar pattern (compare Figure 1J with I). Crystal cells can also be visualized by using the *Black cell* (*Bc*) mutation, which causes premature melanization within the cell. When this mutation was introduced into a *pg33/uas-lz* context, we observed ectopic melanized cells dispersed throughout the embryo by the end of embryogenesis (compare Figure 1L with K). However, hemocytes ectopically expressing Lz morphologically resembled mature plasmatocytes rather than crystal cells (compare Figure 1N with O and M, respectively). Consistent with their morphology, hemocytes ectopically expressing Lz expressed the plasmatocyte markers *peroxidasin* (*pxn*) (Nelson *et al*. 1994) and *croquemort* (*crq*) in a normal pattern (Franc *et al*., 1996) (compare Figure 1Q and S with P and R, respectively). Thus Lz does not seem to repress plasmatocyte differentiation. Note that the plasmatocyte cell fate was not overridden by Lz even when Lz was misexpressed precociously throughout the mesoderm using the *twist-gal4* driver (Supplementary figure 2).

Taken together, these results indicate that Lz can induce in plasmatocytes features specific of crystal cells, namely the expression of genes specific to this lineage as well as the enzymatic cascade required for melanization.

srp is required for lz-mediated activation of crystal-cell-specific genes

In order to test whether Lz could also induce expression of crystal-cell-specific genes in non-hemogenic territories, we misexpressed Lz throughout the embryo using the *da-gal4* driver and analyzed *pro-PO* and *doxA3* expression. As early as stage 9, we detected ectopic expression of *doxA3 in* the hematopoietic primordium as well as in the posterior midgut (compare Figure 2D with A). At later stages, *doxA3* could still be detected in hemocytes and in the posterior midgut as well as in the fat body (a lateral derivative of the mesoderm) and in the amnioserosa (compare Figure 2E and F with B and C). Similar results were obtained when we analyzed *pro-PO* expression (Supplementary figure 3). Thus, the activation of crystal cell markers expression in response to Lz is spatially restricted.

Interestingly, all the tissues that responded to Lz also express the GATA factor Srp (Sam *et al*., 1996). To substantiate this observation, we ectopically expressed Lz under the control of an *srp-gal4* driver that recapitulates most of the embryonic expression of *srp* except that in the fat body. As shown in Figure 2G–L, there was a striking correlation between the pattern of expression of a *lacZ* reporter gene placed under the control of *srp-gal4* and the *doxA3* expression pattern when Lz was ectopically expressed with the same driver or with *da-gal4* (compare Figure 2J, K and L with G, H and I or D, E and F, respectively). Note that ectopic induction of *doxA3* expression was delayed compared with *lacZ*, particularly in the amnioserosa. These results show that Lz-induced activation of the crystal cell program is restricted to *srp*-expressing cells and that all the *srp*-expressing cells can activate this program in the presence of Lz.

The above results suggest that *srp* might be required for Lz-induced gene expression. In order to test this hypothesis, we expressed Lz in an *srp*-mutant background. Expression of the Lz transgene was visualized by *in situ* hybridization with a probe against *lz* and we monitored crystal cell differentiation with a probe against *doxA3*. As expected, expression of neither *doxA3* nor *lz* was detected in an *srp* null mutant (srp^{6G}) (compare Figure 3D with A). Consistent with the above results, when Lz was expressed throughout the mesoderm using the *twist-gal4* driver in a wild-type embryo, ectopic expression of *doxA3* was detected in the anterior mesoderm and posterior midgut by stage 11 (Figure 3F) as well as in the fat body by stage 14 (Figure 3B). In contrast, no *doxA3* expression was observed in an srp^{6G} mutant when Lz was expressed in the mesoderm (Figure 3C). To investigate further the need for *srp*, we then made use of the srp^{3} allele in which hemocyte precursors appear to form (Sam *et al*., 1996; Fossett *et al*.,

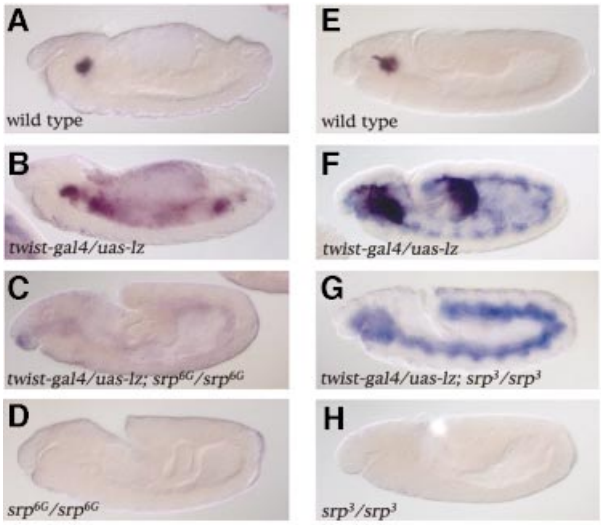

Fig. 3. *srp* is required for *lz*-mediated activation of crystal-cell-specific genes. Side views of *doxA3* (dark purple) and *lz* (blue staining) expression in stage 14 (**A**–**D**) or stage 11 (**E**–**H**) embryos: (A and E) wild-type; (B and F) *twist-gal4/uas-lz*; (C) *twist-gal4/uas-lz*; srp^{6G}/srp^{6G}; (D) srp^{6G}/srp^{6G}; (G) *twist-gal4/uas-lz*; srp^{3}/srp^{3}; (H) srp^{3}/srp^{3}.

2001). In this mutant, neither *doxA3* nor *lz* were expressed (compare Figure 3H with E). As for srp^{6G}, *twist*-driven expression of Lz was unable to induce *doxA3* expression in an srp^{3} mutant (compare Figure 3G with F). These results indicate that *srp* is required for *lz*-induced crystal-cell-specific gene expression.

Lz and Srp interact through a conserved domain

Although it is possible that Lz requires a cofactor induced by *srp* expression, we favored the hypothesis that Lz directly interacts with Srp. Accordingly, we looked for physical interaction between Srp and Lz using a pulldown assay *in vitro*. We found that *in vitro* translated [^{35}S]methionine-labeled SrpC and SrpNC bound to GST-Lz but not to GST alone (Figure 4A). Reciprocally, we observed that *in vitro* translated Lz bound to GST-SrpC but not to GST alone (Figure 4B). To characterize the mutual binding domains within Lz and Srp, we expressed various truncated versions of these proteins either as GST fusions or as *in vitro* translated proteins. As shown in Figure 4A, the GATA zinc finger of SrpC (amino acids 478–533) is required for the interaction with GST-Lz. Moreover, the Runt domain of Lz (amino acids 276–407) is required and sufficient for the interaction with GST-SrpC (Figure 4B). These experiments demonstrate that Lz and Srp bind directly to each other through the RUNT domain and the GATA zinc finger, respectively. Given that these domains are highly conserved in their vertebrate counterparts, we tested the binding of mouse GATA1 to Lz and of mouse RUNX-1 to Srp. As shown in Figure 4C (left panel), *in vitro* translated GATA1 specifically interacted with the Lz Runt domain. Similarly, RUNX1 bound to GST-SrpC (Figure 4C, right panel). Thus the GATA–RUNX interaction is conserved between species.

While this article was in preparation, Elagib *et al.* (2003) reported that RUNX1 interacts with GATA1. Using immunoprecipitations, these authors showed that the N-terminal domain of GATA-1, which is not conserved

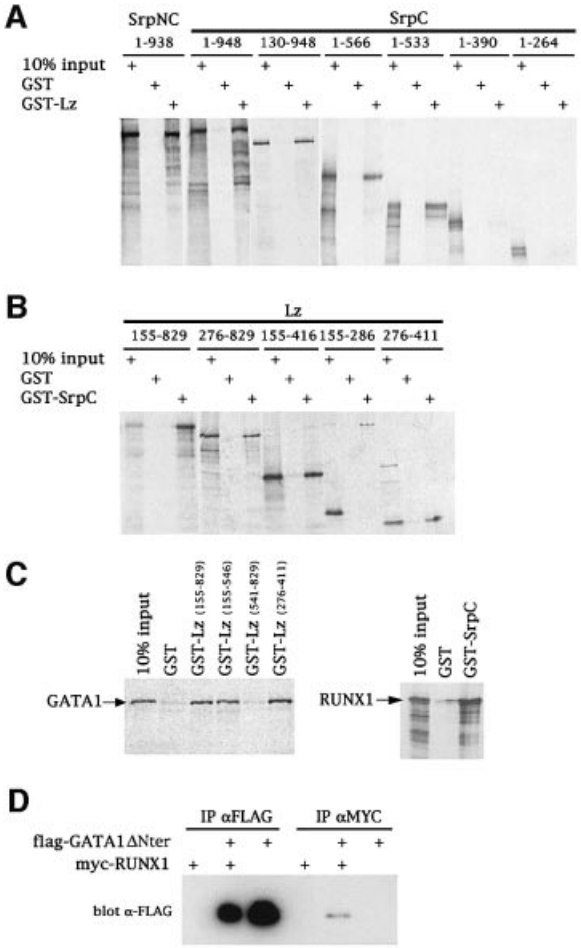

Fig. 4. GATA and RUNX factors interact through their conserved Runt and GATA zinc finger domains. (**A** and **B**) Characterization of the interaction between Srp and Lz by pulldown assays. Equivalent molar amounts of the GST fusion proteins were tested for their interaction with the various *in vitro* translated [^{35}S]methionine-labeled protein fragments as indicated. (**C**) The GATA–RUNX interaction is conserved across species. Pulldown assays between *in vitro* translated mouse GATA1 and various GST-Lz domains (left panel) or between *in vitro* translated mouse RUNX1 and GST-SrpC (right panel). (**D**) RUNX1 and GATA1 deleted of its N-terminal domain interact *in vivo*. COS-7 cells were transfected with pCDNA-mycRUNX1 and pCDNA-flagGATA1ΔNter as indicated in the upper part of the panel. Cell extracts were immunoprecipitated (IP) using either anti-flag or anti-myc and immunoblotted with anti-flag.

between species, was required for the interaction. On the contrary, our results show that GATA factors interact with RUNX proteins through their conserved zinc fingers. In order to clarify this point, we performed immunoprecipitation with GATA1 and RUNX1. As shown in Figure 4D, a flag-tagged version of N-terminally deleted GATA1 expressed in COS-7 cells was specifically co-immunoprecipitated with myc-tagged RUNX1. This result lends further support to our conclusion that GATA and RUNX proteins interact together and that the interaction is mediated though their conserved GATA zinc finger and Runt domain, respectively.

GATA1 and Srp synergize with RUNX1 to activate transcription in vitro

To determine the functional consequences of the GATA–RUNX interaction, we first analyzed the effect of RUNX1

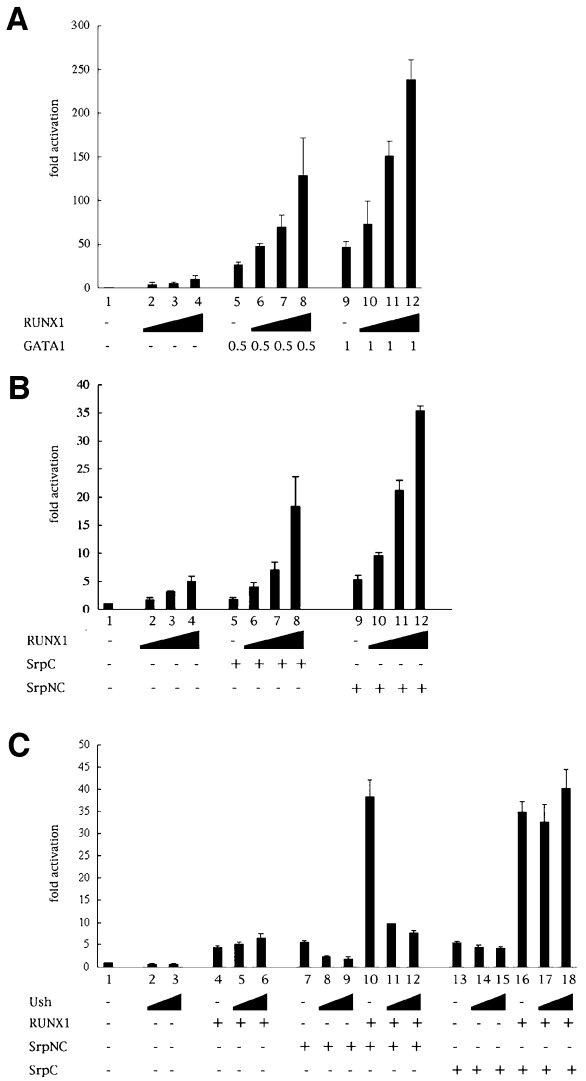

Fig. 5. GATA and RUNX synergize to induce transcription from a GATA-responsive promoter. Luciferase activities of the GATA reporter pGATA-luc in transfected COS-7 cells in the presence of different combination of expression vectors for GATA1, RUNX1, SrpC, SrpNC and Ush. Results are expressed as fold activation relative to empty expression vector. (**A**) RUNX1 synergizes with GATA1 to activate the GATA reporter gene. (**B**) RUNX1 synergizes with SrpC and SrpNC to activate the GATA reporter gene (**C**) Ush antagonizes SrpNC- but not SrpC-mediated transactivation and cooperation with RUNX1.

on GATA1-mediated transactivation. We transfected COS-7 cells with a reporter plasmid containing the luciferase reporter gene placed under the control of nine GATA binding sites linked to a minimal promoter (Newton *et al*., 2001). Upon expression of GATA1, we observed a strong activation of the reporter gene (Figure 5A, lanes 5 and 9). Unexpectedly, RUNX1 weakly activated this reporter gene (Figure 5A, lanes 2–4). Interestingly, when we coexpressed GATA1 with increasing amounts of RUNX1, we observed a dose-dependent synergistic transactivation of the reporter gene (Figure 5A, lanes 6–8 and 10–12). This cooperation was not observed in the absence of the GATA binding site upstream of the reporter gene (data not shown). We then assessed Srp transactivation potential in this heterologous assay. Expression of SrpC or SrpNC modestly, but reproducibly, activated the expression of the reporter gene (Figure 5B, lanes 5 and 9). This activation was dependent on the presence of GATA binding sites upstream of the reporter gene (data not shown). Remarkably, in the presence of increasing amounts of RUNX1, SrpNC and SrpC induced a robust transactivation (Figure 5B, lanes 6–8 and 10–12) that was up to 2-fold greater than the sum of the activation induced by each factor on its own. Thus, like GATA1, Srp can interact and synergize with RUNX1 to induce transcription *in vitro*. On the contrary, despite Lz interacted with Srp and GATA1, it could not substitute for RUNX1 in this transactivation assay (dat not shown). It is possible that Lz does not function normally in this heterologous system. Notably the temperature of 37°C may be detrimental to Lz function as suggested by the existence of a temperature-sensitive allele of *lz*.

Members of the FOG family interact with the GATA N-terminal zinc finger and can either repress or enhance GATA-mediated activation (Haenlin *et al*., 1997; Tsang *et al*., 1997). In *Drosophila*, the FOG factor Ush interacts with SrpNC and represses crystal cell formation as well as *crq* expression (Fossett *et al*., 2001; Waltzer *et al*., 2002). We decided to test the effect of Ush on Srp- and on Srp/RUNX1-mediated activation. As shown in Figure 5C, Ush repressed SrpNC-mediated transactivation (lanes 8 and 9 compared with lane 7). Moreover, the transactivation observed in the presence of both RUNX1 and SrpNC was strongly repressed by Ush (lanes 11 and 12 compared with lane 10). This repression is specific to the interaction between Ush and SrpNC since it is not observed in the case of SrpC- or SrpC/RUNX1-mediated transactivation (compare lanes 14 and 15 with lane 13, and lanes 17 and 18 with lane 16, respectively). Taken together, these data show that the interaction of SrpNC with Ush inhibits SrpNC-induced activation and impedes the cooperation between SrpNC and RUNX1. These results are consistent with previous genetic studies showing that Ush inhibits crystal cell formation (Fossett *et al*., 2001) and SrpNC-induced activation of *crq* (Waltzer *et al*., 2002).

lz and srp synergize to induce crystal-cell-specific gene activation in vivo

Next we evaluated whether GATA and RUNX factors can cooperate *in vivo* during *Drosophila* blood cell development. We have shown previously that *twist*-driven misexpression of *srp* can induce the expression of plasmatocyte-specific genes in most of the mesoderm. In contrast, ectopic *pro-PO* activation was restricted to the head mesoderm (Waltzer *et al*., 2002). Our present results indicate that Lz can induce the crystal-cell-specific program only in *srp*-expressing cells. We surmised that Lz and Srp might cooperate to induce crystal-cell-specific gene expression. In order to test this hypothesis, we coexpressed Lz with SrpC or SrpNC in the mesoderm and we monitored the expression of crystal-cell-specific genes. Whereas Lz, SrpC or SrpNC alone induced restricted ectopic activation of *doxA3* (Figure 6B, C and E compared with A, respectively), the coexpression of SrpC or SrpNC with Lz had dramatic consequences: not only did we observe expression of *doxA3* throughout the mesoderm, but its expression was also much stronger (Figure 6D and F). We observed similar results when we assessed *pro-PO* expression (Supplementary figure 3) or the expression of

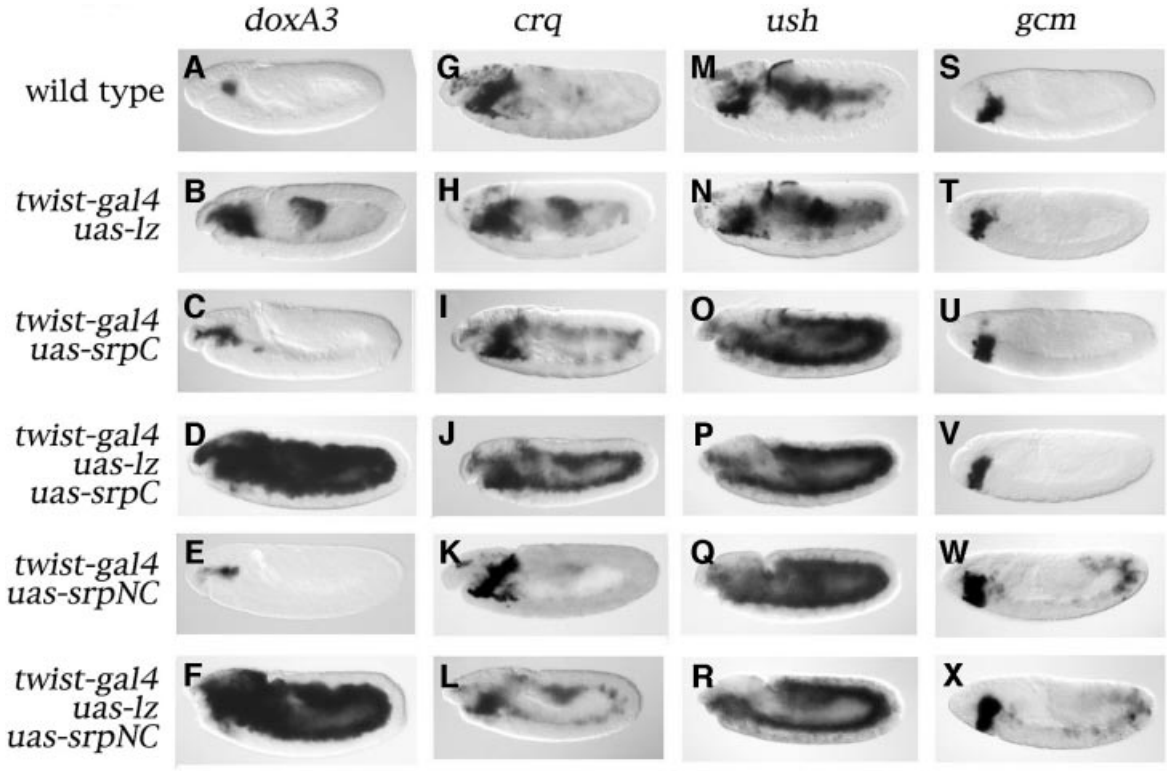

Fig. 6. Srp and Lz cooperate to induce crystal-cell-specific genes *in vivo*. Side views of stage 11 (**A**–**R**) or stage 8 (**S**–**X**) embryos processed to reveal the expression of *doxA3* (A–F), *crq* (G–L), *ush* (M–R) or *gcm* (S–X). (A, G, M and S) wild-type. (B, H, N and T) *twist-gal4/uas-lz*. (C, I, O and U) *twist-gal4*; *uas-srpC*. (D, J, P and V) *twist-gal4/uas-lz*; *uas-srpC*. (E, K, Q and W) *twist-gal4*; *uas-srpNC*. (F, L, R and X) *twist-gal4/uas-lz*; *uas-srpNC*.

two other crystal cell markers, CG1633 and CG8193 (L.Waltzer, unpublished results). These data indicate that Srp cooperates with Lz to induce the activation of the crystal-cell-specific program.

As mentioned above, *srp* can induce ectopic activation of various genes implicated in hematopoiesis. Among them, *crq* can be activated ectopically by SrpC but not by SrpNC, owing to the inhibitory action of Ush (Waltzer *et al*., 2002). Moreover, although *crq* was thought to be specifically expressed in plasmatocytes, we have observed that it is also expressed in crystal cells. Indeed, the main site of expression of *crq* in stage 14/15 embryos is a bilateral cluster of cells next to the proventriculus (Figure 1R, arrow) that also express *doxA3* and are absent in an *lz* mutant (Supplementary figure 1). Thus, at least at the RNA level, *crq* is expressed in both plasmatocytes and crystal cells during late embryogenesis. This suggests that SrpC and Lz may also cooperate to induce *crq* expression as they do to induce crystal-cell-specific genes. Accordingly, when Lz was coexpressed with SrpC, we observed a strong increase in the level of *crq* expression throughout the mesoderm compared with the activation induced by Srp or Lz alone (compare Figure 6J with I and H). However, when Lz was coexpressed with SrpNC, a modest ectopic activation of *crq* throughout the mesoderm was observed (Figure 6L). Since we have shown previously that SrpNC alone does not induce ectopic expression of *crq* owing to the inhibitory effect of Ush (Waltzer *et al*., 2002), these results, as well as those of our transactivation assays, suggest that the balance between Ush and Lz can modulate the transcriptional response to SrpNC *in vivo*.

We also assessed the expression of *ush* and *gcm*, two genes whose expression is initiated in the hematopoietic primordium and is maintained specifically in plasmatocytes (Lebestky *et al*., 2000; Fossett *et al*., 2001). *ush* can be activated throughout the mesoderm in response to SrpC or SrpNC, whereas *gcm* is only activated by SrpNC (Waltzer *et al*., 2002). Interestingly, when Lz was coexpressed with SrpC or SrpNC, Srp-induced activation of *ush* did not seem to be either enhanced or repressed (compare Figure 6P and R with O and Q). Similarly, SrpNC-induced ectopic activation of *gcm* was not affected by the coexpression of Lz, and SrpC could not ectopically activate *gcm* expression even in the presence of Lz (compare Figure 6W with X and U with V). Thus Lz does not cooperate with Srp to induce *ush* or *gcm*, two known antagonists of crystal cell development.

In conclusion, Srp and Lz seem to cooperate only in the activation of genes expressed in crystal cells. This suggests that the Srp/Lz complex provides the molecular selectivity to trigger crystal cell differentiation during hematopoiesis.

srp and lz cooperate to induce crystal cell formation

All the results shown here support the hypothesis that Lz and Srp act in concert to induce crystal cell development. In apparent contradiction, we have previously shown that *lz*-driven expression of Srp in crystal cell precursors inhibits their differentiation (Waltzer *et al*., 2002).

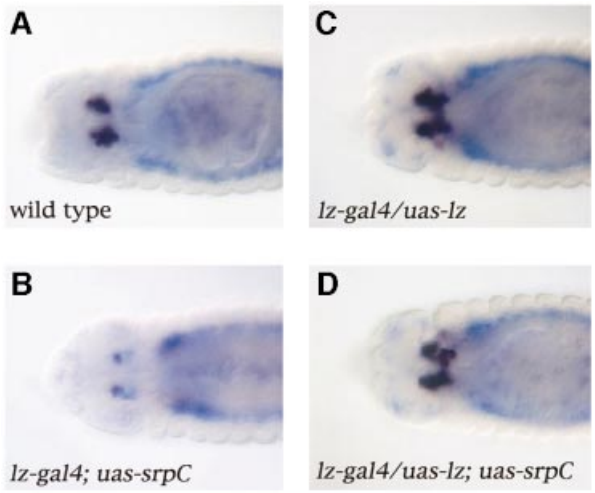

Fig. 7. The balance between Srp and Lz is critical for crystal cell formation. Dorsal view of stage 14 embryos processed to reveal *doxA3* expression (black staining) and *srp* expression (blue staining): (**A**) wild-type; (**B**) *lz-gal4*; *uas-srpC*; (**C**) *lz-gal4/uas-lz*; (**D**) *lz-gal4/uas-lz*; *uas-srpC*.

Intriguingly, Srp expression in crystal cells is lower than in plasmatocytes (Lebestky *et al*., 2000), suggesting that the downregulation of Srp in these cells is important for their differentiation. Moreover, by overexpressing *srp* in crystal cell precursors, we altered the stoichiometry of Srp versus Lz. We hypothesized that increasing the dose of Lz might revert the *srp* gain-of-function phenotype. Accordingly, we used the *lz-gal4* driver to express *uas-srpC* and/or *uas-lz* in crystal cell progenitors and we monitored crystal cell differentiation by assessing *doxA3* expression. As shown in Figure 7, overexpression of Lz did not seem to affect crystal cell determination (compare Figure 7C with A). On the contrary, SrpC reduced the number of crystal cells and repressed their differentiation, judging by the repression of *doxA3* expression (compare Figure 7B with A). However, when Lz was coexpressed with SrpC, formation and differentiation of the crystal cells were restored (Figure 7D). Similar results were obtained with SrpNC or when we assessed *pro-PO* expression (not shown). Thus the relative levels of Srp and Lz are important for crystal cell development and these two factors cooperate to induce their formation *in vivo*.

Discussion

In *Drosophila*, as in vertebrates, members of the GATA and RUNX families play critical roles during hematopoiesis. In this study, we have investigated the mechanisms by which the RUNX factor Lz controls the formation of crystal cells, one of the two embryonic types of blood cell in *Drosophila*. We provide evidence that Lz interacts and cooperates with the GATA factor Srp during this process and that this interaction is conserved through evolution.

Lz induces the crystal cell genetic program

In the embryo, *Drosophila* blood cell progenitors can differentiate into two cell types: plasmatocytes or crystal cells. Crystal cell precursors are first detectable as a small fraction of hemocytes in which Lz is expressed. Whereas Lz function is continuously required for crystal cell development, its mode of action in this process was unknown (Lebestky *et al*., 2000). Our results provide strong evidence that Lz can trigger the genetic program necessary for crystal cell function as its misexpression in plasmatocytes induced the ectopic expression of crystal-cell-specific genes in these cells and their melanization in a *Bc* mutant context. Contrary to our findings, Lebestky and coworkers failed to observe induction of the crystal cell fate when Lz was overexpressed under the control of a heat-shock promoter (Lebestky *et al*., 2000). This difference may be due to the marker they analyzed and/or the method of misexpression used.

It is worth noting that the plasmatocyte cell fate does not seem to be repressed upon ectopic Lz expression. Several factors expressed in plasmatocytes may prevent Lz from repressing their differentiation along this path. For instance *srp*, *gcm* and *ush* are expressed at high levels in pro-hemocytes and in plasmatocytes, while they are normally downregulated (*srp*) or not expressed (*gcm* and *ush*) in crystal cells (Lebestky *et al*., 2000; Fossett *et al*., 2001). Upon ectopic expression of *lz* in plasmatocytes, the expression of these genes was not repressed and thus they might still promote plasmatocyte differentiation. In addition, contrary to crystal cells, plasmatocytes are migratory cells that will be exposed to changing environmental cues; thus their cellular identity must be maintained cell autonomously and may be locked in place early on. Hence the concomitant upregulation of Lz and repression of *ush* and *gcm* in crystal cell progenitors is probably a critical step for normal blood cell differentiation.

Srp and Lz cooperate to induce crystal cell fate

Our results indicate that Srp and Lz act in concert to induce crystal cell development. We demonstrate that the induction of crystal-cell-specific genes by Lz is strictly dependent on the presence of Srp. Moreover, we show that Lz and Srp bind to each other and that Srp synergized with RUNX1 to induce transcription in cell culture. Taken together, these lines of evidence suggest that Srp and Lz form a complex that activates the transcription of the genes required for crystal cell differentiation *in vivo*.

Interestingly, the transcriptional cooperation between Srp and Lz seems restricted to genes expressed in crystal cells, such as *doxA3*, *pro-PO* and *crq*. How this target gene specificity is achieved is not known. In cell culture, Srp can cooperate with RUNX1 to activate transcription from a synthetic promoter containing multimerized GATA binding sites. However, *in vivo*, while Srp alone activates the expression of *crq*, *gcm* and *ush*, cooperation between Srp and Lz was not observed in expression of *gcm* and *ush*, two genes that antagonize crystal cell formation. The Srp/Lz complex may only bind to promoters containing certain GATA binding sites. Alternatively, it is possible that Lz also participates in the tethering of the Srp/Lz complex to RUNX binding sites. In order to bind these sites, RUNX proteins have to form dimers with CBFβ. In *Drosophila*, the CBFβ homologs Brother and Big Brother are ubiquitously expressed during embryogenesis, but it is not known whether they play a role in hematopoiesis (Golling *et al*., 1996; Kaminker *et al*., 2001). Interestingly, *doxA3* and *pro-PO* proximal promoter regions contain both GATA and RUNX consensus binding sites, which

suggests that both partners may participate in the recruitment (L.Waltzer, unpublished observation). Defining the *cis*-responsive elements in crystal-cell- and plasmatocyte-specific genes, respectively, should help to define the molecular mechanisms of cooperation between Srp and Lz.

Our finding that Srp plays a direct part in crystal cell differentiation was unexpected as forced expression of Srp in these cells inhibited their development, suggesting that *srp* has to be downregulated to allow their differentiation (Waltzer *et al*., 2002; Evans and Banerjee, 2003). Instead, we propose that Srp is necessary early on for all hemocyte fate (including that of crystal cell precursors) and subsequently, in balance with Lz, for crystal cell differentiation. A similar situation has been described for GATA2 which is required at a high level for proliferation of blood cell progenitors and at a lower level in differentiated blood cells, such as mature erythrocytes and mast cells (Jippo *et al*., 1996; Harigae *et al*., 1998; Tsai and Orkin, 1997). Our study suggests that the balance between Srp and Lz, rather than the absolute level of Srp, may be critical for crystal cell differentiation. Indeed, the inhibition of crystal cell formation due to the overexpression of Srp was counterbalanced by increasing the levels of Lz. The fact that Srp is required even at stages when its overexpression can be inhibitory highlights that Srp function at a given moment is dependent on its precise level of expression. It also probably reflects the existence of cross-regulation between a combination of transcription factors whose equilibrium in the progenitors is critical for cell fate choice.

Cross-regulations between Ush, Lz and Srp

An important component of this equilibrium is the FOG factor Ush. Members of the FOG family interact specifically with GATA N-terminal zinc fingers and act either as coactivators or corepressors (Haenlin *et al*., 1997; Tsang *et al*.,1997; Wang *et al*., 2002). As such, Ush can only interact with SrpNC, and not with SrpC, and we have previously proposed that Ush controls hematopoiesis by repressing SrpNC-mediated activation (Waltzer *et al*., 2002). Our present data support this hypothesis since Ush directly repressed SrpNC-mediated activation in our transactivation assay. During hematopoiesis, Ush was shown to repress crystal cell formation (Fossett *et al*., 2001). Consistent with this role, we observed that Ush inhibits the transactivation mediated by the SrpNC/RUNX complex. Conversely, the inhibitory effect of Ush on SrpNC-induced activation of *crq* can be partially relieved by Lz *in vivo*. Since Ush is coexpressed with Srp and Lz in crystal cell precursors (Fossett *et al*., 2001), a competition between Ush and Lz to modulate SrpNC activity might influence cell fate choice. Indeed, Lebestky and coworkers observed that a small fraction of the Lz-expressing cells differentiate into plasmatocytes (Lebestky *et al*., 2000). We propose that the cross-interactions between Srp, Lz and Ush might constitute a mechanism whereby one cell type is chosen at the expense of the other. Similar regulation of GATA activity occurs during chicken eosinophil differentiation. Eosinophil progenitors express GATA1, FOG1 and C/EBP. FOG1 was shown to repress the expression of eosinophil-specific markers by blocking GATA-induced transactivation, while C/EBP interacts and cooperates with GATA1 to induce eosinophil differentiation (Yamaguchi *et al*., 1999; Querfurth *et al*., 2000). Thus dose-dependent antagonistic or cooperative interactions between GATA factors and their partners appear to be a conserved means of regulating lineage choice and differentiation.

Conserved interaction and cooperation between GATA and RUNX

We have shown here that GATA and RUNX factors cooperate *in vivo* to control *Drosophila* blood cell formation. Several lines of evidence suggest that the functional relationship between GATA and RUNX factors may be conserved in vertebrate hematopoiesis. First, we have shown that the interaction between Srp and Lz occurs through highly conserved domains. Secondly, we have found that GATA1 binds to Lz and that RUNX1 interacts with Srp. Thirdly, in cotransfection assays, we have demonstrated that RUNX1 and GATA1 bind to each other and synergistically activate transcription. Elagib and coworkers have also recently described RUNX1 binding to GATA1 (Elagib *et al*., 2003). Contrary to what we observed, these authors failed to detect an interaction with RUNX1 when GATA1 N-terminal transactivation domain was deleted. The reason for this discrepancy is unclear. Interestingly though, they showed that these two factors cooperate to induce the expression of megakaryocyte-specific integrin *in vitro*, suggesting that RUNX1 may participate with GATA1 to induce megakaryocytic differentiation. Moreover, haploinsufficient mutations in RUNX1 are associated with familial platelet disorder with predisposition to acute myelogenous leukemia (FPD/AML) (Song *et al*., 1999; Michaud *et al*., 2002), suggesting a role for RUNX1 in human megakaryopoiesis. While it is well established that GATA1 is required for normal megakaryopoiesis (Vyas *et al*., 1999), a role for RUNX1 in this process has not yet been demonstrated. Furthermore, acquired mutations in GATA1 are specifically associated with megakaryocytic leukemia and transient myeloproliferative disorder in children with Down syndrome (Wechsler *et al*., 2002; Groet *et al*., 2003). As RUNX1 is located on chromosome 21, it is tempting to speculate that the interaction between GATA1 and RUNX1 may participate in the development of these megakaryocytoses. Finally, RUNX1 is coexpressed with GATA1, GATA2 or GATA3 at different stages of hematopoiesis (Speck and Gilliland, 2002). Thus the GATA/RUNX complex may regulate various aspects of hematopoiesis from stem cell emergence and proliferation to lineage determination and terminal differentiation.

In conclusion, our results show for the first time that the GATA and RUNX factors cooperate *in vivo* to control hematopoiesis. In *Drosophila*, the cooperation between Srp and Lz is central to the specification of crystal cell fate. We have also highlighted the fact that the GATA–RUNX interaction is conserved between *Drosophila* and mammals. This raises the possibility that a similar functional interaction between GATA and RUNX is important during hematopoietic development in mammals.

Materials and methods

Fly stocks

The *uas-srpC* and *uas-srpNC* transgenic lines were described previously (Waltzer *et al*., 2002). The plasmatocyte-specific driver *pg33-gal4* was

described previously (Bourbon *et al*., 2002). The *uas-lz*, *lz-gal4*, *srp-gal4* and *srp*^{3} lines were kindly provided by P.Gergen, U.Banerjee, M.Meister and R.Reuter, respectively. Other stocks were provided by the Bloomington *Drosophila* Stock Center. All crosses and embryo collections were carried out at 25°C. Blue balancers or *in situ* hybridization against the transgenes were used to genotype the embryos.

In situ hybridizations and antibody stainings

In situ hybridizations were carried out as previously described (Peyrefitte *et al*., 2001). RNA probes for *srp*, *crq*, *pxn*, *pro-PO*, *ush*, *gcm* and *lacZ* have been described previously. To generate RNA probes for *doxA3* exon 3 or *lz* cDNA, the corresponding DNA sequences were cloned by PCR in pGemTeasy. The corresponding antisense RNAs were transcribed *in vitro* using T7 or SP6 RNA polymerase.

Double-fluorescent immunostaining and *in situ* hybridization were carried out using fluorescein-UTP labeled *doxA3* antisense probe, mouse antifluorescein antibody (1/500) (Roche), goat antimouse antibody conjugated to Alexa Fluor 546 (1/400) (Molecular Probe), rabbit anti-β-gal antibody (1/500) (Cappel Inc. Pharmaceutical) and goat antirabbit antibody conjugated to Alexa Fluor 488 (1/400) (Molecular Probe).

Pulldown assays

pBS-SrpC, pBS-SrpNC, pGEX2TK-SrpC and pGEX2TK-SrpNC were described previously (Waltzer *et al*., 2002). These plasmids and pET3c-Lz (Xu *et al*., 2000) were used as templates to subclone various domains of Srp or Lz into pGEX2TK or into pT7βlink by standard cloning techniques. pGEX2TK-derived expression plasmids were used to produce GST proteins in *Escherichia coli* BL21. pBS or pT7βlink-derived plasmids were used as a template to produce *in vitro* [^{35}S]methionine-labeled proteins using a coupled transcription–translation system (Promega). Interaction assays were performed as described (Waltzer *et al*., 2002).

Reporter plasmids and mammalian expression vectors

The mouse RUNX1 expression plasmid pCMV-AML1b (McLarren *et al*., 2000) was provided by S.Stifani. The expression vector for myc-tagged RUNX1 (pCDNA-myc-PEBP2αB1) (Kim *et al*., 1999) was provided by Y.Ito. The mouse GATA-1 expression plasmid pCMV-GATA-1 (Newton *et al*., 2001) was provided by M.Crossley and was used as a template to generate pCDNA3-flag-GATA1ΔNter (81–413). Full-length SrpC and SpNC cDNA were subcloned from pBS-SrpC or pBS-SrpNC into the CMV-based expression plasmid pXJ42 to generate pXJ-SrpC and pXJ-SrpNC respectively. pXJ-Ush was described previously (Haenlin *et al*., 1997). The reporter plasmid pGATA-luc contains multimerized GATA binding sites upstream of the β-globin TATA box and the luciferase reporter gene (Newton *et al*., 2001).

Cells, transfection and reporter assay

COS-7 cells, grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) fetal bovine serum (FBS), were seeded in 12-well culture plates at a density of 0.5×10^4 cells/well and transfected 24 h later using the calcium phosphate method. Fifty nanograms of the β-galactosidase reporter pCMV-βgal was used per transfection as an internal control. Empty pCMV expression vector was added as required to keep the amount of pCMV plasmid constant. pUC plasmid was added as carrier DNA to a total amount of 10 μg DNA per transfection. Cells were harvested 48 h after transfection, washed in PBS and lysed in reporter lysis buffer (Promega). Luciferase assays were performed using a Lumat LB 9501 luminometer. β-Galactosidase activity was measured with a Galacto-Light Plus kit (Applied Biosystems) according to the manufacturer's instructions. Luciferase activities are representative of three independent experiments done in triplicate and have been normalized to β-galactosidase levels.

Immunoprecipitations and immunoblotting

COS-7 cells were plated in 100-mm diameter dishes and transfected using the DEAE/Dextran method. Forty-eight hours after transfection, cells were washed twice in PBS and lysed for 5 min at 4°C in lysis buffer (50 mM Tris–HCl pH 8, 300 mM NaCl, 10 mM $MgCl_2$, 0.4% NP-40) supplemented with protease inhibitors (Roche). Soluble proteins were collected by centrifugation and diluted (v/v) with dilution buffer (50 mM Tris–HCl pH 8, 2.5 mM $CaCl_2$, 0.4% NP-40) supplemented with DNase. Lysates were incubated for 3 h at 4°C with primary antibody-protein A Sepharose complexes. Immunoprecipitated proteins were washed with lysis buffer/dilution buffer and resolved on SDS–PAGE. Western blotting was performed using standard techniques. Monoclonal anti-Flag (M2) and anti-c-*myc* antibodies were purchased from Sigma and Roche, respectively.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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