Functions of Notch and Neuralized in *Drosophila* hematopoiesis

by
Chiyedza Small

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Shubha Govind, Ph.D

Date **Chair of Examining Committee**

Laurel Eckhart, Ph.D

Date **Executive Officer**

Serafin Piñol-Roma, Ph.D.

Diana Bratu, Ph.D.

Thomas Schmidt-Glenewinkel, Ph.D.

Nancy Fossett, Ph.D.

Supervisory Committee

THE CITY UNIVERSITY OF NEW YORK

Abstract

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Adviser: Professor Shubha Govind

In vertebrates, hematopoiesis is commonly divided into two temporal phases, primitive (embryonic) and definitive (adult). Genetic studies in zebrafish and mice have implicated signaling pathways and molecular networks of transcription factors in the control of primitive and definitive hematopoietic programs. Notch signaling is essential for the proper execution of a wide array of cell fate decisions and developmental processes, including hematopoiesis. Many of these same signaling and transcriptional mechanisms also control hematopoiesis in simpler animals, such as the fruit fly *Drosophila melanogaster*. Because of its simple organization and genetic accessibility, *Drosophila* hematopoiesis has recently gained attention.

Drosophila larvae produce three cell types: plasmatocytes, crystal cells and lamellocytes. While plasmatocytes and crystal cells arise in embryonic stages, lamellocytes do not. In fact very few lamellocytes are typically found in healthy third instar larvae. However, their differentiation is induced in large numbers upon oviposition by parasitic wasps. Circulating blood cells divide continuously as the animal grows in size. In addition, a small hematopoietic organ flanking the dorsal vessel supports the growth and development of blood cells.

In larval stages, the Notch pathway regulates the differentiation of crystal cells: loss or reduction of Notch signaling results in the reduction of crystal cells while an increase in Notch signaling leads to the expansion of the crystal cells population in circulation and in the lymph gland (Duvic et al., 2002; Lebestky et al., 2003). Notch encodes the receptor/transcription factor that mediates short range cell-cell signaling. Notch ligand Serrate is expressed in the niche of the lymph gland. Serrate activates Notch in the pro-crystal cells and promotes commitment of the crystal cell fate. This step requires the functions of transcription factor Suppressor of Hairless (Lebestky et al., 2003).

Duvic et al. (2002) also reported that Notch function is essential for lamellocyte differentiation, although how this occurs was not explored in the Duvic study. The goal of this work was to analyze the contributions of Notch and Neuralized in lamellocyte differentiation. Neuralized encodes an E3 ligase for ubiquitination of Notch ligands, and its role in hematopoiesis remains unexplored.

The thesis contains three chapters. In Chapter 1, I report the expression of Notch in the lymph gland and circulating blood cells. Using RNA interference and clonal analysis, I show that Notch maintains lamellocytes in their progenitor state. This requirement is non cell-autonomous. Lamellocytes induced by loss of Notch appear mostly in the peripheral cortical zone of the anterior lobes, that houses both mature cells and progenitors. Notch target genes are expressed in most anterior lobe cells.

In Chapters 2 and 3, the functions of Neuralized are explored in three ways: RNA interference, clonal analysis (Chapter 2), and using putative alleles of *neuralized*, *l(3)hem*^{1} and *l(3)hem*^{2} (Chapter 3). These studies show that Neuralized function is

essential in maintaining hematopoietic stem-like progenitors in their undifferentiated state. In the medullary zone, where these undifferentiated progenitors reside, Neuralized plays an essential role in cell division and differentiation. Further, like Notch, it provides an inhibitory non cell-autonomous influence on pro-lamellocytes in the cortical zone, and keeps them from differentiating in the absence of infection.

In chapter 3, I characterize a classical hematopoietic mutation, *l(3)hem*^{1}, a putative weak allele of *neur*. The lymph glands and blood cells of this homozygous mutant are severely affected, with multiple defects in cell division and differentiation. These studies support the three functions of Neuralized uncovered in Chapter 2.

Our studies provide novel insights into hematopoieitic stem/progenitor division and differentiation. Notch signaling plays an essential role in mammalian hematopoiesis. Misregulation of the Notch pathway leads to hematopoietic malignancies in humans. Because of the high molecular conservation between flies and mammals, understanding the regulation of Notch signaling in *Drosophila* hematopoiesis will yield insights into its role in mammalian hematopoiesis and potentially in developing therapies for treatment of human malignancies.

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Table of Contents

Abbreviations

arm: *armadillo*
AL: Anterior Lobes
Antp: *Antennapedia*
BSA: Bovine Serum Albumin
col: c*ollier*
Cg: *Collagen*
CZ: Cortical zone
da: *daughterless*
cc: crystal cells
Dif: *Dorsal related immunity factor*
dl: *dorsal*
Dl: *Delta*
dome: *domeless*
E(spl): *Enhancer of Split*
F-actin: Filamentous actin
GAL4: Yeast transcription activating protein in galactose metabolism
HSC: Hematopoietic stem cell
Hs: Heat shock
hh: *hedgehog*
Hml: *Hemolectin*
hop: *hopscotch*
Integrin β PS: Integrin beta position specific
JAK: Janus Kinase
L1: Lamellocyte-specific antibody, (recognizes Atilla)
l(3)hem^{1}: *lethal (3) hematopoiesis missing*^{1}
l(3)hem^{2}: *lethal (3) hematopoiesis missing*^{2}
LSM: Laser scanning microscopy
lz: *lozenge*
msn: *misshapen*
MZ: Medullary zone
neur: *neuralized*
NHR: Neur Homology Repeat
NICD: Notch intracellular domain
N: *Notch (*N^{ts1}-temperature sensitive allele, N^{IC} -intracellular domain)
PBS: Phosphate buffered saline
P1: Anti-P1- Plasmatocyte-specific antibody, recognizes Nimrod
PFA: Paraformaldehyde
PL: Posterior Lobes
ProPO: Prophenol oxidase specific antibody that labels crystal cells
PSC: Posterior Signaling Center (niche)
Pvr: Drosophila homolog of the PDGF/VEGF receptor
RING: Really Interesting New Gene; contains zinc finger domain
RNAi: RNA interference
Runx: Runt-related transcription factor

Ser: *Serrate*
SOP: Sensory organ precursor
Su(H): Suppressor of Hairless
STAT: Signal Transducers and Activators of Transcription
UAS: Upstream Activating Sequence
v : vermillion
w : *white*
y : *yellow*

List of Figures

Introduction

The Notch pathway is a conserved cell-cell signaling pathway which is essential for the proper execution of a wide array of cell fate decisions and developmental processes (Glittenberg et al., 2006). In *Drosophila*, Notch functions pleiotropically in many tissues; in neurogenesis it specifies sensory organ precursors (SOPs) that become sensory bristles in the adult epidermis; in oogenesis it is required for proper formation of ovarian follicle cells; in eye development, it determines cone cells, photoreceptors and pigment cells; in wing development Notch signaling specifies the dorsal-ventral wing margin and vein-intervein boundary (Muskavitch et al., 1994 and Cornell et al., 1999). In larval hematopoiesis, Notch signaling is required for formation of embryonic and larval crystal cells (Crozatier and Meister, 2007).

The hallmark function of the pathway occurs through the process of lateral inhibition. In this process, one cell amongst a group of equivalent cells is selected randomly to adopt a specific differentiated fate, while preventing its neighbors from adopting that fate. The cell that will differentiate has the highest levels of ligand expression relative to its neighbors (Lewis, 1998). Lateral inhibition can be understood in the context of stereotypical arrangement of bristles on the dorsal side of the adult fly epidermis. The cell that gives rise to the bristle is selected to become the sensory organ precursor (SOP) cell in the larval imaginal discs. The SOP is specified within a group of cells called the proneural cluster. All cells in the proneural cluster have the potential to become a SOP, as they express the proneural proteins, Achaete and Scute. But only one cell in this cluster will become the SOP as the signals that arise from the pre-SOP cell (with high ligand levels) will turn off expression of the *achaete scute* complex. Thus,

having higher levels of Achete and Scute, the selected cell will acquire the neural fate (bristle), whereas other cells in the cluster will adopt the epidermal cell fate (no bristle in the field of cells immediately adjacent to the bristle, thus patterning the adult epidermis (Bray, 1998).

Notch signaling is activated when the Notch transmembrane receptor in the signal-receiving cell binds to any one of its ligands (Delta, or Serrate in *Drosophila*, and Lag-2 in *C. elegans*), in the signal sending cell. In the fly, activation of the Notch receptor in the signal receiving cell causes proteolytic cleavage of the intracellular domain of the receptor. It translocates to the nucleus and binds to inactive transcription factor Suppressor of Hairless (*Su(H)*), causing the latter to be activated. Active Su(H) binds to the regulatory region of the Enhancer of Split (*E(spl))* group of genes (Figure 2). E(spl) proteins act as repressors of *achaete-scute* genes preventing their transcription, thus preventing the adoption of the differentiated neural fate. Thus, the Notch-bearing cells are prevented from adopting a neural fate, whereas the ligand-containing cell can differentiate into a bristle (Bray, 1998).

neuralized (*neur*) is a neurogenic gene within the Notch signaling pathway, and like *Notch*, is required zygotically for promoting neurectodermal cells to assume epidermal fates. The *neur* gene encodes an E3 ubiquitin ligase which targets Delta for ubiquitination leading to its endocytosis and plasma membrane presentation (Yeh et al., 2001; Pavlopolos et al., 2001; Lai et al., 2001). Neur binds phosphorylated inositol phosphates which are modified membrane lipids important for membrane trafficking and endocytosis of Delta (Skwarek et al., 2007). Endocytic processing of the ligand in the signal-sending cell activates Notch in the signal-receiving cell. According to one model,

endocytosis of Delta (in the signal-sending cell) allows for conformational changes to occur in the Notch extracellular domain that promotes its cleavage by γ secretase in the signal-receiving cell (Windler and Bilder, 2010). The second argues that Delta endocytosis into specialized endosomal compartments is mediated by Liquid facets (*Drosophila* homologue of Epsin) and allows the ligand to be modified and presented at the cell surface in a more active form (Wang et al., 2004). Irrespective of the mechanism, activation of Notch signaling requires *neur*.

The *neur* gene is alternatively spliced and encodes at least two types of protein isoforms, Neur A and Neur C. Both Neur A and Neur C contain a carboxyl terminal RING finger domain (required for the E3 ligase activity) and Neur homology repeats (NHR) 1 and 2. NHR1 mediates Neur binding to Dl and facilitates Neur nuclear envelope localization (Commisso et al., 2008).

Neur C differs from Neur A slightly at the N-terminal end, carrying a consensus sequence for myristylation. Neur A has a unique glutamine/histidine-rich region and a short lysine/arginine-rich region in the N-terminus that is not found in Neur C. This sequence facilitates Neur A plasma membrane localization in S2 cells (Commisso et al., 2007); whereas Neur C lacking this region, exhibits cytoplasmic localization in cultured cells. However, in the wing and eye-antenna imaginal discs, both isoforms can be localized to the plasma membrane (Skwarek et al., 2007).

Drosophila hematopoiesis

Drosophila hematopoiesis involves two waves: an early primitive wave that results in plasmatocyte and crystal cell formation during embryogenesis and a second,

definitive wave that occurs during larval development. During the first wave, phagocytic blood cells called plasmatocytes arise from the head mesoderm 8-10 hours after fertilization (Lebestky et al., 2000). A distinct population of progenitors gives rise to pro-crystal cells. Both these cell types will become part of the circulating blood cell population in the larva (Holz et al., 2003). A cascade of transcription factors designate the two embryonic cell lineages: GATA family protein, Serpent, first determines prohemocytes, Glial Cells Missing (Gcm1 and Gcm2) designate plasmatocytes (Alfonso et al., 2002), while Lozenge specifies the number of crystal cells (Lebestky et al., 2000). Friend-of-GATA, or U-shaped, acts as a negative regulator inhibiting crystal cell formation (Fossett et al., 2003; Bataille et al., 2005).

The definitive wave of hematopoiesis produces adult hemocytes that arise from the larval lymph gland. Lineage tracing and transplantation experiments reveal that these hemocytes persist until adult stages (Holz et al., 2003).

The larval lymph gland itself is formed during mid to late embryonic stages. The embryonic lymph gland, cardioblasts and pericardial nephrocytes are formed from the cardiogenic mesoderm during late embryogenesis. The pericardial cells are similar to mammalian nephrocytes (excretory tissues) and cardioblasts are vascular cells that line the dorsal vessel (Mandal et al., 2004). Genetic evidence has shown that Notch signaling is required twice: early, in 6-8 hour-old embryos, it restricts the specification of cardiogenic mesoderm. Later in 8-10-hour old embryos, Notch signaling limits cardioblast development, ensuring precursors differentiate into lymph gland tissue. Loss of Notch signaling decreases the number of pericardial cells and lymph gland precursors but expands cardioblast fates (Mandal et al., 2004). At this embryonic stage, the lymph

gland comprises a single pair of primary/anterior lobes up until second instar (Mandal et al., 2004).

At third instar, the larval lymph gland contains a large pair of anterior lobes that flank the dorsal vessel (Figure 1). The anterior lobes contain precursors of the three major cell types in the medullary zone (MZ): plasmatocytes, lamellocytes and crystal cells (Jung et al., 2005). Plasmatocytes are the majority of circulating hemocytes, which like mammalian macrophages, function in engulfing and removing apoptotic cells during development. Crystal cells are circulating hemocytes that produce phenoloxidases, enzymes that mediate melanization reactions (Crozatier and Meister, 2007). Lamellocyte differentiation is induced only in the event of wasp infection. Being flat and highly adhesive, these cells function to encapsulate wasp eggs as part of the cellular immune response. Lamellocytes also produce phenoloxidases that facilitate melanization reactions. Recent reports demonstrate that lamellocytes can arise from mature plasmatocytes (Honti et al., 2010; Avet-Rochex et al., 2010; Stofanko et al., 2010). Cells of the cortical zone (CZ) are mitotically more active than cells of the MZ (Jung et al., 2005). The posterior lobes contain undifferentiated hemocytes that will produce differentiated hemocytes needed during pupariation or lamellocytes needed for immune challenges (Lanot et al., 2001).

Notch signaling in the larval lymph gland

During larval development, the Notch signaling pathway has been shown to regulate the differentiation of crystal cells (Duvic et al., 2002; Lebestky et al., 2003). Duvic et al. (2002) showed that loss-of-function mutations in Notch results in decreased

crystal cell numbers. To quantify the crystal cell population, they used a simple "cooking" assay in which sessile crystal cells were counted in the three posterior-most segments of third instar larvae that were gently heat treated. This treatment results *in situ* melanization of crystal cells (Rizki et al., 1980b). They made the following observations:

(i) At permissive temperature, temperature-sensitive N^{ts1} allele-bearing larvae have a wild-type number of circulating plasmatocytes and crystal cells. However, when second instar larvae were shifted to the restrictive temperature (29°C), a sixty percent reduction in the number of crystal cells is observed. The number of plasmatocytes was not affected based on cell counts performed on hemolymph of these mutants.

(ii) When $Notch^{IC}$ (a transgenic line with a cDNA sequence encoding a ligand-independent constitutively-active form of Notch intracellular protein) was expressed with a ubiquitous heat shock protein 70 GAL4 driver (hs-GAL4), a 7-fold increase in the numbers of sessile crystal cells was observed. Gain-of function allele, N^{Mcd8} also resulted in high numbers of sessile crystal cells after "cooking".

(iii) The "cooking" assay also revealed that loss-of-function mutants of other Notch pathway components (Ser^{RX82}) resulted in a severe reduction in crystal cells in the last three larval segments. Loss of *Delta* activity had no effect on plasmatocytes or crystal cells but overexpression of *Ser* or *Dl* with the UAS/GAL4 system caused an increase in crystal cell numbers. Loss-of-function of Notch pathway downstream effector, $Su(H)^{SF8}/Su(H)^{HG36}$ showed loss of crystal cells while plasmatocyte numbers were normal in circulation. Both cell types were normal in larvae carrying the *Deltex* hypermorphic allele, Dx^{1} (Duvic et al., 2002).

Duvic et al., (2002) also analyzed lamellocyte differentiation in these mutants. When N^{ts1} animals were infected with parasitoid wasps, a reduction in lamellocyte numbers was observed at restrictive temperature. In wild-type animals, wasp infection leads to the differentiation of lamellocytes in the anterior lobes. Thus, loss of N activity reduces crystal cells and compromises lamellocyte differentiation.

In a separate study utilizing clonal analysis, Lebestky et al. (2003) showed that Notch signaling is necessary for crystal cell specification. They found that, in addition to Notch itself, mutants of the other Notch pathway components also affected crystal cells: (i) Mutant clones of Ser^{Rx82} lacked Lz-expressing crystal cells whereas control clones with normal Serrate function did not. Occasionally, few Lz+ cells were observed in *Ser−/Ser−* cells at the edge of the mutant clone. Lebestky et al. (2003) also showed that Serrate expression is limited to a small group of cells in the posterior regions of the anterior lobes, that they termed the posterior signaling center (PSC). Because this group of cells maintains progenitors in their quiescent state, this region has subsequently been called the niche (see below). (ii) Flp-out clones of cells with misexpressed *UAS-Ser* using *hsp70-GAL4; Ay-GAL4* caused an increase in crystal cell precursors (Lz^+) in the posterior lymph gland lobes. (iii) Lymph glands of loss-of-function $Su(H)^{SF8}/Su(H)^{AR9}$ mutant larvae also lacked Lz^+ cells. Taken together these results suggest an important role for Notch in the hematopoietic niche and crystal cell determination.

In a subsequent study Crozatier et al. (2004) showed that the niche is specified by transcription factor, Collier (*col*), a *Drosophila* orthologue of vertebrate early B-cell factor. *col* trsnscript is expressed in the embryonic lymph gland in cells destined to become the niche. This expression is prior to Ser expression, and *col* is required for

Serrate expression in the niche (Crozatier et al., 2004). Indeed, crystal cells differentiate (and are even increased) in *col* mutant lymph glands despite abberant Ser expression in a niche whose cells are not as tightly organized as in wildtype lobes. Surprisingly, however even though c*ol* mutant lymph glands possess differentiated crystal cells, the lymph glands fail to differentiate lamellocytes after wasp infestations. These studies suggest an instructive role for c*ol* in the niche via Serrate- expressing niche cells in the anterior lobes (Crozatier et al., 2004).

The hematopoietic niche was also shown to be specified early in the embryo by Antennapedia expression (Mandal et al., 2007). Hedgehog (Hh) signaling appears to be essential in the niche for communication between the niche and MZ. Patched, the Hh receptor is expressed in cells of the MZ, as is activated by Cubitus interruptus (Ci) protein. Loss of Hh signaling via a dominant negative form of Ci (Hh signaling activator) compromised niche signaling and MZ quiescence, resulting in crystal cell differentiation. Cytoplasmic processes which emanate from cells of the niche into the MZ provide a mechanistic basis for Hh signaling from niche into the MZ. Thus, Hh signaling pathway maintains prohemocytes in a quiescent state in the medullary zone (Mandal et al., 2007)..

Krzemien et al. (2007) highlighted a role for JAK-STAT signaling in the niche by showing that signaling from the niche cells maintained prohemocytes in the MZ. Domeless, the receptor for the JAK-STAT pathway is transcribed in the MZ (Jung et al., 2005). Krzemien et al. (2007) using *col* (PSC) and *STAT92E* mutants showed that a loss of PSC or JAK/STAT signaling resulted in increased differentiation in the anterior lobes of the lymph gland. The authors also showed that Notch signaling (via a down regulation of Ser) is required to maintain high levels of *col* transcription in the niche. Therefore, the

PSC maintains JAK-STAT signaling in prohemocytes in the MZ keeping them in undifferentiated states.

These studies have highlighted the importance of hh and JAK-STAT signaling from the niche to the MZ and the effects on differentiation in the CZ. Despite these advances in the definition of lymph gland zones and signaling mechanisms within different cell populations, many questions remain. For example, it is not known how *Serrate* expression (RNA, protein, or modification) is controlled and limited to the niche cells? Furthermore, we do not understand how signaling between the niche and MZ/CZ cells activates the program for crystal cell development. Are these precursors in the MZ or in the CZ? We also do not know if Notch signaling plays a role in plasmatocyte and lamellocyte division/differentiation. Finally, a role for Neuralized in hematopoiesis has not been explored. To address these questions, I examined the roles of Notch and Neuralized in hematopoiesis through genetic and expression analyses. I also characterized the *l(3)hem*^{1} mutation and demonstrated that it is a putative hypomorphic allele of *neuralized*.

Significance

The Notch pathway plays an equally critical role in mammals and is involved in many processes similar to those in the fly. In mice and humans, there are four Notch receptors (Notch 1-4; all found in vertebrate hematopoietic cells). There are six Notch ligands, Jagged-1 and -2 (homologues of Serrate), and Delta-like 1-4 (homologs of Delta). *Drosophila* has one Notch receptor and two ligands (Delta and Serrate) (Ohishi et al., 2003, Radtke et al., 2005).

In mammalian hematopoietic and lymphocyte development, Notch signaling plays a vital role in the generation of the embryonic hematopoietic stem cells (HSC) for the T cell versus B cell lineage decisions in the thymus, and in the development of splenic marginal zone B cells (Malliard et al., 2003). In mice and humans, Notch ligands are expressed in the bone marrow stroma, while Notch receptors are in T cells, B cells, and myeloblasts. Moreover, Notch1 and Notch2 receptors are expressed on primary murine bone marrow cells that will give rise to granulocytes and monocytes but that will suppress erythroid lineages (Walker et al., 2001). Signaling via the Notch 1 receptor has been shown to have a direct influence on the differentiation of common lymphoid progenitors towards a T cell lineage as opposed to B cell lineage (Radtke et al., 2005).

In mammals, Notch signaling is essential for the formation and self renewal of embryonic bone marrow HSCs but to lesser extent for maintenance of HSCs in the adult bone marrow niche (Gering and Patient, 2008). Using gain-of-function experiments, Duncan et al. (2005) showed that if HSCs were treated either with Notch ligands, or with constitutively active Notch alleles, or if Notch target genes such as Hes1 were over-expressed, all resulted in an increase in HSCs that could self renew or repopulate the bone marrow after irradiation. These observations implied a role for Notch pathway in self renewal of HSCs. Notch 1-deficient mice also lack embryonic hematopoietic stem cell specification in the aorta gondal mesonephros (Kumano et al., 2003). Walker et al. (2005) found that embryos mutant for zebrafish *mindbomb*, an E3 ligase similar to *neur* did not specify HSCs, establishing the Notch requirement during stem cell induction. *Neur* and *mindbomb* each contain a RING finger at their C termini, and in flies, they can

compensate for each other to ubiquitinate Delta and promote its endocytosis (Lai et al., 2005).

Given this requirement of Notch signaling in mammalian hematopoiesis, it is not surprising that its aberrant signaling would result in tumorigenesis and blood cancers. Overexpression of Notch1 within hematopoietic bone marrow cells or within T cell progenitors resulted in T cell leukemias indicating Notch's function as an oncogene (Wilson et al., 2006). In humans, activating mutations within the Notch1 receptor was linked to development of T cell acute lymphoblastic leukemia (T-ALL; Wilson et al. 2006). However in the skin, Notch functions as a tumor suppressor and its loss results in the development of basal cell carcinoma-like tumors (Wilson et al., 2006).

Although the Notch pathway is conserved and mechanistically well-understood, molecular redundancy of its components in the mammalian genome makes studying Notch pathway requirements quite complicated. Because *Drosophila* Notch pathway relies on fewer components and mutant alleles are available, it is possible to dissect their biological functions more clearly.

The goal of my thesis was to characterize the function of Notch and Neuralized proteins in the context of Notch signaling in larval hematopoiesis. The thesis has three data chapters, following this section, In Chapter 1, I address the role of Notch in hematopoiesis; in Chapter 2, the role of Neuralized is presented. I then present the characterization of *l(3)hem*^{1} mutation in Chapter 3. A discussion of all data presented in Chapters 1-3 is presented in the last section of the thesis.

Figure 1. Schematic of the larval lymph gland along with a representative organ. Anterior lobes of the third instar larval lymph gland consist of the niche located at the base of the anterior lobes. It releases Hedgehog, required to maintain precursor cells of the medullary zone (MZ) in undifferentiated states. The cortical region (CZ) contains mature differentiated cells (Jung et al., 2005, Mandal et al., 2007). Secondary to these lobes are several pairs of smaller posterior lobes separated by pericardial cells. Cell-specific markers and genes expressed in the different regions are shown. Serrate, Antennapedia, Hedgehog and Collier are expressed in the niche. Domeless which encodes the JAK-STAT pathway receptor is expressed in the MZ (reviewed in Evans et al., 2007).

Figure 1.

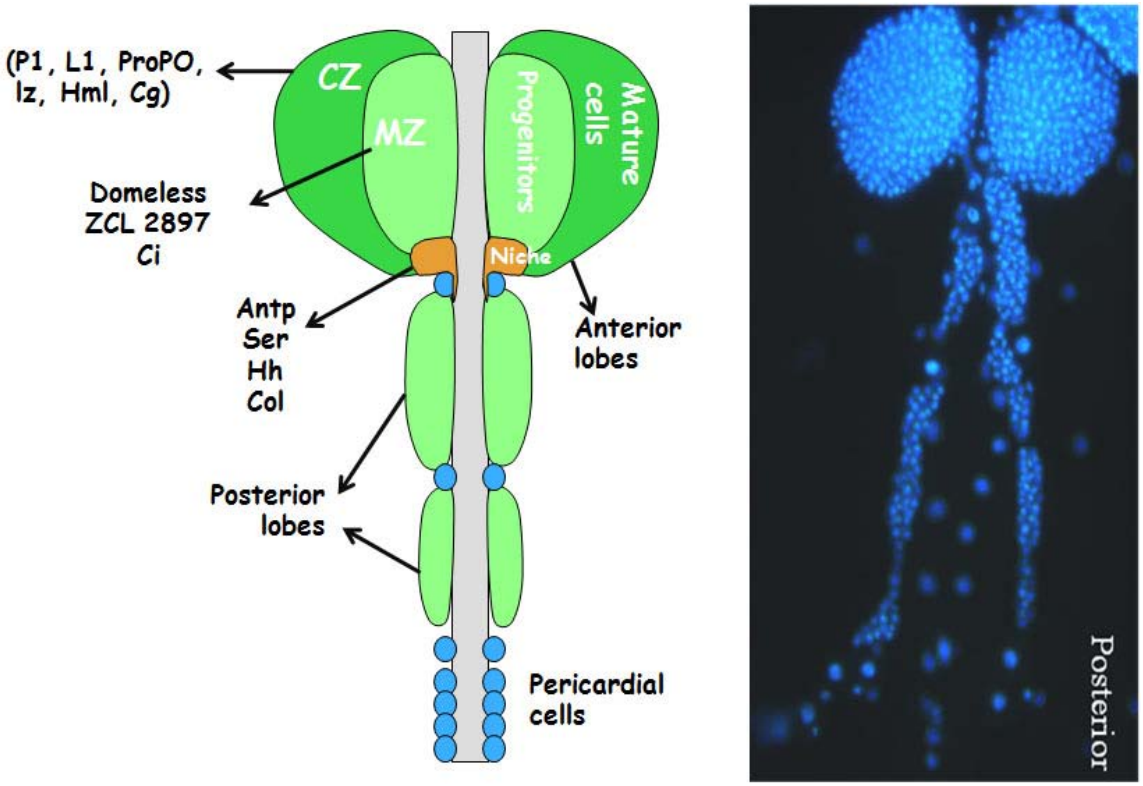
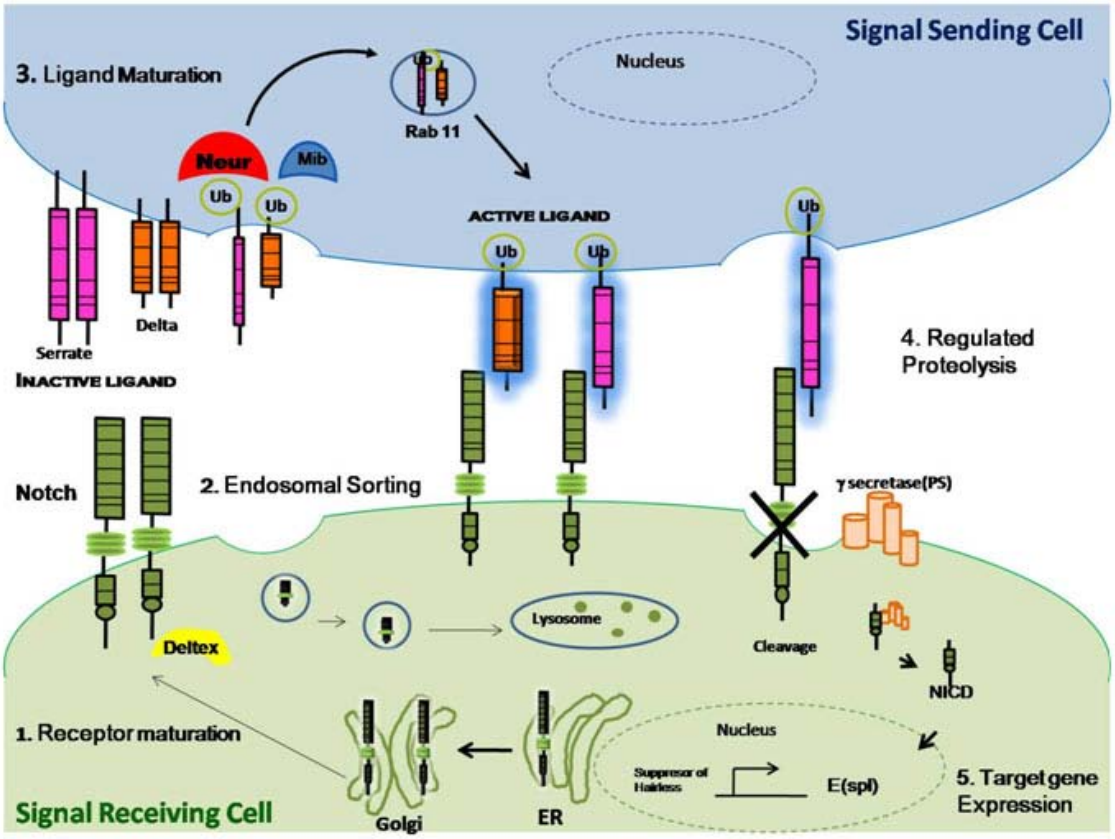

(P1, L1, ProPO,
lz, Hml, Cg)
CZ
MZ
Progenitors
Mature cells
Domeless
ZCL 2897
Ci
Niche
Antp
Ser
Hh
Col
Anterior lobes
Posterior lobes
Pericardial cells
Posterior

Figure 2. Notch signaling pathway. The binding of Notch to its ligands Serrate or Delta on the surface of neighboring cell, initiates signal transduction. Proteolytic cleavage by gamma secretase in the signal-receiving cell causes the nuclear translocation of the intracellular domain of Notch receptor which interacts with the CSL complex of transcription factors (CSL for human CBF1; *Drosophila* Suppressor of Hairless; *C. elegans* Lag-1). The extracellular portion of Notch is taken up by the signal-sending cell in a transcytosis step, mediated by endocytosis (Wang et al., 2005). Notch signaling induces the transcription of downstream target genes, such as the *Enhancer of split complex* [*E(spl-C)*]. Neur E3 ligase ubiquitinates Delta, facilitating its endocytosis and membrane presentation. Figure is modified from Xenia et al., 2007 and Artavanis-Tsakonas et al., 1995.

Figure 2.


Signal Sending Cell
3. Ligand Maturation
Nucleus
Rab 11
Neur
Mib
Ub
Ub
ACTIVE LIGAND
Ub
Ub
Ub
Serrate
Delta
INACTIVE LIGAND
4. Regulated Proteolysis
Notch
2. Endosomal Sorting
γ secretase(PS)
Lysosome
Deltex
Cleavage
NICD
1. Receptor maturation
Nucleus
Suppressor of Hairless
E(spl)
5. Target gene Expression
Signal Receiving Cell
Golgi
ER

Chapter 1

Notch sustains lamellocyte progenitors in an undifferentiated state in *Drosophila* third instar lymph glands

Abstract

Notch (N) signaling influences the diversification of cell fate in animal development. In hematopoietic development, a step-wise genetic and epigenetic program specifies how multipotent progenitors achieve precise identities. Lamellocytes are large, flat, adhesive immune cells, whose primary function in *D. melanogaster* is to encapsulate the egg of parasitic wasps and ensure host survival. While lamellocyte precursors are present in the lymph glands of uninfected third instar larvae, they are prompted to differentiate in high numbers only after parasite infection (Lanot et al., 2001; Sorrentino et al., 2002). Among others, the JAK-STAT (Hanratty et al., 1993; Sorrentino, 2004) and Jun N-terminal kinase pathways (Tokusumi et al., 2009) positively promote lamellocyte differentiation. However, the mechanisms that maintain lamellocyte progenitors in undifferentiated state are not known. We show that while Notch is expressed in all hematopoietic progenitors, its expression is downregulated in mature lamellocytes. Using RNAi-knockdown, we show that Notch is essential for keeping lamellocytes from differentiating. RNAi knockdown blocks progenitors to acquire crystal cell identity. However, unlike its role in crystal cell development, Notch's inhibitory influence on lamellocyte development is non cell-autonomous. Our data suggest that hematopoietic progenitors in the larval lymph gland interpret and coordinate negative regulation of the Notch pathway to acquire the lamellocytic fate while its activation specifies crystal cells.

Introduction

The *Drosophila* innate immune system responds to variety of bacterial, fungal, and metazoan pathogens. When infected by parasitic wasps, the larval host immune response involves a synergistic activation of humoral and cellular responses (Schlenke et al., 2007). The latter process involves activation of blood cells or hemocytes, derived from progenitors to three mature blood cell types. Some of these progenitors are housed in the larval lymph gland (Sorrentino et al., 2002).

At third instar, the larval lymph gland contains a large pair of anterior lobes and several, smaller posterior lobes, that flank the dorsal vessel. The anterior progenitors of the three major hemocyte types, plasmatocytes, lamellocytes and crystal cells, are present in the medullary zone (Jung et al., 2005). Plasmatocytes are the major cell type, and like mammalian macrophages, they engulf bacteria and apoptotic cells. Crystal cells produce phenoloxidase enzymes that mediate melanization reactions (Crozatier and Meister, 2007). Lamellocytes also possess the ability to melanize and their differentiation is induced in the event of wasp infection. These large highly adhesive cells function to encapsulate wasp eggs as part of the cellular immune response (Sorrentino et al., 2004).

The hematopoietic niche or posterior signaling center is located at the base of the anterior lobes. It releases the Hedgehog morphogen into the medullary zone and initiates signaling within progenitors to maintain precursors in their undifferentiated state (Mandal et al., 2007). Progenitors are not only less differentiated, but are also mitotically quiescent (Jung et al., 2005). Genetic data also supports a role of JAK-STAT signaling in progenitor maintenance and homeostasis (Krzemien et al., 2007; 2010). The cortical

region contains mature differentiated cells (Jung et al., 2005). Cells of the cortical zone are mitotically more active than cells of the MZ (Jung et al., 2005).

Several cell-specific genes serve as marker for different regions of the lymph gland. Serrate, Antennapedia, Hedgehog and Collier are expressed in the niche (Crozatier et al., 2004; Jung et al., 2005, Mandal et al., 2007). Domeless (Dome) which encodes the JAK-STAT pathway receptor is expressed in the MZ (Evans et al., 2007), as is the protein trap gene ZCL2897 (Jung et al., 2005). Hemolectin (Hml) and Collagen (Cg) are expressed in cells of plasmatocyte identity, and Lozenge (Lz) and Pro-phenoloxidase (PPO) are expressed in crystal cells in the CZ (Sinenko et al. 2004, Jung et al. 2005). An enhancer in the *misshapen* (*msn*) gene is expressed in lamellocytes (Tokusumi et al., 2009), as is integrin β PS (Stofanko et al., 2008). Monoclonal antibodies against these cell types have also been useful to distinguish these cell types (P1 or Nimrod C for plasmatocytes, and L1 or Atilla for lamellocytes, Kurucz et al., 2007).

Specification and differentiation of all hemocyte lineages in *Drosophila* requires Serpent, a GATA transcription factor whose function specifies prohemocytes. Glial Cells Missing 1, 2, and PVR specify and are essential for plasmatocyte maturation, respectively (Evans et al., 2007). The Toll and JAK-STAT pathways are implicated in lamellocyte differentiation along with Notch (Duvic et al., 2002, Lemaitre et al., 2007, and Evans et al., 2007). Notch pathway components and Lozenge (Runx1 homolog) specify crystal cells (Lebestky et al., 2000; 2003).

Genetic evidence suggests that Notch signaling is required many times for hematopoietic development: early, in 6-8 hour-old embryos, it restricts the specification of the cardiogenic mesoderm. In 8-10-hour old embryos, Notch signaling limits

cardioblast development, ensuring precursors differentiate into lymph gland tissue. Loss of Notch signaling decreases pericardial cells and lymph gland precursors but expands cardioblast fates (Mandal et al., 2004). Recent temperature restriction experiments with temperature-sensitive Notch allele, N^{ts2}, suggest that Notch also plays an additional role in the late embryo in specifying multipotent progenitors to assume limited developmental potential (Krzemien et al., 2010). Wasp infection induced lamellocyte differentiation correlates with the loss of crystal cells in the lymph gland (Krzemien et al., 2010).

Notch (N) signaling is required for crystal cell development in the embryo (Lebestky et al. 2003, Bataille et al., 2005). In larval stages, Notch signaling regulates commitment of crystal cell linage (Duvic et al., 2002; Lebestky et al., 2003), and is also essential for wasp-induced differentiation of lamellocytes in the larval lymph gland (Duvic et al., 2002). Notch pathway is activated in the crystal cell via cell-cell communication, where Serrate, expressed in the niche, directly interacts with the Notch receptor, to activate Supressor of Hairless (Su(H)) target genes (Lebestky et al., 2003).

To examine its role in lamellocyte differentiation, we studied the expression of the intracellular domain of Notch (NICD). Although largely ubiquitous, the intracellular domain of Notch is localized to the membranes of cells in the medullary zone of anterior and posterior lobes, whereas in cells of the cortex, it is both membrane-associated and vesicular. NICD is not detected in Hemolectin-positive cells and is at moderate levels in crystal cells. In lamellocytes, NICD levels are very low or not detected. Genetic experiments discussed here reveal a novel cell non-autonomous role for Notch in maintaining hematopoietic progenitors from differentiating into lamellocytes. We discuss

the mechanisms by which Notch may exert this inhibitory influence on hematopoietic commitment.

Experimental Procedures

Stocks and Crosses

HmlΔ-GAL4 (Sinenko et al., 2004) was obtained from S. Bhattacharya. *Dome-GAL4* (Ghiglione et al., 2002) was a gift from M. Crozatier, *Antp-GAL4* received from S. M. Cohen via S. Minakhina (Emerald and Cohen, 2004). *y w, UAS-mCD8-GFP* (Sinenko et al., 2004) was incorporated into the backgrounds of the listed GAL4 drivers. *lz-GAL4* (Daga et al., 1996) was obtained from U. Banerjee. UAS-RNAi lines for *Notch* (Valium 1 and 10), *Serrate* (Valium 10), and *neuralized* (Valium 10) were obtained from TRiP-Harvard Medical School. *msnf9mocherry* (Tokusumi et al., 2009) was a gift from R. Schulz. a*rm-lacZ 19A /FM7, hsFLP* (Kaplow et al., 2008) was obtained T. Venkatesh (gift from N. Baker). N^{55e11} *FRT19A/FM7 (*Ohlstein and Spradling, 2007*)* was obtained from B. Ohlstein. *UAS-*N^{IC}(Struhl and Adachi, 1998, Duvic et al., 2000) was obtained from K. Irvine. *hopscotch*$^{Tum-l}$ (Sorrentino et al., 2002) was obtained from C. Dearolf. *Espl-Mβ 1.5* (Cooper et al., 2000) was obtained from R. Dasgupta (via E. Bach). *12X Su(H)-lacZ* (Go et al. 1998) was obtained from S. Artavanis-Tsakonas.

Females from respective driver stocks containing (*y w/y w; UAS-mCD8-GFP/UAS-mCD8-GFP; Antp-Gal4/TM6TbSb, y Dome Gal4/FM7; UAS-mCD8-GFP/UAS-mCD8-GFP and y w/y w; HmlΔ–GFP/HmlΔ–GFP*) were crossed to males from respective RNAi stocks ; *y v/Y; UAS -*N^{RNAi}*/UAS-*N^{RNAi} and w^{1118}*/Y ; UAS-*N^{IC}*/TM6 Tb Sb*. Six hour egglays were performed after which cultures were moved to 29^0C until the third instar stage when they were dissected.

To obtain loss-of-function Notch clones, N^{55e11}*FRT19A* females were crossed to *arm lacZ19A; y w hs-FLP* males to create N^{55e11}/N^{55e11} and N^{+}/N^{+}clones that were

marked by the absence of β galactosidase or highest levels of β-galactosidase. Twin clones were generated by heat shock at 37^0 C for 30-40 minutes (Xu and Rubin, 1993).

Wasp infections were performed as follows: from a 6-hour egglay, second instar *Espl* Mβ 1.5 larvae were subjected to infection by *L. boulardi G486* wasps (Sorrentino et al., 2004) for 6 hours, after which wasps were removed and host larvae were allowed to develop to third instar stage. Uninfected controls followed the same growth regimen.

Immunohistochemistry

Antibody staining was performed as follows: Third instar larval lymph glands or hemolymph were air dried on slides, samples were fixed in 4% PFA and incubated with 1% BSA in PBS for 30 minutes at room temperature, followed by incubation overnight with the monoclonal or polyclonal primary antibody. After three washes with PBS (15 min each), samples were incubated for 3 hrs at room temperature with the respective fluorescently-labeled secondary antibodies (Invitrogen and Jackson Immunochemicals). After three washes in PBS, the samples were counterstained with nuclear dye Hoechst 33258 (1:500) and/or F-actin (TRITC-phalloidin) (1:200) where specified, then mounted in glycerol containing antifade (N-propyl gallate). Primary antibody concentrations used were anti-NICD (Hybridoma Developmental Studies Bank) 1:10, anti-L1 (gift from István Ando, Kurucz et al., 2007) 1:10, anti-integrin β PS (Hybridoma Developmental Studies Bank) 1:10, anti- ProPO2 (George Christophides, Imperial College, London, UK; Minakhina et al., 2009) 1:2000. Samples were imaged with Zeiss LSM 510 confocal microscope and images were processed using Zeiss LSM5 and Adobe Photoshop CS3 software.

Dispersal of the Basement membrane

Basement membrane dispersal was determined as described by Sorrentino et al. 2002 and quantified in N^{RNAi} lymph glands (Table 1).

β-galactosidase staining and imaging

Third instar larval lymph glands were dissected in PBS, air dried and placed in 500 µl of solution containing potassium ferri-cyanide (50 mM) (Sigma) in PBS, glutaraldehyde and 30 µl of (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside), (X Gal; (Promega). Samples were incubated at 37^0 C for 2-4 hours. Lymph glands were washed with 1X PBS and mounted in 50% glycerol. Images were taken using a Zeiss Axiovision microscope and processed with Axiovision 4 software.

Results

Expression of Notch intracellular domain in the lymph gland

To address whether Notch's role in lamellocyte differentiation is similar to its contribution to crystal cell development, we examined NICD distribution in wild type control and infected larval lymph glands (Figure 1). NICD is expressed in all hemocytes of the lymph gland (Figure 1). NICD expression is low or it is not detected in *Hml>GFP* positive cells (Figure 1A-B'). Staining in cells of the cortical zone is variable. Some cells have high NICD signal that is localized to the membrane and in puncta, presumably corresponding to vesicles. NICD in *Dome>mCD8-GFP*-positive cells of the medullary zone of anterior lobes (Figure 1C-D') and posterior lobes (data not shown) is high and staining is associated with the mCD8-GFP signal localized to the membrane.

The *Antp>mCD8-GFP*-positive cells of the niche express variable levels of NICD (Figure 1E-F'). The NICD signal is not nuclear in any of these cells. Surprisingly however, NICD levels are very low or undetectable in lamellocytes induced either by wasp infection or from mutant backgrounds (Figure 1G-G"). Numerous lamellocytes are induced in the periphery of anterior lobes, but not in posterior lobes (not shown).

Lamellocytes are large, broad adherent cells that encapsulate wasp eggs. In this study, lamellocytes are identified by staining cells for their shape and cytoskeletal structure (F-actin), or for the expression of Integrin β PS (Irving et al., 2005; Stofanko et al., 2008), or Atilla (L1) (Kurucz et al., 2007). Lamellocytes were also marked by the expression of the *misshapen* transcriptional enhancer *msnf9* linked to mochherry or GFP reporter (Tokusumi et al., 2009; Figure 2 A-B') We confirmed that *msfn9mocherry*

positive lamellocytes were also strongly positive for F-actin with FITC phalloidin (Figure 2B-B').

Expression of Notch intracellular domain in circulating hemocytes

NICD in most circulating hemocytes, presumably plasmatocytes, from uninfected larvae is punctate and cytoplasmic (Figure 4A-C, arrowheads). In these cells, this signal remains unchanged after wasp infection (Figure 4 D-D"). NICD levels are lower in *Hml*-positive circulating cells (Sinenko et al., 2004) (Figure 4B) and in *lz>GFP* positive crystal cells (Figure 4C). As observed in cells of the lymph gland, NICD levels are low or undetectable in circulating lamellocytes induced either by wasp infection (Figure 4 D-D"), or in response to hyperactive JAK signal in $hop^{Tumorous\text{-}lethal}$ larvae (Figure 4E-E" Harrison et. al., 1995). In cells with low NICD expression, the signal is punctate and the levels seem to decrease with an increase in cell size.

$Notch^{RNAi}$ promotes lamellocyte differentiation

We next conducted cell-specific knock down experiments using RNA interference (*UAS-*N^{RNAi}) in the cells of the "niche", cortical and medullary zones, utilizing the *Antp*, *Hml* and *Dome* GAL4 drivers, respectively. The driver stock contains the UAS-GFP reporter transgene, marking the location of GAL4-positive cells. In antibody staining experiments of cells in which N^{RNAi} is expressed, we observed a marked reduction in levels of NICD epitope (Figure 3A-B').

Knockdown of Notch was quantified with both Valium 1 and Valium 10 stocks of N^{RNAi}. These lines differ in the hairpin construct such that knockdown by Valium 10 is

expected to yield stronger effects than Valium 1 knockdown (Ni et al., 2008, Ni et al., 2009).

In each case, the presence of lamellocytes was detected in the anterior lobes of the glands expressing *Notch*^{RNAi}, regardless of whether the RNAi construct was expressed in the niche, MZ or CZ (Figure 5, 6). The strongest effects were observed upon knockdown with either Valium 1or Valium 10 in the niche with the *Antp-GAL4* driver, resulting in greatest numbers of ectopic lamellocytes (Figure 5 H-I). Mature lamellocytes were most often in the cortical zone, rarely in the medullary zone, and never in the niche. Valium10 N^{RNAi} produced a stronger phenotypic effect than Valium 1 N^{RNAi} (Table 1).

Since lamellocytes are migratory cells and appear to make their way past the basement membrane surrounding the anterior lobes (Sorrentino et al., 2004), we inspected the integrity of the basement membrane in both control and experimental glands (Table 1). While the membrane in the control class of glands was largely intact, the basement membranes of the experimental glands were frequently disrupted. We developed the criteria for "immune activated glands," with three or more lamellocytes within the anterior lobes or those that showed basement membrane dispersal (Table 1). This analysis confirmed that in all three compartments, knockdown with Valium 10 *UAS-*N^{RNAi} is stronger than Valium 1 *UAS-*N^{RNAi} knockdown (Figure 5, 6B-D, Table 1).

The percentage of lamellocytes in circulation in experimental animals was more than 10-fold higher relative to control animals (Table 1) in *Antp>*N^{RNAi} Valium 1 and *Hml>*N^{RNAi} Valium 10 populations. In all cases, the lamellocytes themselves were GFP-negative, but were located in the vicinity of the GFP-positive cells in the lymph gland

(see Fig. 5B-C, 5E- F, 5H-I) suggesting a inductive relationship. *Dome>N^{RNAi}* (valium 10) was lethal.

We next confirmed N protein's role in crystal cell development in these glands. As expected, the downregulation of N via *Hml* or *Antp* led to significant reduction in crystal cell numbers (Figure 7B). In contrast, expression of constitutively active N intracellular domain alone resulted in an increase in crystal cells (Fig. 7D). The N^{IC} lobe itself also expands without a significant change in the *Antp>GFP* positive population. Thus, N signaling not only influences crystal cell and lamellocyte fates but apparently also influences proliferation or survival of cells in this region. Active signaling expands lobe size, whereas N^{RNAi} -induced lamellocyte differentiaton disrupts lobe morphology. The number of lamellocytes induced in the lymph glands after RNAi is limited (up to 20 lamellocytes, but 3-6 in most cases) relative to the robust effects observed post infection (Fig. 1G-G''). These results strongly suggest that lamellocyte precursors in the cortical zone are sensitive to the levels of N signaling.

Notch influences lamellocyte differentiation non-cell autonomously

We tested this idea by inducing twin clones in heterozygous $Notch^{55e11}/N^{+}$ glands (Xu and Rubin, 1993, Ohlstein and Spradling, 2007) (Figure 8). In this technique, somatic recombination of a heterozygous cell induces the production of daughters that are either homozygous mutant (N^{55e11}/N^{55e11}) or wild type (N^{+}/N^{+} or N^{+}/Y). Such clonal populations can be distinguished by the dose of the marker protein (β-galactosidase, encoded by *lacZ*, present on the wild type chromosome). As shown in Figure 8, cells with lamellocyte morphology and identity (marked by anti-L1 antibody or integrin β) are

always present with intermediate (heterozygous) or even high (wild type) levels of β-galactosidase protein but never within cells of the N mutant clone itself (Figure 8B-C'). Consistent with the N^{RNAi} experiments, lamellocyte differentiation is non cell-autonomous; newly-differentiated cells always being induced within the cortical zone of the lymph gland and never in the medullary zone or the niche.

Expression of Notch targets in the lymph gland

All together, the staining and genetic experiments support the notion that Notch appears to influence crystal cells and lamellocyte progenitors.This interpretation predicts that Notch pathway targets should be broadly expressed in cells of the anterior lobes. Consistent with this expectation, we found that the *Su(H)lacZ* (Go et al., 1998) and *Espl-lacZ* (Cooper et al., 2000), both known direct targets of Notch activation are expressed in most cells of the cortical zone in wild type backgrounds (Figure 9 B, C). Thus, Notch signaling is widespread in numerous cells of the anterior lobes and appears not to be limited to crystal cell precursors only.

Figure 1. NICD expression in third instar lymph glands with different cell populations marked with GFP.

Cells in cortical zone (*Hml>GFP*; A-B'), medullary zone, (*Dome>mCD8-GFP*; C- D'), and the niche (*Antp>mCD8-GFP*; E-F').

(A-B') Anti-NICD antibody (red) stains cells of the cortical zone of *Hml>GFP* early third instar lymph gland (B-B'). Magnification of cortical zone shows that the NICD signal is weak in *Hml>GFP*-positive cells.

(C-D') NICD colocalizes with *Dome>mCD8-GFP* positive cells of the medullary zone, indicating possible membrane association.

(E-F') Anterior lobes of *Antp>mCD8-GFP* early third instar lymph gland stained with anti-NICD antibody (red).

(F-F') GFP positive niche cells (green) stained with anti-NICD antibody shows colocalization of GFP and NICD.

(G-G") *L. boulardi*-infected wild type lymph gland stained with anti-NICD (red) and FITC-linked phalloidin (green). Lamellocytes (arrows) from wild type third instar lymph glands infected with *L. boulardi* wasps are negative for NICD (G'-G"). Staining is high and punctate in cells in the middle of the lymph gland lobe (G'-G"). All images taken at 40X magnification.

Figure 1.

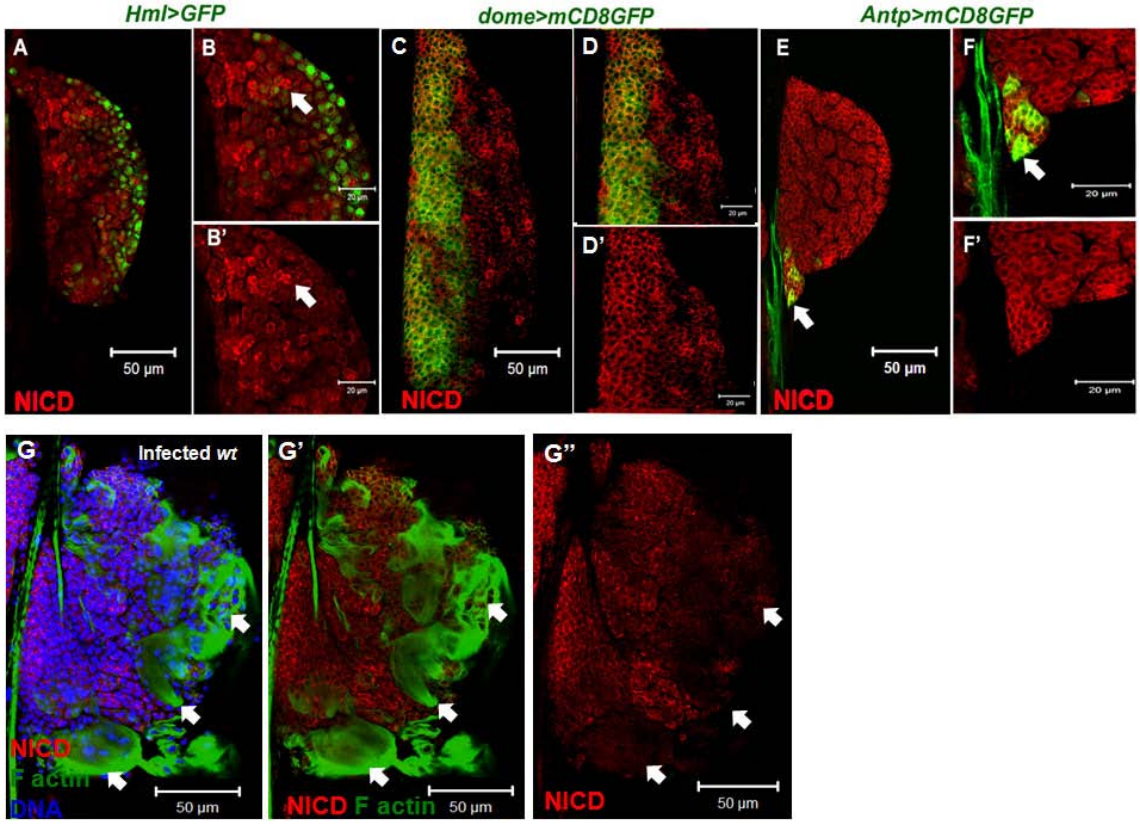

Hml>GFP
dome>mCD8GFP
Antp>mCD8GFP
A
B
B'
C
D
D'
E
F
F'
50 µm
NICD
G
Infected wt
G'
G''
NICD
F actin
DNA
50 µm

Figure 2. Lamellocytes labeled with *misshapen* reporter and F-actin.

(A) Third instar *msnf9mocherry*-expressing lymph gland infected with *L. boulardi* wasp. *msnf9-mocherry* transgene (red) and FITC-labeled phalloidin (F actin; green) along with larger cell and nuclear morphology enable visualization of lamellocytes.

(B) Magnification of a group of four lamellocytes labeled with *msnf9-mocherry* transgene (red) and F actin (green). Hoechst stains DNA (blue) in panels A and B.

(B') Same as B, but only with the red channel to show strong nuclear localization of the mocherry signal. All images taken at 40X magnification.

Figure 2.

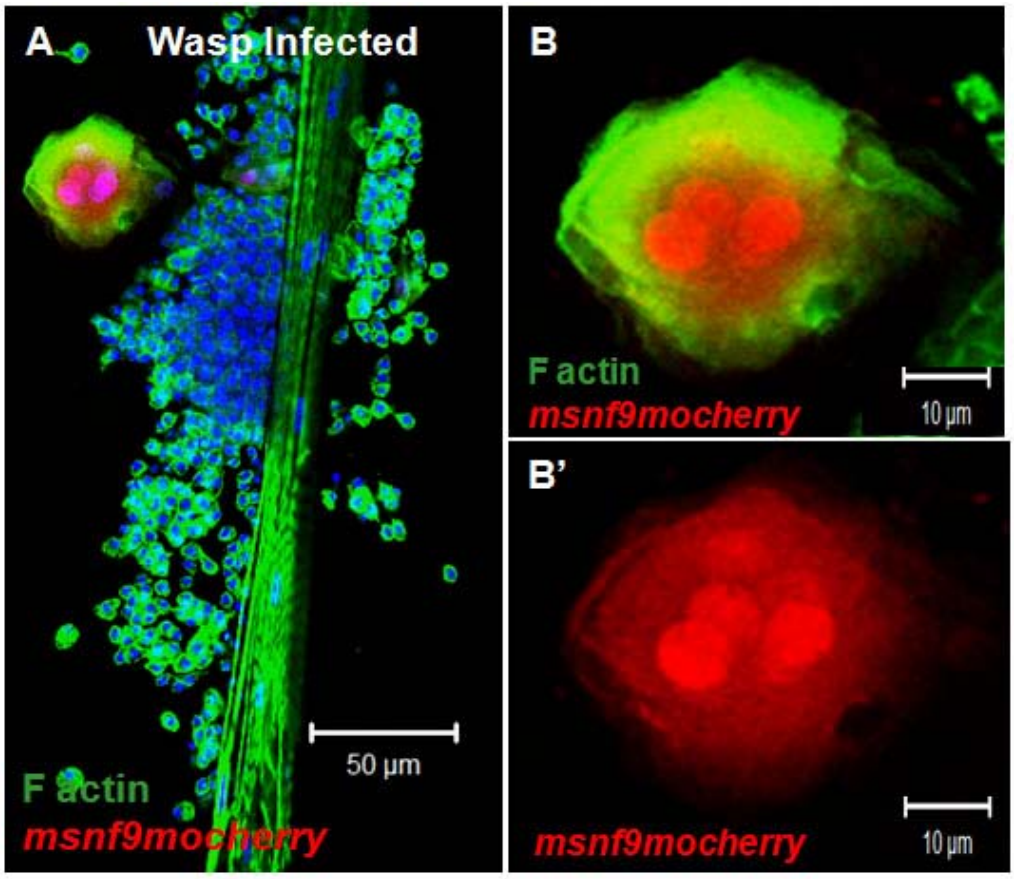

A
Wasp Infected
50 µm
F actin
msnf9mocherry
B
F actin
msnf9mocherry
10 µm
B'
msnf9mocherry
10 µm

Figure 3. NICD signal is reduced or undetected in the *Antp>N^{RNAi}* niche.

(A-A') NICD is expressed throughout the lymph gland of *Antp>N^{RNAi}* except in the niche where it is knocked down via RNAi (arrow).

(B-B') Magnification of the niche region (arrow in panel A') shows NICD expression is undetectable in GFP-positive cells (additional zoom of 2X).

All images taken at 40X magnification.

Figure 3.

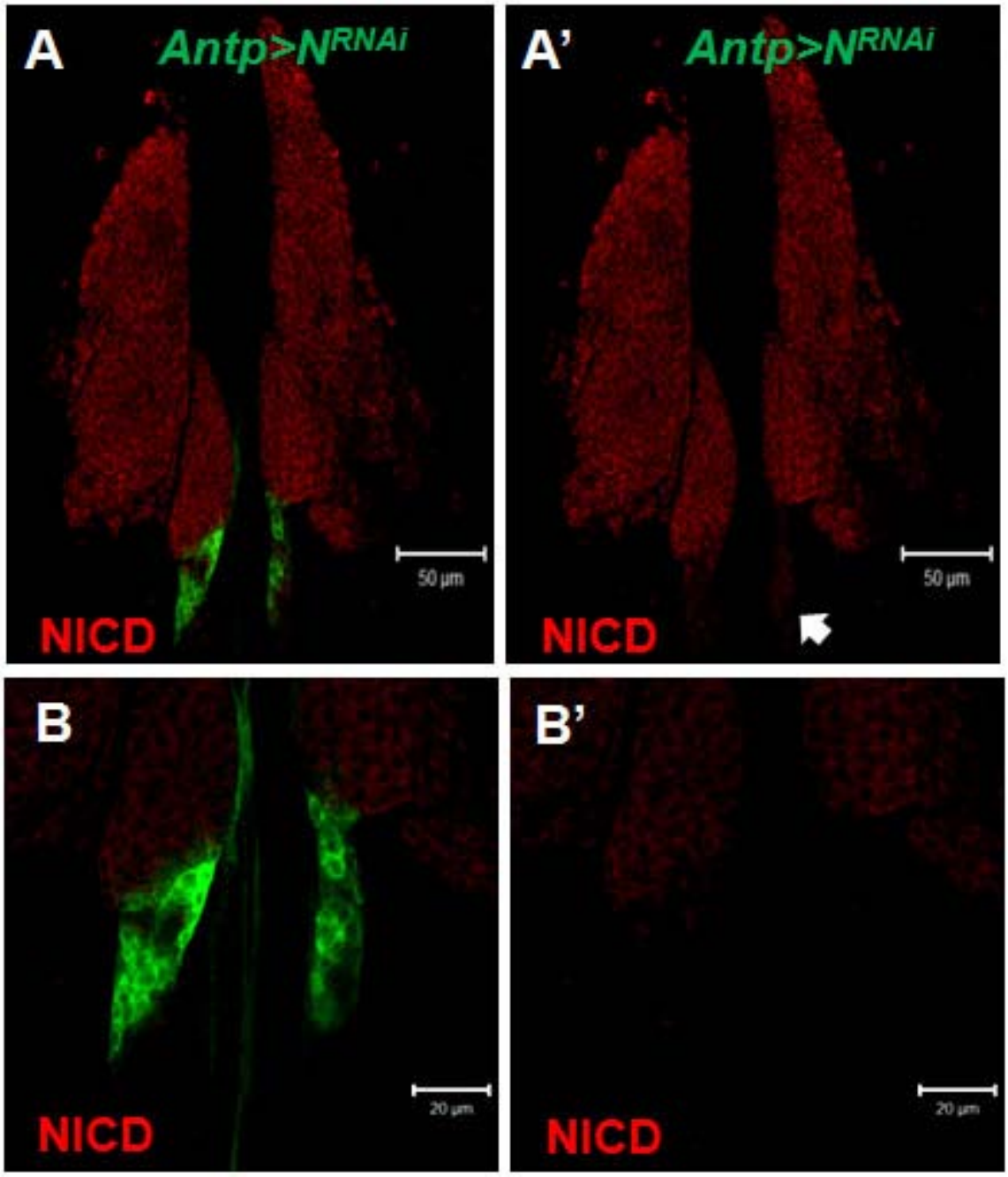

A
Antp>N^RNAi
50 µm
NICD
A'
Antp>N^RNAi
50 µm
NICD
B
20 µm
NICD
B'
20 µm
NICD

Figure 4. NICD expression in circulating hemocytes.

(A) Small GFP-negative circulating hemocytes from the *Hml>GFP* flies express high levels of NICD. This staining signal is punctate (arrows).

(B) *Hml>GFP*-positive cells express low levels of NICD.

(C) *lz>GFP*-positive cells (green, inset) express low levels of NICD.

(D-D') Lamellocytes (positive for F-actin green) induced in wildtype lymph gland after *L. boulardi* wasp infection show different stages of lamellocyte with lower levels of NICD (red). In some cells Notch signal is not detected (arrowhead).

(E-E') Circulating lamellocytes (F-actin green) from $hop^{Tum\text{-}l}$ hemolymph do not express NICD (red). Hoechst stains DNA (blue).

All images taken at 40X magnification.

Figure 4.

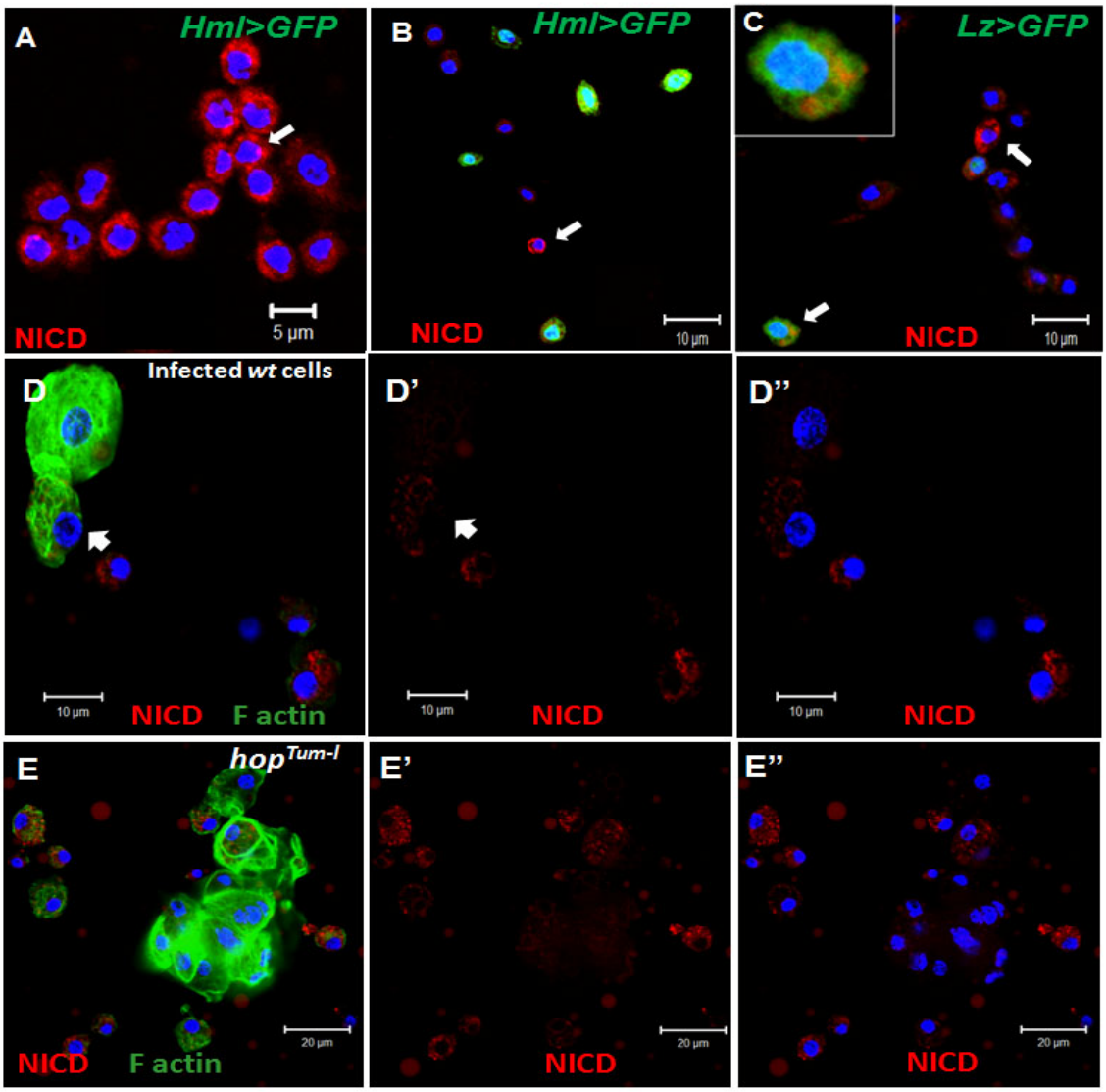

A
Hml>GFP
NICD
5 µm
B
Hml>GFP
NICD
10 µm
C
Lz>GFP
NICD
10 µm
D
Infected wt cells
10 µm
NICD
F actin
D'
10 µm
NICD
D''
10 µm
NICD
E
hopTum-l
NICD
F actin
20 µm
E'
20 µm
NICD
E''
20 µm
NICD

Figure 5. *Notch*^{RNAi} prompts lamellocyte differentiation.

Third instar lymph glands from experimental animals where Notch levels were reduced via RNAi (Valium 1). Drivers used for crosses are shown. Lamellocytes were detected with rhodamine-linked phalloidin (F actin, red).

(A) *Hml>GFP* control lymph gland stained with F actin (red).

(B-C) *Hml*>N^{RNAi} lymph glands produce lamellocytes (red white outline) in the cortical zone stained with F-actin (red).

(C) Magnification of lamellocytes (white outline) (red) are adjacent to *Hml*-positive (green) cells.

(D) *Dome>mCD8-GFP* control lymph gland stained with F-actin (red). MZ is outlined.

(E-F) Dome>N^{RNAi} produces lamellocytes stained with F-actin (red, white outline). (F) Magnification of lamellocytes (red) adjacent to medullary zone cells (green).

(G-I) *Antp>mCD8-GFP* control lymph gland stained for F-actin (red).

(H) *Antp*>N^{RNAi} lymph gland produces lamellocytes stained for F-actin (red, white outline) in the anterior lobes of the lymph gland.

(I) Magnification of lamellocytes (red) adjacent to niche (green) cells.

Hoechst stains DNA (blue).

In no case did N^{RNAi} affect GFP expression, but N^{RNAi} led to dispersal of the anterior lobes.

All images taken at 40X magnification with additional zoom where necessary.

Figure 5.

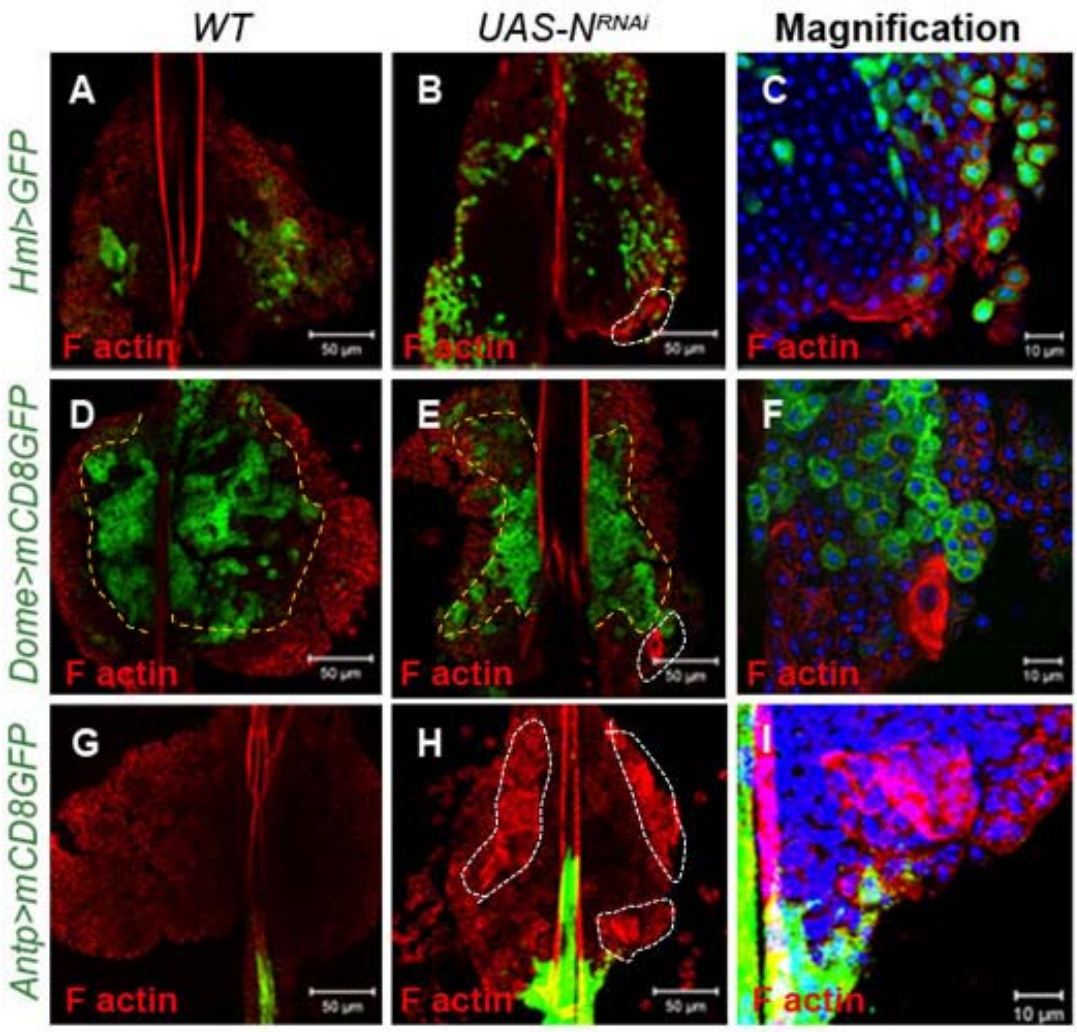

WT
UAS-N^RNAi
Magnification
Hml>GFP
Dome>mCD8GFP
Antp>mCD8GFP
A
B
C
D
E
F
G
H
I
F actin
50 μm
10 μm

Figure 6. N^{RNAi} in the niche promotes lamellocyte differentiation.

Third instar lymph glands from experimental animals where Notch levels were reduced via RNAi (Valium 10).

(A) *Antp>mCD8-GFP* control lymph gland stained with anti-L1 (red).

(B-C) *Antp>*N^{RNAi} (green) produces lamellocytes stained with anti-L1 (red) in the anterior lobes of the lymph gland.

(D) *Hml>*N^{RNAi} (no GFP) produces lamellocytes stained with anti-L1 (red). Hoechst stains DNA (blue).

All images taken at 40X magnification with additional zoom where necessary.

Figure 6.

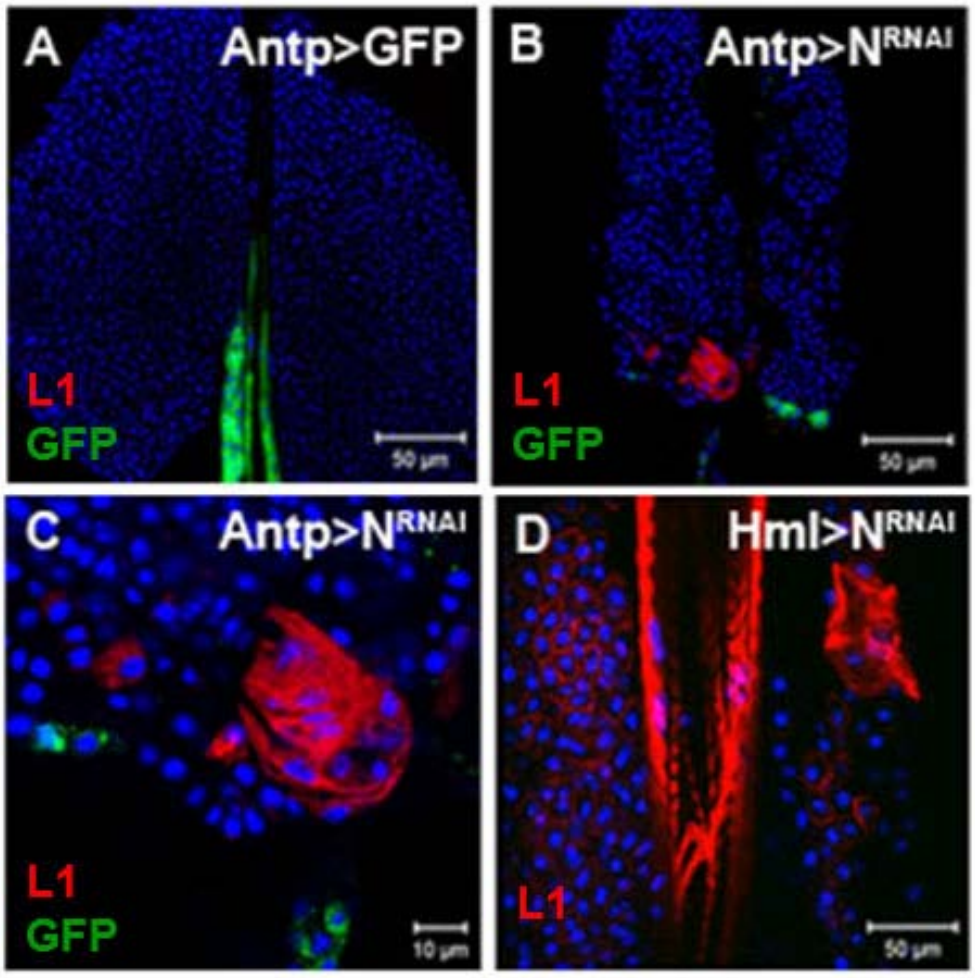

A
Antp>GFP
L1
GFP
50 µm
B
Antp>N^RNAi
L1
GFP
50 µm
C
Antp>N^RNAi
L1
GFP
10 µm
D
Hml>N^RNAi
L1
50 µm

Figure 7. Knockdown of Notch in cortical zone reduces crystal cell number and overexpression of Notch in niche increases crystal cell number in the lymph gland.

(A) Third instar lymph glands were stained with ProPO (red) which labels crystal cells (red) in *Hml>GFP* control lymph gland.

(B) Knockdown of Notch by $Hml>N^{RNAi}$ reduced crystal cell (red) numbers in the lymph gland.

(C) *Antp>GFP* control lymph gland stained with ProPO (red). (D) $Antp>N^{IC}$ lymph glands have increased crystal cells (red). Hoechst stains DNA (blue).

All images taken at 40X magnification.

Figure 7.

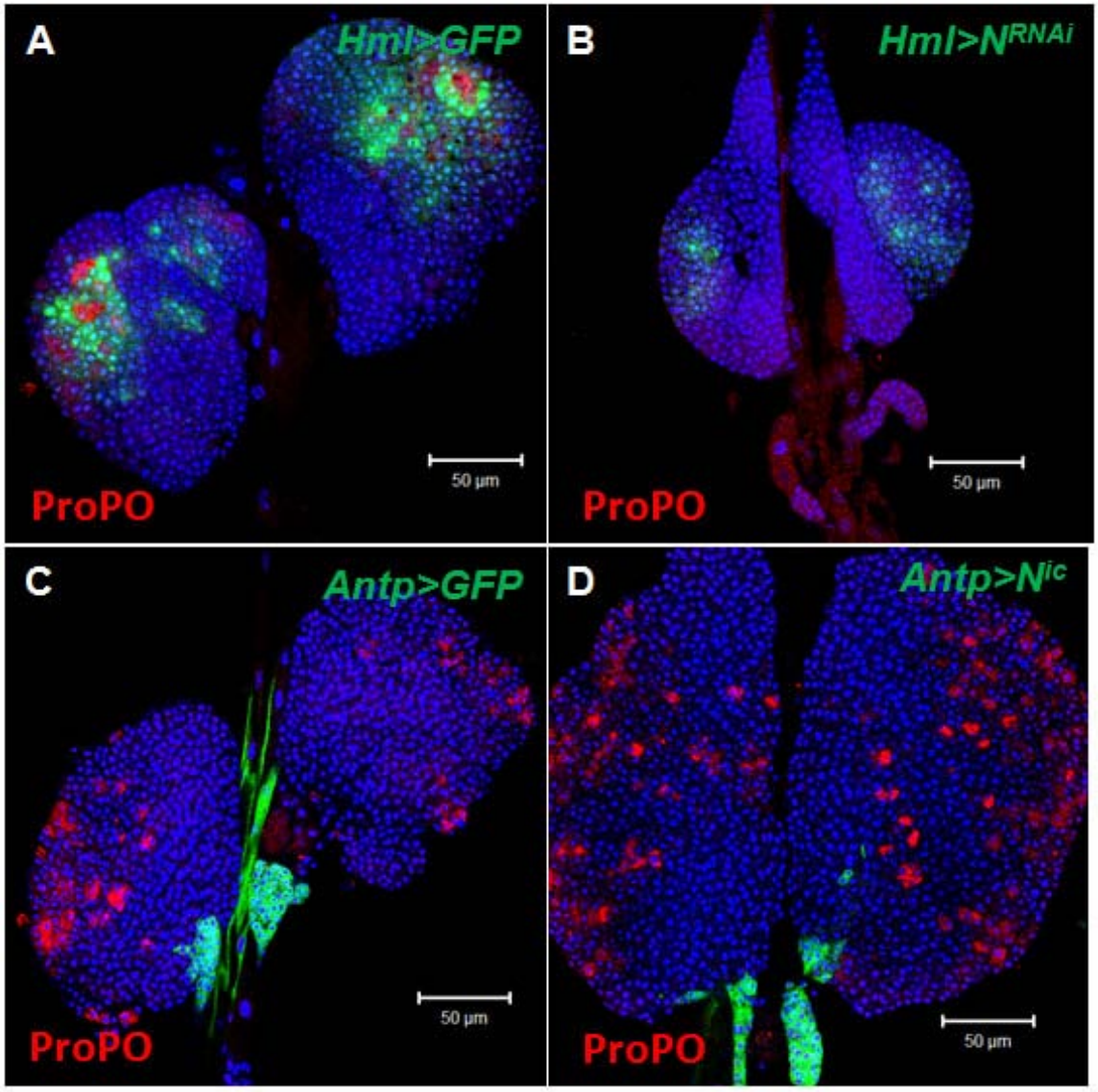

A
Hml>GFP
ProPO
50 µm
B
Hml>N^RNAi
ProPO
50 µm
C
Antp>GFP
ProPO
50 µm
D
Antp>N^ic
ProPO
50 µm

Figure 8. Notch clones reveal non-autonomous function in lamellocyte differentiation.

Notch loss of function mutant clones were generated by crossing N^{55e11}*FRT 19A/FM7* females to *FRT19A; hs-FLP/TM6 Tb Hu* males.

(A) Schematic of clone induction and lamellocyte formation in the cortical zone of the lymph gland.

(B) Mutant clones (N^{55e11}/N^{55e11}) were marked by the absence of β-galactosidase (3 dotted areas, labeled 1-4) whereas homozygous wild type clones (N^{+}/N^{+}) were detected by higher than background levels of β galactosidase (red). Lamellocytes positive for integrin β and β galactosidase (yellow) were found outside of the clones.

(C-C') 40X magnification (additional 2X zoom) of clone #2 shows N^{55e11}/N^{55e11} marked by the absence of β galactosidase (dotted area) and lamellocytes (yellow) outside of the clone. n = 7 clones studied. Hoechst stains DNA (blue).

All images taken at 40X magnification.

Figure 8.

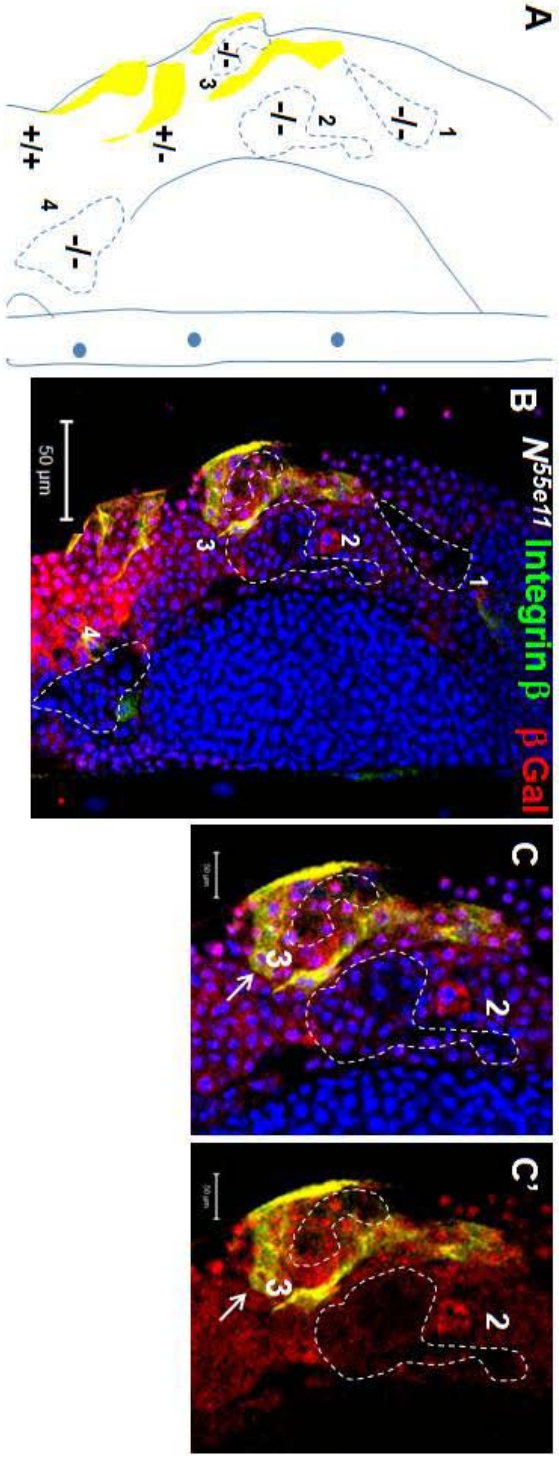


Figure 9. β-galactosidase staining of N pathway reporters.

(A) w^{1118} third instar lymph gland stained for β galactosidase activity.

(B) *E(spl)Mβ 1.5 lacZ* third instar lymph gland stained for β galactosidase. Gene expression is apparent in the outer cortex of the lymph gland.

C) *12X Su(H)lacZ* third instar lymph gland. Blue staining indicates Notch pathway activity.

All images taken at 20X magnification.

Figure 9.

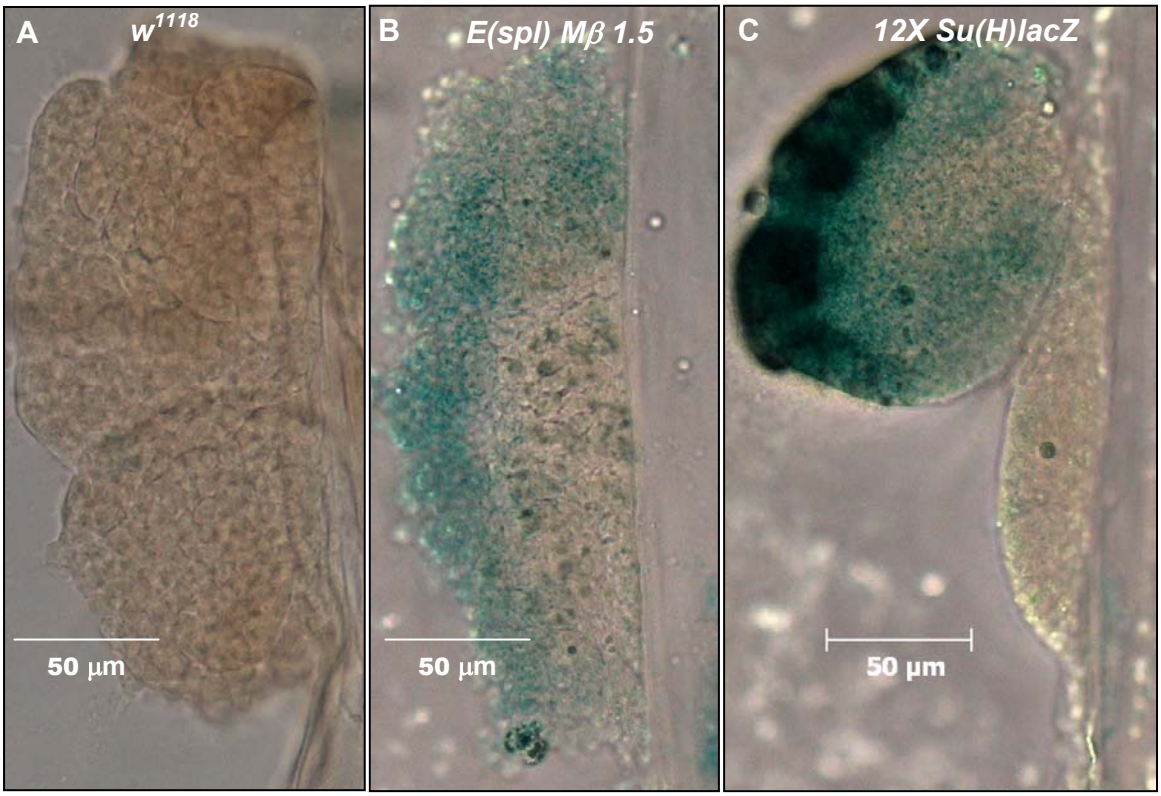


Table 1. The effect of Valium 1 and Valium 10 *Notch* ^{RNAi} in wildtype lymph gland. Dissected lymph glands were analyzed for the presence of three or more lamellocytes, or ruptured basement membrane, both of which are criteria for immune activation. Valium 10 N^{RNAi} had stronger effects on lamellocyte differentiation ($p< 0.05$). In circulation, lamellocytes constituted a larger percentage of the circulating hemocyte population in N^{RNAi} hemocoel compared to controls. LG = lymph gland.

Table 1.

	Genotype	# of LG analyzed (n)	# of LG with lamellocytes	Range of lamellocyte number per lymph gland	# LG with dispersed Basement Membrane
Valium 1	*Antp>w*	10	0	0	1
	Antp>N^{RNAi}	10	4	0-18	4
	Hml>w	10	1	0-2	1
	Hml>N^{RNAi}	10	4	0-10	5
	Dome>w	30	6	0-2	3
	Dome>N^{RNAi}	30	11	0-20	9
Valium 10					
	Antp>w	35	5	0-1	2
	Antp>N^{RNAi}	35	21	0-11	30
	Hml>w	35	2	0-1	4
	Hml>N^{RNAi}	35	17	0-6	31

Chapter 2

Genetic analysis reveals distinct roles for *neuralized* in the division and maintenance of hematopoietic progenitors in the third instar lymph gland

Abstract

neuralized (*neur*), a component of the Notch pathway, encodes an E3 ubiquitin ligase. It plays an essential role in the emission of the juxtacrine signaling ligands, Delta and Serrate. In *Drosophila*, *neuralized* encodes at least four protein isoforms. Of these, Neuralized A (NeurA) and Neuralized C (NeurC) are distinguishable by their amino termini. The role for Neur in *Drosophila* hematopoiesis is not known. Here, we show that Neuralized protein is expressed ubiquitously in the niche, progenitor and mature plasmatocytes and lamellocytes of the third instar lymph gland. The protein is cytoplasmic and associated with the membrane. Using RNA interference knockdown of Neuralized in the niche, cortex and the medulla, we uncovered an inhibitory influence of Neuralized on lamellocyte differentiation. Loss-of-function mitotic clones of *neuralized* promote ectopic differentiation of lamellocytes outside the clone border.

Specific knockdown in the medullary progenitor cells, but not in the niche or in the cortex, also result in cell division defects, with the emergence of binucleate cells. Ectopic expression of full length isoform NeurC or a truncated, dominant-negative form of NeurA lacking the catalytic ring finger (NeurA-ΔRF) had a similar effect on hemocyte division, with less than 10% of the cells in circulation being affected. Ectopic expression of full-length NeurC or NeurA-ΔRF also resulted in a reduction in crystal cell numbers. Our data suggest that Neuralized proteins carry out two functions: first in crystal cell and lamellocyte differentiation, and second, in the division of progenitor cells. We propose that *neuralized* function in mitosis of hematopoietic progenitors is tightly linked to differentiation of cells into different lineages.

Introduction

The transcription factors and signaling pathways governing *Drosophila* hematopoiesis are highly conserved; their orthologs in mammals control blood cell development (Meister and Lagueux, 2003). *Drosophila* is therefore an excellent model for studying blood cell development, not only because of its genetic accessibility, but also because of fewer blood cell types and simple lineage.

In the larval stages, hematopoietic precursors differentiate into three classes of cells: plasmatocytes, crystal cells, and lamellocytes (Meister and Lagueux, 2003). Plasmatocytes, akin to macrophages in mammalian blood lines, make up about 95% of the circulating cells of the hemolymph. Crystal cells, the majority of the remaining 5%, are involved in melanization. Lamellocytes encapsulate metazoan intruders too large to be phagocytosed, only differentiate upon immune challenge (Crozatier, 2004).

The Notch signaling pathway plays a key role in hematopoietic differentiation during larval stages (Duvic et al., 2002; Lebestky et al., 2002; also see Chapter 1). Neuralized (Neur) is one of two ring finger containing E3 ubiquitin ligases (Yeh et al., 2001) involved in the release of the Notch ligands Delta (Dl) and Serrate (Ser) from the signal-sending cell (Pitsouli and Delidakis, 2005; Commisso and Boulianne, 2007; reviewed in Le Borgne, 2006).

The *neuralized* locus is complex and encodes at least four protein isoforms through different transcription initiation and alternative splicing (Commisso and Boulianne, 2007): NeurA, NeurB, NeurC, and NeurD (FlyBase). Both NeurA (3990 amino acids) and NeurC (3754 amino acids) contain three amino acids more than NeurB (3987 amino acide) and NeurD (3751 amino acids), respectively (FlyBase). Because of

the minimal differences, we used NeurA as a model for NeurA and NeurB, and NeurC as a model for NeurC and NeurD. NeurA is 114 amino acids longer in its N-terminal region than NeurC, part of this region contains a phosphoinositide-binding (PIP-binding) motif. PIP-binding of NeurA has been shown to be sufficient for plasma membrane localization and transport of proteins to the external surface of a cell (Skwarek et al, 2007). Both full-length proteins (NeurA and NeurC) retain their RING finger motif (see schematic in Figure 6).

The C-terminus contains RING fingers with ubiquitin ligase activity (Yeh et al., 2001; Pitsouli and Delidakis, 2005). Recently, Koutelou et al. (2008) showed that the mouse ortholog of Neur, Neur 11, is myristoylated at the N-terminus, which targets it to the cell membrane. They showed that the myristoylation-dependent membrane localization was important for the proper membrane function of Neur l1. Skwarek and colleagues (2007) showed that the NHR1 domain of *Drosophila* Neur binds Dl, one of two Notch signaling ligands. Since both NeurA and NeurC contain the NHR1 domain, it is likely that both are able to bind Dl. Delta-binding activity may recruit NeurA to the cell surface (Commisso and Bouliane, 2007; Skwarek et al., 2007), as should myristoylation of the N terminal region (Maurer-Stroh et al, 2004; Koutelou et al, 2008). PIP-binding residues are found only in NeurA, not in NeurC proteins; and only NeurA has PIP-binding functionality (Skwarek et al., 2007). PIP-binding is required for Neur ligase function but not for Dl binding or cell surface localization (Skwarek et al., 2007).

Previous studies have shown that Serrate is expressed in the hematopoietic niche and its function is required for crystal cell development (Lebestky et al., 2003; Duvic et al., 2002). Neur is required in the signal-sending cell; Neur protein targets Delta and

Serrate for ubiquitination leading to its endocytosis and plasma membrane presentation (Yeh et al., 2000; Lai et al., 2001; Pitsouli and Delidakis, 2005).

The objectives of this study were first, to determine if Neur has a role in larval hematopoiesis, similar to Notch and Serrate. We address this question with RNA interference knock-down and clonal analysis. Second, to discriminate if Neur isoforms carry out distinct functions in hematopoietic development, we performed misexpression of Neur isoforms in specific populations. In Chapter 3, we continue the analysis of Neur function via *l(3)hem*^{1} and *l(3)hem*^{2} mutations. A discussion of all observations follows in Chapter 3.

Experimental Procedures

Fly stocks and cultures

RNAi stocks of Neuralized and Serrate were obtained from the TRiP Harvard facility. The UAS responder *neurA* stocks used were standard cDNA in UAS vectors, (1) wild-type *neuralized*A (*UAS-NeurA*), truncated *neuralized*A (RING finger deleted, UAS-*NeurA*-Δ*RF*), and *neuralized* C (UAS-NeurC) (gift from C. Delidakis). *UAS-NeurA* and *UAS-NeurA-ΔRF* were obtained from Eric Lai at MSKCC, New York; *UAS-NeurC* was from Christos Delidakis at FORTH/IMBB in Crete, Greece.

The GAL4 stocks used in the study were: *daughterless* (*da-GAL4*; ubiquitous expression; Bloomington stock ID8641), *armadillo* (*arm-GAL4*; ubiquitous expression; Bloomington Stock ID1560), collagen (*Cg-GAL4*, plasmatocytes; Asha et al., 2003), *l*ozenge (*lz-GAL4*; embryonic and larval crystal cells; Lebestky et al., 2000), *Serpent* (S*rp-GAL4*; all hemocytes), *76B* (*76B-GAL4*; patchy in lymph gland lobes; Harrison et al., 1995), *T98* (*T98-GAL4;* patchy; in lymph gland lobes), *Hemolectin* (*HmlΔ-GAL4*; crystal cells and some plasmatocytes; Sinenko et al., 2003), and *neuralized* (*neur-GAL4* expression in hemocytes not determined; Reddy et al., 1999). The other stocks used in this study are described under the Experimental Methods section of Chapter 1.

Clonal analysis

Mutant clones were generated using females *y w hs-FLP; FRT82B* $neur^{A101}$ *ry* (Lai et al., 2004) crossed to w^{1118}*/Y; {ryneoFRT}82B P{w[+mC]+ piMyc* males. Mutant ($neur^{A101}/neur^{A101}$) clones were marked by the absence of Myc staining whereas homozygous wild type clones ($neur^{+}/neur^{+}$) were detected by higher than background

levels of the Myc epitope as these cells have two copies of the π-Myc gene marker. The genotype of the animals in which the clones are induced is heterozygous for *neur* and heterozygous cells contain only one copy of the myc marker.

Immunostaining, visualization and data analysis

Lymph gland dissections and preparation of smears, their staining, imaging and analysis was done according to Methods in Chapter 1.

Quantification of crystal cells

Crystal cells in third instar larvae were detected by heat induced melanization. Animals were placed in 1xPBS at 70ºC for 10 minutes. Cells from posterior most three segments were counted. Larval genotypes are shown in Table 1.

Results

Expression of Neur protein in hemocytes

Lymph gland staining with polyclonal antibodies against Neur revealed that the protein is largely cytoplasmic in hemolectin-postive cells of the cortical zone (*HmlΔ>GFP;* Figure 1C-D), medullary zone (*Dome>mCD8-GFP*; Figure 1E-F), and the niche (*Antp>mCD8-GFP*; Figure 1G-H). The mCD8-GFP tag on the GFP reporter in Dome and Antp populations allowed us to visualize Neur protein association with membrane. Clear colocalization of the signals is seen in some cells.

In circulating plasmatocytes Neur is uniformly cytoplasmic (Figure 2B-C); in lamellocyte from $hop^{Tum\text{-}l}/hop^{Tum\text{-}l}$ mutant hemolymph, Neur protein levels are also high. (Figure 2D-E). This observation is supported in $hop^{Tum\text{-}l}$ lamellocytes marked by nuclear *msnf9-mocherry* transgenic gene reporter (Figure 2F).

$Neur^{RNAi}$ promotes lamellocyte differentiation

RNA interference knockdown of Neur in *Hml>GFP* (Figure 3B-C), in *Antp>mCD8-GFP* (Figure 3H-I), or *Dome>mCD8-GFP (*Figure 3E-F) cells promotes lamellocyte differentiation. In each case, the presence of lamellocytes was detected at the border of the anterior lobes of the glands expressing $Neur^{RNAi}$, regardless of whether the RNAi construct was expressed in the niche, MZ or CZ. The strongest effects were observed upon knockdown with the *Antp* driver, resulting in the greatest numbers of ectopic lamellocytes (Figure 3H-I). The niche cells appear to also migrate along the dorsal vessel towards the anterior most tip of the gland. Lamellocyte identity was confirmed by staining cells with the anti-L1 marker along with their large, flat shape and

migratory phenotype. As in the case of N^{RNAi}, lamellocyte differentiation is induced in the vicinity of the $Neur^{RNAi}$ producing cells, suggesting non cell-autonomy (Figure 3I).

neuralized inhibits lamellocyte differentiation

We next analyzed the cellular effects of loss-of-function of *neuralized* in third instar lymph glands by doing FLP/FRT clonal analysis (Xu and Rubin, 1993). We generated mutant clones in animals heterozygous for *neur* using females *y w/y w, hsFLP*; *FRT82B* $neur^{A101}$ *ry* crossed to males of genotype w^{1118}*/Y; P{ry neo FRT}82B P{w[+mC]+ piMyc ry.* In this method, mutant clones ($neur^{A101}/neur^{A101}$) are marked by the absence of Myc staining whereas homozygous wild type clones ($neur^{+}/neur^{+}$) are detected by high levels of Myc staining as these cells have two copies of the *pi Myc* gene marker. The genotype of the animals in which the clones are induced is heterozygous for *neur* ($neur^{A101}$/+) and heterozygous cells contain only one copy of the Myc marker.

Twin clones were identified as a group of cells marked by the absence of Myc expression, that were present in close vicinity of another group that showed high Myc levels (Figure 4A-B2). The *neur* mutant clones were located in the periphery/cortical zone region of the lymph gland where mature differentiated hemocytes are normally found. This region is also more highly mitotic (Jung et al., 2005) and is likely to contain the clones. To characterize the identity of mutant cells, we stained lymph glands with anti-integrin-β antibody, which is expressed in high levels in lamellocytes (Stofanko et al., 2008). We observed that a few nuclei in mutant clones were polyploid while one (or more) mutant cells were positive for integrin β (Figure 4B1, B2). Such an integrin β-positive lamellocyte was large in size relative to cells around it and exhibited the thin, flat

morphology, typical of a lamellocyte. Furthermore, lamellocytes appeared to arise only from *neur*^{A101}clone boundary and are unaffected by neighboring wildtype cells. Presence of lamellocytes outside *neur* mutant clones suggests that the function of wild type Neur protein is needed to limit lamellocyte differentiation. Furthermore, the presence of polyploidy/aneuploid cells in the clone suggests that the lamellocyte differentiation step is tied to mitosis. One interpretation of these observations is that loss of Neur function results in aberrant mitosis of a progenitor whose progeny is free to acquire the lamellocyte fate.

Dome>neur^{RNAi} or ectopic expression of NeurC promotes cell division defects

In addition to ectopic lamellocytes, we were surprised to find that *Dome>neur*^{RNAi}, but not *Antp>neur*^{RNAi} or *Hml>neur*^{RNAi} led to the appearance of binucleate cells in the lymph gland and in circulation (Figure 5). This result further supports Neur's role in hematopoiesis, in controlling the cell division of progenitors. We then provided ectopic NeurA, NeurC or NeurA-ΔRF proteins. The latter construct encodes a loss-of-function mutant form of the protein, lacking the ubiquitin ligase activity (Figure 6A, B). We were surprised to find that not only did expression of the NeurA-ΔRF protein (via *arm-GAL4*, *Cg-GAL4* and *lz-GAL4*) yield binucleate cells, but binucleate hemocytes were found when UAS-NeurC was driven by the same drivers (Figure 6C, Table 1, and data not shown). Confocal imaging allowed us to confirm that the cells were binucleate, as both nuclei were on the same plane and shared the same cytoplasm (Fig. 6C-L). Furthermore, *76B>NeurA-ΔRF* but not the *76B>NeurC* produced binucleate hemocytes. Overall, less than 10% of hemocytes were binucleate in all

backgrounds (Table 1). Combined, these results suggest that loss of Neur protein or loss of its ubiquitin ligase activity, or the ectopic expression of the NeurC isoform can induce cell division defects in hematopoietic progenitors.

Serrate^{RNAi} promotes lamellocyte differentiation

Since Neuralized is known to modify the function of Serrate (Pitsouli and Delidakis, 2005), we tested if knockdown of Ser might yield similar hematopoietic defects. *Antp>Ser*^{RNAi} and *Dome>Ser*^{RNAi} led to ectopic lamellocyte differentiation (Figure 7B, C, E, F) although unlike *Neur*^{RNAi}, cell division defects were not observed in *Dome>Ser*^{RNAi} or *Antp>Ser*^{RNAi} samples (data not shown).

Effect of Neur overexpression on crystal cell population

When UAS-NeurA, NeurC and NeurA-ΔRF were expressed in crystal cells via *Cg-GAL4, lz-GAL4* drivers, there was a statistically significant reduction in this cell population (Table 2). Neither *Hml-GAL4* nor *Srp-GAL4* produced a significant change in crystal cell numbers with either full length *neur* transgene. Drivers with ubiquitous expression (*arm-GAL4* and *da-GAL4*) caused an increase in crystal cells in the UAS-NeurA background and a decrease in crystal cells in the UAS-NeurC background, though not to a statistically significant level (data not shown). These results suggest that, consistent with its role in Notch signaling, perturbation of Neur levels or activity modulates crystal cell numbers.

Figure legends

Figure 1. Expression of Neuralized protein in hemocytes of anterior lobes of wild type third instar lymph glands.

(A-B) Negative control for staining where sample was not treated with primary antibody.

(B-H) Confocal images showing Neur expression (red) in third instar lymph glands. GFP signal marks cells in different populations as indicated: cortical zone (*Hml>GFP*) (C-D), medullary zone, (*Dome>mCD8-GFP*) (E-F), and the niche (*Antp>mCD8-GFP*). Note membrane and cytoplasmic expression of Neuralized (red) in all cells.

(B-C) Anti-Neur detects *Hml>GFP* cells; signal is associated with the membrane and cytoplasm.

(E-F) Neur colocalizes with *Dome>mCD8-GFP* positive cells of the medullary zone, indicating membrane association.

(H) Cells of the niche, *Antp>mCD8-GFP* lymph gland express Neur.

Hoechst stains DNA (blue). All images taken at 40X magnification.

Figure 1.

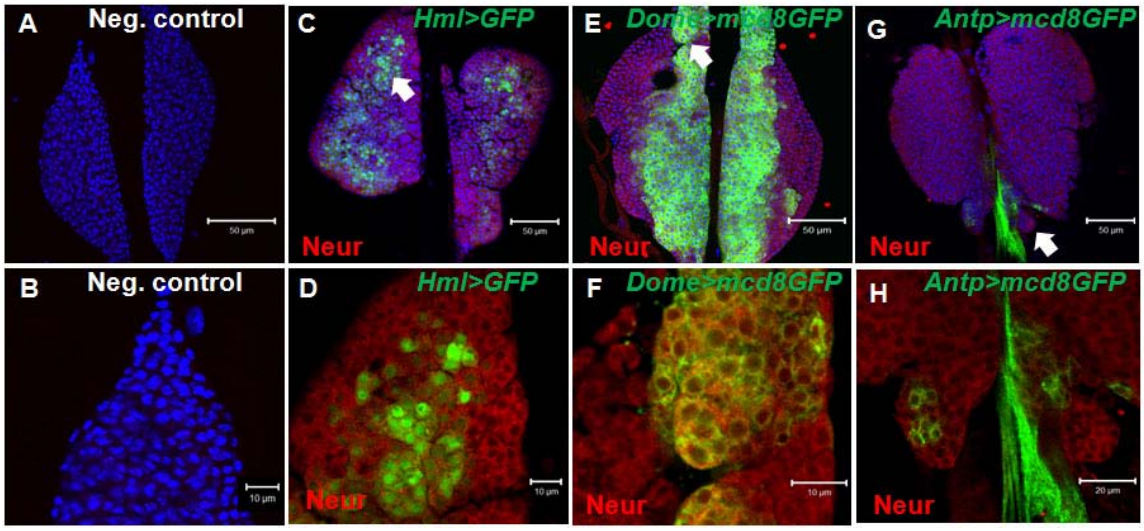

A
Neg. control
C
Hml>GFP
Neur
E
Dome>mcd8GFP
Neur
G
Antp>mcd8GFP
Neur
B
Neg. control
D
Hml>GFP
Neur
F
Dome>mcd8GFP
Neur
H
Antp>mcd8GFP
Neur

Figure 2. Expression of Neuralized protein in circulating hemocytes of third instar larvae.

Samples stained with anti-Neur antibody (red).

(A) Negative control in which primary antibody application was omitted.

(B-C) Neur stains plasmatocytes from wild type in the cytoplasm.

(D-F) Plasmatocytes and lamellocytes from $hop^{Tum\text{-}l}/hop^{Tum\text{-}l}$ hemolymph express Neur.

(D) Lamellocytes from $hop^{Tum\text{-}l}/hop^{Tum\text{-}l}$ express high levels of Neur.

(F) Transheterozygote of ($hop^{Tum\text{-}l}$ /*msnf9-mocherry,* red) express high levels of Neur. *msnf9-mocherr*y is nuclear (red, nucleus). Hoechst stains DNA (blue). All images taken at 40X magnification with additional 3X zoom.

Figure 2.

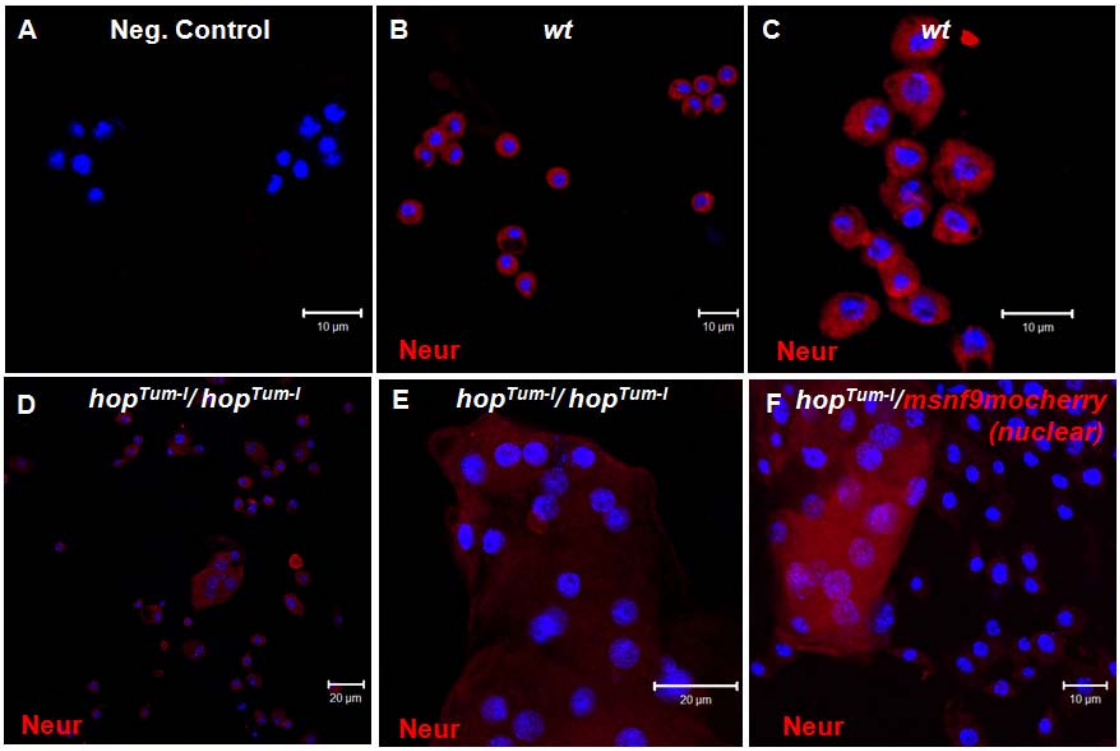

A
Neg. Control
10 µm
B
wt
10 µm
Neur
C
wt
10 µm
Neur
D
hopTum-l/ hopTum-l
20 µm
Neur
E
hopTum-l/ hopTum-l
20 µm
Neur
F
hopTum-l/msnf9mocherry
(nuclear)
10 µm
Neur

Figure 3. Effects of *neur*^{RNAi} on hemocyte development in the lymph gland.

Third instar lymph glands from experimental animals where Neur levels were reduced via RNAi. Drivers used for crosses are shown. Lamellocytes were detected with anti-L1 antibody (red) and (F-actin).

(A) *Hml>GFP* control lymph gland stained with anti-L1 antibody.

(B-C) *Hml>neur*^{RNAi} lymph glands produced lamellocytes in the cortical zone stained with anti-L1 antibody.

(C) 40X Magnification of lamellocytes (white arrow in B) are adjacent to *Hml>GFP* positive cells.

(D) *Dome>mCD8-GFP* control lymph gland stained with anti-L1 antibody.

(E-F) Dome>*neur*^{RNAi} produced lamellocytes (white arrow) stained with anti-L1 antibody.

(F) 40X Magnification of lamellocytes (red) adjacent to medullary zone cells (green).

(G-I) *Antp>mCD8-GFP* control lymph gland stained for anti-L1 antibody.

(H) *Antp>neur*^{RNAi} lymph gland produced lamellocytes (white arrow) stained with anti-L1 antibody in the anterior lobes of the lymph gland.

(I) 40X Magnification of lamellocytes (red) adjacent to niche (GFP) cells, which have migrated from niche area to the cortical zone.

Hoechst stains DNA (blue).

Figure 3.

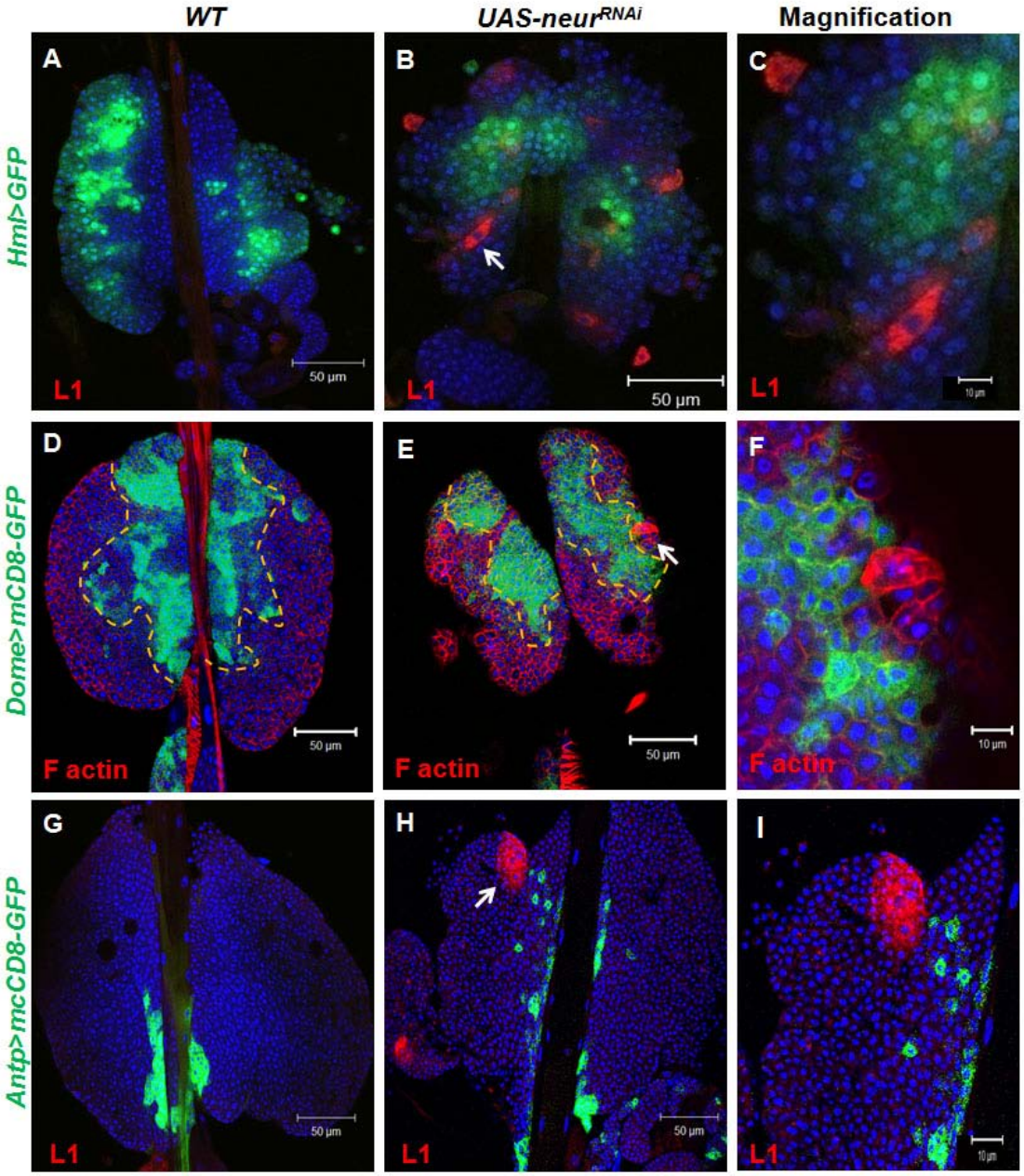

WT
UAS-neur^RNAi
Magnification
A
B
C
Hml>GFP
L1
50 µm
10 µm
D
E
F
Dome>mCD8-GFP
F actin
G
H
I
Antp>mcCD8-GFP

Figure 4. FLP/FRT *neur* ^{A101} clonal analysis reveals non cell-autonomous effects of Neuralized on lamellocyte differentiation.

Clone boundaries (dashed lines) are identified by Myc staining (red) and nuclei marked with Hoechst (blue).

(A) *neur* ^{A101} mutant clone showing polyploid nuclei which are negative for the Myc epitope. *neur* $^{+}$*/neur* $^{+}$ clones are positive for Myc (red) and in white boundary. 40X magnification.

(B1) *neur* ^{A101} mutant clones (arrows) in anterior lobe of lymph gland marked by absence of Myc staining (arrows), and positive for lamellocyte marker integrin β (green) and polyploid (blue).

(B2) Higher magnification of *neur* ^{A101} mutant clone of (B1) shows polyploid lamellocytes stained with integrin β (green). 40X magnification with 3X zoom.

Scale bars: 20 μm (A, B1) and 10 μm (B2).

Figure 4.

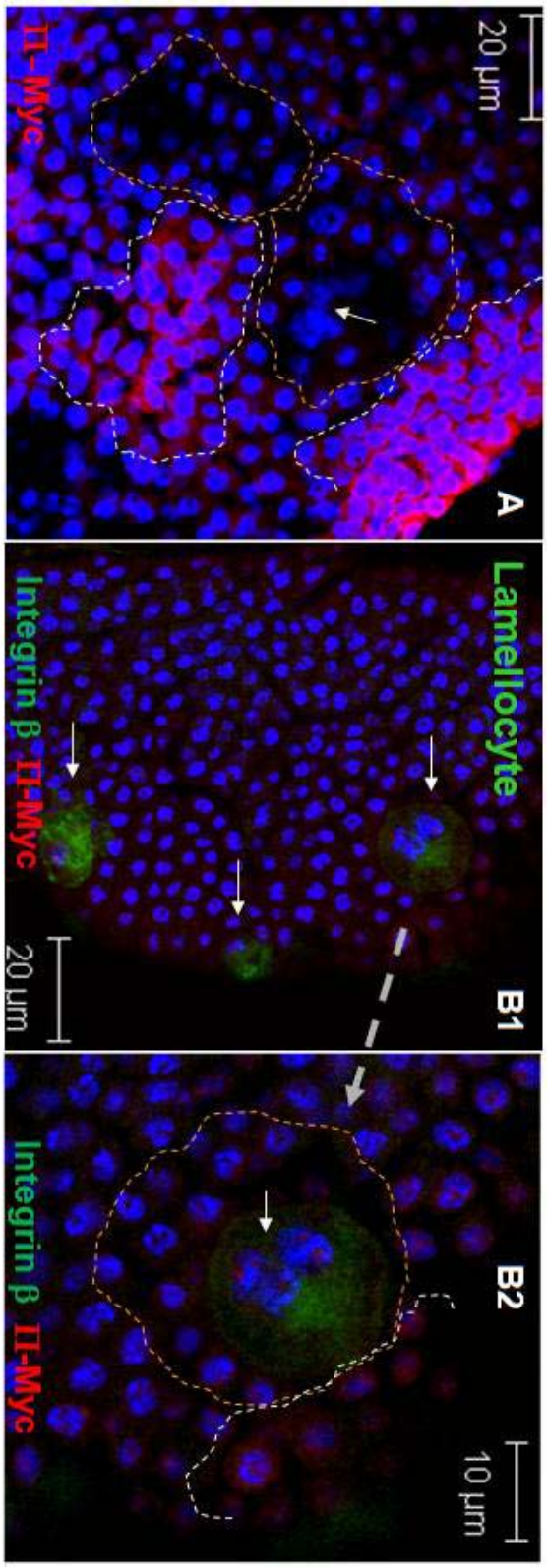

20 μm
Π-Myc
A
Lamellocyte
Integrin β Π-Myc
20 μm
B1
Integrin β Π-Myc
10 μm
B2

Figure 5. *Dome>neur*^{RNAi} induces cell division defects in circulating hemocytes.

Samples stained with anti-integrin β (B-E) and rhodamine phalloidin (F-actin, F-J, both red).

(A) Negative control in which primary antibody application was omitted.

(B) Wild type plasmatocytes express low levels of integrin β protein (red).

(C) Intermediate lamellocytes produced in circulation by *Dome>neur*^{RNAi} express high levels of integrin β protein.

(D-E) Lamellocytes produced in circulation by *Dome>neur*^{RNAi} show integrin β accumulation in the cytoplasm.

(F) *Dome>neur*^{RNAi} produced binucleate cells stained for F-actin (F-J, red).

(F-H) *Dome>neur*^{RNAi} cells with two nuclei in the cytoplasm stained with F-actin (red). Nucleus of some cells appears polyploid.

(H-J) *Dome>neur*^{RNAi} produced lamellocytes that express high levels of F-actin (red).

Cell and nuclear size as well as the amount of integrin β protein in experimental samples can be compared to wild type cells for reference. Hoechst stains DNA (blue).

All images taken at 40X magnification.

Figure 5.

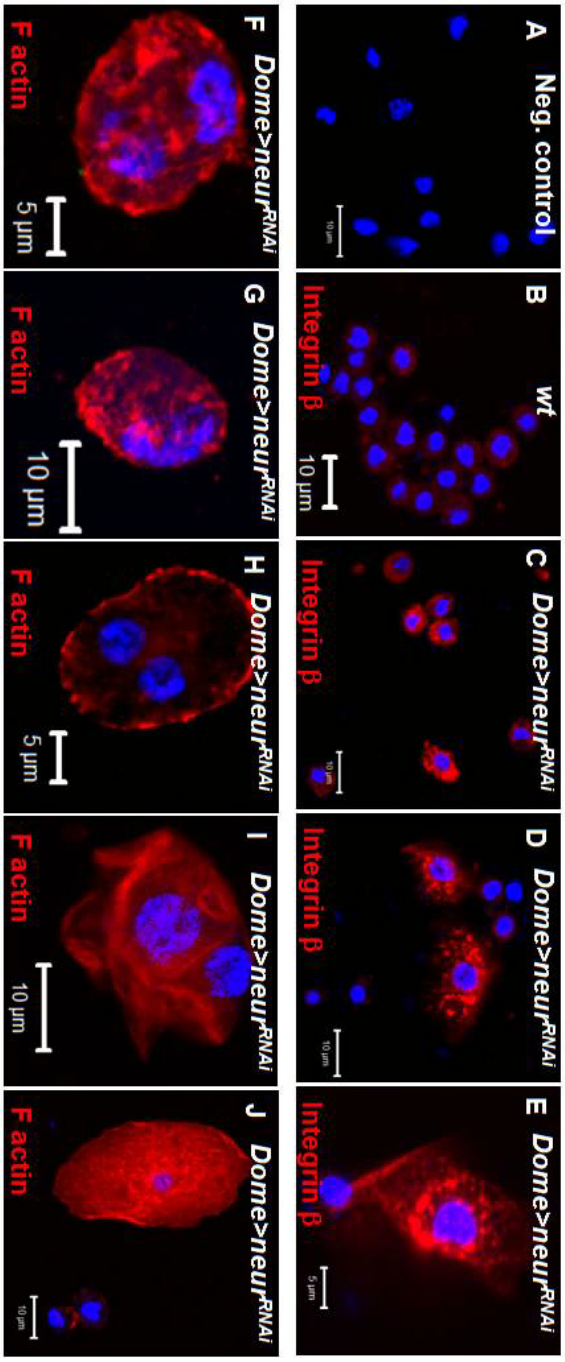

A Neg. control
B wt
C Dome>neur^RNAi
D Dome>neur^RNAi
E Dome>neur^RNAi
Integrin β
10 µm
F Dome>neur^RNAi
G Dome>neur^RNAi
H Dome>neur^RNAi
I Dome>neur^RNAi
J Dome>neur^RNAi
F actin
5 µm
10 µm

Figure 6. Overexpression of Neur promotes some hemocytes to become binucleate.

(A) *neuralized* is located at position 4846K-4865K on the right arm of chromosome 3 (FlyBase FB2009_01). *neuralized* genomic region- Exons 1A and 1C are unique to NeurA and NeurC, respectively. Exons 2 and 3 are common to both. Exon 2 encodes the NHR domains; exon 3 encodes the RING finger. Production of the two isoforms is regulated via different transcriptional initiation and alternative splicing (Commisso and Boulianne, 2007). Exons are indicated in green.

(B) Neuralized proteins: NeurA and NeurC are full-length, wild type proteins. NeurA-ΔRF is truncated NeurA (RING finger deleted). NeurA is approximately 80 amino acids longer than NeurC. This difference is found in the amino terminus, which contains the PIP-binding domain in NeurA.

(C) **Overexpression of Neur C leads to production of binucleate hemocytes in circulation.** All hemocytes stained with rhodamine or FITC phalloidin (F actin, red or green).

(C) Hemocytes from *UAS NeurA* (parental) control.

(D) *lz>NeurA* (E) *Srp>NeurA*

(F) *Hemese >NeurA* (G) *Hml>NeurA* stain with Factin

(H) Hemocytes from Neur C parental control (I*) lz>NeurC*

(J) *Srp >NeurC* (K) *Hemese >NeurC*

(L) *Hml >NeurC* (green, F-actin) produced binucleate cells in the *UAS-NeurC* background. (M) *Srp>NeurC* lymph gland reveals binucleate cells (inset, stars) in the anterior lobes stained with F-actin.

(N) 40X Magnification of *Srp>Neur C* shows cells with two nuclei in the same cytoplasm (white arrows). Hoechst stains DNA (blue).

Figure 6.

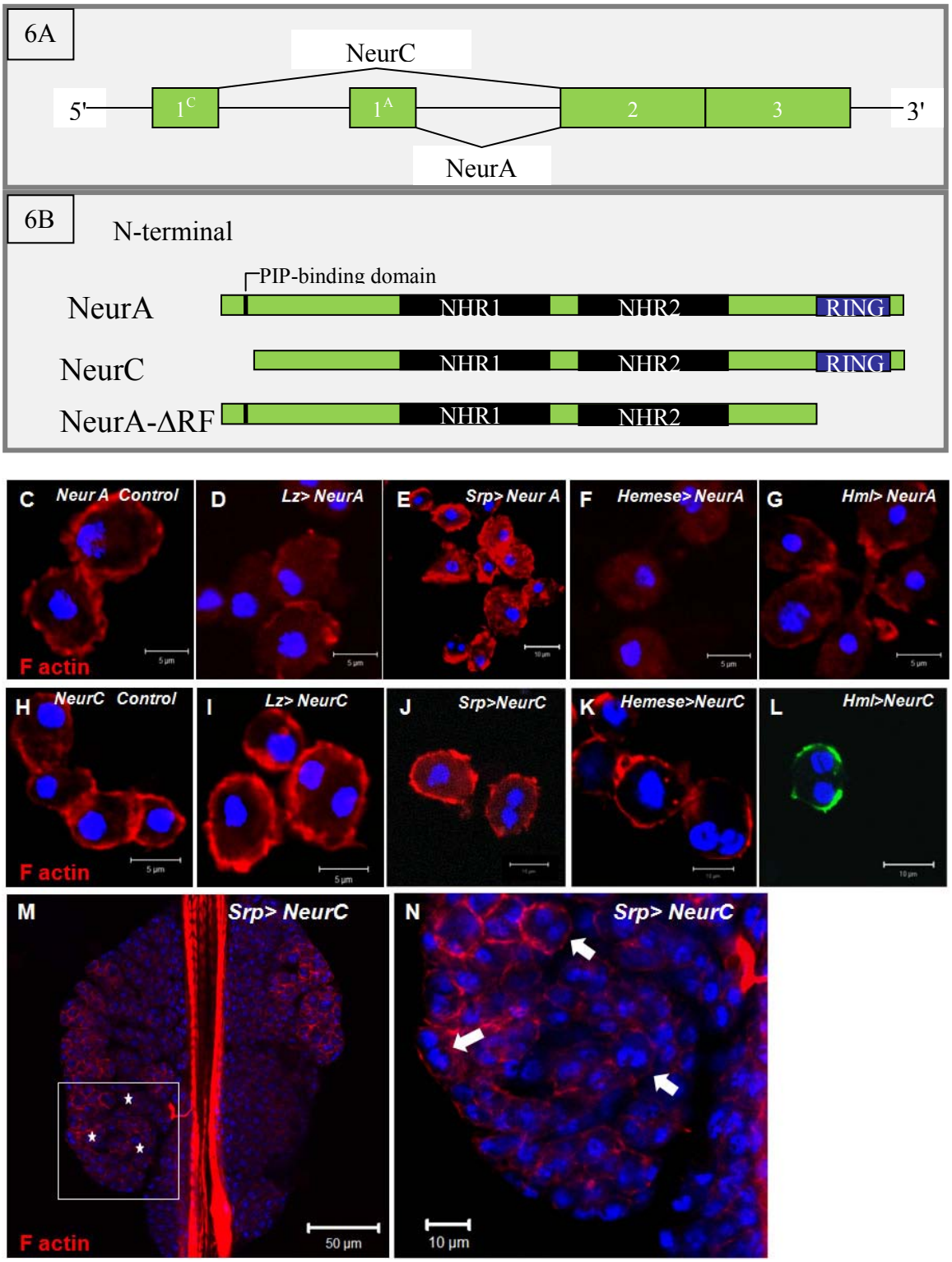

6A
NeurC
5'
1C
1A
2
3
3'
NeurA
6B
N-terminal
PIP-binding domain
NeurA
NHR1
NHR2
RING
NeurC
NHR1
NHR2
RING
NeurA-ΔRF
NHR1
NHR2
C Neur A Control
F actin
D Lz> NeurA
E Srp> Neur A
F Hemese> NeurA
G Hml> NeurA
H NeurC Control
F actin
I Lz> NeurC
J Srp>NeurC
K Hemese>NeurC
L Hml>NeurC
M Srp> NeurC
F actin
50 µm
N Srp> NeurC
10 µm

Figure 7. Effects of *Serrate*^{RNAi} on hemocyte development in the lymph gland.

Lymph glands stained with rhodamine phalloidin (F actin).

(A) *Antp>mCD8-GFP* control lymph gland.

(B) *Antp>Ser*^{RNAi} lymph gland produces lamellocytes in the anterior lobes of the lymph gland.

(C) Magnification of a region in panel B showing lamellocytes adjacent to the niche (GFP) cells.

(D) *Dome>mCD8-GFP* (medullary zone) control lymph gland.

(E) Dome>*Ser*^{RNAi} lymph gland with lamellocytes.

(F) 2X Magnification of lamellocytes in panel E.

Hoechst stains DNA (blue).

All images taken at 40X magnification.

Figure 7.

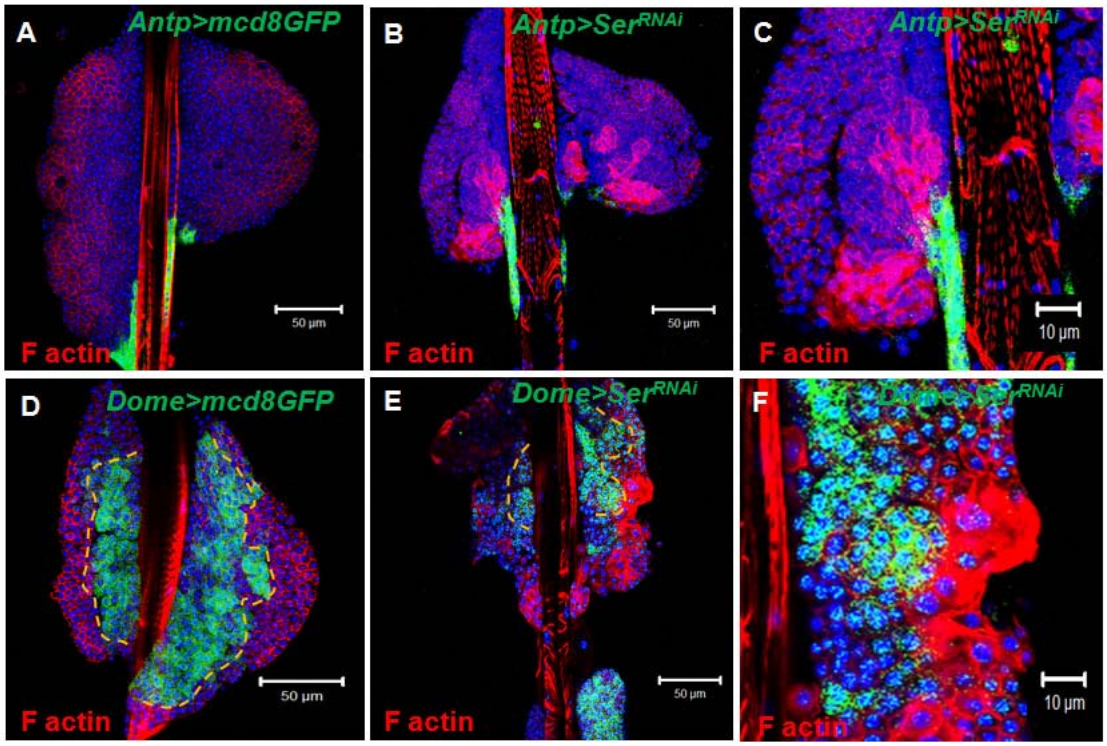

A
Antp>mcd8GFP
50 µm
F actin
B
Antp>Ser RNAi
50 µm
F actin
C
Antp>Ser RNAi
10 µm
F actin
D
Dome>mcd8GFP
50 µm
F actin
E
Dome>Ser RNAi
50 µm
F actin
F
RNAi
10 µm
F actin

Figure 8. *Antp>neur*^{RNAi} reduces crystal cell number and promotes lamellocyte differentiation in the lymph gland.

Third instar lymph glands were stained with anti-ProPO antibody (pink) which labels crystal cells and anti-L1 (red) which labels lamellocytes. Hoechst stains DNA (blue).

(A) Crystal cells (pink) in *Antp>mcd8-GFP* control lymph gland stain with anti-ProPO.

(B) Reduction of Neur by *Antp>neur*^{RNAi} reduced crystal cells (pink, white stars) numbers in the lymph gland and increases lamellocytes (red, white arrows).

(C) 2X Magnification of *Antp>neur*^{RNAi} lymph gland with lamellocytes and a crystal cell.

All images taken at 40X magnification.

Figure 8.

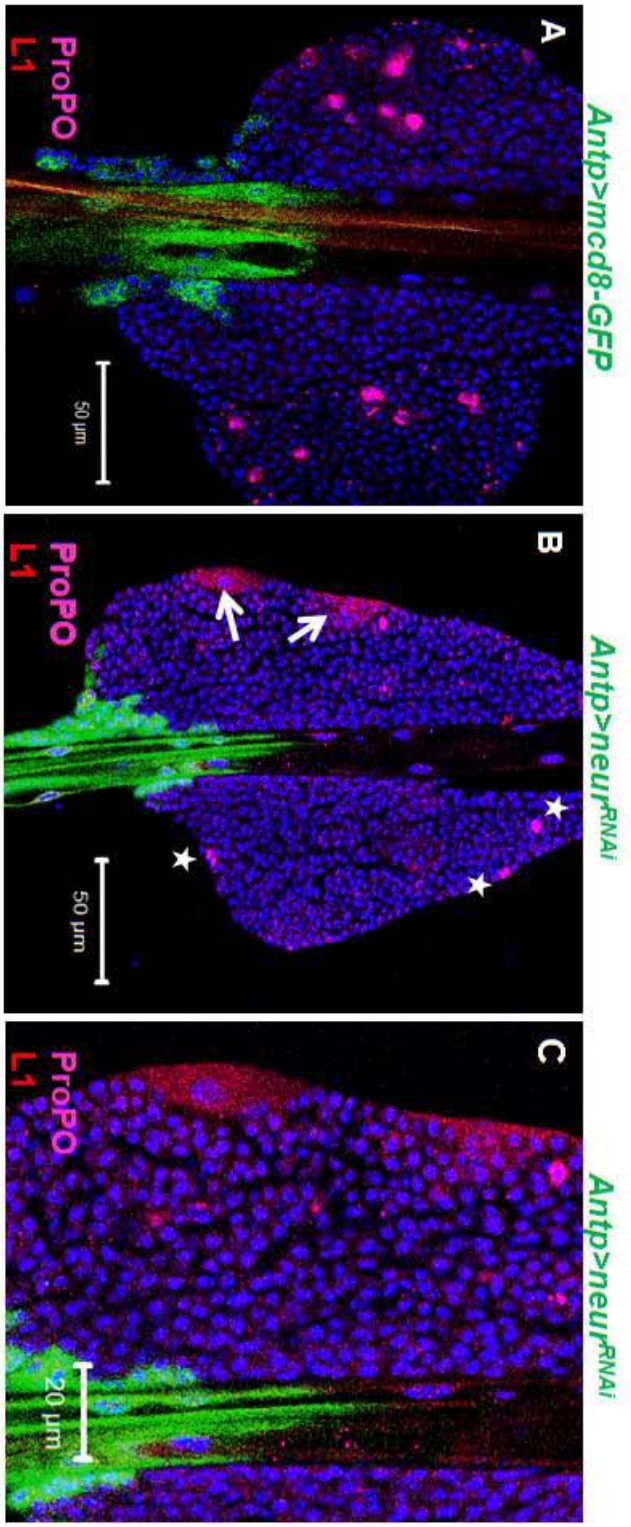

A
Antp>mcd8-GFP
ProPO
L1
50 µm
B
Antp>neurRNAi
ProPO
L1
50 µm
C
Antp>neurRNAi
ProPO
L1
20 µm

Table 1. Percentage of bincucleate cells with ectopic expression of wild type or mutant Neuralized. Parental genotypes (UAS and GAL4 lines) used for crosses are shown in the first two columns. The number and percentage of binucleate cells formed upon ectopic expression of the mutant or wild type version of the protein is shown in the third column. Number of animals and the total number of cells examined in the progeny of each cross examined is indicated in the last column. The expression patterns and original references for the GAL4 drivers are described in the Experimental Procedures.

Table 1

UAS line	**Driver**	**Number of binucleate cells**	**Number of animals (Number of cells)**
UAS-NeurA-ΔRF	*arm-GAL4*	15 (0.47%)	6 (3177)
	Cg-GAL4	18 (1.377%)	6 (1307)
	lz-GAL4	0	4 (436)
	Hml-GAL4	0	6 (374)
	76B-GAL4	26 (0.64%)	6 (4055)
	UAS-NeurA-ΔRF control	5 (0.168%)	6 (2969)
UAS-NeurC	*arm-GAL4*	16 (1.40%)	Not done
	Llz-GAL4	5 (0.81%)	
	Hml-GAL4	1 (0.98%)	
	76B-GAL4	0	
	T98-GAL4	0	

Table 2. Number of melanized crystal cells in Neuralized overexpression animals.

Table shows the average number of crystal cells in the last three segments of a third instar larva. n= 12 larvae. *p* values for all crosses less than 0.05, except *lz>neurA-ΔRF* (*p* = 0.548) and *Srp>neurA* (*p* = 0.155). [*p* values not determined for *UAS-neurC* crosses].

Neur isoform	**Cross**	**Average number of. crystal cells**
UAS neurA	*UAS neurA* control	156.58
	Cg>neuA	75.58
	lz>neurA	15.0
	Hml>neurA	NA
	Srp>neurA	185.75
UAS neurC	*UAS neurC* control	245.0
	Cg>neurC	2.8
	lz>neurC	23.33
	Hml>neurC	188.83
	Srp>neurC	NA
UAS neurA-Δ RF	*UAS neur-Δ RF*	111.92
	Cg>neur-Δ RF	3.17
	lz>neur -ΔRF	85.83
	Hml>neur-Δ RF	190.42
	Srp>neur-Δ RF	NA

Chapter 3

lethal (3)hematopoiesis missing, *l(3)hem*^{1} *and l(3)hem*^{2}, putative alleles of *neutralized,* provide new insights into larval hematopoiesis

Abstract

In healthy, unchallenged *Drosophila* larvae, a majority of hemocytes (plasmatocytes) have the ability to phagocytose microbial pathogens, whereas the remaining cells (crystal cells) mediate immune-related melanization reactions. After eggs of parasitic wasps are deposited into the larval hemocoel, large number of lamellocytes, appear in circulation. We show that *l(3)hematopoiesis missing (l(3)hem^{1}* and *l(3)hem^{2}*) putative novel alleles of *neuralized,* a gene in the Notch pathway that encodes an E3 ubiquitin ligase. Embryos of both *l(3)hem^{1}* and *l(3)hem^{2}* show neurogenic phenotypes, typical of characterized *neur* alleles. The surviving *l(3)hem^{1}* larvae exhibit a dramatic reduction in the number of hematopoietic precursors in the lymph gland and of mature hemocytes in circulation. Mutant hemocytes are larger than wild type hemocytes, aneuploid or polyploid and in some cases, multinucleate. Lymph glands of third instar *l(3)hem^{1}* larvae specify the niche and express markers of the medullary zone. Cells of all three hematopoietic lineages are affected in these mutants suggesting an early requirement for Neur in hematopoietic development. We propose that *neur* function is required for division and development of hematopoietic precursors and this requirement is tightly linked to their differentiation.

Introduction

As is evident from the preceding Chapters, despite substantial progress, some major gaps in understanding the nature of hematopoietic progenitors, their division and differentiation still remain. A traditional genetic approach is to characterize loss-of-function mutants in genes affecting hematopoiesis. *l(3)hem*^{1} is a classical mutation identified by Dr. E. Gateff (Gateff , 1999) and has been used in many studies as one with highly reduced hemocyte populations (Dolezal et al., 2005). The identity of the gene affected in this stock is not known.

l(3)hem^{2} is another uncharacterized mutation, whose relationship to *l(3)hem*^{1} is not known. Our goal in this study was to identify the gene which is mutated in *l(3)hem*^{1}. Previous work has shown that *l(3)hem*^{1} lymph glands exhibit a strong reduction in hemocyte number and they also produce abnormal cell types (Gateff, 1999). This mutation is particularly intriguing as very few recessive mutations are known to reduce hemocytes, while mutations in many genes result in overproliferation. Thus, we hypothesized that a molecular and phenotypic characterization of *l(3)hem*^{1} would shed light on early steps of larval hematopoietic development.

Here, we report that *l(3)hem*^{1} and *l(3)hem*^{2} are putative alleles of *neuralized (neur).* While *l(3)hem*^{2} does not complement known alleles of *neur* such as *neur*^{A101} *or neur*^{11}*, l(3)hem*^{1} shows limited complementation. However, several imprecise excisions of *l(3)hem*^{2} are unable to complement *l(3)hem*^{1}.

The *neur* gene encodes an E3 ubiquitin ligase which targets Notch ligand Delta for ubiquitination leading to its endocytosis and plasma membrane presentation (Lai et al. 2001). We show that *l(3)hem*^{1} is a weak allele. The mutation strongly affects the division

and differentiation of cells in all the hematopoietic compartments in the larval lymph gland. Based on these observations, we propose that *neur* is required for division of hematopoietic precursors and controls differentiation of the crystal lineage and repression of lamellocyte lineage.

Experimental Procedures

Fly Stocks and culture conditions

All crosses were performed and egg lays maintained at 25^0 C in standard yeast/cornmeal/agar medium. All experiments were done using third instar larvae unless specified otherwise. *l(3)hem^1/TM6 Tb Sb* and *l(3)hem^2/TM6 Tb Hu* were obtained from the Bloomington Stock Center. *Tp (3;1) Fa11 e/TM3 Sb Ser* used to rescue the viability of the *l(3)hem^1* mutation was a gift from Dr. Arno Muller. *ZCL2897* was obtained from Yale (GFP Fly Protein Trap, http://flytrap.med.yale.edu).

l(3)hem^2 allele has a transposon within the *neur* locus (see Results). Of the 24 excision stocks (e.g., *Δl(3)hem^2* 402D9), made by imprecise excision of the P element in *l(3)hem^2*, ten failed to complement the *l(3)hem^1* lethality. Excision line 402D9 is referred to as *Δl(3)hem^2/TM3 Sb* fly line by imprecise excision.

To examine the medullary zone, we created fly lines carrying *ZCL2897/ZCL2897*; *l(3)hem^1/TM6 Tb Sb. ZCL2897* expression were analyzed in *l(3)hem^1*/+ and *l(3)hem^1/ l(3)hem^1* backgrounds.

Analysis of cell morphologies and functions

Lymph glands from control and *l(3)hem^1* mutants were dissected and incubated in 40 µl of the trypsin-versene mixture (Bio Whittaker) on a slide in a moist chamber at 37^0C, 15 minutes for the mutant and 30 minutes for the control. Samples were washed in PBS, fixed with 2% formaldehyde, stained with Hoechst 33258 (Invitrogen) and mounted in 50% glycerol. These preparations retained all lymph gland cells.

Immunostaining and microscopy

Immunostainings of lymph glands were performed according to standard protocols with the use of fluorescent secondary antibodies (see Chapters 1 and 2). Third instar larval lymph gland of the appropriate genotype were dissected with forceps in phosphate buffer saline (PBS) on a glass slide, air dried and then fixed with 4% paraformaldehyde. Lymph glands were incubated in 1% BSA with 0.1% Triton X, overnight. Primary antibodies, mouse anti- P1, anti-L1 (Kurucz et al., 2007) and rabbit anti-ProPO antibodies were added at room temp for 4-6 hours, samples were washed thrice with PBS, and incubated for 3 hrs at room temperature with goat tetramethyl rhodamine isothiocyanate (TRITC, Jackson Immuno Inc.) or rabbit fluorescein isothiocyanate (FITC, Jackson Immuno Inc)-labeled secondary antibodies and nuclear dye Hoechst 33258. Samples were mounted in 50% glycerol with 0.4% N-propyl gallate (anti-fade, Sigma). Visualization and imaging were done with confocal microscope LSM510 (Carl Zeiss).

Cuticle preparation of embryos

l(3)hem^{1}, *l(3)hem*^{2}, or *neur*^{A101} mutant embryos were collected on agar plates, dechorionated with 50% bleach and washed in 1X PBS. Washed embryos were placed in 3:1 acetic acid and glycerol solution, inclubated for 2 hrs at 55^{o}C then mounted in Hoyers' solution (Wieschaus et al., 1984). The cuticle phenotypes of these embryos were imaged with Leica MZ FL111 stereomicroscope with 20X objective.

Results

l(3)hem^{1} and *l(3)hem*^{2} are alleles of *neuralized*

The *l(3)hem*^{2} is an embryonic lethal mutation. The *l(3)hem*^{2} mutation did not complement *neur* alleles *neur*^{A101}, *neur*^{1} or *neur*^{11} (Figure 1A) supporting allelism.

Using the *l(3)hem*^{2} stock (with a w^{+} transgene), we generated two classes of excisions that either complemented *l(3)hem*^{1},or did not. The latter category of strains were called *Δl(3)hem*^{2}. Strains from both these classes fail to complement the parental *l(3)hem*^{2} mutation (Figure 1B). Further, complementation studies revealed that *l(3)hem*^{1} and *l(3)hem*^{2} only partially complement one another (Figure 1B). The two stocks themselves are homozygous lethal: Roughly 45% (34/73 *l(3)hem*^{1} animals lacking the balancer) survived into the larval stages. Remainder homozygous mutants (55%) died as embryos with specific cuticle defects (Figure 1C).

l(3)hem^{2}, and Δ*l(3)hem*^{2} did not complement known *neur* allele, *neur*^{A101}. *l(3)hem*^{1} complemented *neur*^{A101} with (> 100%) viability (Figure 1B). Examination of the *l(3)hem*^{1}/*l(3)hem*^{2} trans heterozygote lymph glands revealed a smaller sized anterior lobes with cells containing polyploid nuclei (Figure 1D), a phenotype associated with *l(3)hem*^{1} homozygotes. This result supports the idea that *l(3)hem*^{1} and *l(3)hem*^{2} are potentially allelic.

We compared cuticles of *l(3)hem*^{1}, *l(3)hem*^{2} and *neur*^{A101} embryos from each strain. Embryonic cuticles showed neurogenic defects similar to that seen in Notch pathway mutants, where there is an excess of neural tissue (Figure 1C, Poodry et al., 1990). *l(3)hem*^{2} had more severe cuticle defects (Figure 1C) than *l(3)hem*^{1}. These results suggest that *l(3)hem*^{1} is possibly a hypomorphic allele of *neur*.

To ensure that mutations in other unrelated loci do not contribute to the *l(3)hem*^{1} phenotypes, we used a *Tp (3;1) Fa11 e/TM3 Sb Ser* stock containing a duplication (chromosome 1) and deficiency of the region uncovering cytological positions 85-87 on chromosome 3 (*neur*$^{+}$ is located at 85C2-85C3). When crossed to the *l(3)hem*^{1}*/TM 6Tb Sb e* females, the duplication on the first chromosome replaces *l(3)hem*^{1}*/Df* in half of the progeny rescuing the females (data not shown). Altogether, these results support the idea that mutation in the *l(3)hem*^{1} stock is confined to the deficiency/duplication (85-87) region.

Using reverse transcriptase PCR, we examined the expression of the neur A and neur C transcripts in the *l(3)hem*^{1} mutants using primer sequences specific to the A and C isoforms. Both transcripts were expressed in the *l(3)hem*^{1} mutants and the *l(3)hem*^{1}/+ controls (data not shown). We also amplified and sequenced the genomic region from homozygous *l(3)hem*^{1} mutant embryos. Analysis of the genomic sequence revealed polymorphisms compared to the wildtype; although none are predicted to affect the protein sequence (data not shown).

Effect of *l(3)hem*^{1} on circulating hemocytes

Like their precursors in the lymph gland, mutant hemocytes in circulation also show abnormal morphologies with a progression of related defects. We classified mutant hemocytes into four groups (Figure 2, first row). Type A resembles the control plasmatocytes in shape and size (Figure 2A, first row). Cell types B, C and D are two-to-four, eight-to-sixteen and thirty-two or more times larger than type A, respectively (Figure 2B-D). In these larger cells, the nuclei are larger; in some cells, specially cell

types C and D, there is evidence of polyploidy or aneuploidy (Figure 2D-G, first row). While more than 95% of all hemocytes in circulation are affected by the *l(3)hem*^{1} mutation, less than 10% of all the hemocytes are multinucleate. The relative proportion of the four cell types shows no major fluctuation over this time period (Figure 2B). Type A, constitutes less than 10% of mutant hemocytes in circulation. Cell type B makes up 15-30% of all cells, of which roughly 10% are multinucleate. Cell type C is the most abundant and roughly half of all mutant cells (40-65%) display type C morphology. Less than 10% of type C cells have more than one nucleus. Type D cells are similar to type B in proportion, but are larger than type B in size. All cells in control larvae exhibit the plasmatocyte type A morphology.

l(3)hem^{1} affects lymph gland development.

In order to investigate if the *l(3)hem*^{1} mutation affects all the lymph gland lobes equally, we examined lymph glands from early second instar to wandering stage larvae (Figure 2, second row). Lymph glands from control second-instar *l(3)hem*^{1}/+ larvae are smaller than those of third-instar larvae. This change is most apparent in the size of the anterior lobes (AL) (compare anterior of Figure 2A and 2B, second row). Lymph glands of second-instar *l(3)hem*^{1} mutants appear similar in morphology and size when compared to the second instar control samples (compare Figure 2A and 2E). However, anterior lobes of the mutant third instar lymph glands remain small, and with 126 $\pm$ 21 cells per gland (n = 3) fewer cells than the corresponding controls which had 2218 $\pm$ 289 (n = 3) cells/gland (Figure 2B-D; F-H, arrowhead). Individual mutant cells of the AL are larger in size and the cell density is lower (compare control, Figure 2J and mutant, Figure 2K).

The posterior lobe of feeding third instar mutant larvae appear comparable in size to those in the control (compare PLs of Figure 1D and 1G).Thus, the *l(3)hem*^{1} mutation affects virtually all hemocyte precursors and the mutant lymph glands retain roughly 5% of the control number. However, because of developmental delay in mutants, lymph glands of mutants persist when those from control animals can no longer be found.

The sessile hemocyte populations also had fewer and abnormal cells (Figure 2, third row). We crossed the *l(3)hem*^{1} stock with the *Hml>GFP* strain that allows visualization of mature larval hemocytes. When the sessile hemocyte population is visualized through the transparent larval cuticle, fewer, but larger, hemocytes are apparent (Figure 2O-R).

Expression of Neur protein in *l(3)hem*^{1}

We examined Neur protein expression in *l(3)hem*^{1}/+ and *l(3)hem*^{1} lymph glands. Dissected glands were stained simultaneously with anti-Neur and anti-integrin β antibodies (Figure 3.) Integrin β expression is high in lamellocytes (Stofanko et al., 2008). Neur is expressed throughout the lymph glands of heterozygous controls (Figure 3B) and appears to be in the cytoplasm and associated with the plasma membrane (Figure 3C). Integrin β is not expressed in the *l(3)hem*^{1}/+ lymph gland, but is strongly expressed in some dispersed cells of *l(3)hem*^{1} mutant glands (Figure 3D-F). In these integrin β mutant cells, Neur staining is intense ans speckled (Figure 3G-I). These staining results demonstrate that *l(3)hem*^{1} is a protein coding allele, defective either in the levels and/or the distribution of the protein.

$l(3)hem^1$ affects the niche, medullary and cortical zones

Given that both abundance and the hemocyte morphologies are affected in $l(3)hem^1$ lymph glands, we wondered if the specification or the size of the niche in these glands is altered. It has previously been shown that Serrate expression in the niche is important for niche function (Lebestky et al., 2003) and Collier plays a role in maintaining high *Serrate* RNA levels in the niche (Crozatier et al., 2004). If Neur plays a role in Serrate expression or function, we might expect the niche in the $l(3)hem^1$ lymph glands to be affected. We therefore labeled $l(3)hem^1$ glands with anti-Antennapedia (Antp) antibody (Figure 4). In heterozygous controls, 150-200 Antp-positive cells in both anterior lobes were counted for each gland (Figure 4B). However, only 3-5 Antp-positive cells were detected in $l(3)hem^1$ mutants (n = 4 glands; Figure 4C). These cells were also dispersed in the anterior lobes, suggesting that despite pleiotropic effects of the mutation, the niche is clearly specified.

Loss of niche function has been shown to cause loss of MZ markers, loss of quiescence and increased differentiation of precursors, with a concomitant increase in crystal cell numbers (Mandal et al., 2007). We used *ZCL2897*, a GFP protein trap specific to the MZ (Jung et al., 2005) crossed to $l(3)hem^1$ to examine medullary zone status in mutant glands (Figure 5). We then stained those lymph glands with anti P1 (plasmatocytes) or anti L1 (lamellocytes) (Figure 5).

As expected, *ZCL2897-GFP* expression is clearly detectable in the MZ of heterozygotes and P1 labeled plasmatocytes are found in the cortical zone (Figure 5B). Surprisingly, however, in *ZCL2897/ZCL2897;* $l(3)hem^1/l(3)hem^1$ mutant glands, the proportion of the lobe that was *ZCL2897-GFP*-positive was greater relative to control

lobes (Figure 5C) and P1 positive cells distributed throughout the anterior lobes (Figure 5C). Furthermore, the expression of GFP is strikingly higher in mutants. (Note that *ZCL2897* insertion itself appears to modify the *l(3)hem*^{1} lymph gland phenotype slightly.)

When *ZCL2897/ZCL2897;l(3)hem*^{1}/+ and *ZCL2897/ZCL2897; l(3)hem*^{1}/*l(3)hem*^{1} glands were stained with anti L1 antibody, heterozygote control lymph glands contained no L1 positive cells(Figure 5E) whereas *ZCL2897; l(3)hem*^{1} mutant lymph gland had some L1 positive cells (Figure 5F). These observations support the conclusion that many cells of the *l(3)hem*^{1} anterior lobes express proteins normally expressed in the MZ. We conclude that (1) defects in proliferation of progenitors in the MZ limit the growth of mutant lobes; (2) proliferation of precursors is tightly-linked to their differentiation; and (3) wild type Neur function is essential for both of these processes.

l(3)hem^{1} mutant lymph glands exhibit defects in hemocyte differentiation

Loss of function Neur resulted in lamellocyte differentiation and reduced crystal cell numbers (Chapter 2) and we therefore expected that if *neur* and *l(3)hem*^{1} were allelic, these cell types would similarly be affected. We stained lymph glands with anti-ProPO antibody which labels crystal cells. Whereas, *l(3)hem*^{1}/+ heterozygous glands show crystal cells throughout the cortical zone of the lymph gland (Figure 6B), *l(3)hem*^{1} mutant lymph gland had few or no crystal cells (Figure 6C). This decrease could be a result of overall reduction in hemocyte population. Alternatively, *l(3)hem*^{1} affects specification or differentiation of crystal cell lineage.

Results from *neur* clonal analysis and loss of function (Chapter 2) suggest that *l(3)hem*^{1} mutant lymph glands should have ectopic lamellocytes, even in the absence of

wasp infection. We further examined differentiation of lamellocytes and plasmatocytes in *l(3)hem*^{1}. *msnf9-GFP* (*misshapen* enhancer that marks lamellocytes; Tokusumi et al., 2010) was crossed into the *l(3)hem*^{1} background and lymph glands were stained with anti-P1, L1, or ProPO antibodies (Figure 7). Heterozygote lymph glands contained many P1 positive cells but no *msnf9-GFP* labeled lamellocytes in the cortical zones (Figure 7B). *l(3)hem*^{1} lymph glands had several L1-positive lamellocytes and also contained most P1 positive cells (Figure 7C). These cells were abnormally large, many of which appear polyploid. We checked for cells with mixed identities by co labeling with L1 and ProPO and found many crystal cells in the cortical region of *l(3)hem*^{1}/+ lymph glands (Figure 7E). As expected *l(3)hem*^{1} lymph glands had several L1-positive lamellocytes, and few ProPO positive crystal cells. We also detected rare cells double positive cells (Figure 7F, arrows). These observations support the idea that *l(3)hem*^{1} mutation acts on progenitor cell population, keeping lamellocytes in an undifferentiated state while specifying crystal cells, consistent with Neur's function in this process.

Figure 1. Complementation analysis and neurogenic defects.

(A) Complementation table shows that when homozygous, *l(3)hem*^{2} is embryonic lethal. *l(3)hem*^{2} does not complement *neur* alleles, *neur*^{A101}, *neur*^{1} or *neur*^{11}. ND is not done.

(B) Table shows that homozygous *l(3)hem*^{1}, *l(3)hem*^{2}, *neur*^{A101}, or *Δl(3)hem*^{2} are embryonic or larval lethal and do not complement. More than 50% of *l(3)*hem^{1} mutants are embryonic lethal (EL); and 45% are larval lethal (LL), n = 73 animals. *l(3)hem*^{1} and *l(3)hem*^{2} only partially complement with 38% viability. *l(3)hem*^{1} and *neur*^{A101} complemented each other with 129% viablility. *Δl(3)hem*^{2} failed to complement *l(3)hem*^{1}, *l(3)hem*^{2} or *neur*^{A101}.

(C) Cuticle phenotypes of *l(3)hem*^{1}/+, or homozygous *l(3)hem*^{1}, *l(3)hem*^{2}, *neur*^{A101} embryos. Ventral denticle belts (seen in control, (A) *l(3)hem*^{1}/+) are not seen in (C) *neur*^{A101}or (D) *l(3)hem*^{2} embryos. (B) In some *l(3)hem*^{1} embryos, the neurogenic defects are not as severe (arrowhead, ventral denticle belts are formed), consistent with *l(3)hem*^{1} being a weaker allele. A= anterior, P=posterior, D = dorsal.

(D) (A) *l(3)hem*^{1}/+ lymph gland labeled with rhodamine phalloidin. (B) Cells of *l(3)hem*^{1}/*l(3)hem*^{2} lymph gland labeled with rhodamine phalloidin displays polyploidy associated with *l(3)hem*^{1}and indicates non complementation. (C) Magnification shows cells of *l(3)hem*^{1}/*l(3)hem*^{2} lymph gland are large and polyploid compared to controls. Hoechst stains DNA (blue).

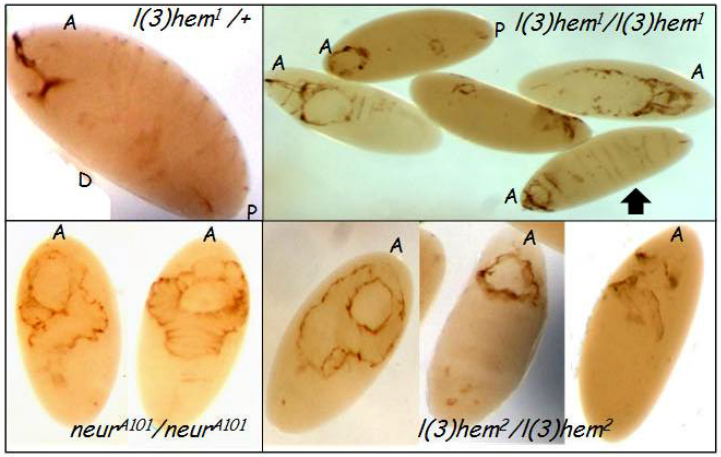
A

	l(3)hem²	*neur¹*	*neur¹¹*	*neur^A101*
l(3)hem²	0			
neur¹	0 (108)	0		
neur¹¹	0 (93)	ND	0	
neur^A101	0 (77)	ND	ND	0

B

Genotype	*l(3)hem¹*	*l(3)hem²*	*neur^A101*	*Δ(3)hem²*
l(3)hem¹	0 (55% EL Rest LL; n = 73)	38 (all LL)	129	0
l(3)hem²		0 (all EL)	0 (all EL)	0
neur^A101			0 (all EL)	0
Δl(3)hem²				0

C



D

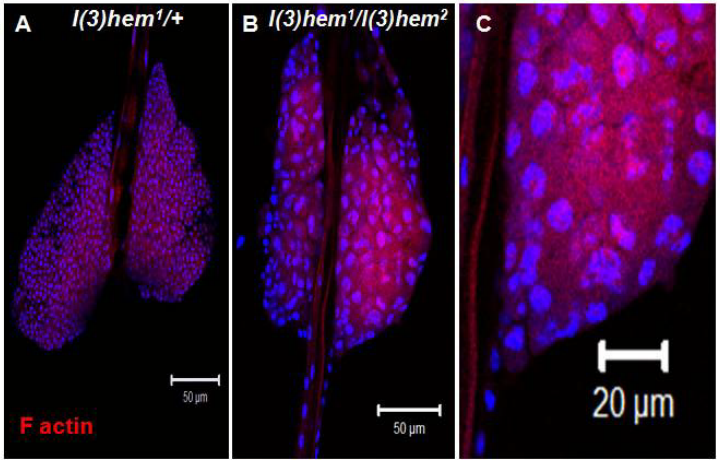


Figure 1.

Figure 2. *l(3)hem*^{1} affects the lymph gland and circulating hemocyte types.

First row: (A-G) Circulating hemocytes from *l(3)hem*^{1} hemolymph. Samples are Hoechst-stained.

(A) Control plasmatocyte, hemocyte type A.

(B-D) Mutant cell types B-D. (B-G) Notice the presence of single or multinucleate, polyploid cells.

Second row: (A-I) General lymph gland morphology of *l(3)hem*^{1} compared to heterozygotes.

(A) Second instar *l(3)hem*^{1}/+ lymph gland.

(B-D) Third instar *l(3)hem*^{1}/+ lymph gland. (E) Second instar *l(3)hem*^{1} mutant lymph gland.

(F-I)Third instar *l(3)hem*^{1} mutant lymph glands. Hemocyte numbers in the anterior lobes of mutant lymph gland is reduced (arrowheads).

Third Row: (O-R) Sessile compartments of heterozygote (O, P), and *l(3)hem*^{1} mutant (Q, R). Sessile population is visualized with the *Hml-GAL4; UAS-GFP* reporter (green).

Scale bars A-D = 400 μm, E-G = 1 mm. Second row scale bars A-I = 100 μm; P, R = 400 μm; O, Q = 40 μm.

Figure 2.

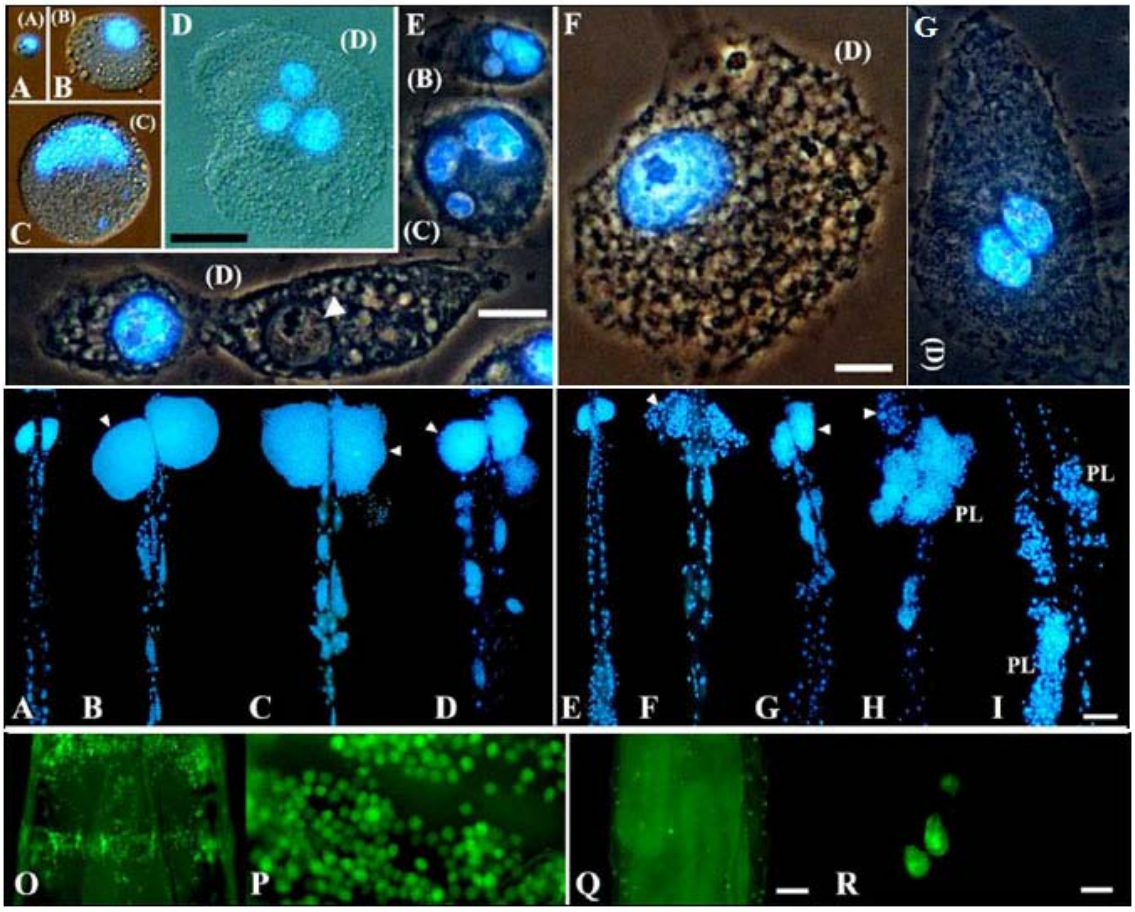


Figure 3. Expression of Neuralized protein in *l(3)hem^{1}* mutant lymph gland.

Lymph glands stained with anti-Neur (red) and anti-integrin β (green) antibodies.

(A) Negative control where primary antibody was not applied.

(B) *l(3)hem^{1}*/+ heterozygous control showed Neur protein is expressed throughout the anterior lobes.

(C) Magnification of a region of the *l(3)hem^{1}*/+ lymph gland shows Neur is cytoplasmic.

(D-F) *l(3)hem^{1}* mutant lymph gland stained with anti-Neur and anti-integrin β antibodies. Neur is expressed highly throughout the cells of *l(3)hem^{1}* mutant lymph gland. Anti-integrin β labels lamellocytes in anterior lobes. Neur (red) appears to accumulate in vesicles in integrin β positive cells.

(G-I) Magnification of a region of *l(3)hem^{1}* mutant lymph gland stained with anti-Neur and anti-integrin β. Neur protein distribution is affected in integrin β positive lamellocytes (white arrows). Neur protein is perinuclear in some cells. Hoechst stains DNA (blue).

Figure 3.

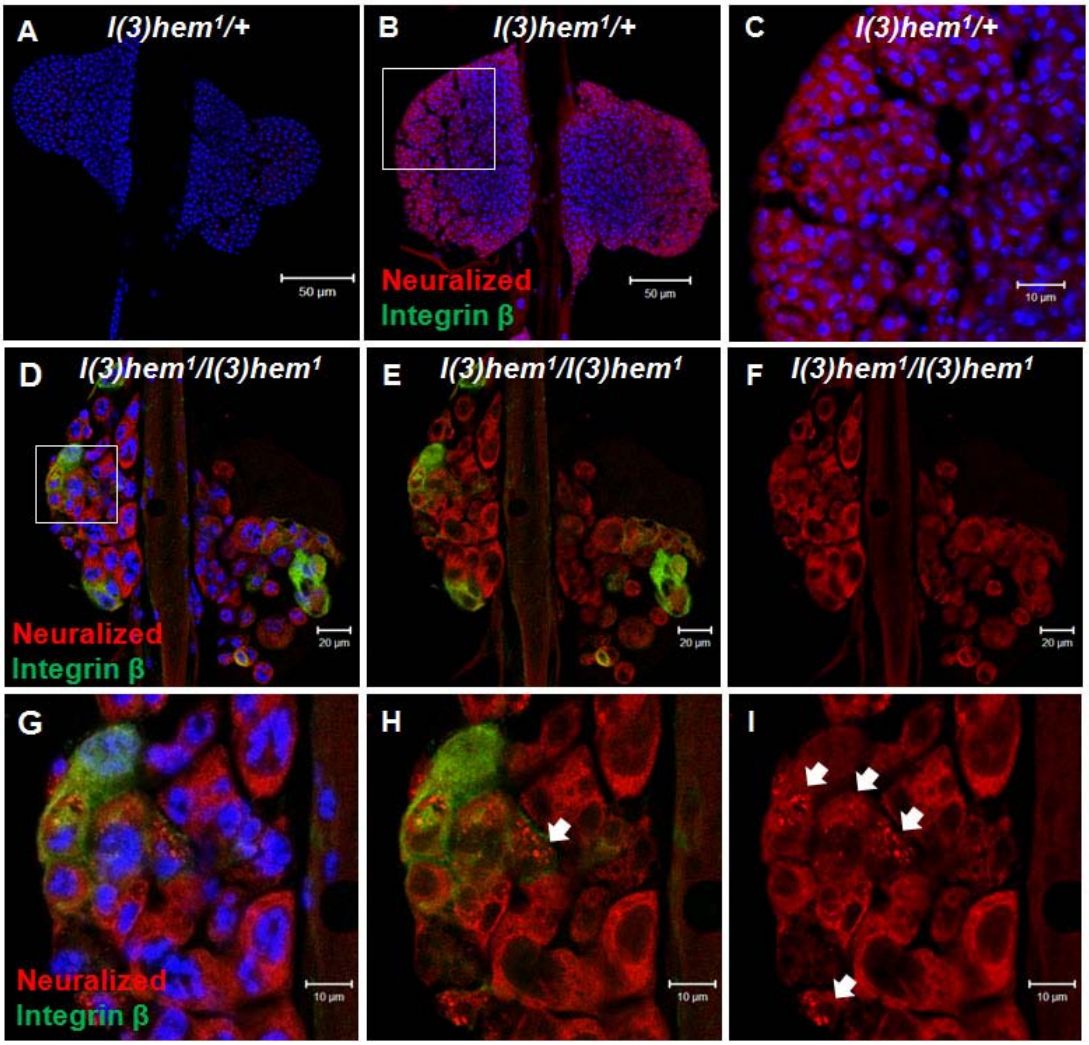

A
l(3)hem1/+
50 µm
B
l(3)hem1/+
Neuralized
Integrin β
50 µm
C
l(3)hem1/+
10 µm
D
l(3)hem1/l(3)hem1
Neuralized
Integrin β
20 µm
E
l(3)hem1/l(3)hem1
20 µm
F
l(3)hem1/l(3)hem1
20 µm
G
Neuralized
Integrin β
10 µm
H
10 µm
I
10 µm

Figure 4. Niche cells are specified in *l(3)hem^{1}* lymph gland.

Anti-Antennapedia (red) antibody labels cells of the niche. Hoechst stains DNA (blue).

(A) Negative control in which primary antibody application was omitted.

(B) *l(3)hem^{1}*/+ heterozygous control with Antp expression in cells of the niche.

(C) *l(3)hem^{1}* mutant lobe shows reduction in Antp-positive population cells. Some cardioblasts in the dorsal vessel are also Antp-positive.

All images taken at 40X magnification.

Figure 4.

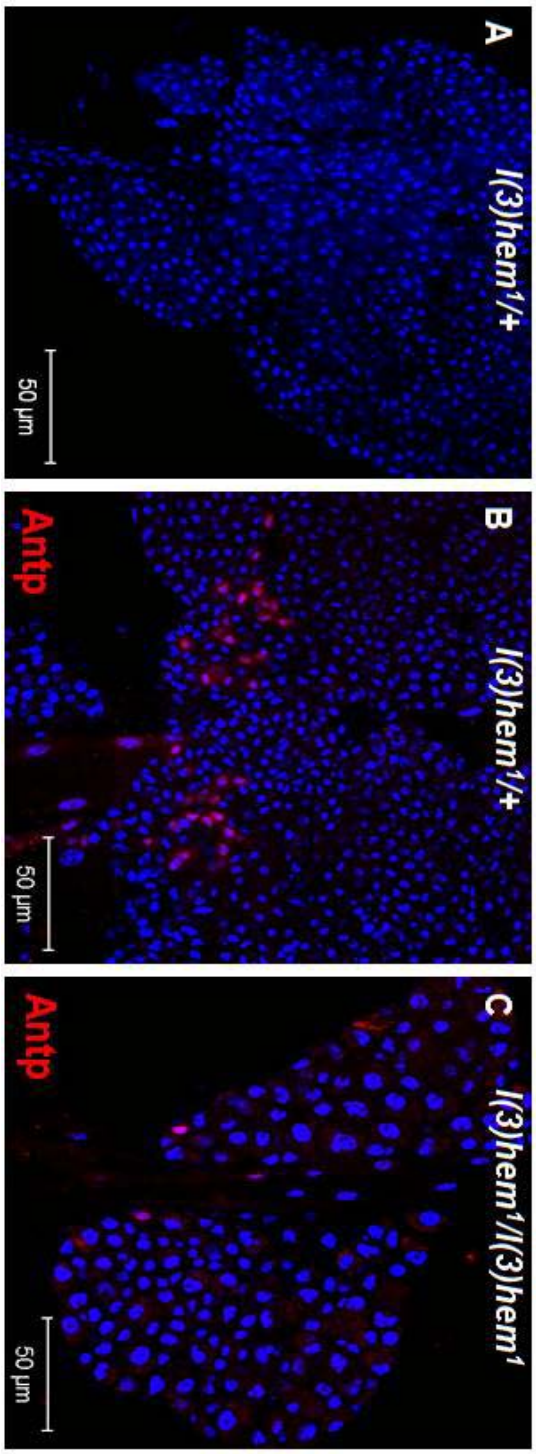


Figure 5. Medullary zone function is changed as measured by *ZCL2897* expression in the *l(3)hem^{1}* background.

Anterior lobe hemocytes express MZ marker, *ZCL2897* (GFP) and are labeled with anti-P1 (red, plasmatocytes) or anti- L1 (red, lamellocytes). Hoechst stains DNA (blue).

(A) Negative control where no primary antibody was added to the sample.

(B) *ZCL2897/ZCL2897*; *l(3)hem^{1}*/+ heterozygous control shows normal GFP expression in the MZ and P1 positive plasmatocytes in the cortical zone.

(C) *ZCL2897/ZCL2897; l(3)hem^{1}/ l(3)hem^{1}* mutant gland shows a significant expansion of MZ, most cells are GFP-positive. P1 positive plasmatocytes are distributed throughout the lymph gland. Few P1 positive cells also express *ZCL2897* (GFP).

(D) Negative control where no primary antibody was added to the sample.

(E) *ZCL2897/ZCL2897*; *l(3)hem^{1}*/+ heterozygous control shows *ZCL2897* expression but do not express L1.

(F) *ZCL2897/ZCL2897; l(3)hem^{1}/ l(3)hem^{1}* mutant gland shows a significant expansion of MZ, most cells are strongly positive for GFP. Few L1 positive lamellocytes are seen in the lymph gland.

All images taken at 40X magnification.

Figure 5.

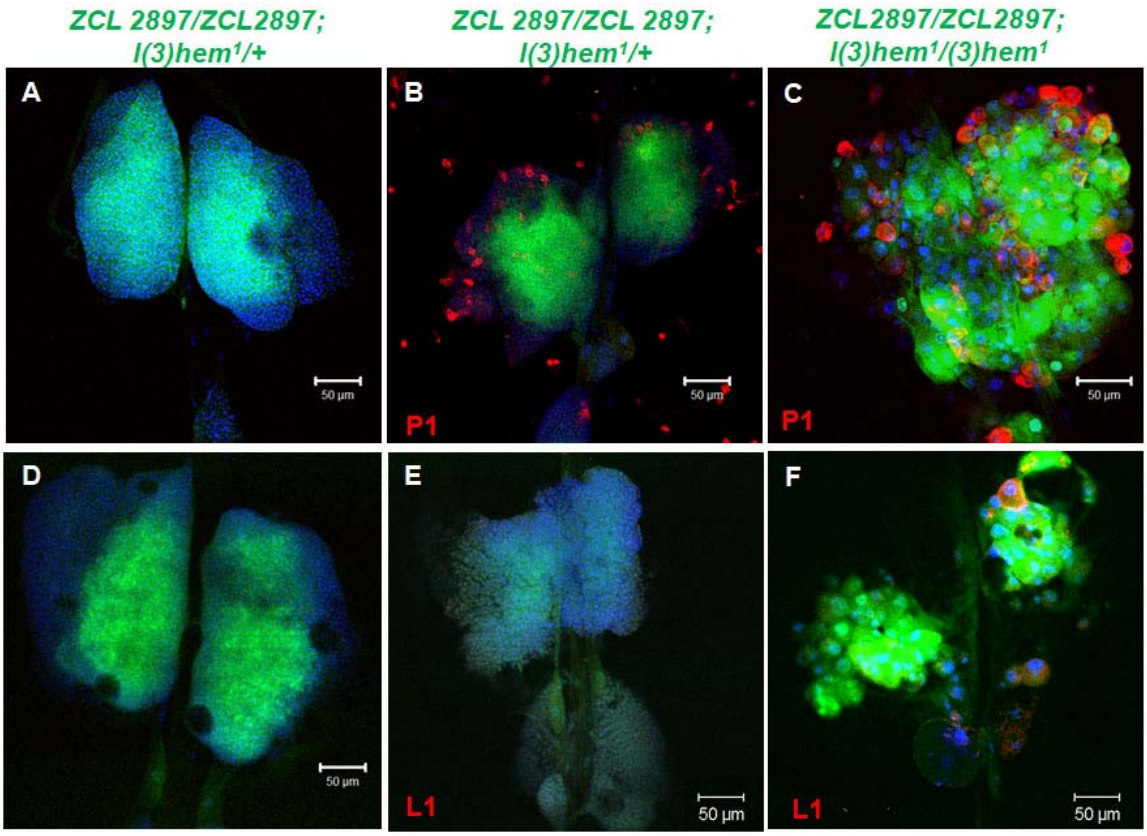

ZCL 2897/ZCL2897;
l(3)hem¹/+
ZCL 2897/ZCL 2897;
l(3)hem¹/+
ZCL2897/ZCL2897;
l(3)hem¹/(3)hem¹
A
B
C
D
E
F
50 µm
P1
L1

Figure 6. Reduced crystal cell numbers in *l(3)hem*^{1} mutant lymph glands.

Hemocytes of lymph gland stained with anti-prophenol oxidase, ProPO antibody (red). Hoechst labels DNA (blue).

(A) Negative control in which primary antibody application was omitted.

(B) *l(3)hem*^{1}/+ heterozygous control shows crystal cells dispersed in the cortical zone of the lymph gland.

(C) *l(3)hem*^{1} mutant lymph gland shows few crystal cells in the lymph gland.

All images taken at 40X magnification.

Figure 6.

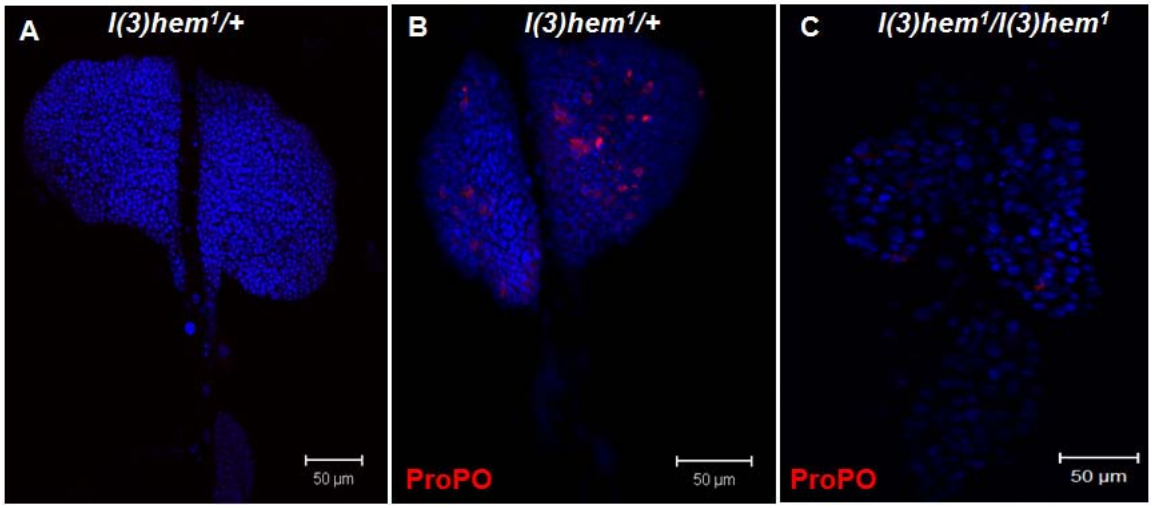


Figure 7. Mature hemocytes are affected in the *l(3)hem^{1}* mutant background.

(A-C) Anterior lobes of lymph glands are labeled with *msnf9-GFP* (green) and anti-P1 (red, plasmatocytes) antibody.

(D-F) Anterior lobes of lymph glands are stained with anti-L1 (green, lamellocytes) and anti- ProPO (red, crystal cells).

(A, D) Negative controls where no primary antibody was added to the samples.

(B) *msnf9-GFP/msnf9-GFP; l(3)hem^{1}*/+ gland shows P1 (red) positive plasmatocytes are distributed throughout the cortical zone gland.

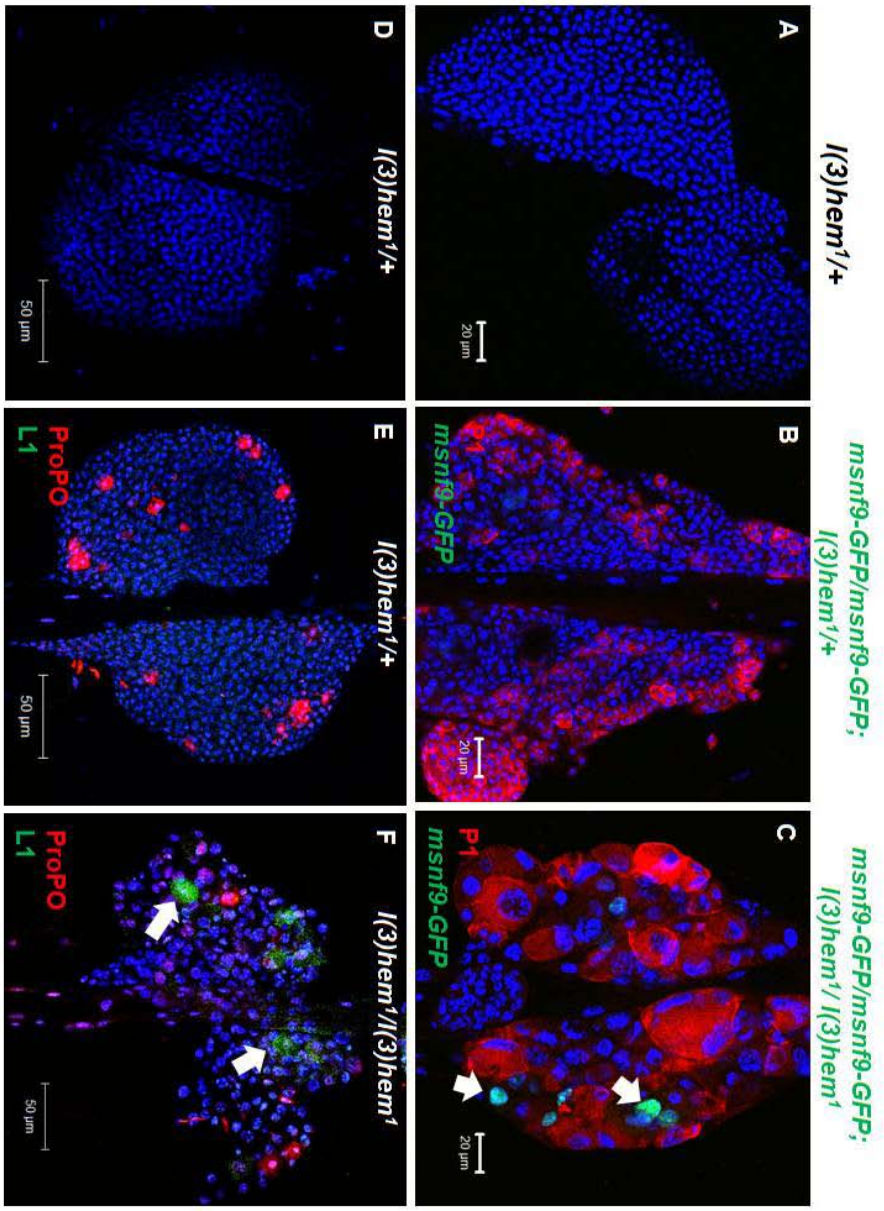
(C) *msnf9-GFP/msnf9-GFP;l(3)hem^{1}/l(3)hem^{1}* mutant gland shows P1 (red) positive plasmatocytes are abnormal, larger in size with polyploid nuclei. *msnf9-GFP* labels several lamellocytes that have polyploid nuclei (large white arrows).

(E) *l(3)hem^{1}*/+ heterozygous control shows ProPO-positive (red) crystal cells throughout the cortical zone of the lymph gland.

(F) *l(3)hem^{1}* mutant lymph gland shows rare double positive cells with ectopic L1 expression and ProPO expression (small white arrows). Hoechst stains DNA (blue).

All images taken at 40X magnification.

Figure 7.


A
I(3)hem1/+
B
msnf9-GFP/msnf9-GFP;
I(3)hem1/+
C
msnf9-GFP/msnf9-GFP;
I(3)hem1/ I(3)hem1
P1
msnf9-GFP
20 µm
D
I(3)hem1/+
E
I(3)hem1/+
F
I(3)hem1/I(3)hem1
ProPO
L1
50 µm

Discussion

Major findings

Lamellocytes are specialized cells dedicated to encapsulate large foreign entities, mainly eggs of parasitic wasps. Lamellocyte differentiation is prompted by wasp oviposition, mostly in the cortical zone of the anterior lobes of the lymph gland (Sorrentino, 2002; Paddibhatla et al., 2010; Chapter 1). The goal of this work was to examine how Notch signaling influences lamellocyte differentiation. Based on previous work (Duvic et al., 2002), we hypothesized that Notch components promote commitment to the lamellocytic fate. RNAi experiments revealed that reduction of Serrate, Neuralized, and Notch, in fact, promotes premature differentiation of lamellocytes in the cortex or in the periphery of the gland. Twin clone analysis of *Notch* and *neuralized* showed that this effect is non cell-autonomous.

Second, Neuralized carries out an essential mitotic function in the stem-like progenitor population. This is evident from cell division defects observed upon $Neur^{RNAi}$ in the medullary zone and overexpression of the Neur C isoform. Third, (1) $l(3)hem^{2}$ mutation maps within the *neur* locus and is likely a new allele. (2) Genetic and molecular analysis of $l(3)hem^{1}$ was less conclusive and additional experiements are ongoing to identify the precise lesion in this strain. (3) Analysis of $l(3)hem^{1}$ hemocytes supports both functions of the gene identified with $Neur^{RNAi}$.

$l(3)hem^{1}$ is a protein-coding hypomorph or neomorph of *neur*

Allelism for a gene is assigned by (1) genetic non-complementation, (2) identification of unique molecular defect(s) within the gene in question, or (3) rescue of

the mutant phenotype. In genetic and molecular tests, we showed clearly that *l(3)hem*^{2} is a new loss-of-function allele of *neur* by two of the three criteria: *l(3)hem*^{2} fail to complement all lethal alleles of *neur* tested (Chapter 3 and data not shown) and the P element insertion maps to non-coding exon 1 of the gene. Significantly, all homozygous embryos die from strong neurogenic defects. In contrast, only a fraction of *l(3)hem*^{1}/*l(3)hem*^{1} animals die as embryos, developing neurogenic defects. The surviving animals proceed into larval stages and die as third instar larvae or early pupae. Thus, the *l(3)hem*^{1} mutation is weaker in its effects than *l(3)hem*^{2}.

Embryonic lethality of *l(3)hem*^{2} precluded its use for the analysis of larval hematopoiesis as such, but the mutation itself (and resulting excisions) were useful in the analysis of the *l(3)hem*^{1} mutation. Hematopoietic defects of *l(3)hem*^{1}/*l(3)hem*^{2} larvae were similar to those in *l(3)hem*^{1}/*l(3)hem*^{1} animals. Moreover, *l(3)hem*^{1}/Δ*l(3)hem*^{2} did not result in complementation.

Molecular analysis of the *neur* locus in *l(3)hem*^{1}/*l(3)hem*^{1} animals spanning the coding region has not revealed defects within the coding region of the locus itself (our unpublished results), suggesting that the mutations in both *l(3)hem*^{1} and Δ*l(3)hem*^{2} backgrounds affect the DNA adjacent to the sequenced locus, possibly affecting its transcription. This conclusion is further supported by the rescue of *l(3)hem*^{1}/*l(3)hem*^{1} animals bearing a duplication of this region on the X chromosome. Clearly, further molecular analysis of the region is warranted to pinpoint the exact cause of the lesion in the *l(3)hem*^{1} strain.

In PCR experiments, we were able to amplify transcripts corresponding to exons for Neur C and Neur A isoforms (Small and Govind, unpublished results). We were

therefore not surprised to find that polyclonal sera against the Neuralized protein detected significant levels of the protein in *l(3)hem*^{1}/*l(3)hem*^{1} hemocytes. Intriguingly, the subcellular localization of the protein in mutant cells is clearly different from wild type cells. In wild type cells, the protein is cytoplasmic and associated with the plasma membrane, whereas in *l(3)hem*^{1}/*l(3)hem*^{1} cells, protein signal is stronger, and in addition to cytoplasmic and membrane association, the signal is patchy, possibly localizing the protein to vesicles. Although this prediction needs to be directly tested in future experiments, the staining results provide an explanation for the hypomorphic nature of the *l(3)hem*^{1} mutation. If this mutation modifies transcription of the locus such that the balance of the isoforms is affected, then the effects of the mutation should mimic defects in Neur^{RNAi} and overexpression backgrounds. Our results from these experiments described in Chapter 2 support such a possibility.

Attempts to rescue the *l(3)hem*^{1} mutation were not successful, possibly because providing NeurA protein via the UAS-GAL4 system (*Antp-GAL4, Dome-GAL4, arm-GAL4, 76B-GAL4, da-GAL4*) in various cell populations did not restore the correct Neuralized protein complement in the cell.

A role for *neur* in the cell cycle of hematopoietic progenitors

Neuralized is one of two E3 ubiquitin ligases that regulate Delta and Serrate signaling in *Drosophila* (Pitsouli et al., 2005 Le Borgne et al., 2005). Neuralized proteins are expressed in all hemocytes, including the Dome/ZCL2897-positive progenitors. The membrane association of Neuralized (Chapter 2) is consistent with similar observations in cells of the imaginal discs (Yeh et al., 2000). These results suggest that in hemocytes,

the proteins are active and engaged in signaling (Yeh et al., 2000). We have recently discovered that this Dome/*ZCL2897*-positive cell population is heterogenous, in that some cells express high levels of one or both markers (Kalamarz et al, submitted). Consistent with this idea of a mixed cell population in the medullary zone is the finding that some progenitors are no longer multipotent and achieve fate restriction in late embryonic stages (Krzemien et al., 2010). This step toward specialization of progenitors requires Notch function (Krzemien et al., 2010), although the mechanism by which Notch carries out this step is not understood.

RNAi knockdown of *neur* in this population (*Dome>neur*^{RNAi}), or the misexpression of NeurA or NeurC, results in appearance of rare binucleate cells (Chapter 2). Furthermore, there is an abundance of abnormal hemocytes (large, polyploid, multinucleate cells with high levels of ZCL2897) in *l(3)hem*^{1} glands (Chapter 3). Based on the data from these two experimental approaches, we propose that Neur plays an important role in the division of a subpopulation of progenitors; aberrant division due to loss-of-Neur function leads to an accumulation of larger, multinucleate progenitors some of which then misdifferentiate. According to this model, progressive rounds of aberrant divisions appear to result in an overall reduction in misdifferentiated hemocyte counts in *l(3)hem*^{1} glands (Figure 1).

The mechanism by which Neur controls the cell cycle is not known. Neuralized is a key player in generating asymmetry in mitosis (Betschinger and Knoblich, 2004; Bardin et al., 2004). Because of its unequal localization in the sensory organ precursor (pI), Neuralized biases Notch activation. Upon cytokinesis of pI, the daughter pIIb cell inherits Neuralized, where it activates Delta and inhibits Notch activation. Delta activates

Notch in its sister pIIa cell (Le Borgne and Schweisguth, 2003). It is possible that similar to SOP precursor, Neur plays a role in controlling asymmetric cell division and differentiation of hematopoietic progenitors. This model predicts that localization of Neur in dividing cells is asymmetric and loss of its function should affect the determination of one or both of the daughter cells. This model is being tested in ongoing experiments and is beyond the scope of the thesis.

Serrate, Notch and Neur: Non cell-autonomy in maintaining pro-lamellocytes in their progenitor state

A majority of lamellocytes are induced in the cortical zone after wasp infection. Lamellocytes are almost never found in the medullary zone or in the posterior lobes of the lymph gland. This specificity in the pattern of lamellocyte differentiation suggests pro-lamellocytes are localized within the cortical zone of the anterior lobes of the organ. Previous work has shown that Notch signaling is not essential for formation of the niche (Lebestky et al., 2003), even though Serrate, Notch and Neuralized are all expressed in the niche (Lebestky et al., 2003 and this study). Furthermore, Notch and Neuralized expression is ubiquitous (Chapters 1 and 2; Figure 2) and Su(H) levels are low in the niche but high in the medullary and cortical zones (Tokusumi et al., 2010). RNAi knockdown of Notch pathway components (Chapters 1, 2) induces ectopic lamellocyte differentiation. Effects of RNAi knockdown in the niche yielded the strongest effects. Ectopic lamellocytes were present in the vicinity of the niche as well as in regions of the lobe distant from the niche. Loss-of-function mitotic clones of *Notch* and *neur* confirmed non cell-autonomous requirement for each.

Combined, these results suggest that (1) local short range signaling between niche cells is sufficient to generate a signal for maintaining pro-lamellocytes in the medulla or in the cortex (orange arrows; Figure 2). (2) A mild negative influence of the Notch signal in the cortex (Hml-positive cells) shelters pro-lamellocytes in the cortex (pink arrows; Figure 2). Non cell-autonomous effects of Ser/Neur/Notch on the multi- or bi-potent progenitors in the medulla lead to alterations in crystal cell counts in the cortex. The nature of the negative signal for progenitor maintenance in the medulla or cortex remains to be determined.

Inverse correlation between crystal cells and lamellocytes

Based on the combination of our results and observations from the literature, it appears that N signaling is essential for the development of both lamellocytes and crystal cells although the effects on the two cells types are opposite: while Notch signaling promotes crystal cell development, it keeps lamellocytes from differentiating prematurely (Figure 3). This observation raises the question whether the pathway acts on a bi-potential cell that can take on one or the other identity. In this scenario, the *same* molecular mechanism can account for lamellocytes differentiation and crystal cell loss. Recently Krzemien et al., 2010 have proposed such a model in the context of wasp infection. They show that wasp infection-induced lamellocyte differentiation accompanies proliferation and loss of crystal cells in the anterior lobes. In this respect, the combined effects of Notch inactivation in an immune competent lymph gland mimics wasp infection.

Figure 4, summarizes the requirements for Notch at multiple steps in hematopoiesis. First, Notch is required in the embryo for the production of fate restricted

progenitors in the medullary zone. At restrictive temperatures during late embryonic stages (stage 16), N^{ts1} embryos are unable to specify crystal cells (Krzemien et al., 2010). This interpretation is consistent with previous reports of Duvic et al. (2002), who showed that the restriction of Notch signaling not only reduced crystal cell development but abolished lamellocyte differentiation after wasp infection. Furthermore, removing N early (using Srp-GAL4) from all precursor cells in the lymph gland abolished sessile crystal cells completely and blocked wasp-induced lamellocyte differentiation (our unpublished results), supporting an early requirement for Notch for either proliferation of progenitors or for restriction of their fates.

Second, our RNAi and clonal analysis support a second non-autonomous effect of Notch signaling, likely in the niche that keeps pro-lamellocytes of the medulla and the cortex from differentiating.

Third, Notch signaling is required cell-autonomously in the commitment of the crystal cell fate as has been proposed by Lebestkey et al. (2003).

Thus, Notch signaling is expected to be active extensively in the body of the gland. Accordingly, we have detected expression of two Notch target genes in most cells of the anterior lobe. Recent reports (Tokusumi et al., 2010) show that Su(H) protein is also expressed extensively in the medulla and cortex.

We also addressed the question of what happens when Notch signaling is constitutively active. In *Antp*> N^{IC} glands, we observed high numbers of crystal cells, as expected (Chapter 1) without any lamellocyte differentiation. When *Antp*>N^{IC} animals were infected with wasps, we were surprised to find lamellocyte differentiation (our unpublished results). This result is consistent with the interpretation that wasp infection

inactivates Notch signaling. Furthermore, progenitor specification (step 1, Figure 4) and crystal cell commitment (step 3; Figure 4) are distinct from the maintenance of pro-lamellocyte step (step 2; Figure 4).

The exact lineage relationships between progenitors and mature cells are not completely understood. We have not investigated how Notch signaling affects plasmatocytes differentiation and this aspect clearly needs to be explored to gain a more complete understanding of the role of Notch signaling in lymph gland hematopoiesis.

Concluding remarks

Our studies, for the first time show a novel role for *neur* in hematopoietic cell proliferation and differentiation, and therefore a potentially new role for the Notch pathway in *Drosophila* larval hematopoiesis. The inhibitory influence of Notch signaling on progenitors is also novel and it will be interesting to examine how other pathways such as the Toll pathway and JAK-STAT pathway, which are activated upon infestation (Schlenke et al., 2007) converge to alter hematopoiesis in infected animals. Understanding the coordination of signaling mechanisms in division and differentiation of progenitors is a major aspect in animal development.

In *Drosophila*, N signaling regulates intestinal stem cell fate and promotes the survival of multipotent stem cells. Modulation of N signaling specifies two distinct cell lineages in the intestine (Ohlstein and Spradling, 2007). Ohlstein at al. (2007) showed that in the absence of a detectable stem cell niche, the fate of stem cell progeny can be determined through Notch signaling by ligands produced by the parental stem cells. Our studies on *neuralized* in the context of Notch signaling in the hematopoietic system will reveal how components of the same pathway control stem cell renewal versus

differentiation. Having this knowledge in two different biological contexts (lymph gland vs gut) and at different life cycle stages (larva vs adult), we will shed light on the strategies involved in stem cell renewal and differentiation. The *Drosophila* hematopoietic system provides a novel paradigm to examine how this integration and coordination is achieved. Given the extensive conservation of Notch and Neur functions in fly and mammals, our studies will shed light on how Notch regulates cell fates and behavior in human hematopoietic development and disorders.

Figure 1. Model for Neuralized function based on genetic analysis of *neur*^{RNAi}, *neur*^{A101} and *l(3)hem*^{1}.

(A) Stem and progenitor cells reside in the Dome- and ZCL-positive cells of the medullary zone. These cells divide slowly and continue to divide and mature into plasmatocytes, lamellocytes and crystal cells in the CZ. The developmental relationship of these cell types is not completely known, although recent studies show that lamellocytes can arise from plasmatocytes.

(B) Loss of Notch signaling in *l(3)hem*^{1} leads to disruption in mitosis resulting in polyploid nuclei and premature lamellocyte differentiation in the MZ. The crystal cell lineage is inhibited, the morphology of some plasmatocytes is altered and the cortical zone is reduced.

Figure 1.

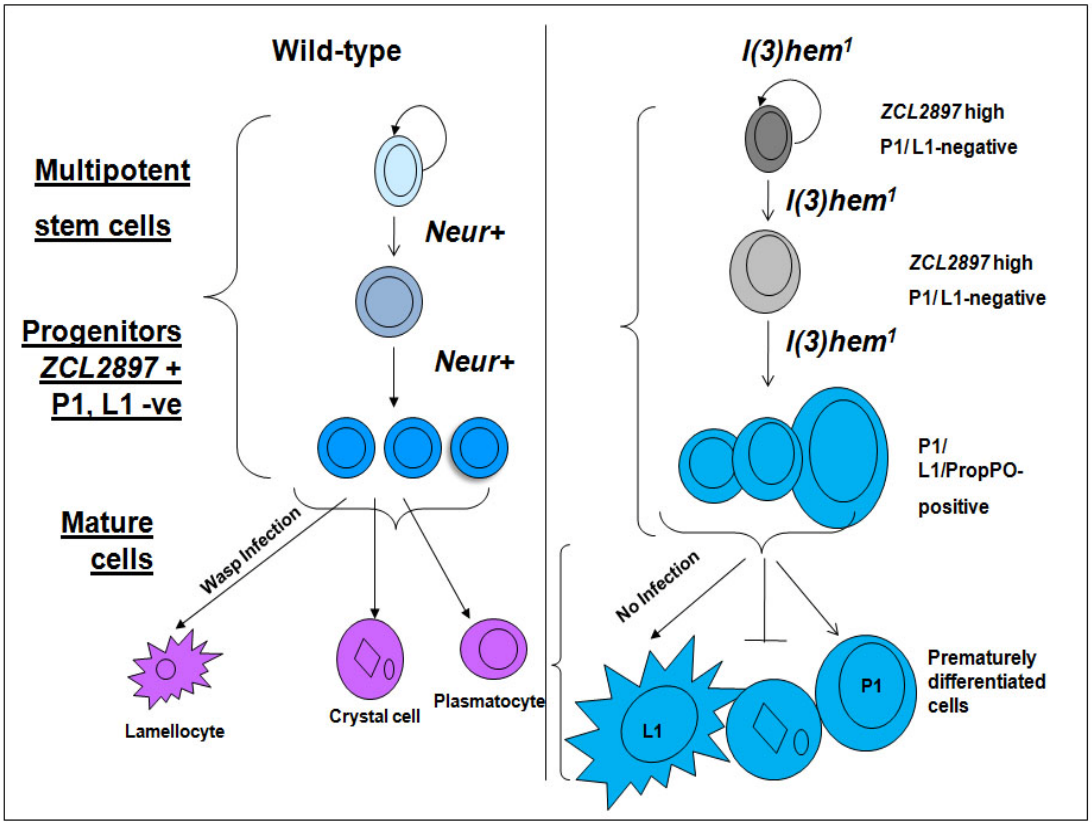

Wild-type
l(3)hem1
Multipotent stem cells
Progenitors ZCL2897 + P1, L1 -ve
Mature cells
Neur+
Neur+
Wasp Infection
Lamellocyte
Crystal cell
Plasmatocyte
ZCL2897 high P1/L1-negative
l(3)hem1
ZCL2897 high P1/L1-negative
l(3)hem1
P1/ L1/PropPO-positive
No Infection
L1
P1
Prematurely differentiated cells

Figure 2. Model for non-cell autonomous effects of Notch signaling in preventing lamellocyte differentiation.

Serrate, Neuralized and Notch are expressed in cells of the multicellular niche, whereas only Notch and Neuralized are expressed in all the remaining cells of the gland. Knockdown of any of these components via RNAi in the niche yields maximal lamellocyte differentiation in the cortical zone. According to this model, local signaling between (or among) cells is sufficient to maintain lamellocyte progenitors in their undifferentiated state. Since Hml-positive cells of the cortical zone are able to keep lamellocyte progenitors from differentiating, it is likely that this inhibitory influence of Notch signaling is also exerted from cells outside of the niche (pink arrows; left lobe).

Figure 2.

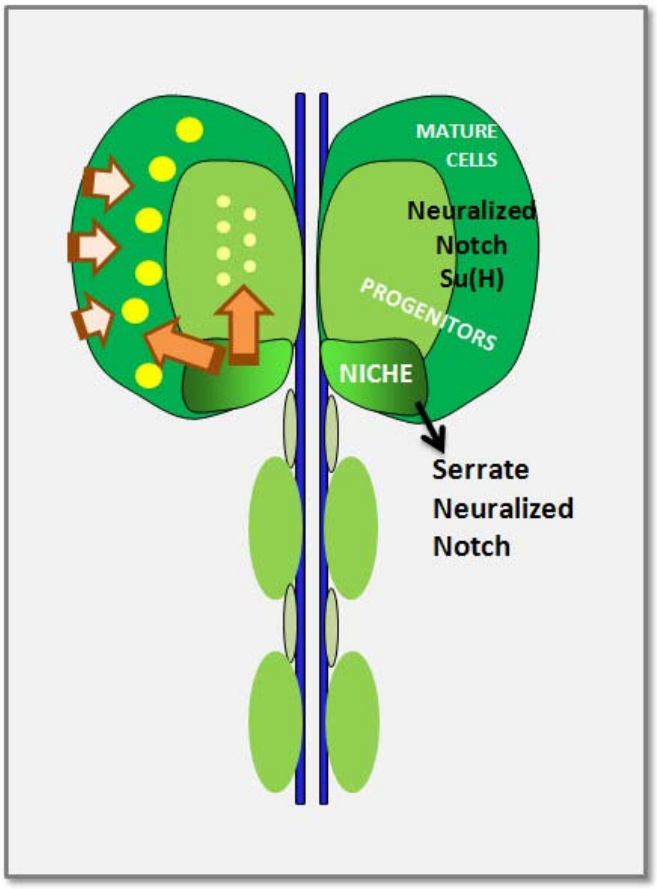

MATURE
CELLS
Neuralized
Notch
Su(H)
PROGENITORS
NICHE
Serrate
Neuralized
Notch

Figure 3. Reciprocal relationship between lamellocytes and crystal cells by Notch signaling.

Low levels of Notch induce lamellocyte differentiation in a non cell-autonomous manner, although Notch signaling is cell autonomously required in commitment of the crystal cell fate in the pro-crystal cell itself (Lebestky et al. 2003). Pro-crystal cells and mature crystal cells express high levels of Lozenge (Lz), a transcription factor essential for crystal cell development (Rizki et al., 1959, 1984, Lebestky et al., 2003). A model for how the inverse correlation of the commitment of the two cell types is realized is shown in Figure 4.

Figure 3.

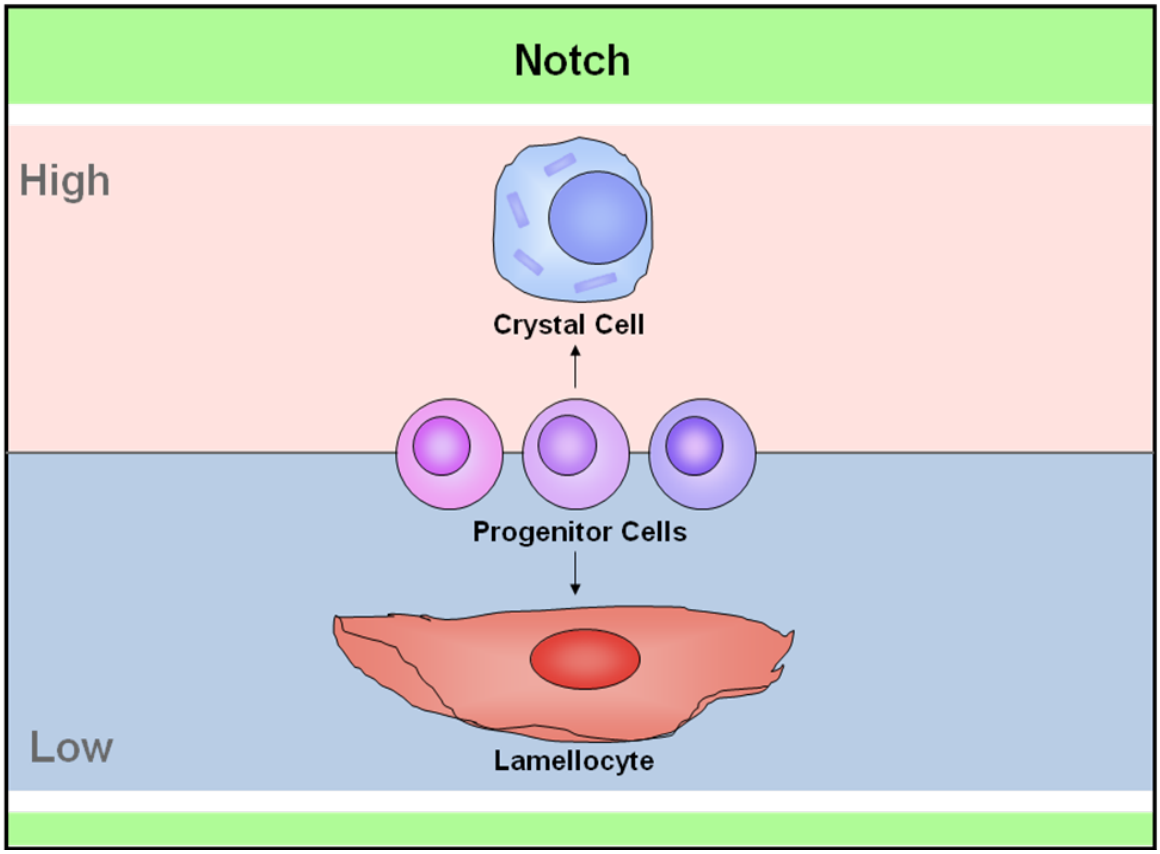
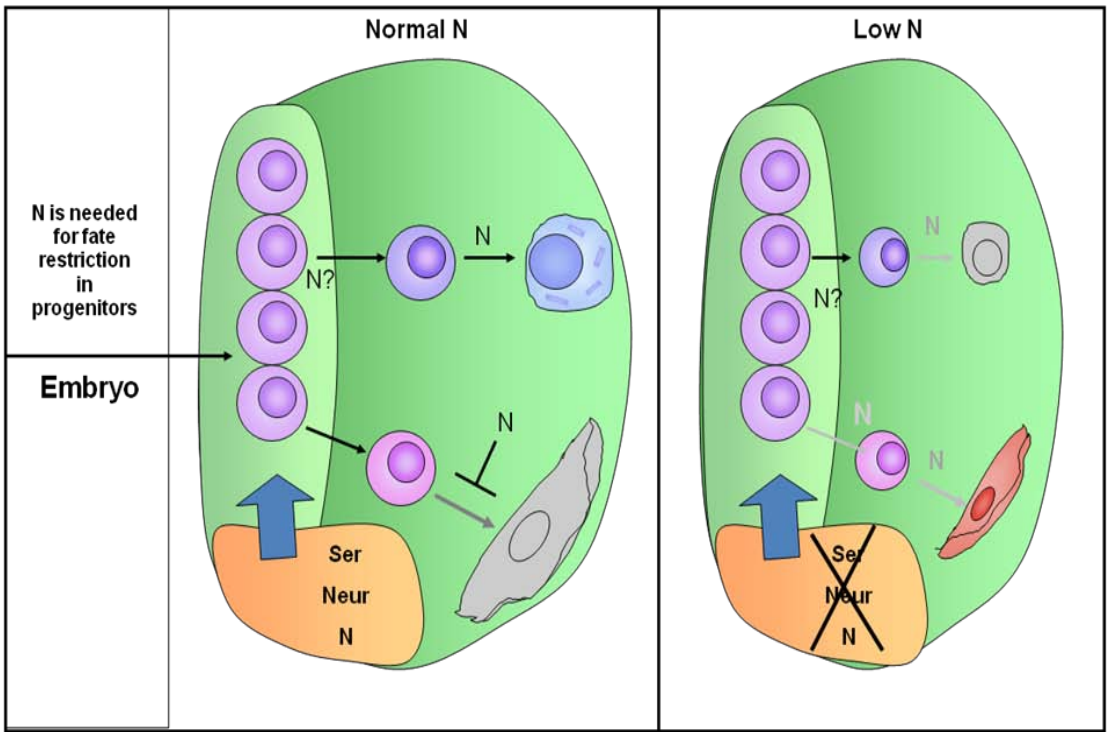


Figure 4. Differences in Notch signaling influences cell fate.

Normal levels of Notch are essential for the normal hematopoieitic complment. Reduction in signaling prompts lamellocyte differentiation and inhibits crystal cell specification. The involvement of the niche is implicated from RNAi studies.

Figure 4.


Normal N
Low N
N is needed for fate restriction in progenitors
Embryo
N?
N
N
Ser
Neur
N
N?
N
N
N
Ser
Neur
N

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