A Serrate-expressing signaling center controls Drosophila hematopoiesis

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The differentiation of Drosophila blood cells relies on a functional hierarchy between the GATA protein, Serpent (Srp), and multiple lineage-specific transcription factors, such as the AML1-like protein, Lozenge (Lz). Two major branches of Drosophila hematopoiesis give rise to plasmatocytes/macrophages and crystal cells. Serrate signaling through the Notch pathway is critical in the regulation of Lz expression and the specification of crystal cell precursors, thus providing a key distinction between the two lineages. The expression of Serrate marks a discrete cluster of cells in the lymph gland, a signaling center, with functional similarities to stromal signaling in mammalian hematopoiesis.

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Runx1, also known as Acute Myeloid Leukemia 1 [AML1], is essential for the development of blood cells arising from all lineages of definitive hematopoiesis in mice (Okuda et al. 1996; Wang et al. 1996). In humans, AML1 is the most frequent target for translocations resulting in acute myeloid leukemias [Werner et al. 1999]. The family of GATA transcription factors is also used reiteratively in multiple stages of blood development [Orkin 1998], and a cofactor for the GATA proteins, FOG1, is required for erythropoiesis and differentiation of megakaryocytes [Tsang et al. 1998]. In studying the regulation of such transcription factors, in vitro differentiation assays have suggested that multiple signaling pathways are coordinately involved in hematopoiesis [Van Den Berg et al. 1998; Yoshida et al. 1998; Milner and Bigas 1999]. Unfortunately, there is a paucity of in vivo loss-of-function data involving signaling pathways that control hematopoiesis in mammals due to difficulties such as pleiotropy, redundancy, and early lethality.

There are significant differences with regard to the variety and function of Drosophila hemocytes relative to mammalian blood cells, however, several molecular aspects of early hematopoiesis and immunity have been evolutionarily conserved (Rehorn et al. 1996; Dearolf 1998; Hoffmann et al. 1999; Lebestky et al. 2000). Two major classes of Drosophila hemocytes are plasmatocytes, which can function as macrophages that phagocytose invading pathogens and debris from apoptotic cells [Tepass et al. 1994], and crystal cells that are involved in the melanization of pathogens [Rizki et al. 1980]. serpent [srp], a Drosophila GATA homolog, is expressed in all hemocyte precursors and is required for the development of both classes of hemocytes [Rehorn et al. 1996; Lebestky et al. 2000]. lozenge (lz) encodes an AML1/Runt domain family transcription factor [Daga et al. 1996] that is expressed in the crystal cell precursors and is required for the specification of this lineage [Rizki and Rizki 1981; Lebestky et al. 2000]. glial cells missing (gcm), a novel transcription factor expressed exclusively in plasmatocytes in the embryo [Bernardoni et al. 1997], is required for their development. Its possible role in larval hematopoiesis is less clear. Srp is essential for the expression of both lz and gcm [Bernardoni et al. 1997; Lebestky et al. 2000], creating a hierarchy of transcription factors controlling the two major branches of hematopoiesis [Lebestky et al. 2000]. u-shaped (ush), a FOG homolog, also functions in Drosophila hematopoiesis [Fossett et al. 2001]. The molecular similarities between Srp/Ush/Lz and GATA/FOG/AML1 suggest that aspects of molecular mechanisms of blood development are shared between mammals and Drosophila. Previous studies in Drosophila have shown a role for JAK/STAT and Toll pathways in the proliferation of hemocytes and immunity [Dearolf 1998; Mathey-Prevot and Perrimon 1998; Qiu et al. 1998]. However, no signaling pathway was known to specify a commitment that distinguishes between lineages.

In this study, we show that localized Notch signaling causes an important early distinction between the crystal cell and plasmatocyte lineages. The Notch pathway controls binary cell fate decisions among undetermined precursor cells in a multitude of developmental systems [Artavanis-Tsakonas et al. 1999]. We also find that a signaling center expressing the ligand Serrate is important for differentiation and proliferation of larval hemocytes in Drosophila.

Results and Discussion

Notch and Su(H) are required for hemocyte proliferation and development

Morphological and molecular differentiation of hemocytes can be monitored in the larval lymph gland (Shrestha and Gateff 1982). Srp is expressed in all hemocyte progenitors, whereas the expression of Lz is largely restricted to a small subset of hemocytes in the first pair (anterior-most) lobes of the lymph gland [Fig. 1A]. In the Notch temperature-sensitive allele, Nts1, Lz expression is eliminated from the lymph gland at the nonpermissive temperature [Fig. 1B]. Lz+ cells are also missing in the lymph glands of Su(H)SF8/Su(H)AR9 larvae [Fig. 1C]. As Su(H) is the transcription factor that activates Notch target genes [for review, see Weinmaster 2000], the canonical Notch/Su(H) pathway is important for Lz expression in crystal cell precursors.
Figure 1. Notch signaling in hematopoiesis. [A] Wild-type first lymph gland lobe from a third instar larva raised at 29°C and stained with antibodies against Lz [green] and Serp [red]. Lz is expressed in a distinct subset of Serp+ hemocytes. (B) Lz raised at 29°C and stained as in A. Lz protein is not expressed, but Serp [red] expression remains unaffected. (C) Su(H)topo/Su(H) service lymph gland stained with Lz antibody. Lz protein is not expressed. (D–F) hsp70-flp/FRT clones [arrows] of Notch pathway members stained with the absence of βGal expression [red]. Lz expressing cells [green] are excluded from mutant clones of NDN1 [D], N1 [E], and Su(H) service [F]. (G) hsp70-flp; Ay-Gal4, UAS-βGal+. In this control “flip-out” experiment [Ito et al. 1997], βGal-expressing clones were induced in the first larval instar and analyzed in the third larval instar lymph gland. Nuclear βGal [green] marks cells in which Gal4 is expressed. Many examples of colocalization of Lz and βGal can be seen [inset]. [H] hsp70-flp; Ay-Gal4, UAS-βGal/USN. In this genotype, βGal+ cells [green] also express NDN. Lz [red] and NDN [green] are mutually exclusive [inset], suggesting a requirement for Notch in the development of Lz+ cells. Additionally, the lymph gland size is reduced relative to wild-type controls. (J) Cell counts from the Notch clonal analysis described as in G and H. For wild-type clones [histogram 1], 20 lymph gland lobes were counted and of 162 total Lz+ cells, 768 were also βGal+. NDN clones were either counted from equivalent number of lymph gland lobes [histogram 2] or equivalent number of Lz+ cells [histogram 3]. From 20 lymph gland lobes counted [histogram 2], of 153 total Lz+ cells, 148 did not express βGal and, therefore, NDN+. Similarly, of 1370 total Lz+ cells counted from 68 lymph gland lobes [histogram 3], only 55 expressed βGal. In either case, ~96% of the Lz+ cells did not express βGal. [I] hsp70-flp; Ay-Gal4, UAS-βGal+. Experimental conditions are the same as in G. TUNEL staining [red] marks apoptotic cells. (K) hsp70-flp; Ay-Gal4, UAS-βGal/USN. Experimental conditions are the same as in H. TUNEL staining [red] is similar to wild type. [L] Dorsal view of a wild-type stage 12 embryo. Anterior is to the left. Expression of Lz protein is observed in crystal cell precursors [circled] of the head mesoderm. Additional ectodermal expression of Lz [arrow] is unrelated to its role in hematopoiesis (Lebestky et al. 2000). Cell counts were performed for 30 bilateral clusters of Lz+ cells. [M] Dorsal view of a NDN1 stage 12 embryo. Fewer Lz expressing crystal cell precursors [circled] are seen. In contrast, Lz expression in the ectoderm is expanded [arrow], due to the neurogenic phenotype of Notch, unrelated to its role in hematopoiesis. In C, G–H, and J–K, the dotted line marks the outline of the lymph gland lobe. Bars: A–C, G–H, J–M, 25 µm; D–F, 10 µm.

Using the FLP/FRT system [Golic 1991], we generated mutant clones of NDN1 [Fig. 1D], NDN service [Fig. 1E], and Su(H) service [Fig. 1F] in the lymph gland. Clones were marked by the absence of βGal expression and were always small, reflecting an early requirement for the Notch pathway in cell proliferation. Importantly, Lz+ cells were always excluded from the mutant clones. Approximately 200 Lz+ cells were counted for each genotype. We also generated positively marked clones within the lymph glands that express an extracellular, dominant negative form of Notch [DN], Rebay et al. 1993. As in the loss-of-function clones, marked βGal+ cells expressing DN do not coexpress Lz [Fig. 1G,H]. Therefore, Lz+ cells are derived preferentially from wild-type precursors, once again suggesting a requirement for Notch in the development of crystal cell precursors. Rare exceptions to this rule have been seen [Fig. 1I], presumably reflecting minor variability in the level of expression of USN in individual βGal+ cells. Additionally, lymph glands that misexpress DN are smaller than wild-type controls and fewer βGal+ cells are seen. This is not a result of increased apoptosis, as the fraction of cells that stain with TUNEL is not increased in DN lymph glands compared with controls [Fig. 1J,K]. Taken together, the above genetic analyses establish that Notch signaling is critical for the specification of the crystal cell lineage, but also has an early role in the proliferation of hematopoietic cells in the lymph gland, similar to the proliferative role of Notch in imaginal discs [Go et al. 1998; Artavanis-Tsakonas et al. 1999].

Notch function is also required for crystal cell development in the head mesoderm region during embryogenesis. The average number of Lz+ crystal cell precursors in each bilateral cluster is reduced significantly in NDN embryos [from 18 in wild-type to 9 in mutant stage 12 embryos [n = 30], Fig. 1L,M]. The residual crystal cell development in NDN embryos likely reflects the maternal contribution of Notch [Morel and Schweisguth 2000].

Serrate is the ligand for Notch in hematopoiesis

To identify the Notch ligand responsible for the development of crystal cells, we investigated the possible role of the two Notch ligands, Delta [Dl] and Serrate [Ser] in larval hematopoiesis. Expression of Dl protein was not detected in the lymph gland, and expression of Lz was not altered in the Dl [ME2]/Dl service genetic background [data not shown]. In contrast, Lz expression was absent in lymph glands of SerBd3 larvae [Fig. 2A,B], and as a consequence, significantly fewer circulating crystal cells were observed [Fig. 2C,D]. As SerBd3 is a dominant-negative allele, we used a null allele, SerBd8, to generate loss-of-function clones. Mutant clones usually did not contain Lz-expressing cells [Fig. 2E]. However, consistent with Ser being required in signaling cells, rare examples of SerBd8 mutant cells at the edge of a clone were found to express Lz [Fig. 2F, arrowhead]. These results establish Ser as a ligand for Notch in the specification of crystal cells.

To investigate the sufficiency of the Notch pathway in crystal cell development, we used the Gal4/UAS system
Expression of Ser was monitored using an antibody raised against the Ser protein and using a Ser-βGal reporter (Bachmann and Knust 1998; Fig. 3). Many Ser-expressing cells were found to be clustered at the posterior end. Additionally, scattered Ser+ cells were also seen throughout the gland [Fig. 3A]. A high level of Ser-βGal expression is also observed in the cells at the posterior tip, immediately adjacent to a pericardial cell [Rugendorff et al. 1994; Fig. 3B–D]. Given the unique pattern of Ser expression in this discrete cluster of cells, we designate this region as the Posterior Signaling Center (PSC). A number of Ser+ cells that are adjacent to the PSC appear to have broken off from the cluster [Fig. 3A,F, inset], suggesting that Ser+ cells may all be derived from the PSC.
Notch activation is upstream of Lz function. When tants (Fig. 4F), this further substantiates the fact that denton cells that receive Notch signal are marked by \( \text{Su(H)} \) protein causes expression of Gal reporter line (Go et al. 1998) can be used as a read-out for Notch signaling. Upon activation of the Notch pathway, the Su(H) protein causes expression of Ser (GATA; Fig. 3E–G). The expression of Ser is the first observed indication that distinguishes the PSC from the remainder of the Ser' progenitors within the lymph gland. Additionally, the PSC displays a number of interesting characteristics that distinguish this region from the rest of the lymph gland. First, although Ser is required for the expression of Lz, the PSC is spatially distant from the region in which the majority of crystal cells differentiate (Fig. 3I). Second, Ser expression in the PSC is unaffected in \( \Delta^{12X} \) mutants raised at the nonpermissive temperature (Fig. 3K). Also, in creating clones of \( N^{+/N} \) in the lymph gland (Fig. 1H), the majority of cells that express \( N^{+/N} \) are found in the PSC region, and these cells do not apopose compared with cells in the rest of the lymph gland (Fig. 3L). These results suggest that although the Ser' cells of the PSC signal through the Notch pathway, they themselves are refractory to the Notch signal and do not require Notch for their own development. Finally, the Ser' cells of the PSC do not incorporate BrdU administered in vitro for 1 h (Fig. 3M) or through overnight (18 h) feeding of third instar larvae (Fig. 3N), unlike the majority of cells within the lymph gland that actively proliferate in the third instar (Fig. 3M,N). Thus, the Ser' cells of the PSC represent a distinct signaling cell population that rarely proliferates, but is important for proliferation of hemocyte precursors and for their differentiation into crystal cells.

We next investigated the temporal relationship between Notch signaling and Lz expression. Ser is expressed robustly in the second instar larval lymph gland (Fig. 4A), however, very few Lz' cells, if any, are seen at this stage (Lebestky et al. 2000). Whereas Lz is spatially restricted to the first lobe of the lymph gland, Ser is also seen in further posterior lobes (Fig. 4B). Consistent with these temporal and spatial relationships, expression of Ser is independent of Lz (Fig. 4C). The expression of a 12XSu[H]–βGal reporter line (Go et al. 1998) can be used as a read-out for Notch signaling. Upon activation of the Notch pathway, the Su[H] protein causes expression of βGal. In the first lobe of the lymph gland, a small number of cells that receive Notch signal are marked by βGal expression (Fig. 4D). The expression of βGal is independent on Notch (Fig. 4E), but is unaffected in Lz null mutants (Fig. 4F). This further substantiates the fact that Notch activation is upstream of Lz function. When lymph glands expressing 12XSu[H]–βGal were stained with the α-Lz antibody (Fig. 4G–I), many examples of βGal' cells that also express Lz protein are seen (Fig. 4I), further supporting that signaling by Notch is important for cells to become Lz'. However, cells that express βGal, but not Lz, and those that express Lz alone are also seen. The simplest explanation for this pattern is that a cell receiving a Notch signal is a prohemocyte. As a cell initiates Lz expression, it seems refractory to the Notch signal but the perdurance of the stable βGal protein is still evident. Finally, as this cell initiates a program for crystal cell differentiation, it continues to express Lz. Mature crystal cells in circulation have been shown to be Lz' (Lebestky et al. 2000). Whether Drosophila hemo-

![Figure 4](image_url)

Figure 4. Activation of the Notch pathway precedes Lz expression. (A) Ser–βGal (green) and Ser [red] expression in second instar larval lymph glands. (B) Ser protein expression is also observed within the smaller posterior lymph gland of a third instar larva. Lz is rarely expressed (Fig. 2). (C) \( \Delta^{12X} \), Ser–βGal+/-. Ser expression [green] is unaffected in a Lz null mutant background. DAPI [blue] marks nuclei within the lymph gland. (D–I) \( N^{+/+}, 12XSu[H]–βGal+/+ \) first lobe from third instar lymph gland. The 12XSu[H]–βGal transgene marks cells receiving the Notch signal (Go et al. 1998). (E) When raised at 29°C, the nonpermissive temperature, βGal is no longer expressed. (F) \( \Delta^{12X} \), 12XSu[H]–βGal+-. βGal expression [green] is maintained in Lz null mutant background. (G–I) 12XSu[H]–βGal. Identical confocal images of a third instar lymph gland stained for Lz protein [red, G], βGal [green, H], and merged [I]. (J) Arrowheads point to cells that coexpress βGal and Lz (see text for details). (J) Schematic representation of third instar larval lymph gland. Four to six pairs of lymph gland lobes along the dorsal vessel (heart) are separated by pericardial cells. Ser is expressed in hemocyte precursors in lymph gland lobes and also in pericardial cells. Lz is expressed in crystal cell precursors in the first lobe and rarely, if at all, in the further posterior lobes. Ser is expressed at the PSC and also inside the first lobes and other posterior lobes. Bars: A–F, 25 µm; F–I, 10 µm.
cytes undergo maturation as in mammals, can be tested once suitable markers are identified.

Previous studies have shown that a hierarchy of transcription factors is responsible for the differentiation of two independent classes of hemocytes [Lebestky et al. 2000]. Results presented here demonstrate that Notch signaling among Srp+ hemocyte precursors directly or indirectly regulates the expression of Lz. This signaling creates a molecular distinction between the two major branches of hematopoietic lineage and results in the differentiation of crystal cells [Fig. 4].

The expression and function of Ser in the lymph gland marks a putative signaling center that highlights certain developmental and molecular similarities between hematopoietic tissues of Drosophila and mammals. The existence of discrete hematopoietic microenvironments within the bone marrow to stromal cells influence the proliferation and differentiation of the HSCs has been proposed in many mammalian studies [Bianco et al. 2001]. In fact, stromal signaling via the mammalian Ser homolog, Jagged1, has been implicated in the expansion and self-renewal of HSCs [Varnum-Finney et al. 1998]. However, in vivo identification and characterization of such signaling microenvironments within the bone marrow is difficult to achieve. Although Drosophila hemocytes are quite distinct from mammalian blood cells, spatial relationships with regards to signaling pathways in the Drosophila lymph gland will lead to a better understanding of how similar patterning events affect HSC populations in the marrow.

In mice, AML1/Runx1 is expressed in the embryonic dorsal aorta in hematopoietic clusters associated with an endothelial layer [North et al. 1999]. The Notch ligand, Jagged1, is also expressed in the embryonic aorta at this stage [Loomes et al. 1999], but any possible role it may have in the generation of hematopoietic clusters has not been investigated. Furthermore, independent studies have implicated both Notch and AML1 in the progressive commitment and maturation of T cells [Aster and Pear 2001; Reizis and Leder 2002]. The regulatory relationship between Notch and Ix in Drosophila suggests similar interactions may exist between the Notch pathway and AML1 in mammalian hematopoiesis. Investigating such regulatory relationships is especially valuable, as AML1 is critically needed for all definitive hematopoiesis and is also the most frequent target in acute myeloid leukemias [Downing et al. 2000].

Materials and methods

Fly-out clones expressing UAS-Nọc or UAS-Ser were generated using the Ay-Gal4 system [Ito et al. 1997] and marked with either UAS-nucleofactor [FN] or UAS-GFP. To activate hsp70-flp, progeny were maintained at 18°C until the first larval instar, heat-shocked at 37°C for 40 min, and then returned to 18°C until the dissection. hsp70-Gal4/UAS-Nọc or hsp70-Gal4/UAS-Ser were maintained at 18°C until early third instar and heat-shocked twice at 37°C for 40 min, then returned to 18°C until dissection. or SerRx82 mutant clones were generated using the hsp70-FLP/FRT system [Golic 1991]. Crosses were maintained at 18°C, heat-shocked for 1 h in the second instar for Nọc and Su(H)flp, and in the first instar for Nocl and SerRx82. For Figure 1, Nọc was raised at 29°C either from first instar until third instar, or for 18 h in the early third instar before dissection in the third instar, in both cases showing loss of Lz cells.

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Note added in proof

Duovic et al. [2002] have also recently studied the role of Ser and Notch in hematopoiesis.

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