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## The GATA factor Serpent cross-regulates *lozenge* and *u-shaped* expression during *Drosophila* blood cell development

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### Abstract

The *Drosophila* GATA factor Serpent interacts with the RUNX factor Lozenge to activate the crystal cell program, whereas SerpentNC binds the Friend of GATA protein U-shaped to limit crystal cell production. Here, we identified a *lozenge* minimal hematopoietic cis-regulatory module and showed that *lozenge-lacZ* reporter-gene expression was autoregulated by Serpent and Lozenge. We also showed that upregulation of *u-shaped* was delayed until after *lozenge* activation, consistent with our previous results that showed *u-shaped* expression in the crystal cell lineage is dependent on both Serpent and Lozenge. Together, these observations describe a feed forward regulatory motif, which controls the temporal expression of *u-shaped*. Finally, we showed that *lozenge* reporter-gene activity increased in a *u-shaped* mutant background and that forced expression of SerpentNC with U-shaped blocked *lozenge*- and *u-shaped-lacZ* reporter-gene activity. This is the first demonstration of GATA:FOG regulation of *Runx* and *Fog* gene expression. Moreover, these results identify components of a Serpent cross-regulatory sub-circuit that can modulate *lozenge* expression. Based on the sub-circuit design and the combinatorial control of crystal cell production, we present a model for the specification of a dynamic bi-potential regulatory state that contributes to the selection between a Lozenge-positive and Lozenge-negative state.

### INTRODUCTION

Hematopoiesis is a dynamic process that produces the various blood cell lineages from a single hematopoietic stem cell and is regulated by key lineage-specific factors (Orkin, 2000; Zhu and Emerson, 2002; Warren and Rothenberg, 2003). When viewed in the context of all known genetic interactions, the complexity of the processes that control hematopoiesis can be appreciated, but not readily understood (Swiers et al., 2006). An understanding of the interactions in terms of gene activation or repression, coupled with information about *cis*-regulatory inputs, has revealed mechanistic details about the modular sub-circuits that together describe these processes and development in general (Swiers et al., 2006; Oliveri and Davidson, 2007). Moreover, simple genetic model organisms provide an opportunity to analyze these interactions *in vivo*, thereby providing a direct link between the genomic programs that encode them and the biological functions they control. The *Drosophila* model system has been used to identify conserved key factors and investigate their function during hematopoiesis (Dearolf, 1998; Fossett and Schulz, 2001; Evans et al., 2003; Meister and Lagueux, 2003; Sorrentino et al., 2005; Hartenstein, 2006; Crozatier and Meister, 2007). In order to more fully understand

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*Drosophila* hematopoiesis, we characterized the role of Serpent (Srp) cross-regulation of Lozenge (Lz) and U-shaped (Ush) in the crystal cell lineage.

The blood system of the fly lacks the complexity seen in vertebrates. Nevertheless, cross-species comparisons have shown that fundamental aspects of hematopoiesis are conserved across taxa (Fossett and Schulz, 2001; Evans et al., 2003; Fossett et al., 2003; Meister and Lagueux, 2003; Sorrentino et al., 2005; Hartenstein, 2006; Crozatier and Meister, 2007). *Drosophila* has two primary blood cell types, plasmatocytes and crystal cells, which have similar functions to the vertebrate myeloid lineages (Rizki, 1978; Dearolf, 1998; Evans et al., 2003). Crystal cells, named for their crystalline inclusion bodies, are required for wound repair and xenobiotic encapsulation (Rizki, 1978). Plasmatocytes are operational macrophages and synthesize antimicrobials (Rizki, 1978; Tepass et al., 1994; Dearolf, 1998). Like their vertebrate counterparts, these cells develop from a common hematopoietic progenitor (Rizki, 1978; Tepass et al., 1994; Dearolf, 1998; Lebestky et al., 2000; Lanot et al., 2001). Both vertebrate and *Drosophila* hematopoiesis consists of two spatially and temporally distinct periods or waves. In *Drosophila*, the first hematopoietic wave begins in the early embryonic head mesoderm. The second wave begins in embryogenesis and continues throughout larval development within a specialized hematopoietic organ, called the lymph gland (Dearolf et al., 1998; Lebestky et al., 2000; Lanot et al., 2001; Fossett and Schulz, 2001; Evans et al., 2003; Hartenstein, 2006; Crozatier and Meister, 2007).

Srp, similar to vertebrate GATA-2, is positioned at the apex of hematopoiesis and, as such, is required for the production of hemocyte precursors (Rehorn et al., 1996; Sam et al., 1996). In this role, Srp acts upstream of Glial cells missing (Gcm) and the RUNX factor Lz, which are required later for plasmatocyte and crystal cell production, respectively (Bernardoni et al., 1997; Lebestky et al., 2000; Kammerer and Giangrande, 2001; Alfonso and Jones, 2002). Of the conserved hematopoietic regulators, the GATA, Friend of GATA (FOG), and RUNX protein families are of particular interest because pair-wise interactions between GATA and FOG or GATA and RUNX regulate both vertebrate and *Drosophila* hematopoiesis (Tsang et al., 1997; 1998; Querfurth et al., 2000; Elagib et al., 2003; Fossett et al., 2003; Waltzer et al., 2003; Cantor and Orkin, 2005; Ferjoux et al., 2007). Moreover, Srp acts as a contextual switch between RUNX activation and FOG repression of the crystal cell lineage (Fossett et al., 2003). GATA transcriptional regulators generally have two zinc-finger domains. The C-terminal zinc-finger binds the DNA recognition sequence, WGATAR (Cantor and Orkin, 2005). The N-terminal zinc-finger interacts with FOG proteins; and the GATA:FOG complex modifies transcription by either activating or antagonizing activity, depending upon the gene regulatory context (Crispino et al., 1999; Lu et al., 1999; Svensson et al., 1999; Tevosian et al., 1999; Chang et al., 2002; Letting et al., 2004; Hong et al., 2005; Cantor and Orkin, 2005; Lowry and Mackay, 2006). The *srp* gene is alternatively spliced to produce either a single C-terminal zinc-finger isoform (SrpC) or the canonical dual zinc-finger protein (SrpNC). The FOG protein Ush interacts with SrpNC, but not SrpC, which lacks the N-terminal zinc-finger (Walter et al., 2002; Fossett et al., 2003). RUNX proteins bind DNA through the conserved Runt domain (Tracy and Speck, 2000; Speck and Gilliland, 2002; Rennert et al., 2003; Anglin and Passaniti, 2004). In general, RUNX activity is influenced by a variety of interacting factors, including GATA factors (Coffman, 2003; Elagib et al., 2003; Fossett et al., 2003; Waltzer et al., 2003; Ferjoux et al., 2007). Of the three mammalian *Runx* genes, *Runx1* is required for hematopoiesis (Otto et al., 2003), and is one of the most frequent targets of chromosomal translocations associated with human leukemia (Okuda et al., 1996; Speck and Gilliland, 2002; deBruijn and Speck, 2004). Currently, there is a lack of information about the role of *Runx1* in hematopoietic gene regulatory networks (Swiers et al., 2006; Otto et al., 2003).

Srp, Lz and Ush act combinatorially to regulate crystal cell production. Both SrpC and SrpNC can interact with Lz to activate the crystal cell program (Fossett et al., 2003; Waltzer et al.,

2003), whereas only SrpNC interacts with Ush to repress crystal cell production (Fossett et al., 2003). This suggests that Srp acts as a contextual switch, mediating cross-talk between crystal cell activation and repression pathways. In order to increase our understanding of the mechanistic basis for this contextual switch, and how it regulates crystal cell production, we investigated the cis- and trans-regulation of *lz* and *ush*. Collectively, our data provide evidence for a Srp cross-regulatory sub-circuit that regulates *lz* and *ush* expression. Based on these results, we present a model for the specification of a dynamic bi-potential regulatory state that contributes to the selection between a Lz-positive and Lz-negative state.

## MATERIALS AND METHODS

### Fly strains

Fly stocks were maintained at 23°C on standard food, and *w<sup>1118</sup>* was used as the wild-type stock. The following fly lines were used in this study and are described elsewhere: *ush<sup>1</sup>/SM6*, *Roi*, *eve-lacZ*; *upstream activation sequence (UAS)-ush*; *UAS-srpNC*; *UAS-srpNC<sup>V421G</sup>*; and *twi-Gal4* (Fossett et al., 2001; 2003). The following strains were generous gifts from colleagues: *lz-Gal4;UAS-GFP* (J. Pollock, Duquesne University); *UAS-lz* (J. Canon and U. Banerjee, University of California, Los Angeles); *UAS-srpC* (D. K. Hoshizaki, University of Nevada, Las Vegas, NV); *UAS-srpNC;UAS-lz* (K. M. Gajewski, University of Texas, M. D. Anderson Cancer Center, Houston, TX). *UAS-srpNC,UAS-ush* stock was constructed using genetic recombination between *UAS-transgenes* located on chromosome II. The generation of strains carrying *lz-lacZ* transgenes is described below.

### Generation of transgenic animals carrying *lz-lacZ* fusion constructs

Overlapping DNA fragments of the *lz* 1.5 kb 5' UTR genomic DNA region were analyzed for their ability to direct *lacZ* reporter-gene expression in crystal cells. This was accomplished by generating PCR fragments that were either cloned directly into the P-element CaSpeR-Hsp43-AUG-βgal (Chab) germline transformation vector (Thummel et al., 1988), or first cloned into pCR-II TOPO cloning vector (Invitrogen) and subsequently into Chab vector. Site-directed mutations (SDM) were introduced into DNA fragments as described previously (Muratoglu et al., 2006). The oligonucleotide primers used to generate point mutations in DNA fragments are available upon request.

The DNA sequence of each recombinant vector was verified prior to injection. *w<sup>1118</sup>* embryos were injected with the recombinant vectors by Model Systems Genomics, Duke University, or Rainbow Transgenic Flies, Inc., Newbury Park, CA. Germline transformants were established according to previously described methods and were screened for tissue-specific *lacZ* expression using immunohistochemical staining analysis as previously described (Gajewski et al., 1997). At least six independent lines were generated and tested for each construct.

### Determination of the *lz-Gal4* chromosomal insertion site

Plasmid rescue was used to identify the *lz* genomic sequences that flank the pGawB insertion (Pirrota, 1986). Briefly, genomic DNA was isolated from *lz-Gal4* fly lines. The DNA was cut with *MspI* or *DraI*, each having a single restriction site within the pGawB plasmid insert. Ligation of this restriction digest produced a circular template for PCR. Specific primers for the pGawB plasmid, but facing the flanking sequences, were used to produce a pGawB/flanking-sequence chimeric fragment. The fragment was cloned into the pCR-II TOPO cloning vector (Invitrogen) and sent to The Biopolymer Core Facility at the University of Maryland, Baltimore for sequence analysis.

## Immunohistochemical and fluorescent antibody staining of embryos and larvae

Collection, fixation, and immunohistochemical staining of embryos were performed as previously described (Schulz and Fossett, 2005). The following primary antibodies were used: mouse anti- $\beta$ -galactosidase, 1:1000 (Promega); rabbit anti-U-shaped, 1:750 (Fossett et al., 2001); rabbit anti-Serpent, 1:1000 (Hu, 1995). Either biotinylated (Vector Laboratories) or fluorescent (Molecular Probes, Invitrogen) conjugated secondary antibodies were used for primary antibody recognition, according to the manufacturer's recommendation. All embryos were visualized with Zeiss Axioplan optics.

Lymph glands from third instar wandering larvae were removed by making an incision below the head region and removing the lymph gland, which was attached to the dorsal vessel and brain. Fixation and immunostaining were performed as previously described (Schulz and Fossett, 2005). Stained preparations were mounted in PBS under cover slips and visualized using Zeiss Axioplan optics.

## Gene expression analysis in mutant and *Gal4/UAS* embryos

Embryos were cultured and collected at 23°C. Gain of function studies were conducted using the *Gal4/UAS* binary system (Brand and Perrimon, 1993). *lz-lacZ* activity in gain of function backgrounds required a two generation cross. Each *lz-lacZ* line was crossed to *twi-Gal4* virgin females to produce *twi-Gal4/+; lz-lacZ/+* transheterozygous progeny. The F<sub>1</sub> virgin females were collected and then crossed to one of the following: *UAS-srpNC* or, *UAS-lz* or, *UAS-srpC* or, *UAS-srpNC;UAS-lz* or, *UAS-srpNC,UAS-ush* homozygous males or *UAS-srpC/+; UAS-lz/+* heterozygous males. To assess *lz-lacZ* activity in *ush<sup>1</sup>* homozygous embryos also required a two generation cross. Males carrying *lz-lacZ* constructs on chromosome III were crossed to *ush<sup>1</sup>/SM6, Roi, eve-lacZ* virgin females. F<sub>1</sub> *ush<sup>1</sup>/+; lz-lacZ/+* transheterozygous progeny were then intercrossed. Because *Ush* is required for germ band retraction, homozygous mutants are easily identified by the altered morphology that results from failure of the germ band to retract. In all cases, the F<sub>2</sub> progeny were collected during embryogenesis and assayed for  $\beta$ -galactosidase activity using immunohistochemistry as previously described (Gajewski et al., 1997).

To assess *Ush* expression in *Srp* and *Lz* gain of function backgrounds, homozygous *twi-Gal4* virgins were crossed to homozygous *UAS-srpNC;UAS-lz* males or, heterozygous *UAS-srpNC<sup>V421G</sup>/+;UAS-lz/+* males. *ush-lacZ* activity in gain of function backgrounds required a two generation cross. Each *ush-lacZ* line was crossed to *twi-Gal4* virgin females to produce *twi-Gal4/+; ush-lacZ/+* transheterozygous progeny. The F<sub>1</sub> virgin females were collected and then crossed to one of the following: *UAS-srpNC;UAS-lz* or, *UAS-srpNC,UAS-ush* homozygous males or, *UAS-srpNC<sup>V421G</sup>/+;UAS-lz/+* or, *UAS-srpNC,UAS-ush/+; UAS-lz/+* heterozygous males. The progeny from these crosses were collected and immunohistochemically stained with either the *Ush* or  $\beta$ -galactosidase antibody as previously described (Gajewski et al., 1997).

## RESULTS

### Identification of a *lz* minimal crystal cell cis-regulatory module

Both *Srp* isoforms can interact with *Lz* to activate the crystal cell program (Fossett et al., 2003; Waltzer et al., 2003). Conversely, *SrpNC* acting with *Ush* converts *SrpNC* from an activator to a repressor of crystal cell production (Fossett et al., 2003). These transcription factors may control crystal cell production by directly regulating *lz* gene expression. In this case, we expect that both activation by *Srp* and repression by *SrpNC:Ush* would be mediated through GATA sites within the *lz* crystal cell cis-regulatory module (CRM). To test this

hypothesis, we identified a 1.2 kb *lz* crystal cell CRM and then determined which cis-elements are required for *lz* expression.

We employed a three-tiered strategy to narrow our search for the *lz* crystal cell CRM. First, we used the *lz-Gal4* fly line to determine the approximate location of the CRM. In this fly line, the promoter-less *Gal4* cDNA (pGawB) is inserted into the *lz* locus (Crew et al., 1997), which directs reporter-gene expression in a pattern that recapitulates endogenous *lz* expression (Lebestky et al., 2000). We determined that the pGawB insertion site was 160 bp upstream of the transcription start site (Fig. 1A). Based on this information, we then analyzed the region upstream of the transcription start site for highly conserved sequences between the *melanogaster* sibling species, *sechellia*, *yakuba* and *erecta*. Such comparisons can help identify evolutionarily conserved cis-elements. We identified three such regions at positions -1169 to -827, -643 to -320, and -206 to the coding region (Fig. 1A). Finally, because of our interest in Srp regulation of *lz* gene expression, we searched this 1.2 kb region for GATA motifs. This search identified 13 GATA motifs located between positions -1207 and -768. Thus, we isolated and tested a DNA fragment between positions -1263 and -18 for its ability to drive crystal cell-specific reporter-gene (*lacZ*) expression in vivo (Fig. 1A). *lz-lacZ* expression was first detected in embryonic crystal cell precursors during stage 9 and continued throughout development in this lineage (data not shown). Together, these results indicate that a crystal cell CRM is located within the 1.2 kb region upstream of the transcription start site. However, by stage 10, we also observed ectopic *lz-lacZ* expression that continued throughout development in the ectoderm (data not shown). Ectopic expression is defined as fragment-directed reporter-gene activity that differed significantly from the pattern produced by *lz-Gal4* driving *UAS-lacZ* (Lebestky et al., 2000).

To identify the *lz* minimal crystal cell CRM, we subdivided the 1.2 kb region into 5 overlapping fragments. Fragment designations are based on their positions relative to the transcription start site (Fig. 1A). Embryos harboring the -1236/-737 fragment exhibited *lz-lacZ* expression in the crystal cell lineage starting from stage 9 and continuing to stage 16 (Fig. 1B). We also observed *lz-lacZ* expression in plasmatocytes for two of the six lines tested, consistent with results seen with *lz-Gal4;UAS-lacZ* embryos (Lebestky et al., 2000). In addition, one line showed ectopic *lz-lacZ* expression in the amnioserosa and garland cells (Fig. 1B, data not shown). A similar expression pattern was detected in embryos harboring the -1019/-737 fragment (Fig. 2B). As seen with the parental fragments, the -1019/-931 fragment directed crystal cell-specific *lz-lacZ* expression throughout embryonic development. However, embryonic expression was first detected slightly later than with the parental fragments, beginning in stage 10 rather than stage 9 (Fig. 1B). In addition, one of the eight lines tested showed ectopic expression in the amnioserosa (Fig. 3B, data not shown). We tested two additional fragments from the 1.2 kb region. These two fragments, -1236/-1018 and -737/0, were not able to direct crystal cell-specific *lz-lacZ* expression (Fig. 1A). However, some of the lines generated from each fragment exhibited ectopic and plasmatocyte activity (data not shown).

While we were analyzing the 1.2 kb 5' non-coding region of *lz*, Bataille and co-workers published the crystal cell expression pattern of a larger fragment located between -1509 and -59 (Bataille et al., 2005). They reported fragment-directed *lz-lacZ* expression as early as stage 7 in hemocyte precursors. We generated and analyzed 9 independent fly lines harboring this fragment. All of the lines directed expression in the crystal cell lineage throughout development. Seven lines had spatiotemporal expression similar to the -1236/-737 fragment, with expression being first detected during stage 9, whereas *lz-lacZ* expression was first detected during stage 8 in two of the lines (Fig. 1B). However, these two lines also exhibited ectopic expression, beginning during stage 10 and continuing throughout embryonic development (data not shown). The observed differences for the 1.5 kb-driven *lz-lacZ*

expression pattern may be due to methodological differences between laboratories. The difficulty in detecting a signal from a low level transcript could explain the variance seen with staging in this study. In any case, the results with the  $-1509/-59$  and  $-1236/-737$  fragments indicate that *lz* expression is upregulated by stage 9. The possibility of earlier activity during stage 7 or stage 8 prompted us to determine if an additional crystal cell-specific CRM was located upstream of the 1.2 kb fragment. We tested the region between positions  $-1509$  and  $-1210$ , but did not detect fragment-driven *lz-lacZ* activity in the crystal cell lineage (Fig. 1A, data not shown).

Our analysis of the *lz* 5' non-coding region indicated that genomic fragments lacking the  $-1019/-931$  region were unable to drive *lz-lacZ* expression in the crystal cell lineage. Thus, the  $-1019/-931$  fragment most likely contains a minimal CRM that is required for *lz* expression during crystal cell development. However, *lz*  $-1019/-931$  *lacZ* activity was detected during stage 10, slightly later in development than that of the larger  $-1509/-59$  or  $-1236/-737$  fragments (Fig. 1B). This suggests that regions outside of the  $-1019/-931$  CRM are also required for early activation of *lz* expression in crystal cell precursors.

We also tested the activity of the  $-1509/-59$ ,  $-1236/-737$ , and  $-1019/-931$  fragments in the third larval instar lymph gland (Fig. 1B). The third larval instar lymph gland is a set of bilateral primary lobes and a series of smaller secondary lobes that flank the dorsal vessel (insect heart). Morphological, functional, and gene expression analyses indicate that the primary lobes are divided into three distinct regions, namely, the cortical zone, the medullary zone and the posterior signaling center. *Lz* is expressed in the cortical zone, but not in the medullary zone (Lebestky et al., 2000; Jung et al., 2005). Consistent with these reports, all three fragments directed *lz-lacZ* expression in the cortical zone, but not in the medullary zone (Fig. 1B). Together, these data show that the *lz*  $-1019/-931$  *lacZ* expression pattern recapitulates the endogenous *lz* expression pattern, indicating that it is required for *lz* expression during both embryonic and larval lymph gland hematopoiesis.

### Clustered GATA and RUNX sites are required for *lz* minimal crystal cell CRM activity

Our analyses of the *lz* cis-regulatory region showed that a minimal CRM located between positions  $-1019$  and  $-931$  can direct *lz* expression in crystal cells. In order to identify trans-acting regulators of *lz* expression, we surveyed the  $-1019$  to  $-931$  sequence for cis-regulatory elements that may be required for crystal cell activity. This sequence contains four GATA motifs with the following distribution: two overlapping motifs at positions  $-1010$  and  $-1008$ , one at  $-957$ , and one at  $-947$ . We also identified two RUNX motifs at positions  $-995$  and  $-977$  (Fig. 2A). To determine if these elements are required for *lz* crystal cell expression, we introduced point mutations into either the GATA or RUNX motifs. We mutated all four GATA sites between positions  $-1010$  and  $-947$  and assigned this mutation the name G4m (Fig. 2A). The  $-1019/-931$  G4m fragment failed to direct *lz-lacZ* expression in crystal cells (Fig. 2B). This indicates that Srp binds these sites to upregulate *lz* in the crystal cell lineage. Based on the functional requirement of these four sites, we designated this cluster the GATA Core.

Of the 13 GATA motifs within the  $-1236/-737$  region, nine are located outside of the GATA Core. Four of the nine are located upstream of the Core, between positions  $-1207$  and  $-1081$ , and are designated the Upstream Cluster. The remaining five are located downstream of the Core, between positions  $-848$  and  $-768$ , and are designated the Downstream Cluster (Fig. 2A). To determine the functionality of these sites, we constructed two different fragments. Both fragments carried mutant versions of the GATA Core. Using the convention described above, the fragments were designated by their positions relative to the transcription start site, with the suffix G4m used to indicate disruption of the GATA Core. Thus, the  $-1019/-737$  G4m fragment had a mutated GATA Core, but a wild-type version of the Downstream Cluster (Fig. 2A). This fragment was unable to direct *lz-lacZ* expression in crystal cells (Fig. 2B). Thus, the

Downstream Cluster does not activate *lz* expression in crystal cells in the absence of the GATA Core. In contrast, we detected crystal cell activity in the  $-1236/-931$  G4m fragment (Fig. 2B). This indicates that the Upstream Cluster can compensate for the loss of the GATA Core and may function in the activation of *lz* expression. The functional difference between the Upstream and Downstream Clusters may reflect the fact that three of the four Upstream GATA motifs are conserved consensus (WGATAR) motifs across sibling species. In contrast, the Downstream Cluster contains only one consensus motif and two conserved motifs (Fig. 2A, data not shown). Together, these observations are consistent with studies that show GATA-1 activates  $\beta$ -globin gene expression using a subset of GATA motifs within the locus, and that GATA occupancy occurs more often among conserved consensus motifs (Im et al., 2005; Bresnick et al., 2006). Finally, these data strongly indicate that Srp directly upregulates *lz* activity in crystal cells by binding to specific GATA motifs.

Although the  $-1236/-931$  G4m fragment directed *lz-lacZ* expression in crystal cells, the  $-1236/-1018$  sub-fragment failed to do so (Figs. 1A, 2). The difference in activity between these two fragments is due to the cis-elements located between positions  $-1019$  and  $-931$ . As stated above, this region contains the GATA Core and two RUNX motifs. Because the Upstream GATA Cluster was able to compensate for the four GATA motifs within the Core, the RUNX motifs are most likely essential elements that are required to direct crystal cell activity. Furthermore, the functional Upstream Cluster is closer to the RUNX motifs than the nonfunctional Downstream Cluster, suggesting that cooperation between Lz and Srp may be necessary for *lz* expression. Based on these observations, we disrupted the two RUNX motifs within the  $-1019/-737$  fragment and assigned this mutation the name R2m (Fig. 2A). This mutation completely abolished the crystal cell activity of the  $-1019/-737$  fragment in nine out of ten lines tested, suggesting that Lz functions as an auto-activator in a positive feedback loop (Fig. 2B). Together, the mutational analysis strongly suggests that both GATA and RUNX motifs within the minimal  $-1019/-931$  CRM are required for *lz* crystal cell CRM activity. Moreover, these data are consistent with studies showing that crystal cell-specific CRMs in a number of genes, including *lz*, contain at least one essential RUNX motif (Muratoglu et al., 2006; Gajewski et al., 2007; Ferjoux et al., 2007). However, the functional Upstream GATA Cluster may act with either the two RUNX motifs and/or the GATA Core to direct crystal cell-specific activity, and this question is the subject of continuing studies.

### Dual role of SrpNC as a positive and negative regulator of *lz* crystal cell expression

SrpNC may function as a contextual switch by acting with Lz to activate the crystal cell program or with Ush to block activation (Fossett et al., 2003). Lz is required for crystal cell lineage commitment (Lebestky et al., 2000), and GATA and RUNX motifs within the *lz* minimal  $-1019/-931$  CRM are most likely required for its expression (Fig. 2B). Together, these observations strongly suggest that SrpNC cross-regulates *lz* expression through the GATA and RUNX motifs within the minimal  $-1019/-931$  CRM, thereby controlling crystal cell lineage commitment. To address this question, we assayed the *lz* minimal  $-1019/-931$  CRM for activity in embryos with altered levels of SrpNC, Lz, or Ush, either individually or in combination.

Forced mesodermal expression of SrpNC increased *lz*- $-1019/-931$  *lacZ* expression. Expression was detected in the stage 9 head mesoderm, earlier than in a wild-type background (Fig. 3A). The expanded expression pattern is reminiscent of the supernumerary crystal cell pattern produced by forced expression of both Srp isoforms (Fossett et al., 2003; Waltzer et al., 2003). Similar results were observed with SrpC (data not shown). Forced expression of Lz also increased the *lz-lacZ* expression domain in a pattern similar to the expression pattern of endogenous Srp (Fig. 3A). This indicates that Srp and Lz interact to activate *lz*  $-1019/-931$  *lacZ* through the GATA and RUNX motifs. This was confirmed by showing that co-expression

of SrpNC and Lz synergistically upregulated *lz* -1019/-931 *lacZ*. We observed strong *lz* -1019/-931 *lacZ* expression throughout the head mesoderm and weak expression in trunk mesoderm beginning in stage 9. By stage 12, strong expression was observed throughout the mesoderm, in stark contrast to results obtained with forced expression of either factor alone (Fig. 3A). Similar results were obtained with SrpC (data not shown). These results indicate that both Srp isoforms can interact with Lz to upregulate *lz* expression in crystal cells through the GATA and RUNX motifs located within the minimal CRM.

The functional role of the GATA motifs within the *lz* crystal cell CRM suggested that the SrpNC:Ush complex binds these motifs to block *lz* expression. To test this hypothesis, we examined *lz* -1019/-931 CRM activity in *ush* loss of function and SrpNC:Ush gain of function genetic backgrounds. Compared to the wild-type control, *lz* -1019/-931 CRM activity increased in a *ush* mutant background (Fig. 3B). Similar results were observed with the larger -1236/-737 fragment that contained all 13 GATA motifs (data not shown), indicating that Ush is required to limit the *lz* expression domain. Consistent with these results, forced expression of SrpNC and Ush blocked *lz* -1019/-931 CRM activity (Fig. 3B). We observed similar results with the larger -1509/-59 and -1236/-737 fragments (data not shown). Thus, the SrpNC:Ush complex blocked *lz* expression through the GATA sites within the minimal CRM. Together, these data indicate that SrpNC acts as cross-regulatory switch by interacting with Lz to maintain *lz* expression or with Ush to block *lz* expression. Collectively, our new data, coupled with our previously published results, indicate that the SrpNC:Ush complex blocks crystal cell production by blocking *lz* expression (Fig. 3B; Fossett et al., 2001;2003). Finally, forced expression of SrpNC and Ush did not block *gcm-lacZ*-expressing hemocyte precursors or plasmatocytes (data not shown). These results indicate that the SrpNC:Ush complex does not limit the production of these hemocyte classes. To increase our understanding of the cross-regulatory process and how it modulates crystal cell production, we expanded our investigation of *ush* expression during crystal cell lineage commitment.

### Ush expression is upregulated after crystal cell lineage specification

Ush is expressed in a variety of tissues throughout embryogenesis, including the following hematopoietic tissues: hemocyte precursors, plasmatocytes, and crystal cells (Fossett, et al., 2000; 2001). Previously, we identified a *ush* -7462/-25 CRM, which recapitulates endogenous Ush embryonic expression. Our analyses of sub-fragments from the -7462 to -25 region showed that two hematopoietic CRMs are located within 1.2 kb of the transcription start site. The distal -1243/-956 CRM drives expression in hemocyte precursors and plasmatocytes but not crystal cells, whereas the proximal -174/-25 CRM is required for expression in all three cell types. Genetic and mutational analyses of these hematopoietic CRMs showed that Srp activates expression in hemocyte precursors and plasmatocytes through the GATA motifs within both CRMs, whereas both Srp and Lz activate expression in crystal cells through clustered GATA and RUNX motifs unique to the -174/-25 CRM (Muratoglu et al., 2006).

During embryonic development, Srp is first detected in stage 5 hemocyte precursors (Rehorn et al., 1996; Sam et al., 1996). Lz is upregulated as early as stage 7 (Bataille et al., 2005) and as late as stage 9 (Fig. 1B). And, Ush is detected in hemocyte precursors during stage 8 and in crystal cell precursors again later during stage 10. During stage 13 and continuing through the end of embryogenesis, Ush is increasingly downregulated in the crystal cell lineage (Fossett et al., 2001). However, it is not known whether *ush* expression is maintained as hemocyte precursors develop into crystal cell precursors, or if expression is upregulated after lineage specification. To more accurately assess the timing of Ush expression, we determined the level of co-expression of Ush with *lz* -1236/-737 *lacZ* in early crystal cell precursors. We observed *lz* -1236/-737 *lacZ* expression in crystal cell precursors as early as stage 9. In contrast, Ush expression was not detected in these cells. However, beginning in stage 10 and continuing



through stage 11, we observed extensive co-expression of *lz*-1236/-737 *lacZ* and Ush in crystal cell precursors (Fig. 4, data not shown), consistent with our previous observations (Fossett et al., 2001). Similar results were observed with the larger -1509/-59 fragment (data not shown). Thus, Ush expression is not maintained as hemocyte precursors develop into crystal cell precursors, but is upregulated after lineage specification.

During stage 12, we continued to observe co-expression of Ush and *lz*-1236/-737 *lacZ*. However, adjacent to *lz*-1236/-737 *lacZ*-positive cells and within the crystal cell cluster, we also observed cells that expressed only Ush (Fig. 4). The morphology and position of these cells suggest that they are derived from *lz*-*lacZ*-positive precursors. Moreover, these cells were primarily Ush-positive, *lz*-*lacZ*-positive during stages 10 and 11 (Fig. 4; Fossett et al., 2001). Together, these observations are consistent with downregulation of *lz*-1236/-737 *lacZ* by the endogenous SrpNC:Ush complex to specify a Ush-positive, *lz*-*lacZ*-negative state. This interpretation is also in agreement with our results that showed forced expression of SrpNC and Ush blocked *lz* CRM activity and that *lz*-*lacZ* activity increased in a *ush* mutant background (Fig. 3B). Finally, during stage 13 and continuing until the end of embryogenesis, we observed an increasing number of *lz*-*lacZ*-positive cells with greatly diminished Ush expression (data not shown), consistent with previous studies (Fossett et al., 2001).

### Ush acting with SrpNC downregulates *ush* hematopoietic expression

We previously showed that Ush is downregulated in embryos with forced expression of SrpNC and Lz. In contrast, forced expression of SrpC and Lz did not inhibit expression. This suggests that the N-terminal zinc-finger of Srp is essential for repression (Fossett et al., 2003). The GATA N-terminal zinc-finger stabilizes DNA binding and serves as a protein interaction domain (Pedone et al., 1997; Crispino et al., 1999; Newton et al., 2001). Ush interacts with SrpNC through a conserved valine residue at position 421 (Crispino et al., 1999; Fossett et al., 2003). To investigate the mechanism of *ush* gene repression, we co-expressed Lz with the N-terminal zinc-finger mutant SrpNC<sup>V421G</sup> and assayed for endogenous Ush expression. Similar to results obtained with SrpC and Lz, SrpNC<sup>V421G</sup> and Lz did not repress Ush expression (Fig. 5A). We repeated these studies using the *ush*-7462/-25 *lacZ* CRM and, as with endogenous Ush, observed downregulation of *ush*-*lacZ* in embryos with forced expression of SrpNC and Lz, but not in those with forced expression of SrpNC<sup>V421G</sup> and Lz (Fig. 5B). Together, these data suggest that Ush acting with SrpNC is an auto-repressor of its hematopoietic expression. Furthermore, forced expression of SrpNC<sup>V421G</sup> and Lz upregulated, but did not repress, endogenous Ush or *ush*-7462/-25 *lacZ* expression (Fig. 5). This is consistent with our previous work that showed at least one Srp isoform acts with Lz to upregulate *ush* expression in crystal cells (Muratoglu et al., 2006). Together, these data suggest that SrpNC and Lz initially activate *ush* expression. Subsequently, Ush acts with SrpNC to downregulate its hematopoietic expression. Consistent with a cross-regulatory process, forced expression of SrpNC and Lz produced individuals with either downregulated or upregulated *ush*-7462/-25 *lacZ* activity when compared to the wild-type control (Fig. 5B, data not shown).

### Ush competes with Lz to control SrpNC regulation of *ush* gene expression

Ush auto-repression may occur by one of two possible mechanisms. First, Ush could interact with SrpNC and Lz to form a complex that blocks *ush* expression. Alternatively, Ush may compete with Lz by converting SrpNC from an activator to a repressor of *ush* gene expression in a similar manner to control of SrpNC regulation of *lz* gene expression. To determine if Lz is required for inhibition, we ectopically expressed SrpNC and Lz, SrpNC and Ush, or all three factors together and assayed *ush*-7462/-25 *lacZ* activity. As with forced expression of SrpNC and Lz, forced expression of all three factors downregulated *ush*-7462/-25 *lacZ* in embryonic hemocytes (Fig. 6A). We also observed embryos with increased *ush*-7462/-25 *lacZ* activity (data not shown). In contrast, forced expression of SrpNC and Ush completely blocked *ush*

-7462/-25 *lacZ* expression in hemocytes throughout the entire embryo population. Moreover, SrpNC and Ush completely blocked *ush -7462/-25 lacZ* activity in plasmatocytes, whereas residual activity remained in a sub-population of plasmatocytes with either forced expression of SrpNC and Lz or forced expression of all three factors (Fig. 6A). Collectively, these results show that SrpNC and Ush repressed *ush -7462/-25 lacZ* expression more effectively in the absence of ectopic Lz. In addition, forced expression of SrpNC and Ush did not block production of *gcm-lacZ*-expressing plasmatocytes but did block *ush* expression (data not shown). Finally, two observations show that the SrpNC:Ush complex can block *ush* expression without first downregulating the trans-activator Lz. First, the SrpNC:Ush complex blocked *ush -7462/-25 lacZ* activity in plasmatocytes, which do not express Lz. Second, the complex blocked *ush -7462/-25 lacZ* activity even in the presence of ectopic Lz (Fig. 6A). Together, these data support cross-regulatory control of *ush* expression, with activation by SrpNC and Lz and repression by SrpNC and Ush.

As stated above we previously identified a *ush* minimal -174/-25 CRM, which directs *lacZ* expression in hemocyte precursors, plasmatocytes, and crystal cells. This minimal hematopoietic CRM has a single RUNX motif and three GATA motifs, which are required for activity in the crystal cell lineage (Muratoglu et al., 2006). We next asked whether activation of *ush* expression by SrpNC and Lz and repression by SrpNC:Ush were mediated through this CRM. Forced expression of SrpNC and Lz upregulated the *ush -174/-25* CRM, producing pan-mesodermal expression by stage 12. This activity was somewhat reduced when all three factors were expressed together. In contrast, forced expression of SrpNC and Ush, in the absence of ectopic Lz, inhibited *ush -174/-25 lacZ* expression when compared to the wild-type control (Fig. 6B). These results indicate that both activation and repression can be mediated through the *ush -174/-25* CRM. Together, these results and those obtained with the larger -7462/-25 fragment indicate that SrpNC and Lz activate, whereas SrpNC and Ush repress, *ush* hematopoietic expression.

We observed differences between the *ush* minimal -174/-25 CRM and the larger -7462/-25 fragment that suggest the upstream -7462 to -174 region is important for repression of *ush* by SrpNC and Ush. Specifically, in contrast to the -7462/-25 CRM, forced expression of SrpNC, Lz and Ush did not downregulate the -174/-25 CRM to levels below that of wild-type (Fig. 6, compare A and B). This difference suggests that the SrpNC:Ush complex may function to repress *ush* hematopoietic expression through binding to GATA motifs within the -7462 to -174 region, thereby blocking activation of *ush* expression by competing with Lz. In this regard, there is a stark contrast between the -7462/-174 and the -174/-25 regions with respect to GATA/RUNX motif clustering. The -174/-25 region has one RUNX and three GATA motifs clustered within 83 bp. This is similar to the *lz* minimal CRM, which has four GATA and two RUNX motifs within 88 bp. In contrast, the -7462/-174 region has 19 GATA motifs and two RUNX motifs. However, the RUNX motifs are separated from the nearest GATA motif by at least 80 bp. The proximity of the GATA and RUNX motifs appears to be a key feature in the co-activation by Srp and Lz (Ferjoux et al., 2007). Thus, the architecture of the -7462/-174 region may favor SrpNC:Ush complex binding over Srp/Lz binding, whereas the -174/-25 region appears to accommodate both SrpNC:Ush and Srp/Lz binding. Increased binding of the SrpNC:Ush complex to the GATA motifs within the -7462 to -174 region may increase the ability of the SrpNC:Ush complex to repress *ush* expression in the presence of ectopic Lz. Overall, these results show that the SrpNC:Ush complex negatively regulates *ush* expression and that Ush competes with Lz to control SrpNC regulation of *ush* gene expression.

## DISCUSSION

Here, we present a gene regulatory basis for combinatorial control of crystal cell production by Srp, Lz, and Ush. Our results show that Srp cross-regulates *lz* and *ush* through the GATA/RUNX motifs within the hematopoietic CRMs of both genes. This gene regulatory sub-circuit functions to modulate *lz* expression. The sub-circuit specifies a dynamic bi-potential regulatory state in crystal cell precursors by co-activating *lz* and its repressor *ush*. Subsequently, the sub-circuit contributes to the selection of a Lz-positive or Lz-negative state, which regulates crystal cell production.

Lz is upregulated in hemocyte precursors and is required for crystal cell lineage specification and differentiation (Lebestky et al., 2000; Bataille et al., 2005). To more fully understand the control of crystal cell production, we began by investigating the transcriptional regulation of *lz* expression. We identified a *lz* minimal CRM and showed that it directs expression in the crystal cell lineage. Our results showed that Srp activates *lz-lacZ* reporter-gene expression by binding to the GATA motifs within this CRM. Specifically, mutational analyses of the *lz* CRM showed that the GATA motifs were required for activation. In addition, forced expression of Srp produced both early activation of the *lz* CRM and an expanded expression domain when compared to the wild-type control. Moreover, this expression pattern was reminiscent of the supernumerary crystal cell pattern produced by forced expression of Srp (Fossett et al., 2003; Waltzer et al., 2003). Together, these observations indicate that *lz* is directly activated by Srp as a prerequisite for crystal cell specification.

In addition to the GATA motifs, *lz* CRM activity requires canonical RUNX motifs. This indicates that positive autoregulation of *lz* is involved during crystal cell commitment and development. Autoregulation is likely a conserved property of the *Runx* gene family. RUNX2 has been shown to both positively and negatively autoregulate its expression in osteoblasts (Ducy et al., 1999; Drissi et al., 2000). In addition, all three mammalian *Runx* genes carry canonical RUNX motifs within their respective cis-regulatory regions, suggesting that *Runx1* and *Runx3* are autoregulated as well (Otto et al., 2003; Smith et al., 2004). However, Lz autoregulation requires cooperative interaction with Srp, evidenced by the fact that mutation of either the GATA or RUNX motifs completely blocked *lz* CRM activity throughout embryonic development. Moreover, forced co-expression Srp and Lz synergistically activated the *lz* CRM. Together, these results and the recent report of Ferjoux and colleagues (Ferjoux, et al., 2007) indicate that both Srp and Lz interact to maintain *lz* expression during crystal cell commitment and development.

Srp and Lz are also required to activate *ush* expression in crystal cell precursors (Muratoglu et al., 2006). This gene regulatory sequence, where Srp activates *lz* and then both Srp and Lz are required to co-activate *ush*, constitutes a feed forward regulatory motif. This type of motif determines the order of gene expression and controls the temporal expression of the downstream target (Mangan et al., 2003; Swiers et al., 2006). Accordingly, we observed Ush expression in the crystal cell lineage after the onset of *lz-lacZ* expression. Thus, this design insures that the crystal cell precursors are specified by Lz before upregulation of the repressor Ush. This is essential because premature activation of the SrpNC:Ush complex completely blocked *lz* CRM expression and crystal cell production.

After the initial specification of the crystal cell precursors, Srp and Lz maintain *lz* and upregulate *ush* through clustered GATA/RUNX motifs within the CRMs of both genes. As a result, *lz-lacZ* and Ush were co-expressed in the crystal cell lineage from embryonic stage 10 through 14. The co-expression of Lz and its antagonist Ush forms the basis for the bi-potential nature of the crystal cell precursor. As such, this regulatory state provides the precursor with the option of progressing towards either the Lz-positive or Lz-negative state. Thus, Srp and Lz

co-regulation of crystal cell development involves more than just activation of the crystal cell program, but rather includes the specification of a dynamic bi-potential regulatory state. Furthermore, Srp and Lz activate two crystal cell-specific genes (prophenoloxidasases; *proPOs*) in stage 11 precursors through GATA/RUNX sites within the CRMs of each gene (Gajewski et al., 2007; Ferjoux et al., 2007). The co-expression of Ush with these crystal cell-specific genes may be consistent with lineage priming, showing that regulators of opposing pathways are expressed in bi-potent precursors prior to lineage commitment (Hu et al., 1997; Orkin, 2000; Warren and Rothenberg, 2003; Ye et al., 2003; Huang et al., 2007). Collectively, these results indicate that Srp and Lz direct *ush* expression as part of a feed forward motif, which insures the bi-potential regulatory state is established after crystal cell specification and before lineage commitment. Finally, in the dynamic bi-potential regulatory state, where Ush and Lz compete with SrpNC to cross-regulate each other, the expected phenotypic heterogeneity is reflected in the co-localization of *lz-lacZ* and Ush during embryonic stages 12 through 14. Initially, we observed *lz-lacZ*-positive, Ush-positive precursors. By embryonic stage 12, the population included cells that expressed only Ush (*lz-lacZ*-negative, Ush-positive). During stage 13, we observed a third cell type (*lz-lacZ*-positive with diminished Ush expression).

As part of the Srp cross-regulatory sub-circuit, both Srp isoforms can act with Lz to maintain *lz* expression and activate *ush* to produce the bi-potential regulatory state. However, only SrpNC can mediate cross-antagonism between Lz and Ush and, thereby, the selection of the Lz-positive or Lz-negative state. This illustrates the importance of alternative splicing of *srp* (Fossett et al., 2001; Waltzer et al., 2002) as a determinant of *lz* gene regulation. The role of the SrpNC:Ush complex in selecting the Lz-negative state is supported by our results that showed forced expression of SrpNC and Ush blocked *lz* CRM activity, whereas CRM activity increased in *ush* mutant embryos. Thus, Ush converts SrpNC from an activator to a repressor that blocks *lz* expression through the GATA motifs within the crystal cell CRM. This contributes to the selection of the Lz-negative state, thereby preventing the bi-potential precursor from becoming a terminally differentiated crystal cell. Overall, this limits crystal cell number. Thus, these results provide a gene regulatory basis for our previous studies that showed the SrpNC:Ush complex limits crystal cell production (Fossett et al., 2003). Moreover, Lz-expressing crystal cell precursors can give rise to either Lz-positive crystal cells or Lz-negative plasmatocytes (Lebestky et al., 2000; Bataille et al., 2005), and the modulation of *lz* expression is most likely a critical determinant of cell fate choice. Therefore, the SrpNC:Ush complex may contribute to selection of the plasmatocyte lineage by downregulating *lz* expression, thereby terminating the crystal cell developmental program to stabilize plasmatocyte cell fate choice.

SrpNC-mediated cross-antagonism of Lz and Ush is a dynamic process. SrpNC and Ush not only blocked *lz* expression, but the SrpNC:Ush complex can also block *ush* expression in crystal cells and plasmatocytes. This limits the formation of the SrpNC:Ush complex and, thereby, its capacity to regulate gene expression in these cells. In plasmatocytes the SrpNC:Ush complex also blocks expression of *croquemort*, an important component of the programmed cell death pathway (Franc et al., 1999; Waltzer et al., 2002). Thus, negative regulation of *ush* may relieve *croquemort* repression preparing plasmatocytes to function during programmed cell death. In crystal cells, downregulation of *ush* limits the capacity of the SrpNC:Ush complex to block *lz* expression. This may provide an additional regulatory tier that prevents the complete repression of *lz* expression in bi-potent crystal cell precursors. The net effect would be to increase the availability of SrpNC to act with Lz to maintain the Lz-positive state. As part of the Srp cross-regulatory sub-circuit, subtle changes in the relative levels of SrpC and SrpNC may be involved in locking on the Lz-positive, Ush-negative regulatory state. These differences would be mediated through the distinct characteristics of the individual CRMs, within the overall architecture of the cross-regulatory sub-circuit, resulting in the shift to the Lz-positive,

Ush-negative state. This subject is also being investigated in our laboratory as part of the larger gene regulatory network that controls crystal cell commitment and development.

Together, these studies provide the basis for a model of *Drosophila* hematopoiesis that describes the regulation of *lz* expression and, as a result, blood cell production. Although the totality of the regulatory process remains to be determined, this model builds on previous studies of crystal cell specification and development and describes the events that occur after the initial activation of *lz* by *Srp*. Subsequently, the Lz-positive state is maintained by cooperative interaction between *Srp* and Lz. These transcriptional regulators also activate *ush* expression in Lz-positive crystal cell precursors to produce a dynamic, bi-potential regulatory state. This provides for the selection of either a Lz-positive or Lz-negative state, which is determined in part by *SrpNC*-mediated cross-antagonism of Lz and Ush (Fig. 7). This model is also consistent with the observation that a number of factors involved in cell fate control function as components of cross-regulatory and auto-regulatory sub-circuits (Orkin, 2000; Swiers et al., 2006; Huang et al., 2007). Furthermore, the *Srp* cross-regulatory sub-circuit controls blood cell development as one part of a larger gene network. This sub-circuit is undoubtedly interlinked to additional sub-circuits that control related functions, such as the regulation of alternative splicing of the *srp* transcript to produce either the *SrpNC* or *SrpC* isoform (Fossett et al., 2001; Waltzer et al., 2002). Finally, the dynamic regulation of these hematopoietic factors is consistent with the observed population heterogeneity as crystal cell precursors progress from a bi-potential regulatory state to either a Lz-positive or Lz-negative state.

Aspects of GATA, FOG and RUNX functions are conserved between the fly and vertebrates. Our studies provide new information describing how these factors may interact to regulate *Runx* and *Fog* gene expression in *Drosophila*. Specifically, this study presents the first demonstration of GATA:FOG regulation of *Runx* gene expression. This may be particularly important to the understanding of vertebrate hematopoiesis because GATA, FOG and RUNX factors are co-expressed and required for megakaryopoiesis (Tsang et al., 1997; 1998; Song et al., 1999; Gaines et al., 2000; Nichols et al., 2000; Freson, et al., 2001; Mehaffey et al., 2001; Cantor et al., 2002; Chang et al., 2002; Michaud et al., 2002; Walker et al., 2002; Yu et al., 2002; Elagib et al., 2003; Tanaka et al., 2004). In addition, little is known about how *Runx* gene expression is regulated during this process (Otto et al., 2003; Swiers et al., 2006). We also present the first demonstration of FOG autoregulation. This finding may provide insights into regulation of vertebrate *Fog* genes, thereby increasing our understanding of the mechanisms by which these transcriptional regulators modify the function of GATA factors.

During vertebrate hematopoiesis, multipotent progenitors express lineage specific factors prior to lineage commitment (Hu et al., 1997; Orkin, 2000; Warren and Rothenberg, 2003; Ye et al., 2003). This constitutes a dynamic regulatory state that provides for multiple developmental options (Orkin, 2000). As a result, lineage commitment can involve cross-antagonism to select one developmental program and repress alternative lineage programs (Orkin, 2000; Cantor and Orkin, 2002). Our studies have identified a gene regulatory sub-circuit that produces a dynamic, bi-potential regulatory state that contributes to selection of one of two possible regulatory pathways, consistent with these models of vertebrate hematopoiesis. Finally, this provides a mechanistic basis for the combinatorial control of blood cell development by *Srp*, Lz, and Ush and a framework for future investigations of *Drosophila* hematopoiesis.

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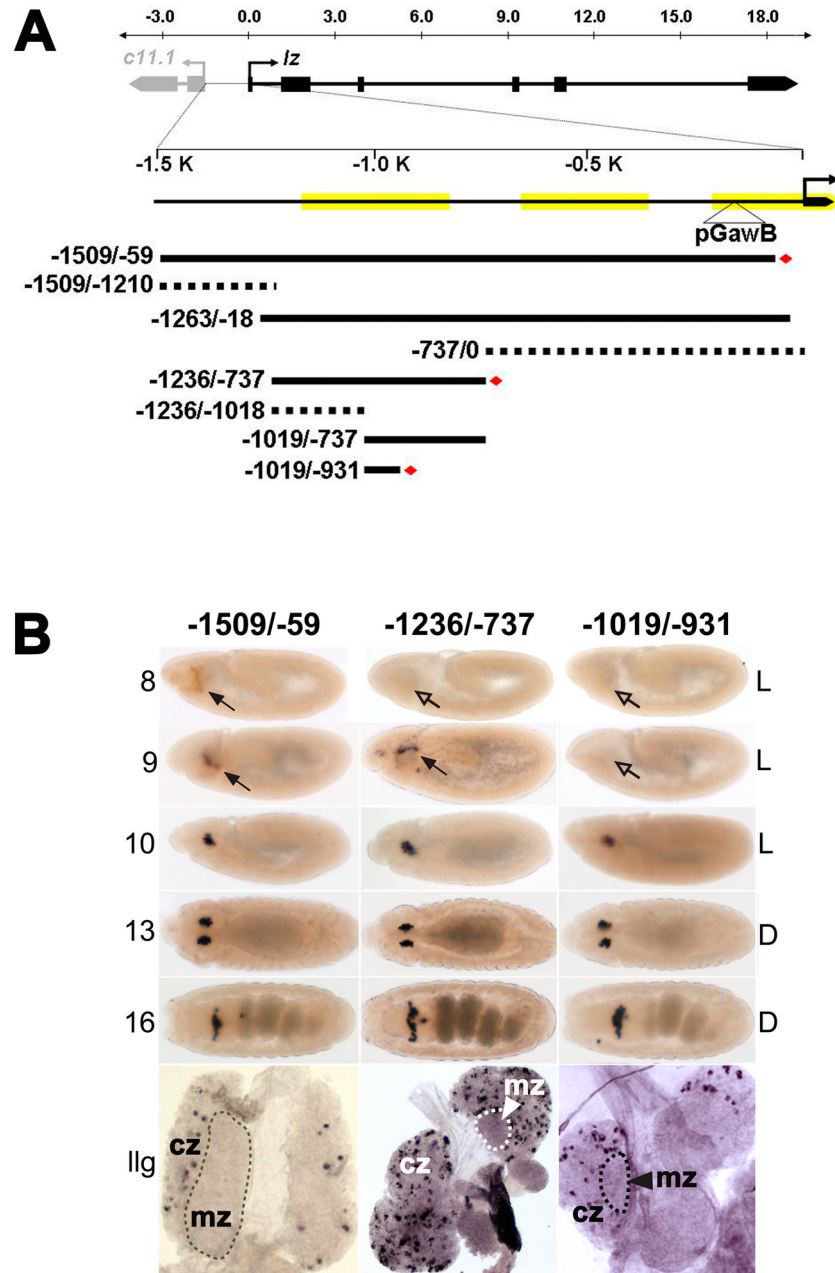
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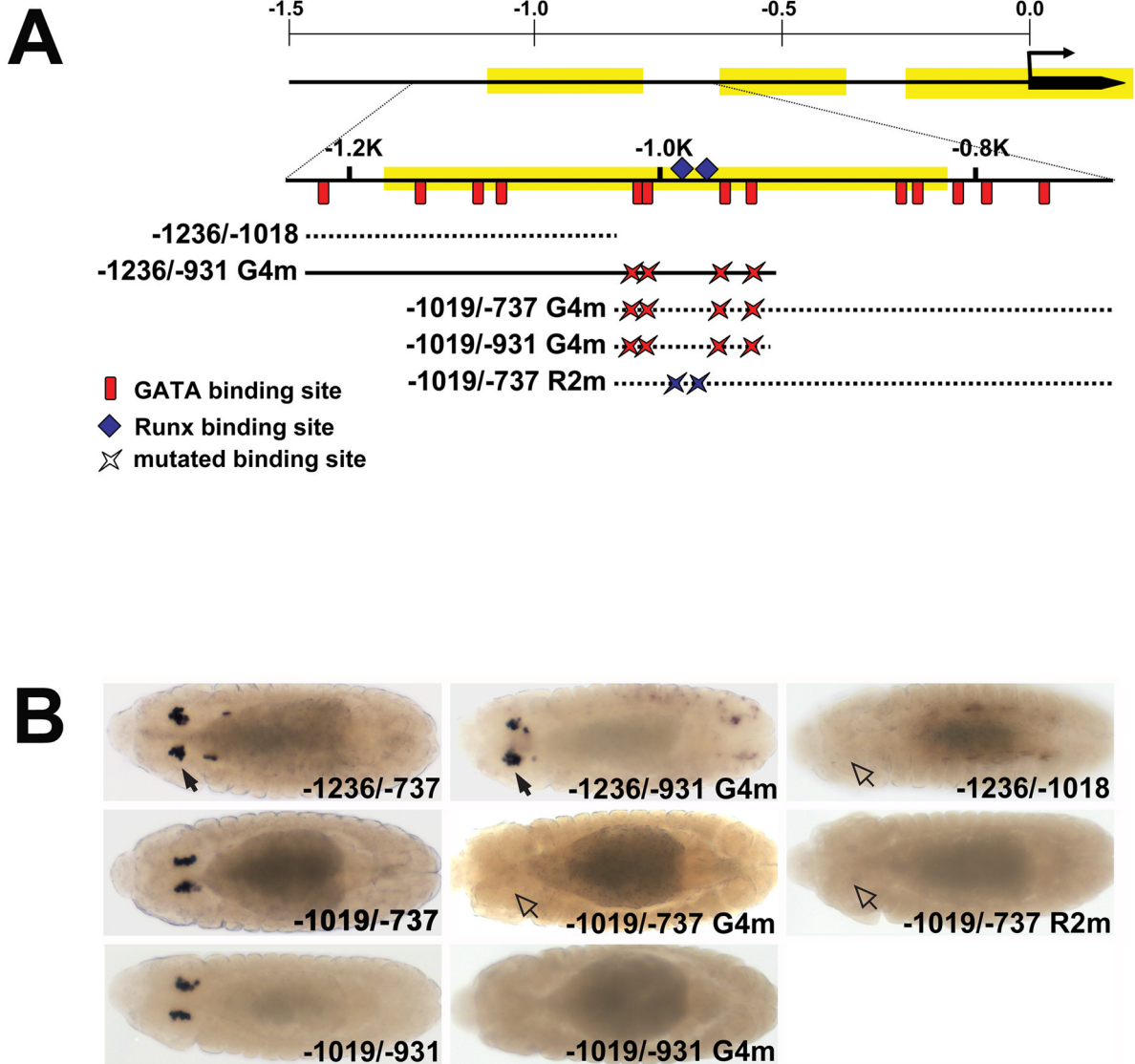


**Fig. 1. Identification of the *lz* crystal cell *cis*-regulatory module**

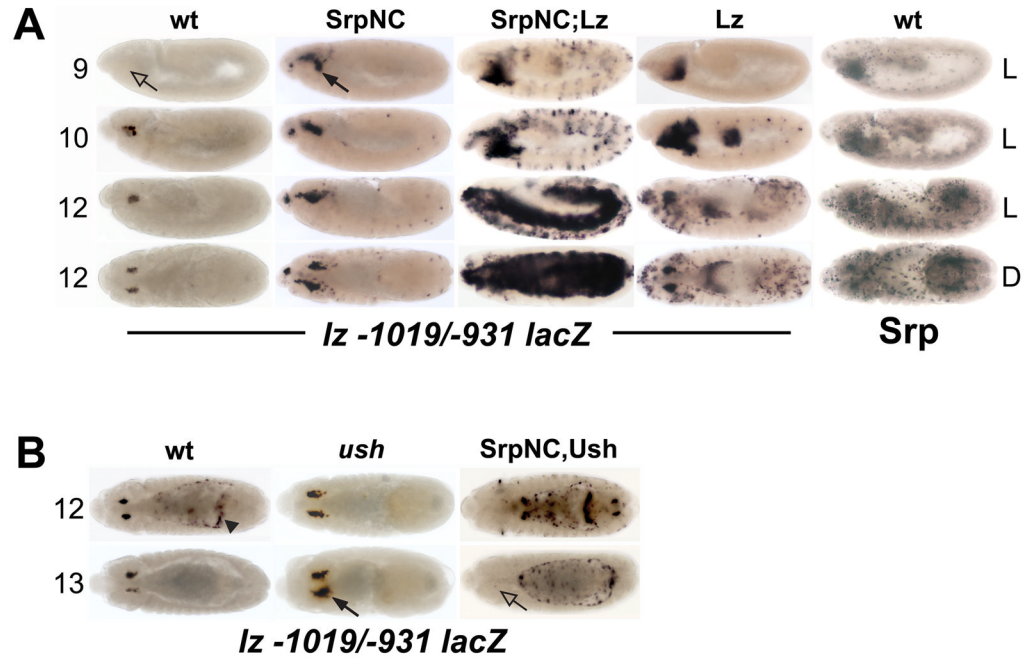
(A) Schematic of the *lz* locus and screen for the crystal cell CRM. A horizontal arrow marks the transcriptional start site, designated position 0. Solid lines indicate introns, whereas boxes indicate exons. The upstream *c11.1* gene is depicted in grey. Beneath the 21 kb map is an expanded map of the 1.5 kb region upstream of the *lz* transcription unit. Evolutionarily conserved regions are highlighted in yellow. The relative position of the P-element pGawB insert is indicated. The cryptic promoter used by this transgene likely lies just 5' to the transcription start site. The DNA fragments used to screen for crystal cell CRMs are indicated by black lines and are positioned and numbered relative to the transcription start site. Solid lines represent fragments with crystal cell activity, whereas dashed lines represent fragments lacking crystal cell activity. The red diamonds designate representative data presented in part

B. (B) Fragment-driven reporter-gene (*lacZ*) expression during embryogenesis and in third larval instar lymph gland. The stage of embryogenesis is indicated at the left of each row. The *lz-lacZ* strain designations are indicated at the top of each column. Orientation of the embryo is indicated to the right of each row. Solid arrows mark activity and open arrows mark the lack of activity in stage 8 hemocyte precursors and stage 9 crystal cell precursors. The boundaries between the cortical and medullary zones of the larval lymph glands are outlined.

Abbreviations: *lz*, *lozenge*; llg, third larval instar lymph gland; *cz*, cortical zone; *mz*, medullary zone; L, lateral view; D, dorsal view.

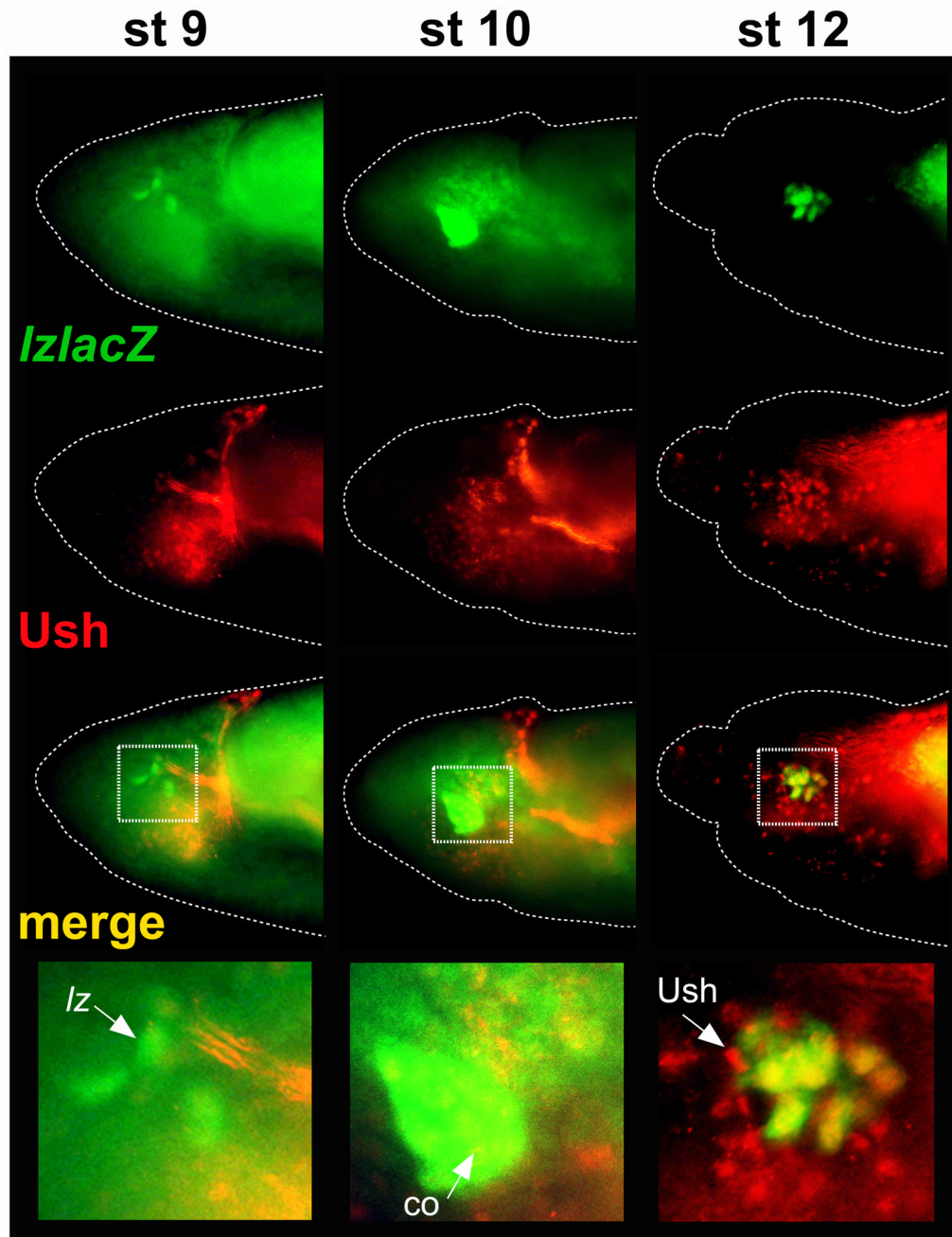


**Fig. 2. Clustered GATA and RUNX motifs are required for *lz* crystal cell CRM activity**  
 (A) Schematic showing fragments with mutated GATA and RUNX motifs used to evaluate *lz-lacZ* CRM activity. The 1.5 kb region upstream of the *lz* transcription start site is depicted with the evolutionarily conserved regions highlighted in yellow. Beneath the map of the 1.5 kb region is an expanded map of the region from -1250 to -727. Red vertical lines show the relative positions of the 13 GATA motifs between positions -1207 and -768. The blue diamonds mark the positions of the two RUNX motifs located at -995 and -977. Mutations in the four GATA motifs, between positions -1010 and -947, are designated G4m, whereas the mutations in the two RUNX motifs are designated R2m. The mutated fragments are numbered and positioned relative to the transcription start site, with the suffix G4m or R2m to indicate disruption of the GATA or RUNX motifs, respectively. Solid line represents a fragment with crystal cell activity, whereas dashed lines represent fragments lacking crystal cell activity. (B) Comparison of wild-type and GATA or RUNX mutant CRMs. Dorsal views of stage 13 embryos. *lz-lacZ* strains are indicated in the lower right hand corner of each panel. Closed arrows mark activity in crystal cells; open arrows mark lack of activity. Abbreviations: G4m, GATA Core mutants; R2m, RUNX mutants.



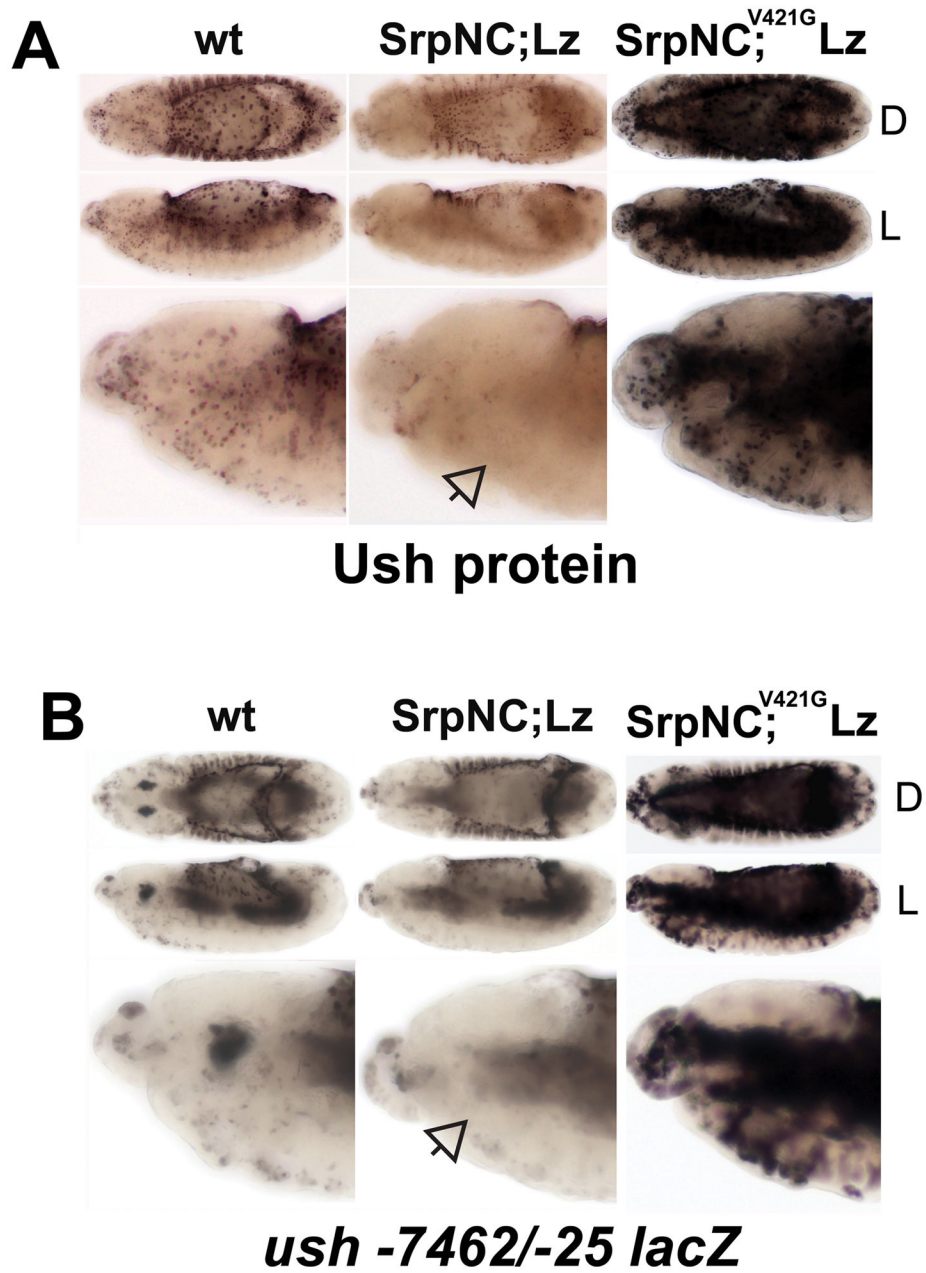
**Fig. 3. SrpNC is a positive and negative regulator of *lz* crystal cell CRM activity**

(A) SrpNC and Lz synergistically upregulate *lz* -1019/-931 *lacZ* activity. From left to right: embryos in the first four columns are stained with  $\alpha$ - $\beta$ -galactosidase antibody; embryos in the last column are stained with  $\alpha$ -Srp antibody. (B) SrpNC and Ush block *lz* -1019/-931 *lacZ* activity. *lz* -1019/-931 *lacZ* activity was assessed in different genetic backgrounds and developmental stages. The stage of embryogenesis is indicated at the left of each row. The genetic background is indicated at the top of each column. Orientation of the embryo is indicated at the right of each row (panel A only). SrpNC indicates *twi-Gal4* driving *UAS-srpNC*. Lz indicates *twi-Gal4* driving *UAS-lz*. SrpNC;Lz represents *twi-Gal4* driving *UAS-srpNC* and *UAS-lz* located on chromosomes II and III, respectively. SrpNC,Ush represents *twi-Gal4* driving *UAS-srpNC* and *UAS-ush* located on chromosome II. Closed arrows mark increased activity in crystal cells; open arrow marks lack of activity; arrowhead marks ectopic expression in the amnioserosa, which is used to identify embryos carrying the reporter-gene. Abbreviations: wt, wild-type; Srp, Serpent; Lz, Lozenge; Ush, U-shaped; L, lateral view; D, dorsal view.



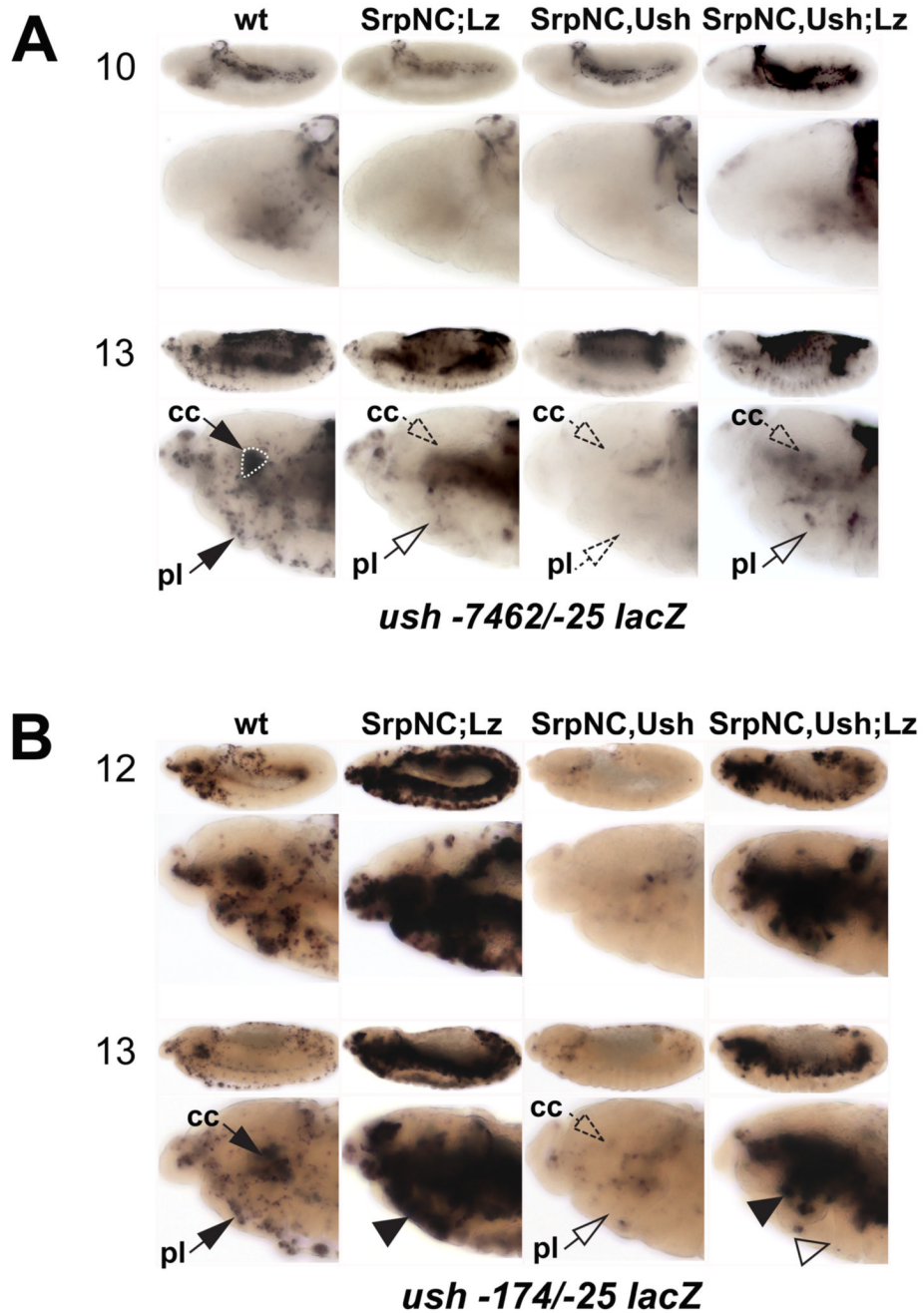
**Fig. 4. *lz-lacZ* and Ush expression during crystal cell development**

The *lz*-1236/-737 CRM was used to assess *lz* expression and is depicted in green. Ush protein expression is depicted in red. Co-localized expression is depicted in yellow. The stage of embryogenesis is indicated at the top of each column. The enlarged area is marked by a white dotted box. Arrows mark the following: stage 9 cells expressing only *lz-lacZ*; stage 10 cells with co-localized expression of *lz-lacZ* and Ush; and stage 12 cells expressing only Ush. Abbreviations: *lz-lacZ*, *lz*-1236/-747 *lacZ*; Ush, U-shaped; co, co-localization.



**Fig. 5. N-terminal zinc-finger conserved valine residue is required for SrpNC and Lz repression of *ush* expression**

(A) Ush protein expression and (B) *ush -7462/-25 lacZ* activity was assessed in stage 13 embryos with different genetic backgrounds. The genetic background is indicated at the top of each column. Orientation of the embryo is indicated at the right of each row. SrpNC;Lz represents *twi-Gal4* driving *UAS-srpNC* and *UAS-lz* located on chromosomes II and III, respectively. SrpNC;<sup>V421G</sup>Lz represents *twi-Gal4* driving *UAS-srpNC<sup>V421G</sup>* and *UAS-lz* located on chromosomes II and III, respectively. Open arrows mark lack of expression in hemocytes. Abbreviations: wt, wild-type; Srp, Serpent; Lz, Lozenge; Ush, U-shaped; L, lateral view; D, dorsal view.

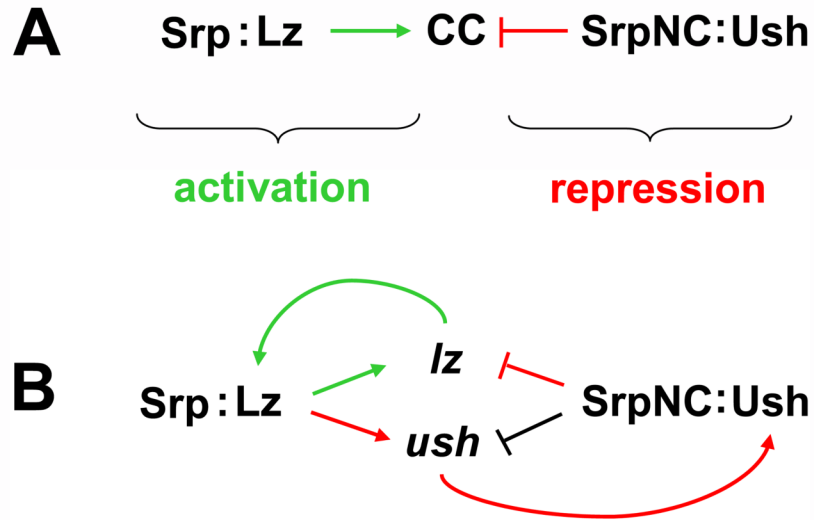


**Fig. 6. Ush competes with Lz to control SrpNC regulation of *ush* expression**

(A) *ush -7462/-25 lacZ* and (B) *ush -174/-25 lacZ* was assessed in different genetic backgrounds and developmental stages. The stage of embryogenesis is indicated at the left of each row. All embryos are lateral views. The genetic background is indicated at the top of each column. SrpNC;Lz represents *twi-Gal4* driving *UAS-srpNC* and *UAS-lz* located on chromosome II and III, respectively. SrpNC,Ush represents *twi-Gal4* driving *UAS-srpNC* and *UAS-ush* located on chromosome II. SrpNC,Ush;Lz indicates *twi-Gal4* driving *UAS-srpNC,UAS-ush* and *UAS-lz* located on chromosomes II and III, respectively. Plasmatocytes and crystal cells (outlined in panel A) are marked with arrows: closed arrows indicate wild-type CRM activity; open arrows indicate reduced activity; open dotted arrows indicate lack of



activity. The level of CRM activity in the head mesoderm is marked with arrowheads: closed arrowheads indicate increased activity; the open arrowhead indicates lack of activity. Abbreviations: wt, wild-type; Srp, Serpent; Lz, Lozenge; Ush, U-shaped; cc, crystal cells; pl, plasmatocytes.



**Fig. 7. Model of Srp cross-regulatory control of crystal cell lineage commitment**

(A) Combinatorial control of crystal cell lineage commitment by Srp, Lz, and Ush. Srp acts as a contextual switch, interacting with Lz to activate crystal cell lineage commitment and with Ush to block crystal cell production. (B) Srp cross-regulatory control of *lz* and *ush* expression. Srp and Lz interact to maintain the Lz-positive regulatory state. These transcriptional regulators also activate *ush* expression in Lz-positive crystal cell precursors to produce a dynamic, bi-potential regulatory state. SrpNC mediates cross-antagonism of Lz and Ush, acting with Lz to maintain the Lz-positive state or with Ush to select the Lz-negative regulatory state. Additionally, the SrpNC:Ush complex can downregulate *ush* expression, thereby limiting the capacity of the complex to block *lz* expression. Srp cross-regulatory control of *lz* and *ush* is mediated through the GATA and RUNX binding sites within the minimal hematopoietic CRMs of each locus. Green arrows indicate the activation pathway. Red arrows and blocked red lines indicate the repression pathway. The blocked black line indicates relief of repression. Abbreviations: Srp, Serpent; Lz, Lozenge, CC, crystal cells; Ush, U-shaped.