Molecules and Cells

Minireview

The Role of Lozenge in *Drosophila* Hematopoiesis

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***Drosophila* hematopoiesis is comparable to mammalian differentiation of myeloid lineages, and therefore, has been a useful model organism in illustrating the molecular and genetic basis for hematopoiesis. Multiple novel regulators and signals have been uncovered using the tools of *Drosophila* genetics. A Runt domain protein, lozenge, is one of the first players recognized and closely studied in the hematopoietic lineage specification. Here, we explore the role of lozenge in determination of prohemocytes into a special class of hemocyte, namely the crystal cell, and discuss molecules and signals controlling the lozenge function and its implication in immunity and stress response. Given the highly conserved nature of Runt domain in both invertebrates and vertebrates, studies in *Drosophila* will enlighten our perspectives on Runx-mediated development and pathologies.**

Keywords: crystal cells, *Drosophila melanogaster*, hematopoiesis, lozenge, lymph gland, melanization, prophenoloxidase, RUNX

INTRODUCTION

The fruit fly is a holometabolous insect which undergoes four distinct phases in its development with each stage emphatically different in both anatomy and physiology (Fig. 1A) (Snodgrass, 1954). In brief, the adult female produces fertilized eggs after copulation, which then undergoes stages of internal rearrangements to form an embryo. Upon hatching, a larva goes through two more molts while consistently feeding before turning into a pupa. Active feeding at this stage ceases, allowing internal and external structures to be generated or reorganized within the pupal case (Ashburner and Novitski, 1976). A winged fly emerges after four to five days from the point of pupa formation, but the whole cycle takes about ten days under normal conditions. The emergent fly can fly and feed after a couple of hours and generally lives up to fifty days, within which the cycle is repeated several times (Linford et al., 2013; Robertson, 1936; Stocker and Gallant, 2008).

HEMATOPOIESIS IN *DROSOPHILA*

Hematopoiesis in the fruit fly, though very multifaceted, is mainly classified into two waves: primitive and definitive hematopoiesis (Evans et al., 2003). In the first wave or primitive hematopoiesis, hemocytes are derived from the embryonic head mesoderm from which point they migrate to designated areas and facilitate organogenesis and immune responses (Holz et al., 2003; Moreira et al., 2010; Olofsson and Page, 2005; Tepass et al., 1994; Wood et al., 2006). Upon hatching, hemocytes are released either into circulation or to specialized sites including the hematopoietic pockets. During larval stages, these hemocytes perform diverse functions including phagocytosis of debris, immune responses, and metabolic regulation (Agaisse et al., 2003; Elrod-Erickson et al., 2000; Lanot et al., 2001; Lebestky et al., 2000; Makhijani et al., 2011; Márkus et al., 2009; Tepass et al., 1994).

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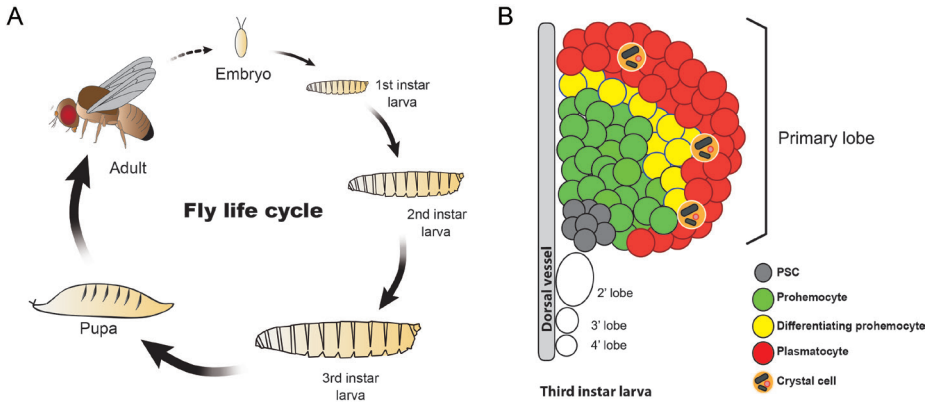

Fig. 1. Life cycle of *Drosophila* and constitution of the lymph gland. (A) The fly undergoes four distinct phases of change that is commenced by the embryo which is formed after fertilization. The hatched embryo produces the first instar larva, which molts into second and then third instar larva, eventually forming a pupa. The pupa ecloses to the adult fly and the cycle repeats. (B) The lymph gland is the venue for definitive hematopoiesis. It comprises of four pairs of lobes and the primary lobe is divided into four regions: the posterior signaling center (PSC), the medullary zone (MZ), the intermediate zone (IZ), and the cortical zone (CZ). The prohemocytes of the MZ differentiate into plasmatocytes and crystal cells of the CZ. Lamellocytes are barely seen in healthy animals. The IZ contains differentiating prohemocytes that expresses both MZ and CZ markers.

The second wave or definitive hematopoiesis occurs in a specific organ called the lymph gland (Rugendorff et al., 1994). The lymph gland originates from the cardiogenic mesoderm, distinctive from the embryonic lineage, which later differentiates into hemangioblast-like cells that give rise to the posterior signaling center (PSC) and pre-prohemocytes (Crozatier et al., 2004; Krzemień et al., 2007; Mandal et al., 2004; 2007; Rugendorff et al., 1994). Pre-prohemocytes turn into prohemocytes which produces three types of mature hemocytes: plasmatocytes, crystal cells, and lamellocytes (Jung et al., 2005; Krzemien et al., 2010; Lebestky et al., 2000; Shrestha and Gateff, 1982). The late-third-instar larval lymph gland is comprised of four pairs of lobes of which the biggest—the primary lobe—is further divided into four areas: the PSC, the medullary zone (MZ), the cortical zone (CZ), and the intermediate zone (IZ) (Fig. 1B) (Ferguson and Martinez-Agosto, 2014b; Krzemień et al., 2007; Krzemien et al., 2010; Mandal et al., 2007). The posterior lobes express similar markers as the primary lobe, yet, their detailed functions remain uncharacterized (Grigorian et al., 2011; Jung et al., 2005). The PSC, located at the medio-posterior region of the lymph gland, serves as a signaling center for the maintenance of prohemocytes (Crozatier et al., 2004; Krzemień et al., 2007; Lebestky et al., 2003; Mandal et al., 2004). Recent studies have suggested that the dorsal vessel plays additional signaling roles for the regulation of prohemocytes (Morin-Poulard et al., 2016). Closest to the PSC and the dorsal vessel is the MZ, possessing potentials to generate mature hemocytes of the lymph gland (Jung et al., 2005). The MZ is connected to the CZ via the IZ (Blanco-Obregon et al., 2019). The IZ is identified by reactive oxygen species (ROS), *domeless*, and *Hemolectin* as well as their transition states (Krzemien et al., 2010; Owusu-Ansah and Banerjee, 2009; Sinenko et al., 2009). The CZ which lies on the outermost region of the lymph gland, contains mature hemocytes (Blanco-Obregon et al., 2019; Crozatier et al., 2004; Jung et al., 2005).

During normal development, both circulation and the lymph gland maintain constant ratios of mature hemocytes. Plasmatocytes generally make up approximately 95% of the total hemocytes whereas crystal cells cover 5% (Bangs et al., 2000; De Gregorio et al., 2002; Holz et al., 2003; Lanot et al., 2001; Ramet et al., 2002; Rizki, 1957; Shrestha and Gateff, 1982; Tepass et al., 1994). Another group of hemocytes, which are seen under severe immune responses, is lamellocytes (Brehelin, 1982; Lanot et al., 2001; Rizki, 1957). Lamellocytes encapsulate eggs deposited by parasitic wasps into the larva and neutralize them as an active defense mechanism (Carton et al., 2008; Keebaugh and Schlenke, 2013; Russo et al., 1996).

RUNX IN *DROSOPHILA* DEVELOPMENT

Runt is a DNA-binding domain first identified in *Drosophila runt* and is highly conserved in invertebrates and vertebrates (Crute et al., 1996; Daga et al., 1996; Gergen and Wieschaus, 1986; Rennert et al., 2003). In mammals, acute myeloid leukemia 1 (AML1), also known as RUNX1, is involved in the regulation of hematopoiesis and its transposition causes myeloid leukemia (Lo Coco et al., 1997; Okuda et al., 1996; Rabbitts, 1994; Speck and Terryl, 1995). There are three paralogues of RUNX: RUNX1, RUNX2, and RUNX3, in mammals. Alternatively, there are two well-known Runt domain proteins in *Drosophila*, runt and lozenge (Rennert et al., 2003). Loss of *runt* or *lozenge* leads to comparable pleiotropic phenotypes during *Drosophila* development which include defects in: 1) neurogenesis (Dormand and Brand, 1998; Duffy et al., 1991), 2) eye development (Crew et al., 1997; Daga et al., 1996; Flores et al., 1998; Oliver, 1946), 3) segmentation (Ingham and Gergen, 1988), and 4) sex determination (Duffy and Gergen, 1991; Sánchez and Nöthiger, 1983). However, loss of *lozenge* causes two additional phenotypes: reproduc-

tive disorders and defects in hematopoiesis (Anderson, 1945; Milchanowski et al., 2004; Wang et al., 1996). Investigating the role of *lozenge* in *Drosophila* has provided mechanistic details on Runt domain protein functions in fly hematopoiesis and related signaling pathways (Canon and Banerjee, 2000; Milchanowski et al., 2004). Here on, we discuss the role of *lozenge* in *Drosophila* hematopoiesis and its genetic interactions.

LOZENGE IN *DROSOPHILA* HEMATOPOIESIS

In *Drosophila* hematopoiesis, *lozenge* controls the lineage specification of prohemocytes into crystal cells (Fig. 2). This unique role of *lozenge* is observed in both waves of *Drosophila* hematopoiesis in the embryo and larval lymph gland (Holz et al., 2003; Lanot et al., 2001). In the embryo, a group of cells identified as crystal cell precursors (CCPs) express lozenge and are initially seen at the head mesoderm of stage 11 embryos (Lebestky et al., 2000). If CCPs lose *lozenge* expression, they give rise to plasmatocytes while CCPs with high *lozenge* become crystal cells (Lebestky et al., 2003). This implies that generation of crystal cells is dependent on *lozenge* expression in embryonic hemocytes. In addition to developmental studies, previous mutant screens of *lozenge* identified several *lozenge* alleles with variant phenotypes including loss of crystal cells resulting from *lozenge*^{ts} and *lozenge*^{r15} (Daga et al., 1996; Galko and Krasnow, 2004; Lebestky et al., 2000; Rizki et al., 1980). This reaffirms the assertion that early expression of *lozenge* is required for the formation of crystal cells in the embryo (Lebestky et al., 2000). Besides crystal cells generated from the embryonic head mesoderm, studies by Leitao and Sucena (2015) have demonstrated that *lozenge*-expressing primed cells can differentiate into crystal cells in a Notch-dependent manner at larval hematopoietic pockets.

Similarly, in the second instar larval lymph gland, *lozenge*-positive cells emerge from differentiating prohemocytes in the IZ and divide into crystal cells (Ferguson and Martinez-Agosto, 2014a). Consequently, these *lozenge*-positive cells generate up to a hundred crystal cells per one lobe (Lebestky et al., 2000). lozenge, in determining the proportion of divided cells that become crystal cells, relies on an intricate circuit of interactions with other proteins. Over the years, though significant research is still required in this aspect, several transcription factors and related signaling proteins have been uncovered in *Drosophila*. First, the GATA-like transcriptional factor—*serpent* (*srp*), was identified as a marker for prohemocytes as well as an upstream regulator of *lozenge* that is crucial for the formation of crystal cells. Indeed, a small subset of *srp*-positive cells become *lozenge*-positive crystal cells and loss of *srp* turns off *lozenge* expression (Bataille et al., 2005; Lanot et al., 2001; Lebestky et al., 2000). Second, *u-shaped* (*ush*), a Friend of GATA homologue, was found to regulate the population size of crystal cells. Increasing or reducing *u-shaped* expression significantly diminishes or augments the number of crystal cells produced respectively (Fos-

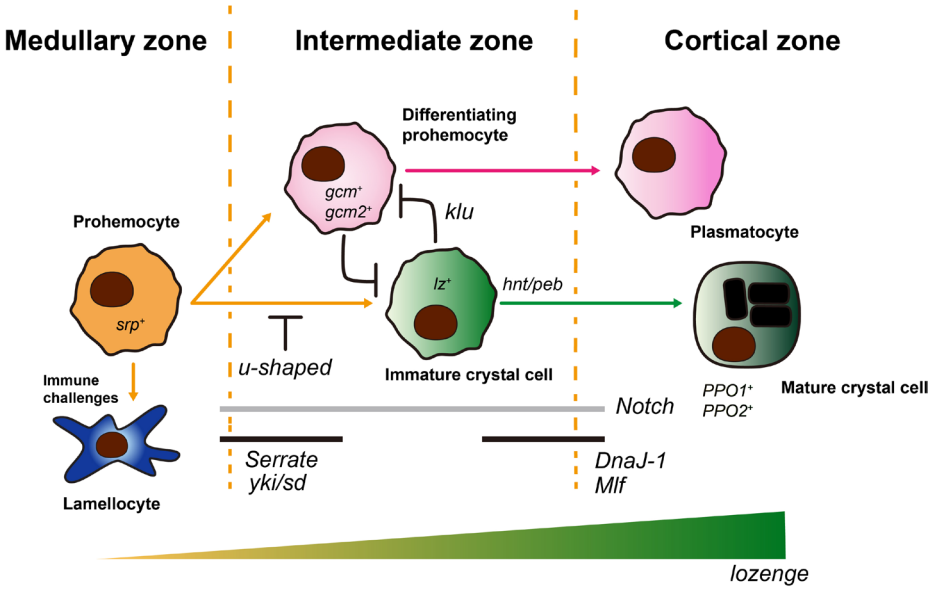

Fig. 2. lozenge in crystal cell formation. *serpent*-positive (*srp*$^+$) prohemocytes differentiate into *gcm*$^+$/*gcm2*$^+$ differentiating prohemocytes or *lz*$^+$ immature crystal cells. Differentiation of prohemocytes into immature crystal cells is mediated by the *Notch*/*Serrate* interaction and moderated by *u-shaped*. Additionally, *yki* and *sd* control the crystal cell specification in a *Notch*/*Serrate*-dependent manner. High *gcm*/*gcm2* expression reduces the number of crystal cells, however, fated crystal cells are inhibited by *klu* from becoming plasmatocytes. This high *gcm*/*gcm2* cells become plasmatocytes. Increased *lz* in immature crystal cells coupled with *hnt*/*peb*, *DnaJ-1*, *Mlf* and *Notch* leads to formation of mature crystal cells which possess crystalline inclusions and express *PPO1* and *PPO2*. The process of mature crystal cell formation is heavily dependent on *lozenge* expression from the onset to the late stage. The medullary, intermediate, and cortical zones demarcate three regions of the primary lymph gland lobe. Healthy animals do not actively generate lamellocytes. Though, prohemocyte differentiation is lamellocyte-biased upon immune challenges.

sett et al., 2001). Further investigation showed that a synergistic confluence of *serpent* and *lozenge* inhibits *u-shaped* to allow formation of crystal cells from its precursors (Fossett et al., 2003; Muratoglu et al., 2007; Waltzer et al., 2003). Third, *glial cell missing* (*gcm*)-positive cells later differentiate into plasmatocytes (Bernardoni et al., 1997; Lebestky et al., 2000). The absence of *gcm* in prohemocytes induces formation of *lozenge* positive cells which become crystal cells (Bataille et al., 2005; Lebestky et al., 2000). This process is identical in both embryonic and larval contexts.

In addition to GATA and Zinc finger transcription factors, *srp, ush* and *gcm* and *gcm2* respectively, one of the main signaling molecules that has been shown to impact the formation of crystal cells is Notch. The numbers of crystal cells or prohemocytes are shown to be reduced or absent in Notch-mutant clones of the lymph gland (Dey et al., 2016; Lebestky et al., 2003). In-depth studies in the lymph gland showed that a group of cells that are positive for Serrate, a Notch ligand, trigger the expression of *lozenge* and hence determine the crystal cell lineage (Duvic et al., 2002; Lebestky et al., 2003). Recently, endogenous expression of Serrate was revealed in the IZ, where Notch-mediated crystal cell differentiation takes place, emphasizing that Notch-Serrate activity in differentiating prohemocytes is critical for crystal cell specification (Cho et al., 2018). Of note, *yorkie* (Berson et al., 2019)- and *scalloped* (*sd*)- activation of *lozenge* is another important factor in Notch-dependent crystal cell determination. Loss of *yki* or *sd* reduces the crystal cell differentiation, and expression of *yki* does not rescue $Serrate^{DN}$-mediated crystal cell depletion (Ferguson and Martinez-Agosto, 2014b). These results imply that *yki/sd* function requires Serrate-Notch signaling (Ferguson and Martinez-Agosto, 2014a). Lastly, *pebbled* (*peb*), *myeloid leukemia factor* (*Mlf*), *DNA-J-like1* (*DnaJ-1*), *klumpfuss* (*klu*) have been implicated in Notch-*lozenge* dependent crystal cell formation. While *DnaJ-1* and *Mlf* regulate Notch activation, *peb* regulates nuclear size of crystal cells. *Klu*, on the other hand, inhibits pre-destined crystal cells from becoming plasmatocytes (Miller et al., 2017; Terriente-Felix et al., 2013). The stabilization of *lozenge* by these proteins: klu, Mlf, and DnaJ-1, and their interaction with Notch supports proper differentiation of crystal cells (Miller et al., 2017; Terriente-Felix et al., 2013). It is worth noting that the Notch-RUNX collaboration is conserved in mammals and therefore, further investigations could elucidate their roles in mammalian hematopoiesis (Bras et al., 2012; Kulkarni et al., 2011; Miller et al., 2017; Terriente-Felix et al., 2013).

LOZENGE, CRYSTAL CELLS IN *DROSOPHILA* STRESS RESPONSES

Crystal cells are responsible for a significant part of the *Drosophila* innate immunity and stress responses (Cho et al., 2018; Sorrentino et al., 2002). Though the population of crystal cells is sparse, upon invoking an immune reaction through wounding (Galko and Krasnow, 2004; Ramet et al., 2002; Rizki, 1960) or injection of wasp eggs into a larva, crystal cells rupture and melanization takes place (Fig. 3) (Dudzic et al., 2015; Tang et al., 2006). In melanization, crystal cells are mobilized via the c-Jun N-terminal Kinase (JNK), small GTPases, and Eiger (tumor necrosis factor, TNF) pathways, and they burst to activate the Serine protease cascade which is facilitated by phenoloxidases (Bidla et al., 2007; Peeples et al., 1968; 1969a; 1969b; Rizki, 1957). Within this cascade, proteolytic enzymes convert Prophenoloxidase 1 (PPO1) and Prophenoloxidase 2 (PPO2) released by crystal cells into active phenoloxidases. Phenoloxidases produce phenols and free radicals that eventually inactivate pathogens while building melanin via non-enzymatic polymerizations (Binggeli

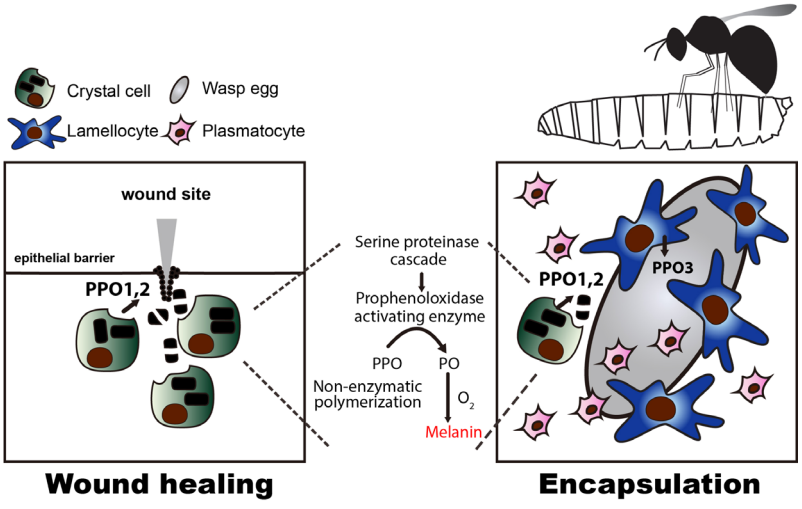

Fig. 3. Crystal cell mediated stress responses. When larva gets a sterile wound, crystal cells migrate towards the wound site, and rupture to initiate the melanization cascade (left). This promotes the formation of a scab at the wound site leading to a subsequent healing process. Upon wasp infestation, wasps lay eggs inside *Drosophila* larva that trigger innate immune responses including differentiation of lamellocytes. Lamellocytes encapsulate wasp eggs and activate PPO3 to neutralize them (right). During encapsulation, crystal cells co-opt PPO1 and PPO2 to facilitate melanization. In melanization, rupturing of crystal cells is coupled with the activation of Serine proteinase cascade which triggers the conversion of PPOs into POs initiating non-enzymatic melanin formation (middle).

et al., 2014; Cerenius et al., 2008; Laifook, 1966; Ramet et al., 2002). Specifically, in wound healing, sterile wounding triggers calcium waves that in turn activates an NADPH oxidase, DUOX, required for the recruitment of hemocytes (Razzell et al., 2013). At this site, crystal cells rupture and induce melanization for the formation of a scab that blocks the punctured region. This precedes the crucial step of restoring epithelial integrity (Galko and Krasnow, 2004). Also, upon wasp infestation, lamellocytes inter-dependently contribute to melanization by secreting Prophenoloxidase 3 (PPO3) which augments crystal cells' phenoloxidase activity, and mediate encapsulation (Dudzic et al., 2015; Gold and Brückner, 2015; Nam et al., 2008; Rizki, 1960; Rizki, 1957; Rizki and Rizki, 1974; 1980). Hence, in the absence of *lozenge* or *PPOs* in *lozenge*^{r15}, *lozenge*^{ts}, *PPO1*$^{\Delta}$ or *PPO2*$^{\Delta}$, these immune reactions are abrogated (Binggeli et al., 2014; Dudzic et al., 2015; Ferguson and Martinez-Agosto, 2014a). Different from these phenomena, hypoxic stress activates crystal cell differentiation while keeping the cell intact (Mukherjee et al., 2011). While the innate immune functions of crystal cells and their PPO inclusions are well characterized, the function of crystal cells in hypoxia is still obscure. Considering that crystal cells are significantly induced by manipulation of CO_2- or O_2-sensing neurons, it is plausible that crystal cells may play a non-canonical role in this condition (Cho et al., 2018).

PERSPECTIVES

Extensive and thorough studies on *Drosophila* hematopoiesis have unraveled novel insights into mechanisms underlying *lozenge* and its intricate molecular and genetic links. We have discussed a number of these factors, however, going forward additional analyses will be expedient in broadening our perspectives on lozenge/RUNX. Specifically, spatio-temporal interactions between lozenge and Notch/Serrate, srp, gcm, gcm2, ush, yki/sd, klu, peb, DnaJ-1, and Mlf in the embryo or the lymph gland contexts will require in-depth future investigations. Furthermore, explorations on additional functions of the crystal cell, other than its melanization effect, will be worth interrogating.

Disclosure

The authors have no potential conflicts of interest to disclose.

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