# Hematopoiesis in the *Drosophila* larva: beyond the lymph gland

**Alexandre Castanho Barata Leitão**

Dissertation presented to obtain the Ph.D degree in Biology

Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

Oeiras,
July, 2014

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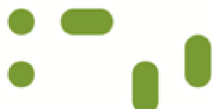

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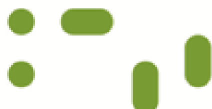

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## Declaração/Declaration

Esta dissertação é o resultado do meu próprio trabalho desenvolvido entre Março de 2010 e Julho de 2014 no laboratório do Dr. Élio Sucena, Instituto Gulbenkian de Ciência em Oeiras, Portugal, no âmbito do Programa Gulbenkian de Doutoramento (edição 2009-2010). Parte deste trabalho encontra-se em preparação para ser publicado.

This dissertation is the result of my own research, carried out between March 2010 and July 2014 in the laboratory of Dr. Élio Sucena, Instituto Gulbenkian de Ciência in Oeiras, Portugal, under the Gulbenkian Doctoral Programme (2009-2010 edition). Part of this work in being prepared for publication.

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# Summary

All coelomate animals possess a population of cells that do not make part of an organ and instead freely flow inside the body cavity. These cells, termed hemocytes (in invertebrates) or blood cells (in vertebrates), are involved in varied functions including immune response, clearance of apoptotic cells and distribution of nutrient and gases (Grigorian & Hartenstein 2013). To perform all these functions correctly, blood cells need to achieve an optimal concentration and proportion between cell types (Almeida et al. 2005). This balance is achieved by several mechanisms of cell fate choices and proliferation in a process collectively termed hematopoiesis.

In vertebrates, immune cells differentiate from Hematopoietic Stem Cells (HSC) residing in hematopoietic organs. Several layers of genetic control dependent on cytokines and cell contacts ensure the correct proportion of produced cell types. Invertebrates produce fewer blood cell types but almost all species possess a hematopoietic organ (Hartenstein 2006). In *Drosophila melanogaster* hematopoiesis is best studied in larval stages where a distinct hematopoietic organ (the lymph gland) is easily accessible. In homeostasis two different types of hemocytes are produced in the lymph gland: plasmatocytes and crystal cells. One striking feature of the lymph gland is its compartmentalization (Jung et al. 2005). Prohemocytes reside in the medullary zone and receive signals from the posterior signaling center cells to maintain their undifferentiated state or to start migrating into the cortical zone and differentiate into plasmatocyte or crystal cell.

The same mature cell types can be found in circulation. Importantly, both plasmatocytes and crystal cells found in circulation are not derived from the lymph gland because without an immune challenge this organ does not release hemocytes until pupariation (Holz et al. 2003).

Also, tough hemocytes can be found in circulation, up to two thirds of them are sessile and reside in the epithelium underneath the larval cuticle (Lanot et al. 2001). These sessile cells are not randomly dispersed but aggregated in clusters in every segment of the larva. Recently, it has been shown that the peripheral nervous system supports hemocyte homing to these sites and influences their proliferation/survival (Makhijani et al. 2011). It has been suggested that these sessile hemocytes clusters function as hematopoietic compartments because cells taken from them can differentiate into lamellocytes (Márkus et al. 2009). However, to define the hemocyte clusters as hematopoietic compartments one needs evidence to show that the clustering of cells is necessary to induce cell fate choices.

The number of plasmatocytes and crystal cells increases dramatically throughout larval development and maintains a relatively constant ratio (Rizki 1957). Given that plasmatocytes do proliferate and crystal cells are post-mitotic cells the maintenance of the correct proportions is an interesting developmental question. Notch signaling is one of the first genetic pathways shown to be essential for crystal cell differentiation (Duvic et al. 2002; Lebestky et al. 2003). Inspired by the fact that hemocytes in clusters make cell contacts for long periods of time, we conducted a series of experiments to test if the hemocyte clusters function as hematopoietic sites where crystal cells are differentiated. Our results show that crystal cells are formed in these clusters and that, similarly to the lymph gland, the cells

responsible for inducing crystal cells differentiation are themselves hemocytes. Moreover, we show that the clusters are necessary to achieve a correct proportion between plasmatocytes and crystal cells. Nevertheless, a striking difference exists between crystal cell development in hemocyte clusters and in the lymph gland. Whereas, in the lymph gland crystal cells differentiate from prohemocytes, in the hemocyte clusters they differentiate from plasmatocytes.

With the confirmation that hemocyte clusters function as hematopoietic tissue we conducted a Genome Wide Association Study (GWAS) to search for genetic players involved in the regulation of hemocyte numbers/proportions. Our results show that the *G protein-coupled receptor kinase 1* (*Gprk1*) is essential to achieve the correct number of hemocytes. Although the function of *Gprk1*is poorly studied in Drosophila the vertebrate ortholog (*GRK2*) plays several roles in hematopoiesis and is essential for the correct number of monocytes and neutrophils (Otten et al. 2013). This indicates that *Gprk1* is another example of the evolutionary conservation in genetic control of hematopoiesis between insects and vertebrates.

Together, our results show that the regulation of hemocyte numbers/proportions in the *Drosophila* larva is achieved by a hematopoietic tissue formed by aggregation of mature hemocytes in clusters. We show that the number of differentiated cells in this hematopoietic tissue varies greatly between different genetic backgrounds. This gives us the opportunity to study which genetic pathways are important to regulate hemocyte numbers.

# *Sumário*

Todos os animais celomados têm uma população de células que não fazem parte de um órgão mas, ao invés, estão em livre circulação dentro da cavidade do corpo. Estas células, denominadas células sanguíneas (em vertebrados) ou hemócitos (em invertebrados), estão envolvidas em diferentes funções incluindo: resposta imunitária, fagocitose de corpos apoptóticos e distribuição de nutrientes e gases (Grigorian & Hartenstein 2013). Para que estas funções sejam efetuadas corretamente pelas células sanguíneas é necessário que estas atinjam a concentração e proporção ótima entre diferentes tipos celulares (Almeida et al. 2005). Este balanço é conseguido através de diferentes mecanismos de determinação de diferenciação celular e proliferação, um processo denominado hematopoiese.

Em vertebrados, as células imunitárias diferenciam-se a partir de células estaminais hematopoiéticas. A produção de diferentes tipos celulares nas proporções corretas é conseguida através de diferentes níveis de controlo genético dependente de citoquinas e contactos celulares. Os animais invertebrados produzem menos tipos celulares mas quase todas as espécies têm um órgão hematopoiético (Hartenstein 2006). Em *Drosophila melanogater*, o estudo da hematopoiese está mais bem desenvolvido nos estádios larvares, quando um órgão hematopoiético, a glândula linfática, é facilmente dissecado. Em condições de homeostasia são produzidos dois tipos de hemócitos na glândula linfática: plasmatócitos e células cristal. Uma característica marcante da glândula linfática é a sua compartimentalização (Jung et al. 2005). Os prohemócitos, localizados na zona medular, recebem sinais das células do centro

sinalizador posterior para manterem o seu estado indiferenciado ou para migrarem para a zona cortical e começarem a sua diferenciação.

Os mesmos tipos de hemócitos encontram-se em circulação. Um aspeto importante a notar é que tanto os plasmatócitos como as células cristal em circulação não derivam da glândula linfática porque, sem um infeção, este órgão não liberta os hemócitos produzidos até se formar a pupa (Holz et al. 2003).

Fora da glândula linfática os hemócitos encontram-se em circulação mas dois terços dos hemócitos estão num estado séssil no epitélio da cutícula larvar (Lanot et al. 2001). Estas células sésseis não estão distribuídas aleatoriamente mas sim agregadas em conjuntos nos vários segmentos da larva. Recentemente, foi demonstrado que o sistema nervoso periférico influencia a atracão dos hemócitos para estes locais e influencia a sua proliferação/sobrevivência (Makhijani et al. 2011). Já foi sugerido que os agregados de hemócitos sésseis funcionam como tecido hematopoiético porque células retiradas destes agregados conseguem diferenciar-se num terceiro tipo de hemócito, o lamelócito (Márkus et al. 2009). No entanto, para definir os agregados de hemócitos como tecido hematopoiético é necessário mostrar que a estrutura destes agregados é necessária para induzir escolhas de destinos celulares.

O número de plasmatócitos e células cristal aumenta drasticamente durante o desenvolvimento larvar mantendo um rácio relativamente constante (Rizki 1957). Uma vez que os plasmatócitos proliferam e as células cristal são células pós-mitóticas a manutenção das proporções corretas torna-se uma interessante

questão de desenvolvimento. A sinalização Notch foi uma das primeiras cascatas genéticas demonstrada ser essencial na diferenciação de células cristal (Duvic et al. 2002; Lebestky et al. 2003). Inspirados pelo facto que os hemócitos dentro dos agregados estão em contacto por longos períodos de tempo, nós delineámos uma série de experiências para testar se os agregados funcionam como tecido hematopoiético onde as células cristal seriam diferenciadas sob a influência de outros hemócitos. Os nossos resultados mostram que as células cristal são formadas nestes agregados e que, tal como na glândula linfática, as células que induzem a sinalização Notch são hemócitos. Também demonstramos aqui que os agregados são necessários para manter a proporção correta entre plasmatócitos e células cristal. Com a confirmação de que os agregados funcionam como tecido hematopoiético conduzimos um estudo de associação genómica para procurar genes envolvidos na regulação dos números/proporção de hemócitos. Os nossos resultados mostram que o gene *G protein-coupled receptor kinase 1* (*Gprk1*) é essencial para desenvolver o número correto de hemócitos. Apesar deste gene ser pouco estudado em *Drosophila,* o ortólogo em vertebrados (*GRK2*) desempenha importantes funções em hematopoiese e é essencial para o estabelecimento do número correto de monócitos e neutrófilos (Otten et al. 2013). Isto é uma indicação que *Gprk1* é outro exemplo de conservação evolutiva no controlo genético da hematopoiese entre insetos e vertebrados.

Em conjunto, os nossos resultados mostram que a regulação dos números/proporções de hemócitos na larva de *Drosophila* é conseguida por um órgão hematopoiético formado por agregados de hemócitos maduros. O número de células diferenciadas neste tecido hematopoiético varia grandemente em diferentes fundos genéticos.

Isto proporciona a oportunidade de estudar quais as cascatas genéticas envolvidas na regulação dos números de hemócitos.

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# 1 Introduction

The term “hematopoiesis” results from the conjugation of two Greek words: *haimat* (blood) and *poiesis* (to make). Understanding how animals *make blood* presupposes the study of two intertwined mechanisms, cell specification and cell proliferation. Although some mature blood cells proliferate in circulation, at the fundamental level, the regulation of blood cell numbers is controlled because most of the blood cell differentiation and proliferation occurs in hematopoietic organs (Grigorian & Hartenstein 2013). These organs are compartmentalized structures composed of different cell types that assure the correct formation of new blood cells. Importantly, the cellular organization, the architecture, of the hematopoietic organ is necessary for achieving the two functions mentioned above: it enhances or inhibits cell proliferation and influences cell fate choice decisions (Grigorian & Hartenstein 2013). This is achieved with a myriad of paracrine and cell contact signaling molecules. In vertebrates the number and location of hematopoietic organs changes during ontogeny and evolution (Zon 1997). For example, in mammals there are two waves of hematopoiesis during development. The first one occurs early in development in an extraembryonic tissue, the yolk sack. This first wave of hematopoiesis produces only a limited number of blood cell types. Secondarily, Hematopoietic Stem Cells (HSC), differentiated in the aorta-gonad-mesonephros mesoderm region, disperse and colonize other tissues such as the liver, spleen, and bone marrow where definitive hematopoiesis occurs (Zon 1997). In contrast with mammals, definitive hematopoiesis in fishes occurs in the kidney and not in the bone marrow. This is demonstrative of the different hematopoietic niches

that evolved in animals and alludes to the fact that this may be a very plastic process.

In invertebrates, blood cells are more commonly termed hemocytes. The number of different hemocyte types in invertebrates is smaller than blood cell types in vertebrates. This reflects the fact that a great part of vertebrate immune cells are dedicated to the adaptive immune response, a branch of the immune system that is not present in invertebrates. Nevertheless, it is possible to distinguish different hemocyte types and hematopoietic organs in most of coelomate invertebrate species studied so far (Grigorian & Hartenstein 2013). The number, location and structure of these hematopoietic organs changes greatly between species (Grigorian & Hartenstein 2013). They range from very simple structures where hemocyte proliferation is enhanced, such as the hematopoietic centers of polychaete annelids, to more complex gland like structures, exemplified by the *Drosophila* lymph gland (Hartenstein 2006). Hematopoiesis in the *Drosophila*'s larval lymph gland produces the two major hemocyte types found in homeostatic conditions: plasmatocytes and crystal cells. However, in homeostatic conditions, hemocytes produced in the lymph gland do not contribute to the hemocyte population found in the haemocoelic compartment during larval stages (Holz et al. 2003; Honti et al. 2010). The majority of haemocoelic hemocytes are sessile cells that form clusters in the subepidermal layer of the body cavity (Lanot et al. 2001). The two cell compartments, circulating and sessile cells, are in a dynamic equilibrium (Babcock et al. 2008; Welman et al. 2010; Makhijani et al. 2011). The regulation of hemocyte types is certainly influenced by the behavior of cells when they are in circulation or in clusters.

In this introduction to the theme of hematopoiesis in *Drosophila melanogaster* we will start to define the different types of hemocytes found at the different life stages and follow with the current knowledge of hematopoiesis during embryogenesis and larval stages.

## 1.1 Hemocytes in Drosophila

In insects it is common to classify hemocytes into four morphologically distinct types: prohemocytes, granulocytes, plasmatocytes and oenocytoids (Lavine & Strand 2002). Confusingly, the terminology for functional equivalent cells in *Drosophila* is different (Ribeiro & Brehélin 2006). Prohemocytes are cells that can differentiate into the other three types of hemocytes. Granulocytes, termed plasmatocytes in Drosophila, are phagocytic cells. Large cells dedicated to encapsulation of entities too large to be phagocytized are termed plasmatocytes in most insect species and lamellocytes in Drosophila. Finally, oenocytoids are cells that produce Phenoloxidase precursors necessary for the melanization response and in *Drosophila* they are called crystal cells (Ribeiro & Brehélin 2006). Throughout this thesis we will use the *Drosophila* terminology when referring to hemocyte types, described as: prohemocytes, plasmatocytes, lamellocytes and crystal cells (Rizki 1957; Roshana & Gateff 1982; Ribeiro & Brehélin 2006). This hemocyte classification into four types was validated recently using molecular markers (Kurucz, Váczi, et al. 2007).

Some variations on this theme can be found at different levels. For example, there are references to another hemocyte type in *Drosophila* termed podocyte but they are often considered morphological variants of plasmatocytes when entering pupariation

(Rizki 1957; Rizki 1962). Also, it has been reported that the obscura group flies do not produce lamellocytes (Havard et al. 2009). A fusiform cell termed nematocyte has been recently proposed to play a role against parasitoid egg infections in *Zaprionus indianus*, a phylogenetic close relative of *Drosophila melanogaster* (Kacsoh et al. 2014). At a larger scale, it is also possible to distinguish another hemocyte type termed spherule cell in Lepidoptera for which the function remains unknown (Lavine & Strand 2002). Life-stage also determines the blood cell composition of *Drosophila*. Hemocytes can be found in every developing stage of *Drosophila melanogaster* but only during larval stages may we find all four types of hemocytes. Embryo, pupa and adult stages only contain a subset of hemocyte types.

A summary of hemocyte types and their functions is represented in Figure 1.1.

### *1.1.1 Prohemocyte*

Prohemocytes are quiescent cells that can differentiate into the other three hemocyte types. They are found in the medullary zone of the larva hematopoietic organ, the lymph gland. Prohemocytes are characterized by their lack of maturation markers (Jung et al. 2005). In addition, they express genes that are down-regulated once they start to differentiate into mature hemocytes. Examples of these genes are *DE-cadherin* (*DE-Cad*), *domeless* (*dome*), *unpaired3* (*upd-3*), and *patched* (*ptc*) (Jung et al. 2005; Mandal et al. 2007). Some of these genes are essential to maintain the prohemocyte state. For example, prohemocytes will start to upregulate differentiation markers if *hedgehog*, the ligand for the receptor Patched, is mutated (Mandal et al. 2007). It is also possible to consider the existence of pro-

hemocytes during embryogenesis when the first wave of hematopoiesis occurs. During hemocyte development in the embryo the cells pass through a stage when they only express early hemocyte markers and still are bipotent cells that may differentiate into plasmatocytes or crystal cells (Waltzer et al. 2010). Nevertheless, this is only a transient state that lasts few hours during embryogenesis. After that, all cells have either plasmatocyte or crystal cell markers (Waltzer et al. 2010).

Some reports suggest the presence of prohemocytes in circulation (Lanot et al. 2001; Sinenko et al. 2010). For example Lanot and colleagues report that hemocytes dividing in circulation do not phagocyte Indian ink particles (Lanot et al. 2001). This could indicate that there are two subpopulations of plasmatocytes, one with the capacity to divide and another devoted to phagocytosis. An alternative explanation would be that plasmatocytes do not phagocyte during cell division but a recent study shows that cells phagocyte in equal proportion whether they are synthesizing DNA or not (Makhijani et al. 2011). All larval hemocytes outside the lymph gland expresses *Hemolectin* and *Peroxidasin* GAL4 drivers (Goto et al. 2003; Stramer et al. 2005), two live genetic drivers that are not expressed in lymph gland prohemocytes (Jung et al. 2005). This indicates that no equivalent of lymph gland prohemocyte exists in the haemocoelic compartment. Nevertheless, a small population of hemocytes that express low levels of hemolectin are positive for Wingless antibody (Sinenko et al. 2010). This could constitute a prohemocyte subpopulation since Wingless signaling is important for prohemocyte maintenance (Sinenko et al. 2009). However, to our knowledge, there is no functional evidence to show that a population of prohemocytes exists outside the lymph gland in larval stages.

### *1.1.2 Plasmatocyte*

Plasmatocytes are present in all developmental stages of *Drosophila* and are always the most abundant hemocyte type. They are spherical cells varying in diameter from 7 to 12 µm and containing several vacuoles (Rizki 1957). Plasmatocytes first develop at early embryonic stages from the head mesoderm and shortly after start to migrate and disperse into the entire embryo (Tepass et al. 1994). Plasmatocytes are very efficient phagocytic cells and for that reason they are often functionally compared to vertebrate macrophages (Evans et al. 2003). Phagocytosis is an active process of recognition, engulfment and destruction of any particle bigger than 0.5 µm (Flannagan et al. 2012). This process is essential during the immune response to destroy invading microorganisms and during ontogeny to clear apoptotic bodies. These two functions can be partitioned at the molecular level. Indeed, to recognize bacteria plasmatocytes use receptors such as Eater (Kocks et al. 2005) and NimrodC1 (Kurucz, Márkus, et al. 2007), while Croquemort (Franc et al. 1999) and Draper (Manaka et al. 2004) are used to recognize dying cells. At the end of embryogenesis almost all plasmatocytes contain apoptotic bodies and this phagocytic function is essential for the proper development of the Central Nervous System (CNS) (Tepass et al. 1994; Defaye et al. 2009). Moreover, the proper establishment of a condensed nerve chord also depends on plasmatocytes, which deposit extracellular matrix (ECM) components essential for this process (Martinek et al. 2008). In larval stages plasmatocytes are known to perform three more functions: they produce Anti-Microbial Peptides (AMPs) (Samakovlis et al. 1990), clotting factors (Goto et al. 2003) and signal to the fat body to induce or enhance the humoral immune response triggered by the presence of invading pathogens

(Shia et al. 2009; Charroux & Royet 2009) (Figure 1.1). In contrast to their larval function, plasmatocytes are dispensable for the activation of humoral response in the adult fat body (Defaye et al. 2009). Later, when entering pupariation, an ecdysone peak increases the mobility of plasmatocytes and their chemo-attraction towards damaged epithelia (Regan et al. 2013). Also, concomitantly with this increase in phagocytic activity, plasmatocytes become bigger and increase the number of granules (Regan et al. 2013). During this period they change their expression profile by down-regulating *Hemese* expression and becoming positive for the Ad1 antigen (Honti et al. 2014). In flies where hemocytes are depleted, by over-expressing a apoptotic gene, increased mortality rate is observed during pupal stages (Defaye et al. 2009; Charroux & Royet 2009). However, this phenotype is rescued by raising the larvae under sterile conditions, demonstrating the necessity for hemocytes for a immune response in the pupa but excluding a need for plasmatocytes in pupal development (Defaye et al. 2009; Charroux & Royet 2009). Embryonic and larval derived plasmatocytes persist into the adulthood in a post-mitotic state (Holz et al. 2003). Plasmatocyte is virtually the only hemocyte type found in adult and when they are depleted, the flies are more susceptible to bacterial challenge (Defaye et al. 2009; Charroux & Royet 2009).

### *1.1.3 Crystal Cell*

Crystal cells make up to 10% of the circulating hemocytes of the larva. They are spherical cells slightly bigger than plasmatocytes and contain cytoplasmatic crystalline inclusions (Figure 1.1). These are composed of pro-Phenoloxidase, the zymogen of Phenoloxidase that is cleaved during the melanization proteolytic cascade. The

*Drosophila melanogaster* genome encondes three pro-Phenoloxidase (PPO) genes. A recent report demonstrates that crystal cells are the sole source of PPO1 and PPO2 but only PPO2 is present in the crystals (Binggeli et al. 2014). The melanization cascade is used as an immune response in several invertebrate groups whereby the production of toxic intermediates in the cascade kills bacteria and/or melanin deposition sequesters microbes and hardens capsules formed around parasitoid eggs (Lemaitre & Hoffmann 2007; Jiravanichpaisal et al. 2006). Since crystal cells are non-phagocytic cells and produce pro-Phenoloxidase crystals it is most likely that they are dedicated to the melanization response. This is evident when larvae are bled and crystal cells rupture right away releasing the crystal contents. The rupture of crystal cells *in vivo* depends on JNK pathway and on *Eiger*, a gene with high homology to Tumor Necrosis Factor (TNF) family members (Bidla et al. 2007).

Crystal cells first appear in the embryo in the same anlage where plasmatocytes are formed but, unlike plasmatocytes, they do not disperse throughout the embryo, remaining clustered in the proventricular zone. In the larva they are formed in the lymph gland from prohemocytes and, although they do not divide, their numbers in circulation increase as larval development proceeds (Rizki 1957; Lanot et al. 2001). To explain the increase of crystal cell numbers Rizki proposed that crystal cells in circulation are differentiated from plasmatocytes (Rizki 1957). It is also possible that a population of pro-crystal cells or prohemocytes proliferates to later give rise to crystal cells. To our knowledge none of these hypotheses has been tested thus far. Soon after pupariation crystal cell numbers decrease drastically (Lanot et al. 2001). In adult it is believed that crystal cell

are not present but some rare cells do express the crystal cell marker C1 (Kurucz, Váczi, et al. 2007).

### 1.1.4 Lamellocyte

Lamellocytes are only found during larval stages and they are dedicated to respond to parasitoid wasp infections. They are extremely flattened cells that vary in form from discoid to a very elongated shape. They can reach 50 μm in length whilst not exceeding 1 μm in thickness (Rizki 1957). Lamellocytes are rarely seen in larval hemolymph but their numbers increase drastically once a female parasitoid wasp lays an egg inside the larva (Rizki 1992). After lamellocyte induction they start to accumulate around the wasp egg and eventually form a capsule. The first layer of cells in the capsule is formed by plasmatocytes that adhere to the egg chorion and form septate junctions between them (Carton et al. 2008). After massive lamellocyte production they surround the egg and finally become melanized. Lamellocytes produce a cell specific pro-Phenoloxidase gene that is enzymatically active in its uncleaved form (Irving et al. 2005; Nam et al. 2008). For this reason it was believed that capsule melanization was only dependent only on lamellocytes. In fact, a recent report shows that in a double mutant for PPO1 and PPO2, the two pro-Phenoloxidases expressed in crystal cells, the capsule is formed but does not became melanized (Binggeli et al. 2014). This result suggests a requirement for crystal cells in capsule melanization. After the encapsulation response, lamellocytes decrease in number and they are no longer found in the surviving adult.

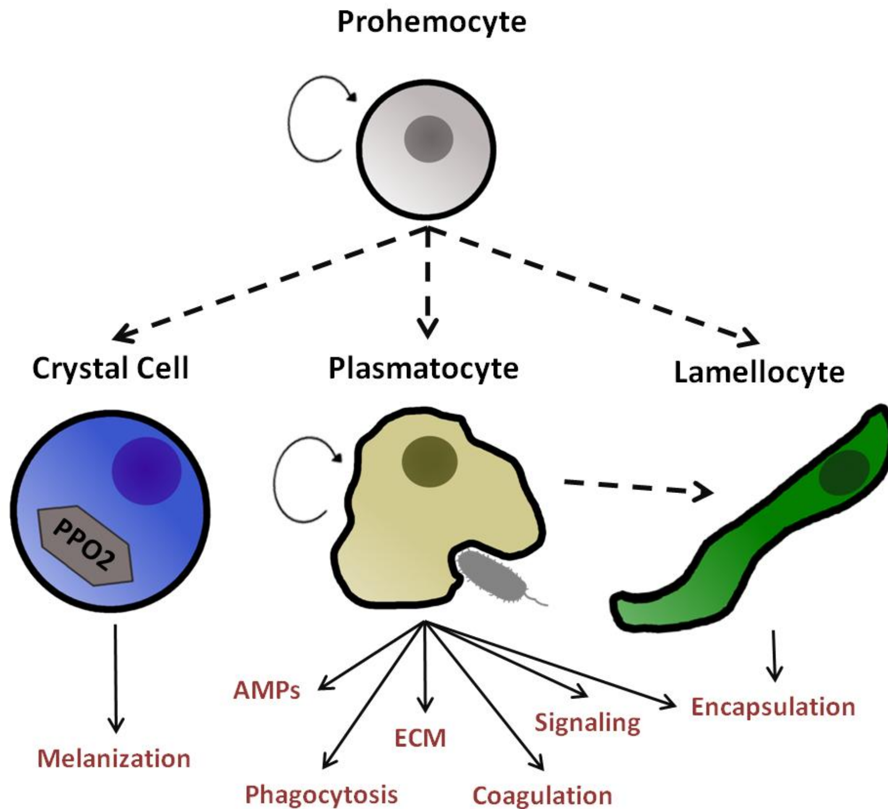


**Figure 1.1 – Hemocyte types and their functions in *Drosophila melanogaster*.** Prohemocytes are cells with the capacity to proliferate and to differentiate into the three mature hemocyte types. Crystal cells are characterized by their crystals containing proPhenoloxidase 2 (PPO2). Plasmatocytes can proliferate and are involved in several function including Anti-Microbial Peptides (AMPs) production and Extra Cellular Matrix (ECM) deposition. Upon parasitoid infection plasmatocytes can differentiate into lamellocytes. Lamellocytes are elongated cells rarely seen in homeostasis but are produced in considerable numbers during parasitoid egg infection.

## 1.2 Embryonic hematopoiesis

In *Drosophila*, hematopoiesis occurs in two distinct phases of development. During embryogenesis hemocytes differentiate from cells in the head mesoderm and during larval stages they are produced in the lymph gland (Tepass et al. 1994; Roshana & Gateff 1982). In impressive single cell transplantation experiments, Holz and colleagues have shown that, as early as the blastoderm stage, a small anlage is already cell-autonomously committed to hemocyte fate (Holz et al. 2003). This commitment corresponds to the domain and time of expression of *serpent*, the first transcription factor known to be essential for hemocyte differentiation (Rehorn et al. 1996). Serpent is one of the five GATA transcription factors found in *Drosophila*, so called because they bind to the consensus DNA sequence WGATAR (Waltzer et al. 2010). Nonetheless, it is worth noting that this commitment to hemocyte fate cannot be explained solely on *serpent* expression since it is expressed in other mesodermal tissues such as fat body (Waltzer et al. 2010).

Two other zinc finger transcription factors are expressed in all pro-hemocytes at embryonic stage 5*: Glial cells missing* (*Gcm*) and *Glial cells missing 2* (*Gcm2*) (Bataillé et al. 2005; Alfonso & Jones 2002; Bernardoni et al. 1997). This expression of *Gcm* and *Gcm2* is dependent on *serpent* (Bernardoni et al. 1997). Overexpression of *Gcm* is sufficient to induce expression of plasmatocyte markers and in *Gcm/Gcm2* mutants the number of plasmatocytes is strongly reduced (Bernardoni et al. 1997; Alfonso & Jones 2002). This indicates that Gcm has instructive roles in plasmatocyte differentiation. Shortly after, at stage 6 of embryogenesis, *Gcm* is down-regulated in the most anterior row of *serpent* expressing cells, which turn on *lozenge* expression at the next embryonic stage

(Bataillé et al. 2005) (Figure 1.2). In 40% of these *lozenge* positive cells, expression is discontinued and these cells differentiate into plasmatocytes. The other 60% maintain *lozenge* expression and differentiate into crystal cells (Figure 1.2). In *Gcm/Gcm2* mutants all *lozenge* positive cells differentiate into crystal cells (Bataillé et al. 2005). Moreover, forced expression of *Gcm* in *lozenge* positive cells is sufficient to provoke a full differentiation into plasmatocytes (Bataillé et al. 2005). Together these results show that *Gcm* plays a repressive role in crystal cell development and at the same time it promotes plasmatocyte differentiation.

How the expression of *lozenge* is initiated and limited to the first rows of cells of the embryonic anlage is not determined. Although Notch signaling is sufficient and required in hemocytes for *lozenge* expression in larval lymph gland cells (Duvic et al. 2002; Lebestky et al. 2003), this is not the case during embryonic development (Bataillé et al. 2005). Nevertheless, Notch signaling plays a role in determining the number of embryonic crystal cells (Lebestky et al. 2003; Bataillé et al. 2005). *lozenge* expression is also dependent on Serpent and its maintenance can be assured by a regulatory loop through an enhancer activated by the Lozenge/Serpent complex (Ferjoux et al. 2007). Moreover, other crystal cell specific *loci* such as pro-Phenoloxidases contain such Lozenge/Serpent binding sites (Ferjoux et al. 2007). However, *lozenge* expression is maintained in crystal cells during larval stages while *serpent* is no longer detectable in these cells.

The binding of Serpent to DNA is dependent on a highly conserved zinc finger motif. Similarly to all GATA factors, Serpent has two of these motifs, the C-finger is essential for DNA biding and the N-finger has been shown to mediate the interaction with the Friend of

GATA (FOG) family gene *u-shaped* (*Ush*) (Waltzer et al. 2010). By stage 10 all prohemocytes express *Ush* (Fossett et al. 2001). Later in development, by embryonic stage 13, Lozenge positive cells start to reduced *Ush* expression while in plasmatocytes *Ush* continues to be maintained at high expression levels. In *Ush* mutants the number of crystal cells increases significantly (Fossett et al. 2001). Moreover, the interaction of Serpent N-Zinc finger with Ush modulates the expression of *Crq* in plasmatocytes (Waltzer et al. 2010). Hence, *Ush* adds another layer in the gene regulatory network that governs the cross-talk between specification of crystal cells and plasmatocytes.

At early stage 12, plasmatocytes start to migrate throughout the embryo along stereotypical routes (Tepass et al. 1994) and eventually colonize the germ band in a process that requires the dissolution of epithelial cell-cell junctions (Siekhaus et al. 2010). Pvf2 and Pvf3 are two ligands of the PDGF-and VEGF-receptor related (Pvr), that are expressed in epithelial cells along the migration routes of embryonic hemocytes and are proposed to guide this process (Cho et al. 2002). Later work has shown that the Pvf ligands are used as trophic factors that are essential for germband invasion (Brückner et al. 2004; Parsons & Foley 2013). During migration, plasmatocytes start to phagocyte dead cells and are essential for the proper development of the central nervous system (Defaye et al. 2009). In contrast, crystal cells do not migrate and remain in a cluster close to the anterior midgut. No role has been attributed to crystal cells during embryogenesis. It is possible that they form a pool of cells ready to act after larval hatching. This is the first observed behavioral difference seen between plasmatocytes and crystal cells.

In late embryogenesis, plasmatocytes stop *serpent* and *Gcm* expression and start to express other larval plasmatocyte markers

such as *Hemolectin* (Goto et al. 2003). Once the larva hatches the plasmatocytes populate the body cavity and continue to proliferate. Crystal cells maintain *lozenge* expression and similarly enter the body cavity of the larva (Lebestky et al. 2000) but have never been observed proliferating (Lanot et al. 2001; Rizki 1957).

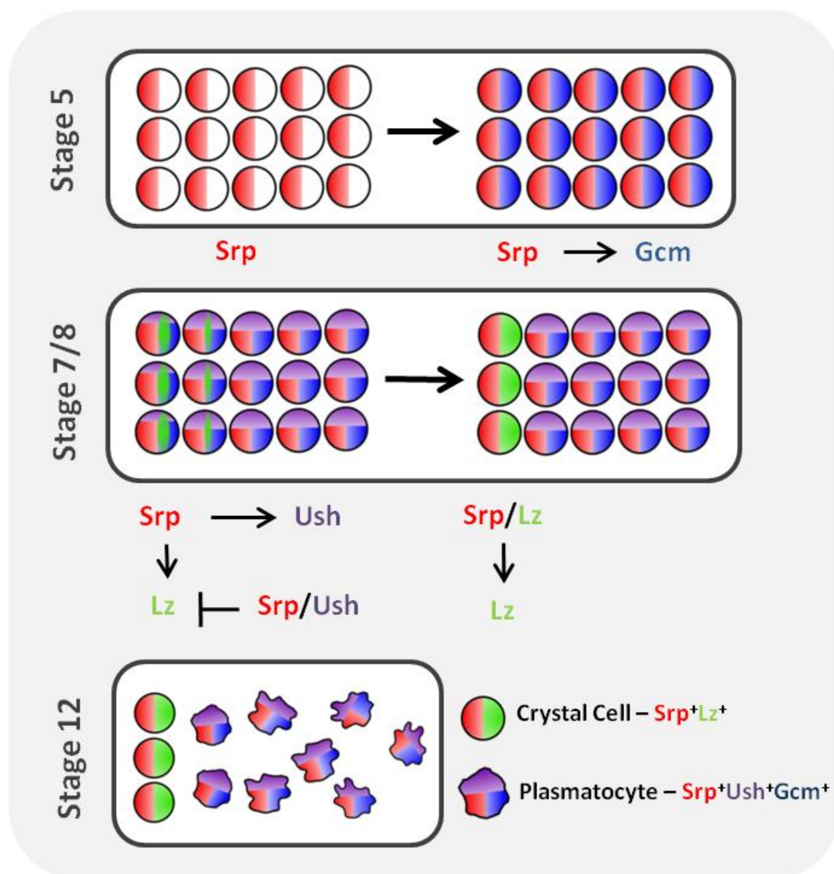


**Figure 1.2. – Embryonic hematopoiesis.** At embryonic stage 5 an anlage of cells start to express *serpent* (*srp*) in the head mesoderm. These srp$^+$ cells are at this stage committed to differentiate into hemocytes. Following serpent expression all prohemocytes induce *glial cell missing* (*Gcm*) expression. At stage 7 all prohemocytes initiate *u-shaped* (*ush*) expression. At the same time the anterior-most rows of prohemocytes are *lozenge* (*lz*) positive. 40% of Lz$^+$ prohemocytes discontinue lozenge expression, maintain high *ush* expression and differentiate into plasmatocytes as the majority of prohemocytes. The other 60% of Lz$^+$ prohemocytes continue with lozenge expression, diminish *ush* expression and differentiate into mature crystal cells. By embryonic stage 12 plasmatocytes start to migrate in stereotypical routes while crystal cells remain clustered.

## 1.3 Larval hematopoiesis

### *1.3.1 Lymph gland development and structure*

In the *Drosophila* larva, hematopoiesis proceeds within the lymph gland. It consists of three to four paired lobes surrounding the anterior part of the dorsal vessel (Jung et al. 2005). The primary lobes of the lymph gland are bigger and, in homeostatic conditions, contain prohemocytes as well as differentiating and mature plasmatocytes and crystal cells. However, the lymph gland is an immune responsive organ and if a parasitoid wasp female punctures the cuticle of the larva to deposit an egg, the lymph gland will trigger the production of lamellocytes at the expense of prohemocytes (Makki et al. 2010). The lymph gland is differentiated in the embryo from thoracic lateral mesoderm cells (Holz et al. 2003). At stage 11 the transcription factor *collier* (*col*) is expressed in two clusters of cells in T2 and T3 segments that eventually coalesce to form the paired lobes of the lymph gland (Crozatier et al. 2004). Looking at the expression pattern of another transcription factor, *odd-skiped* (*Odd*), one can see the same T2 and T3 clusters plus some cells in T1 that will also make part of the lymph gland (Krzemien, Crozatier, et al. 2010). Similarl to embryonic hemocytes, *serpent* is expressed in lymph gland hemocytes but only after *collier* (Crozatier et al. 2004). Unlike the embryonic hemocytes, that lose *serpent* expression after hatching, lymph gland hemocytes will maintain *serpent* expression throughout larval stages. In late embryogenesis the lymph gland precursor consists of a pair of lobes, around 20 cells each, flanking the dorsal vessel. Only the 3 to 4 most posterior cells of each lobe maintain high *collier* expression (Crozatier et al. 2004). This differential expression pattern starts the regionalization of the lymph gland that is evident in

third instar larva, when one can distinguish three regions: the Posterior Signaling Center (PSC) formed by the cells maintaining *collier* expression, the medullary zone composed of prohemocytes and the cortical zone with differentiated hemocytes (Jung et al. 2005). These three zones can be distinguished by the expression of different markers (Jung et al. 2005).

The PSC in third instar larva is composed of 30 to 45 cells and is defined by the expression of *Antennapedia*, *collier* and *Serrate* (Mandal et al. 2007; Lebestky et al. 2003). Both *Antennapedia* and *collier* mutants lose the PSC (Figure 1.2). Without a functional PSC, prohemocytes of the medullar zone abandon their characteristic quiescence and begin differentiating into plasmatocytes and crystal cells (Mandal et al. 2007). Moreover, in the absence of PSC lymph glands cannot produce lamellocytes upon challenge, probably by the lack of prohemocytes (Krzemień et al. 2007). This set of evidences show that the PSC is necessary to maintain the hematopoietic precursors in the lymph gland medullary zone (Mandal et al. 2007). This role of the PSC involves also the Hedgehog pathway. *hedghog* is expressed in PSC cells and the receptor, *patched* (*ptc*) in medullary zone cells. Mutants for *hedgehog* still have the specification of the PSC but similarly to *collier* and *Antennapedia* mutants lose the prohemocytes in the medullary zone (Mandal et al. 2007). One other genetic cascade essential to maintain the prohemocytes undifferentiated state is the JAK/STAT pathway. The PSC acts in a non-cell-autonomous way to maintain high levels of JAK/STAT activity in the medullary zone cells (Krzemień et al. 2007). When a larva is infected with a parasitoid wasp egg it is necessary to downregulate JAK/STAT activity in the medullary zone to differentiate lamellocytes (Makki et al. 2010). The complete switch off of

JAK/STAT activity in the medullary zone is dependent on Latran, a protein that forms a heterodimer with Domeless, the receptor for JAK/STAT cytokines, and antagonizes its activity (Makki et al. 2010).

### *1.3.2 Hematopoiesis within the lymph gland*

Hemocytes inside the medullary zone, where they lack differentiation markers, migrate into the cortical zone and they start to express early differentiation markers such as *Hemolectin* and *Peroxidasin* in second larval instar (Jung et al. 2005). Mature molecular markers for plasmatocytes (P1) and crystal cells (pro-Phenoloxidase) are expressed only in the late second instar larvae. However, cell lineage tracing experiments show that in second instar prohemocytes in the medullary zone are already restricted to plasmatocyte or crystal cell fate (Krzemien, Oyallon, et al. 2010). Activation of *lozenge* expression requires Notch signaling with Serrate acting as ligand (Lebestky et al. 2003). Although PSC cells express *Serrate* they are not the source of the ligand for crystal cell formation (Crozatier et al. 2004). Instead, *Serrate* expressing cells scattered inside the lymph gland may be the ones inducing Notch signaling (Crozatier et al. 2004; Lebestky et al. 2003) (Figure 1.2). Notch activated cells will induce *lozenge* expression and both proteins will cooperate in the transcription of crystal cell specific genes (Terriente-Felix et al. 2013). After maturation, crystal cells maintain a vital high *Notch* expression, since without Notch crystal cells burst (Mukherjee et al. 2011). The maintenance of Notch signaling in mature crystal cells in no longer dependent on Serrate but dependent on *similar* (*sima*), a hypoxia-inducible factor (Mukherjee et al. 2011). This ligand independent mechanism can help

to explain how crystal cells survive in circulation, where they do not have cells in contact.

In vertebrates, hematopoietic stem cells (HSC) residing in hematopoietic organs give rise to all blood cell types. Stem cells are defined by their unlimited capacity of cell renewal and to generate multiple cell types (Seaberg & van der Kooy 2003) and their maintenance is dependent on niche signals (Martinez-Agosto et al. 2007). Since the PSC in the lymph gland produces signals to maintain the undifferentiated state of prohemocytes in the medullary zone it has been tempting to postulate the existence of stem cells inside the lymph gland. Cell lineage analysis only suggests the existence of stem cells early in lymph gland development (Minakhina & Steward 2010). The absence of stem cell markers and BrDU chase experiments suggest that no slowly dividing stem cell is present in the medullary zone of the lymph gland during larval stages (Krzemien, Oyallon, et al. 2010). Interestingly in these experiments some cortical zone crystal cells retain BrDU. Since mature crystal cells do not divide this suggests that during their differentiation process replicate DNA or are differentiated from cells after DNA replication. Supporting these results, more recently it has been suggested that crystal cells enter endoreplication (Terriente-Felix et al. 2013).

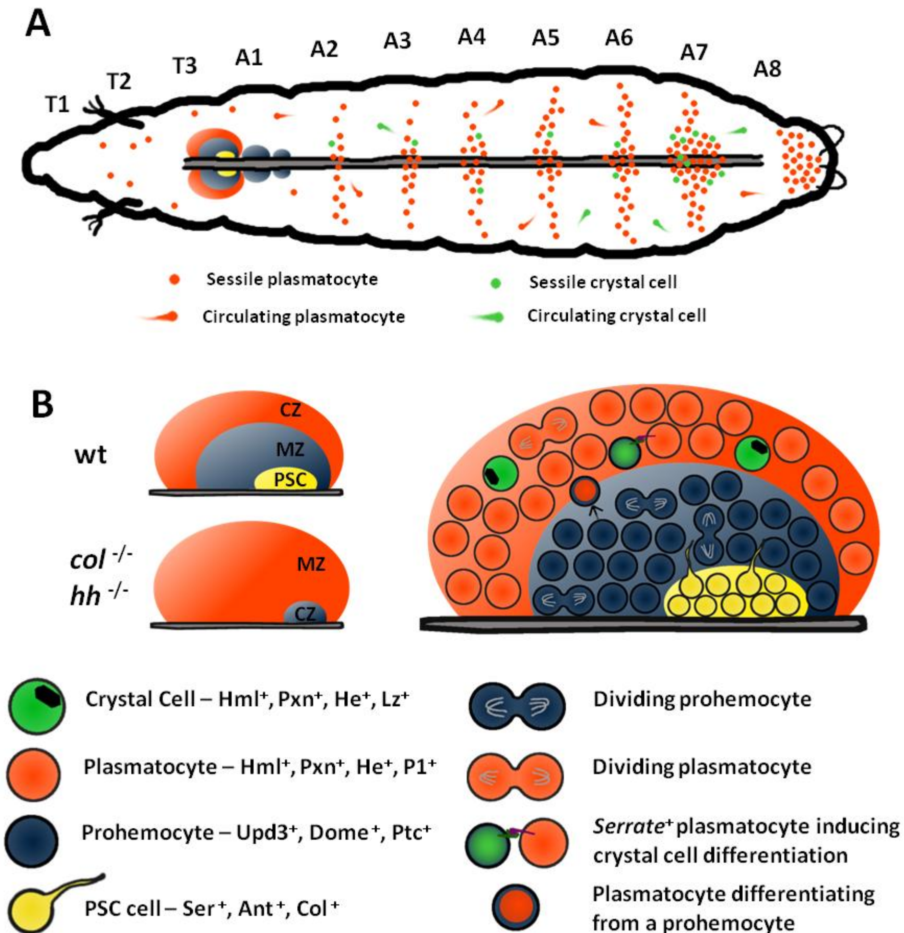


**Figure 1.2 – Hemocytes during larval stages (A)** The majority of plasmatocytes (orange dots) and crystal cells (green dots) during larval stages are clustered in patches underneath the epidermis. The number of clustered hemocytes is higher in the posterior segments of the larva. The lymph gland is located in the anterior part of the dorsal vessel. **(B)** The lymph gland is structured in three distinct zones: Posterior Signaling Center (PSC), Medullary Zone (MZ) and Cortical Zone (CZ). PSC cells produce long protrusions that may serve to enhance signaling to the MZ cells. Prohemocytes in the medullary zone divide mostly in first and second larval instar becoming more quiescent in the third instar. In the CZ it is possible to find mature plasmatocytes and crystal cells.

### 1.3.3 Hematopoiesis outside the lymph gland

After hatching embryonic hemocytes persist in the larva and can be found in circulation or immotile in direct contact with the epidermis forming hemocyte sessile patches/clusters (Lanot et al. 2001) (Figure 1.2). Hemocyte load increases drastically throughout larval development from less than 200 cells in early first instar up to 6000 cells just before pupariation (Lanot et al. 2001; Makhijani et al. 2011). In homeostatic conditions, the number of sessile hemocytes is always higher than circulating ones, and constitute up to two thirds of the total cell count up until mid third instar larva (Lanot et al. 2001). In first instar larvae, the majority of hemocytes accumulate in the last segment and in lateral patches surrounding the oenocytes (Makhijani et al. 2011). Throughout development, with the increase in hemocyte load, they start to form dorsal patches in the epidermis and accumulate along the dorsal vessel. A discrete population of hemocytes also populates the proventriculus (Zaidman-Rémy et al. 2012) and a considerable number is attached to imaginal discs (Lanot et al. 2001).

Hemocyte homing to lateral patches and dorsal patches is partially dependent on Peripheral Nervous System (PNS) neurons (Makhijani et al. 2011). Also, PNS neurons contribute with unknown trophic signals towards hemocyte survival (Makhijani et al. 2011). Yet, the higher proliferation rate observed in sessile hemocytes when compared to circulating is not dependent on PNS neurons. Haemocoelic hemocytes form a dynamical population of cells with sessile hemocytes entering circulation and circulating hemocytes attaching to hemocyte clusters (Babcock et al. 2008; Welman et al. 2010).

As stated above, lymph gland hemocytes do not enter circulation in homeostatic conditions until pupariation (Holz et al. 2003; Honti et al. 2010). The haemocoelic hemocyte load increases throughout development because hemocytes are mitotically active. However, only plasmatocytes have been shown to divide, mature crystal cells do not (Lanot et al. 2001; Rizki 1957). This suggests that crystal cells are differentiated in circulating and/or sessile hemocytes. The formation of crystal cells in the larva is dependent on Notch signaling (Duvic et al. 2002; Lebestky et al. 2003). This is evident when Notch signaling is disrupted in larvae carrying a *Notch* thermo sensitive allele raised at restrictive temperature (Duvic et al. 2002). In this experiment crystal cell numbers decreases in hemocyte clusters and in the lymph gland.

Notch receptor is activated by two different ligands in Drosophila: Serrate and Delta. Only Serrate plays a role in crystal cell differentiation both in the lymph gland and in sessile hemocytes (Duvic et al. 2002; Lebestky et al. 2003). Since a *Notch* expressing cell requires cell contact with a *Serrate* expressing cell for its activation, it is tempting to propose that crystal cells are differentiated in hemocyte clusters where they make cell contacts with other hemocytes, epidermal cells and neurons (Lanot et al. 2001; Makhijani et al. 2011).

Embryonic derived hemocytes also have the capacity to differentiate into lamellocytes, a phenomenon first demonstrated by the work of Markus and colleagues in 2009 (Márkus et al. 2009). In challenging cell transfer experiments the authors show that cells taken from the most posterior hemocyte cluster have the capacity to differentiate into lamellocytes. The authors concluded that a pool of lamellocytes progenitor cells exists in the sessile hemocyte patches

and suggest the patches act as hematopoietic tissue. However, the existence of *bona fide* lamellocyte progenitors is questionable since later work demonstrates that lamellocytes can differentiate from mature plasmatocytes (Honti et al. 2010; Stofanko et al. 2010). During a parasitoid wasp egg infection plasmatocytes down-regulate plasmatocyte specific genes and start to express lamellocyte specific factors (Honti et al. 2010).

Overall, the evidences gathered so far indicate that hematopoietic cell fate decisions occur in hemocytes outside the lymph gland. While plasmatocytes are mitotically active, crystal cells must differentiate outside the lymph gland. Furthermore, similarly to the lymph gland case, haemocoelic hemocytes can change their normal development upon parasitoid infection and produce lamellocytes. How the number of crystal cells and lamellocytes is controlled is certainly an important topic for further investigation that should reveal how circulating cells in animals achieve the correct concentration/proportion to perform their functions at optimal efficiency.

## 1.4 Objectives of this work

The maintenance of the plasmatocyte/crystal cell ratio during *Drosophila melanogaster* development is striking since plasmatocytes are mitotically active cells while mature crystal cells do not divide (Rizki 1957). One possibility to explain this phenomenon is that a population of prohemocytes exists in circulation or in sessile clusters with the ability to differentiate into crystal cells. Another possibility is that some mature plasmatocytes change their fate during development into crystal cells. Although the differentiation of crystal cells outside the lymph gland is poorly studied we know that Notch

signaling is essential for their differentiation. One interesting aspect of Notch signaling is that the activation of the receptor requires stable cell contacts (Fiúza & Arias 2007). The aggregation of cells in clusters may provide the opportunity for Notch activation in hemocytes. With this in mind we conceived a set of experiments to test if hemocyte clusters are essential for crystal cell development. At the same time we wanted to know if crystal cells derive from a population of cells predetermined to form crystal cells with expansion capacity or if they are derived from mature plasmatocytes. Since hemocytes in clusters constitute the biggest compartment of hemocytes in the larva we also searched for genetic players that affect the number of hemocytes in clusters. Overall the information resulting from these experiments should help us better define where and how hematopoiesis occurs in such an important model system for the study of the innate immune system.

# *Hemocyte clusters function as a hematopoietic tissue*

## 2.1 Summary

Blood cells can be found in virtually all species of coelomate animals. Their functions are usually compartmentalized into different cell types for which the correct establishment of proper numbers and ratios is essential for homeostasis (Almeida et al. 2005). This depends upon a regulated balance between proliferation and differentiation mostly carried out in the hematopoietic organs (Hartenstein 2006). In *Drosophila melanogaster*, the larval hematopoietic organ (lymph gland) produces two types of mature hemocytes (blood cells), plasmatocytes and crystal cells. Strikingly, in homeostatic conditions, hemocytes produced in the lymph gland are not released until pupariation. Yet, as larval development proceeds, the numbers of circulating hemocytes of both types increase, an observation difficult to reconcile with the post-mitotic character of crystal cells (Rizki 1957; Lanot et al. 2001). In this light, it has been proposed that hematopoietic properties must reside elsewhere, namely within the clusters of hemocytes found in close association with epidermal and neuronal cells along the larval body axis. Here, we show that hemocyte clusters function as a *bona fide* hematopoietic tissue as their structure is necessary for Notch-dependent differentiation of crystal cells. Moreover our results suggest that, in contrast to the lymph gland, crystal cells formed in clusters do not derive from prohemocytes but from the transdifferentiation of plasmatocytes. The existence of this novel hematopoietic tissue, relying on structure-dependent signaling events to promote blood homeostasis, creates a new paradigm for addressing outstanding questions in *Drosophila* hematopoiesis and establish further parallels with vertebrate systems.

## 2.2 Introduction

Blood cells can be found in almost any species of coelomate animals. Their functions are varied and include: gases transport, phagocytosis, extracellular matrix deposition and antibody production. The different functions performed by blood cells are to some degree compartmentalized in different cell types. Thus, the correct establishment of different blood cell numbers/ratios is essential for their proper function (Almeida et al. 2005). Some mature blood cells retain the ability to proliferate when in circulation but the majority of blood cell proliferation and differentiation occurs in hematopoietic organs (Grigorian & Hartenstein 2013). These organs provide the correct 'molecular environment' for the control of cell proliferation and differentiation. Thus, the study of hematopoietic organ structure and function is essential to understand how different mature blood cells numbers are controlled.

In *Drosophila melanogaster*, embryonic hematopoiesis produces two different types of mature hemocytes (blood cells): plasmatocytes and crystal cells. Plasmatocytes are phagocytic cells that are often functionally compared to vertebrate macrophages (Evans et al. 2003). Crystal cells are non-phagocytic cells dedicated to produce proPhenoloxidase, an essential component of the melanization cascade (Binggeli et al. 2014). Both plasmatocytes and crystal cells are generated during embryogenesis and persist into larval stages forming a population of cells termed haemocoelic hemocytes (Holz et al. 2003; Meister & Ferrandon 2012). In a seminal work describing hemocyte changes during larval development, Rizki reports that haemocoelic hemocytes increase in number through time while maintaining the proportion of crystal cells at roughly 4% (Rizki

1957). Plasmatocytes increase in number because they are mitotically active cells but mature crystal cells do not divide (Rizki 1957; Lanot et al. 2001; Krzemien, Oyallon, et al. 2010; Makhijani et al. 2011). This observation implies that crystal cells differentiate/mature during larval stages.

The larva possesses a hematopoietic organ, the lymph gland, where plasmatocytes and crystal cells are differentiated from prohemocytes. Prohemocytes residing in the medullary zone of the lymph gland are influenced by cells from the Posterior Signaling Center (PSC) to maintain their quiescent state or to differentiate into mature plasmatocytes and crystal cells (Crozatier et al. 2004; Mandal et al. 2007). During the differentiation process, cells migrate and occupy the most cortical zone of the lymph gland (Jung et al. 2005; Krzemien, Oyallon, et al. 2010). Similarly to what happens in haemocoelic hemocytes, in the lymph gland cortical zone, mature plasmatocytes are still capable to divide but mature crystal cells enter a post-mitotic state (Krzemien, Oyallon, et al. 2010). An essential aspect of *Drosophila*'s larval hematopoiesis is that hemocytes produced in the lymph gland do not disperse from the organ until pupariation or upon immune challenge such as parasitoid wasp egg infection (Holz et al. 2003; Honti et al. 2010). Hence, in homeostatic conditions, differentiated hemocytes in the lymph gland do not contribute to haemocoelic hemocyte population. Without contribution from the lymph gland or cell division, how haemocoelic crystal cells augment in number during larval development remains an open question.

Although we do not have much information on how crystal cells are formed outside the lymph gland one important aspect is that Notch signaling is necessary to form these cells (Duvic et al. 2002;

Lebestky et al. 2003). In the lymph gland the role of Notch signaling in crystal cell formation is cell autonomous. (Lebestky et al. 2003; Mukherjee et al. 2011; Small et al. 2014). Notch activation is sufficient in hemocytes to induce the expression of *lozenge*, the first known transcription factor in crystal cell development (Lebestky et al. 2000). One requirement of Notch signaling is that it requires cell contact because the two Notch ligands of *Drosophila*, Serrate and Delta, are membrane bound proteins (Fiúza & Arias 2007). In the haemocoelic compartment hemocytes can make cell contacts because the majority of them are sessile cells that form clusters in the subepidermal layer of the body cavity (Lanot et al. 2001). Hemocytes in clusters are densely packed and linked through interdigitations. Neurons from the Peripheral Nervous System (PNS) attract hemocyte to the cluster location and provide trophic signals for their maintenance (Makhijani et al. 2011). Moreover, when they are sessile in clusters, plasmatocytes have a higher division rate than when they are in circulation.

In the last two abdominal larval segments, hemocytes are clustered in higher densities and for this reason they were named Posterior Hematopoietic Tissue (PHT) (Kurucz, Váczi, et al. 2007). To test the origin of lamellocyte precursors Márkus and colleagues took cells from these two last clusters and injected them into another larva (Márkus et al. 2009). Transferred cells have the ability to differentiate into lamellocytes. For these reasons hemocyte clusters are now considered hematopoietic tissue (Honti et al. 2014). This is a very attractive hypothesis because sessile hemocytes in clusters constitute the bigger compartment of hemocytes in the larva (Lanot et al. 2001). However, to consider the hemocyte clusters as hematopoietic tissue, we need evidence that their structure is

necessary to control cell proliferation and/or cell fate choice decisions.

The hemocytes in clusters are in a dynamic equilibrium with circulating hemocytes (Babcock et al. 2008; Welman et al. 2010). Hemocytes can enter and leave the clusters, which gives the opportunity for hemocytes produced in clusters to contribute to the increase in circulating crystal cells. Thus, hemocyte clusters are a good candidate location to study haemocoelic crystal cell development. In this chapter we ask if crystal cell precursors differentiate/mature in haemocoelic hemocytes and if the structure of hemocyte clusters is important for this differentiation.

## 2.3 Material and Methods

### *2.3.1 Fly stocks and parasitoids maintenance*

All fly stocks were maintained in standard fly food at room temperature. Experiments were performed at 25°C except for RNAi experiments that were performed at 29°C. The following stocks with stock numbers in brackets were obtained from the Bloomington Stock Center: Lz-GAL4 UAS-mCD8GFP (X) (6314); Lz-GAL4 (X); UAS-GFP (II) (6313); HmlΔ-GAL4 UAS-2xEGFP(II) (30140); UAS-String$^{RNAi}$ (34831); UAS-White$^{RNAi}$ (33613); UAS-FLP UbiFRTSTOPStinger (28282); Trio-GAL4 (48798); Notch-GMR29F08-GAL4 (49492); Notch-GMR29H07-GAL4 (45189); Notch-GMR30A01-GAL4 (45554); Notch-GMR30C04-GAL4 (41342); Notch-GMR30C06-GAL4 (49528); Notch-GMR30D10-GAL4 (49632); Notch-GMR30G07-GAL4 (45561); Notch-GMR57F04-GAL4 (46387). The line UAS-White$^{RNAi}$ was used as control for RNAi experiments. The following stocks with stock numbers in brackets were obtained from

the Vienna Drosophila Resource Center: Cg9313$^{RNAi}$ (103600) Notch$^{RNAi}$ (100002), Serrate$^{RNAi}$ (108348) Delta$^{RNAi}$ (109491). The line Cg9313$^{RNAi}$ was used as a control line for RNAi experiments. The line HmlΔ-nuclearDsRed was a kind gift from Marc Dionne. The Eater-GAL4 (X) and Eater-GAL4 (II) were a kind gift from Robert A Schultz. Leptopilina boulardi strain G486 females were allowed to lay eggs in second instar *Drosophila Dif* mutants at room temperature. Adult parasitoids were maintained in fly food vials with a drop of honey.

### *2.3.2 Larva staging*

Around 20 female flies were placed in a cage with a food plate with yeast. Egglays took place at 25ºC for 6 hour. At ~72h midpoint after egglay 2$^{nd}$ instar larvae were selected based on spiracle morphology and transferred into a new food plate. After 2h larvae that molted into 3$^{rd}$ instar were selected and transferred into a food tube. This first time point is referred 2h after 3$^{rd}$ instar.

### *2.3.3 Flow cytometry analysis and cell viability assay*

Larvae were bled in 200-400 µl Ringer's solution. The number of larvae greatly depends on the experiment but at least 10 larvae were used in each sample. The hemocyte dilution was passed through a 30µm filter to exclude cell aggregates. The samples were maintained on ice until acquisition. Acquisition was performed in CyAn ADP cytometry Analyzer (DAKO Cytomation, Beckman Coulter) with Summit software (DAKO). Hemocyte population was gated in Forward Scatter (FSC) and Side Scatter (SSC) channels and single events in FSC and Pulse Width channels. GFP and DsRed were measured in the appropriate channels. The results were analyzed using the Flowing Software (version 2.5.0). To analyze cell viability a

stock solution of Propidium Iodide (PI) was diluted in 200µl of Ringer’s solution to a final concentration of 2µg/ml. Positive events for PI were considered dead or dying cells.

### *2.3.4 Cell cycle analysis*

Hemocytes were centrifuged for 5 minutes at 500g at 4ºC and ressuspended in 400µl Ringer’s solution. 4,6ml of ice cold ethanol was added to each sample while vortexing. Cells were incubated for 2h at 4ºC and centrifuged for 5 minutes at 500g. The pelleted cells were ressuspended in 1ml PBS and centrifuged again for 5 minutes at 500g. Cells were ressuspended in 400µl PI staining solution (10µg/ml PI + 100µg/ml RNAse A) and incubated form 30 minutes at room temperature. Samples were immediately analyzed by flow cytometry.

### *2.3.5 Movie analysis*

Male larvae raised at 25°C on standard fly food plates 72h±3h were selected. They were briefly washed in Ringer’s solution and dried on a filter paper. Double sided scotch tape was glued on a cover slip and a spacer of tape was added on the edges. The selected larva was glued in the tape with the ventral side down and a cover slip was put in the dorsal part of the larva. Hence, the larva is stuck between two cover slips. This way to mount the larva does not allow for larva movement but maintains the larva alive for more than 12h in a humid chamber. The pressure from the cover slip affects the A7 dorsal cluster, most probably because this affected the hemolymph circulation. Hence we imaged more anterior clusters that were not so affected. The larva was mounted in an inverted confocal spinning microscope (Andor Revolution xD). The temperature of the

slide chamber was maintained at 25°C and 95% relative humidity. A Z-stack of pictures raging 28μm was taken every 1m30s for the GFP and RFP channel for 3h. At the end of the movie each larva was checked if it was still alive by ascertaining a beating dorsal vessel and movement of mouth parts. Only one larva died during the process. Z-stacks were then analyzed manually in FIJI software (Schindelin et al. 2012).

### *2.3.6 Total hemocyte loads counts and crystal cells counts*

To estimate hemocyte concentration in the hemolymph six wandering male or female larvae were selected, briefly washed in Ringer's solution and dried in filter paper. The six larvae were pooled in a glass well and bled by rupturing the cuticle in the ventral side to avoid disturbance of sessile hemocytes in the dorsal part where they are more abundant. All the hemolymph was collected and pooled in a 0,5ml microcentrifuge tube. 1μl of hemolymph was diluted in 9μl of Ringer's solution. 9,5 μl of hemolymph dilution was loaded into a Neubauer chamber and hemocytes counted in all squares of $1mm^2$ area. This way the hemocyte concentration can be estimated by the formula: [number of counted cells]*$10^5$ cells/ml. To estimate the total number of hemocytes in circulation per larva we bled one larva in 15μl Ringer's solution in a 10 reaction well slide (∅ 5mm Marienfeld). To count crystal cells in larva we performed a 70°C heat shock treatment for 10min. With this treatment mature crystal cells auto-melanize and it is possible to count crystal cell numbers trough the cuticle.

### *2.3.7 Hemocyte immunohistochemistry*

Hemocytes were allowed to settle on a glass slide in a humid chamber for 10min and fixed with 4% formaldehyde solution for 20 min. After fixation cells were washed three times with PBS and blocked with PBST (PBS + 0,1% TritonX, +1% normal goat serum) for 30 min. After washing the cells with PBS the primary antibody was added at the correct dilution and cells incubated overnight at 4°C. Cells were then washed 3 times with PBS for 15 min and the secondary antibody added in the correct concentration. Cells were incubated for 3h at room temperature or at 4ºC overnight with the secondary antibody. The secondary antibody was washed three times with PBS. DAPI was added and incubated for 3 min followed by 3 washes with PBS. Slides were mounted with 80% glycerol solution and kept at 4°C before image acquisition. Antibodies used: anti-*lozenge* (1:100 dilution, Developmental Studies Hybridoma Bank) and anti-*Nimrod* (1:100 dilution, mixture of P1a and P1b antibodies, kind gift from Instiván Andó)

### *2.3.8 Imaging*

To count $Hml^+$ and $Lz^+$ cells, each larva was immobilized between two slides. This permits to focus the hemocytes that are right underneath the cuticle along the body axis but in the last segment they are out of focus. For this reason the hemocytes in this segment were excluded. Pictures were taken in a Zeiss' Stereo Lumar fluorescence stereoscope (Zeiss SteREO Lumar.V12). Larvae were rolled to take pictures from 4 different angles. Hemolymph smears with live or fixed cells were imaged in a Leica DMRA2 microscope coupled with a CoolSNAP HQ CCD camera. Objectives used were 10x, 40x and 63x.

### *2.3.9 Phagocytosis assay*

To test phagocytosis activity early third stage larvae were injected with 69nl of pHrodo Red *E.coli* BioParticles (1mg/ml; Molecular Probes). Injected larvae were maintained in yeast for 1h hour before ~10 larvae were bled in 20µl Ringer's solution. Hemocytes were allowed to settle for 20min at room temperature in a humid chamber and pictures were taken immediately.

### *2.3.10 Cluster disruption assay*

Pools of ~20 early third instar male larvae were selected and transferred to fresh yeast in a plastic petri dish maintained in a humid chamber. Every 1h30m larvae were taken from yeast, cleaned in Ringer's solution and dried on filter paper. Groups of ~5 larvae were rolled several times by pressing a cover glass to disrupt hemocyte clusters. Control larvae were maintained on yeast. Before larva bleeding the two groups were subjected to cluster disruption to sample both circulating and sessile hemocytes.

### *2.3.11 Statistical analysis*

Students t-tests were used to compare two treatments. ANOVAS were performed when several comparisons were necessary and Dunnett's multiple comparison tests were applied to test differences between pairs of treatments. Statistical test and graphics were performed in Prism v5.01 (GraphPad Software) and R v2.15.2

## 2.4 Results

### 2.4.1 The number of crystal cell precursors increase during larval development without cell proliferation

In homeostatic conditions, the larval haemocoelic hemocyte population is constituted by plasmatocytes and crystal cells. It is possible to distinguish these two cell types with several combinations of cell markers (Kurucz, Váczi, et al. 2007). To analyze hemocyte development throughout larval development we chose two live genetic drivers: HmlΔ-nuclearDsRed and Lozenge-GAL4 in combination with UAS-EGFP/UAS-mCD8GFP (Makhijani et al. 2011; Lebestky et al. 2000). With this combination of markers we can distinguish $Hml^{+}Lz^{-}$ and $Hml^{+}Lz^{+}$ cells in sessile hemocytes through the larval cuticle (Figure 2.1A-A'). $Hml^{+}Lz^{-}$ cells are plasmatocytes while $Hml^{+}Lz^{+}$ cells are fully mature crystal cells or differentiating crystal cells (Lebestky et al. 2000). When analyzing total hemocyte counts by cell morphology it is indisputable that both plasmatocytes and crystal cells increase throughout larva development (Rizki 1957; Lanot et al. 2001). Since no crystal cell is dividing it is reasonable to assume that new crystal cells are differentiated throughout development. This can be achieved by inducing new crystal cell precursors during larva development or simply by maturation of precursors cells already present in the larva body cavity.

Lozenge is the first marker known in the genetic cascade that leads to crystal cell differentiation (Lebestky et al. 2000). Hence, we checked to see if $Hml^{+}Lz^{+}$ cells increase in number throughout the third instar larva or if its number is fixed and crystal cell number increases due to maturation of $Lz^{+}$ precursors. We counted the sessile hemocyte population in the larva except the tail that is hard to

image (Makhijani et al. 2011) (see methods). The total number of Hml$^{+}$ cell increases throughout the third instar larva development in males and females (Figure 2.1B). The number of Lz$^{+}$ cells also increases in the same time period (Figure 2.1C). In females there is no difference in the number of Lz$^{+}$ in the last 16h of development. With this, late third stage larva females have less crystal cells than males which is not common in the majority of fly stocks where females tend to have a higher number of crystal cells than males (see results below). Nevertheless, the results clearly show that Hml$^{+}$Lz$^{+}$ cells are increasing in number during the third instar larval development. The proportion of Hml$^{+}$Lz$^{+}$/Hml$^{+}$Lz$^{-}$ cells even increases in the first 24h of third larval instar development while Hml$^{+}$Lz$^{-}$ cells are also increasing (Supplementary Figure 2.1). Therefore, we can conclude that there is no fixed number of Hml$^{+}$Lz$^{+}$ cells throughout development.

Although mature crystal cells do not divide it is still possible that Lz$^{+}$ precursors proliferate before full crystal cell maturation. To check if proliferation of Lz$^{+}$ cells plays a role in the increase of crystal cell loads we inhibited cell division using RNAi for *string*. String is an essential phosphatase for entering mitosis (Edgar & O'Farrell 1990). When we drive *string* RNAi with HmlΔ-GAL4 driver there is a significant reduction in the concentration of hemocytes, which demonstrates the necessity of *string* for hemocyte proliferation (Figure 2.1E). Only females were tested in this experiment because the number of males is much reduced in this cross. Next, we asked if *string*$^{RNAi}$ expressed only in Lz$^{+}$ cells would decrease the hemocyte concentration and/or specifically the number of crystal cells. *String*$^{RNAi}$ driven by Lz-GAL4 does not affect the hemocyte concentration (Figure 2.1E). To visualize crystal cells we used a heat-shock

treatment that blackens mature crystal cells (Rizki 1957; Neyen et al. 2014). To estimate crystal cell numbers changes we counted the number of cells in the dorsal part of the seventh abdominal segment (see methods and below). When driven by Lz-GAL4 driver the number of crystal cells is not reduced (Figure 2.1F). This result suggests that once a cell becomes Lz$^+$ it enters a postmitotic state. Hence, the increase in Hml$^+$Lz$^+$ cell numbers observed in third larval instar is due to *de novo* differentiation of Hml$^+$Lz$^+$ cells and not their proliferation.

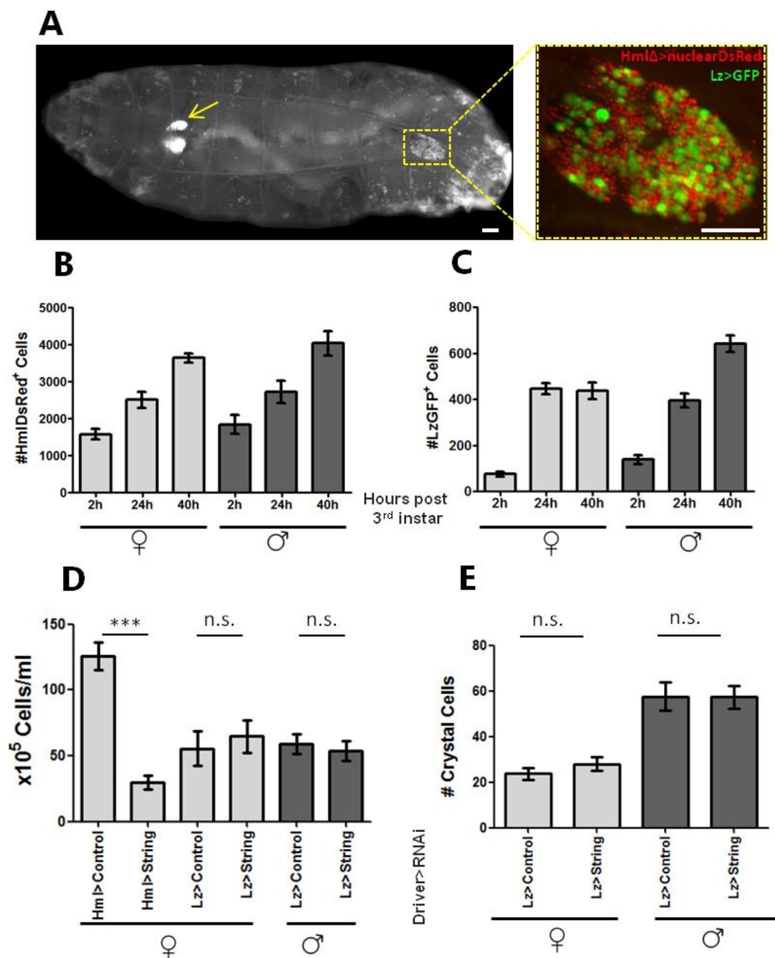

**Figure 2.1 (*preceding page*) – Hml$^{+}$Lz$^{+}$ cells increase during larval development without cell proliferation. (A)** Dorsal view of a third instar larva where hemocyte nuclei are marked with HmlΔ-nuclearDsRed. It is possible to see the lymph gland (arrow) sessile hemocytes along the body axis and a big cluster of cells in the A7 segment (square). **(A')** In the magnification of hemocyte cluster it is possible to see that is constituted by Hml$^{+}$Lz$^{-}$ and Hml$^{+}$Lz$^{+}$ cells. **(B)** It is possible to see a increase throughout third instar larval development of Hml$^{+}$ cells both in females (light grey) and males (dark grey). **(C)** Lz$^{+}$ cells also increase during third instar larval development. **(D)** *string* knockdown with RNAi reduces the hemocyte concentration when driven in all hemocytes (HmlΔ-GAL4) but not when driven by Lz-GAL4 driver. **(E)** The number of crystal cells is not reduced in larvae when string$^{RNAi}$ is driven in Lz$^{+}$ cells. Error bars represent SEM n.s. = non-significant p-values *** = p-value <0.0001

### *2.4.2 Serrate expression in Hml$^{+}$Lz$^{-}$ cells is essential to induce crystal cell differentiation*

Crystal cell numbers are reduced in larvae raised at restrictive temperature in a thermo sensitive *Notch* allele background (Duvic et al. 2002).This reduction is visible in haemocoelic hemocytes and in the lymph gland (Duvic et al. 2002; Lebestky et al. 2003). In the lymph gland Notch signaling has a cell autonomous role in Hml$^{+}$Lz$^{-}$ precursors (Mukherjee et al. 2011). To check if the role of Notch is also cell autonomous in haemocoelic hemocytes we expressed *Notch*$^{RNAi}$ specifically in all hemocytes making use of the HmlΔGAL4 driver. Since it is complicated to count the total number of crystal cells in the larva due to the overlap of cells in the different pictures we compared the results of total numbers with the numbers in the dorsal part of the seventh abdominal segment (A7). The number of crystal

cells is reduced in the same proportions in all treatments when comparing total crystal cells with the A7 dorsal cluster (Supplementary Figure 2.2A). Hence, from this point onwards the number of crystal cells was only estimated from the dorsal part of the A7 segment. *Notch* downregulation reduces the number of sessile crystal cells both in males and females (Figure 2.2A-B). The knockdown of *Notch* does not disrupt the hemocyte clusters nor changes the concentration of hemocytes in circulation (Figure 2.2C-D). Two other GAL4 drivers expressed in all larval hemocytes (Pxn and Trio) reduce the number of crystal cells when driving *Notch*$^{RNAi}$ (Supplementary Figure 2.3A). When expressed in plasmatocytes only during embryogenesis, using the driver CrqGAL4 (Honti 2010), there is no difference in the number of larva crystal cells (Supplementary Figure 2.3A). In the lymph gland *Notch* activation is essential to induce *lozenge* upregulation (Lebestky et al. 2003). To confirm that *Notch* knockdown inhibits the induction of Lz$^{+}$ in haemocoelic cells and not the maturation of Lz$^{+}$ cells into crystal cells, we measured the proportion of Lz$^{+}$ cells with anti-Lozenge antibody while inhibiting *Notch* expression in all hemocytes (Trio-GAL4>*Notch*$^{RNAi}$). The proportion of Lz$^{+}$ cells in this case is clearly reduced (Figure 2.2E). Altogether these results confirm that Notch activation is essential to induce *lozenge* expression in larval hemocytes that will mature into crystal cells.

In *Drosophila*, Notch is activated by two different ligands: Serrate and Delta. Only *Serrate* mutants have reduced haemocoelic crystal cell numbers (Duvic et al. 2002; Lebestky et al. 2003). Interestingly, knocking down *Serrate* in hemocytes (HmlΔ$^{-}$GAL4x*Serrate*$^{RNAi}$) reduces the number of crystal cells to similar level of *Notch*$^{RNAi}$ (Figure 2.2A-B). This indicates that the ligand necessary

to induce Notch signal in hemocytes is expressed itself in hemocytes. Similarly to what has been described before (Duvic et al. 2002), the downregulation of *Delta* does not lead to a reduction in crystal cell numbers (Figure 2.2A-B). Since we have used the HmlΔ-GAL4 driver it is possible that *Serrate* is necessary in $Hml^{+}Lz^{-}$ or in $Hml^{+}Lz^{+}$ cells. To distinguish between these two hypotheses we performed knockdown of *Serrate* with Lz-GAL4 driver. In this case there is no reduction in the number of crystal cells (Figure 2.2F). Hence $Hml^{+}Lz^{-}$ cells (plasmatocytes) are responsible for *Serrate* signaling to activate *Notch*. Unexpectedly, *Notch* knockdown in $Lz^{+}$ cell does not lead to reduction in the total number of crystal cells (Supplementary Figure 2.4A). In the lymph gland it is reported that Lz-Gal4 driving *Notch*$^{RNAi}$ leads to crystal cell bursting (Mukherjee et al. 2011). In our experiments we did not observe crystal cell disruption in the lymph gland (Supplementary Figure 2.4B). One possible way to explain the differences in results is the use of different RNAi lines between our experiments and the ones from the work of Mukherjee and colleagues (Mukherjee et al. 2011) (see methods).

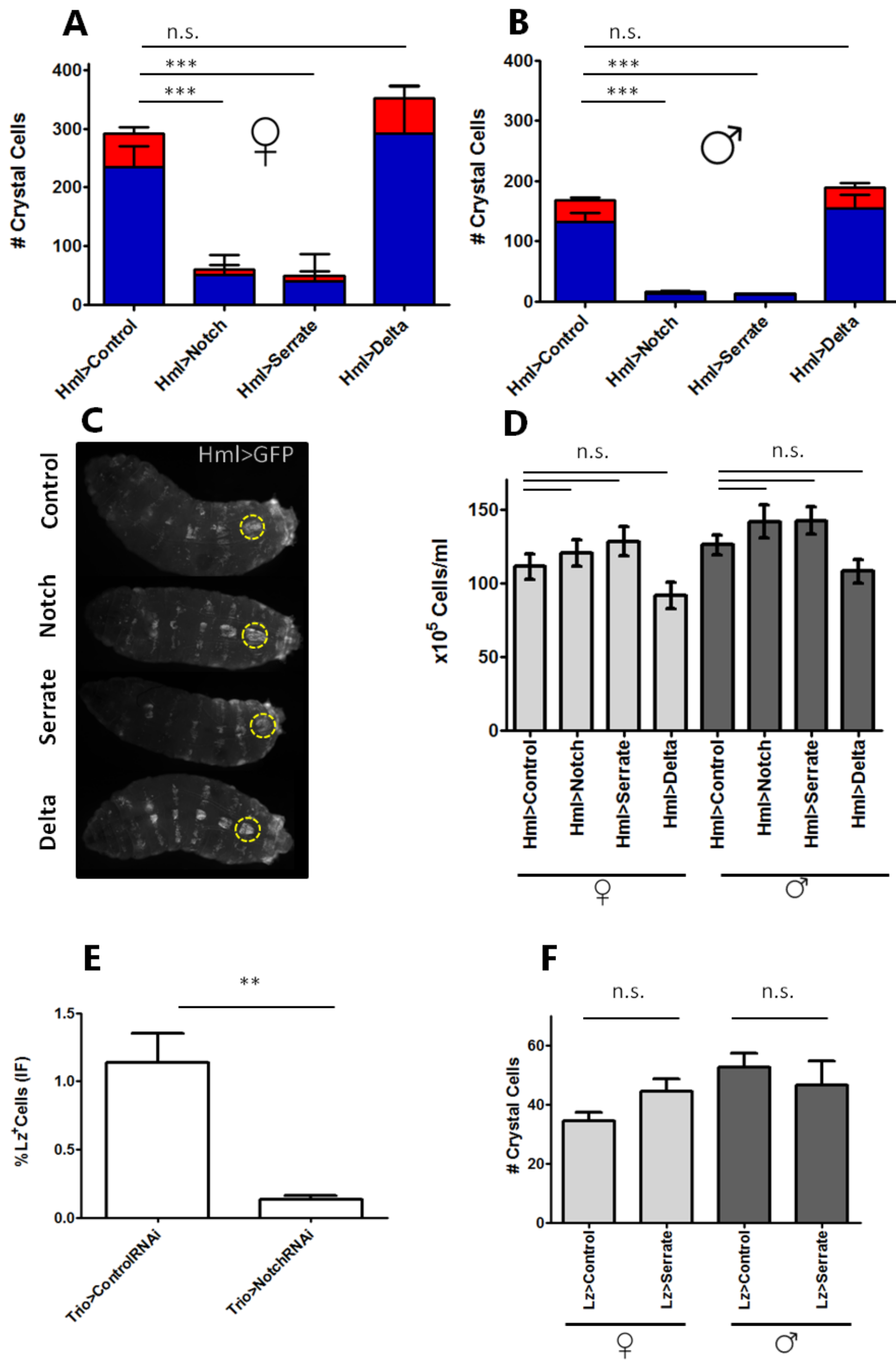
A
n.s.
***
***
# Crystal Cells
Hml>Control
Hml>Notch
Hml>Serrate
Hml>Delta
B
C
Hml>GFP
Control
Notch
Serrate
Delta
D
x10^5 Cells/ml
E
%Lz+Cells (IF)
Trio>ControlRNAi
Trio>NotchRNAi
**
F
Lz>Control
Lz>Serrate

**Figure 2.2 (*preceding page*) – Serrate downregulation in hemocytes leads to crystal cell numbers reduction. (A)** Notch$^{RNAi}$ driven in all hemocytes reduces the number of crystal cells in all body (Blue) and specifically in the dorsal part of A7 segment (Red). A similarly level of reduction is seen with Serrate$^{RNAi}$ but not with Delta$^{RNAi}$. **(B)** The results in males are similarly than in males with the exception that males have fewer crystal cells than females. **(C)** Notch pathway manipulation in hemocytes does not disrupt hemocyte clusters. **(D)** Notch pathway manipulation does not alter hemocyte concentrations. **(E)** Notch$^{RNAi}$ driven in all hemocytes reduces the proportion *lozenge* positive cells measured with antibody staining. **(F)** Serrate$^{RNAi}$ driven only in Lz+ cells does not reduce the number of crystal cells. Error bars represent SEM n.s. = non-significant p-values ** = p-value <0.001 *** = p-value <0.0001

### *2.4.3 Plasmatocyte/crystal cell ratio in clusters is influenced by the number of contacting hemocytes*

For Notch pathway activation, cells need to be in contact because the ligand Serrate is membrane bound (Guruharsha et al. 2012). The fact that *Serrate* expression in Hml$^{+}$Lz$^{-}$ hemocytes is necessary for crystal cell development suggests that Lz$^{+}$ cells are induced when hemocytes are in contact in hemocyte clusters. If this is the case, we can predict that it is more probable to find Hml$^{+}$Lz$^{+}$ cells in close contact with other cells than when they are isolated. To test this hypothesis we first measured the distances between nuclei in cells that are in contact. Nuclei were marked by nuclear-DsRed and the whole cell by cytoplasmatic GFP (HmlΔ-nuclearDsRed;HmlΔGAL4>UAS-GFP). The mean distance between nuclei in contacting cells is 11.1 µm and the maximum distance is 23µm (Figure 2.3A). Next, we measured the distance between nuclei

in Hml$^+$Lz$^-$ and Hml$^+$Lz$^+$ sessile hemocytes and considered how many nuclei are less than 23µm away. This distance was used as a proxy for the number of contacting cells. The probability of a cell to be Hml$^+$Lz$^+$ increases with the number of cells it is in contact with (Figure 2.3B). Because we used the distance between nuclei to estimate the number of cells that are in contact there is a clear overestimation of cells in contact. However, this caveat does not affect our interpretation because it only turns our cell contacts estimation more conservative. The fact that we see a positive correlation between cells that are in contact and the percentage of those cells that are Lz$^+$ goes in line with the fact that induction of Notch pathway is dependent on other hemocytes. The dependence on other hemocytes to induce *Notch* signaling influences the proportion of induced Hml$^+$Lz$^+$ and thus cluster structure.

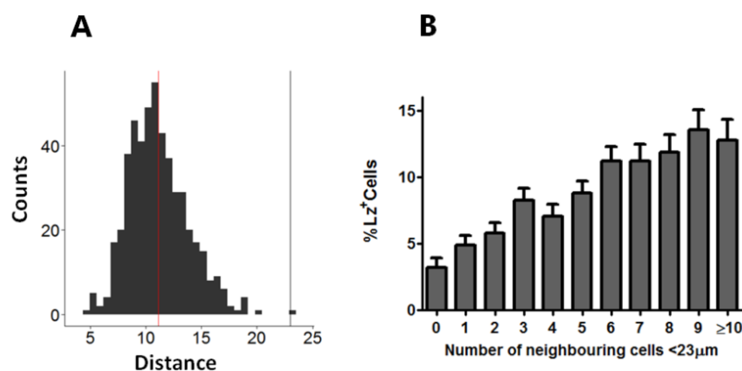


**Figure 2.3 – The number of clustered hemocytes influences their constitution. (A)** Histogram representing the distribution of distances between nuclei in contacting cells. Cytoplasm was visible by GFP driven by HmlΔ-GAL4 and nuclei visible by HmlΔ-nuclearDsRed **(B)** Probability of a cell to be Lz$^+$ increases linearly with the number of cells that has in contact.

### 2.4.4 Differentiation of $Hml^+Lz^+$ cells occurs in clusters

All the descriptions before lead us to propose that $Hml^+Lz^+$ cells are induced from $Hml^+Lz^-$ cells in clusters of sessile hemocytes. A way to test this hypothesis is to directly visualize the transformation of $Hml^+Lz^-$ cells into $Hml^+Lz^+$ cells with live time-lapse imaging. To achieve this we developed a method for imaging hemocytes in clusters in live larvae for periods of three hours. HmlΔ-nuclearDsRed; LzGAL4>EGFP/mCD8GFP early L3 male larvae (<12h after L3 ecdysis) were selected and prepared for imaging (see methods). Larvae had pressure from a cover slip in the dorsal part, which affects A7 dorsal hemocyte cluster most probably because this alters hemolymph circulation. Hence, we imaged more anterior clusters that were not so affected. As expected, it was possible to see the division of $Hml^+Lz^-$ cells nuclei (Arrows in Figure 2.4A). The proportion of dividing cells in these movies was ~7% of $Hml^+Lz^-$ cells present in the beginning of the movie (n=13 movies with mean 50 Hml+Lz- and 6 Hml+Lz+ cells per movie). No case of $Hml^+Lz^+$ cell division was seen in any movie, once again confirming that $Lz^+$ cells are postmitotic cells while plasmatocytes are in proliferation in sessile clusters. Notably, it was possible to see induction of GFP in $GFP^-$ cells, demonstrating that an $Hml^+Lz^-$ cell is turning into an $Hml^+Lz^+$ cell (Arrowhead in Figure 2.4A). The new $Hml^+Lz^+$ cells can be in contact with other $Hml^+Lz^+$ cells or only in contact with $Hml^+Lz^-$ cells. This supports the idea that $Serrate^+$ inducing cells are $Hml^+Lz^-$ cells. In our movies cells that are $LzGFP^+$ never become $GFP^-$ and cells that start with low GFP tend to increase it with time (Supplementary Figure 2.5A).

When we bleed larvae and analyze $Hml^+Lz^+$ cells we see a tendency for larger cells to have more GFP measured by mean grey

value (Figure 2.4 B). If larger cells had the same GFP signal they would have a lower mean grey value because the signal would be diluted. In conjugation these results indicate that *lozenge* expression starts in $Hml^{+}Lz^{-}$ cells morphologically indistinct from plasmatocytes and increase in size during crystal cell maturation.

The proportion of $Hml^{+}Lz^{+}$ induced cells in our movies is ~3,5% in 3 hours. Knowing the rate of differentiation of $Hml^{+}Lz^{-}$ into $Hml^{+}Lz^{+}$ we can extrapolate the number of $Hml^{+}Lz^{+}$ cells differentiated from $Hml^{+}Lz^{-}$ cells at given time using the formula:

$$\mathrm{Hml^{+}Lz^{+}}_{t+1} = \mathrm{Hml^{+}Lz^{+}}_{t} + \mathrm{Hml^{+}Lz^{-}}_{t} \times \text{(Proportion of Lz induced cells)}$$

The proportion of Lz induction calculated in our movie analysis is sufficient to explain the increase in $Hml^{+}Lz^{+}$ cell number observed in the first 24h of third instar larval development The mean increase of $Lz^{+}$ cells measured during development is 256.4 cells (Figure 2.1C) and the extrapolation gives an increase of 447 $Lz^{+}$ cells.

On the whole, the analysis of these movies showed that in fact $Hml^{+}Lz^{+}$ cells are differentiated from $Hml^{+}Lz^{-}$ cells in hemocyte clusters and the differentiation rate from $Hml^{+}Lz^{-}$ to $Hml^{+}Lz^{+}$ is sufficient to explain the increase in this cell type.

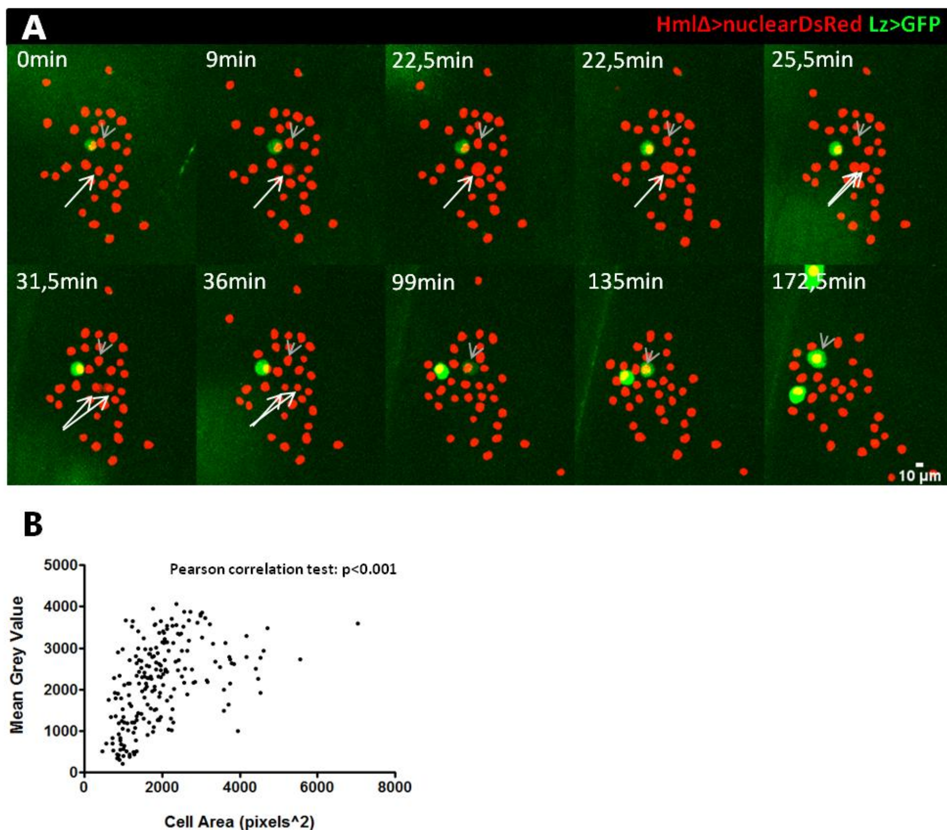


**Figure 2.4 – *lozenge* expression induction in Hml+ cells. (A)** In 3 hours it is possible to division of Hml+ nuclei (Arrows) and induction of GFP expression in GFP negative cells. **(B)** There is a correlation between cell size and GFP intensity, as measured by Mean Grey Value, in Lz+ cells.

### *2.4.5 Hemocyte clusters are necessary for the normal development of different hemocyte types*

All the results described previously indicate that the clustering of hemocytes is important for hematopoietic decisions. One way to test the necessity of hemocyte clusters is to check proportions of crystal cells to plasmatocytes in larvae without clusters. At the time

these experiments were undertaken, no mutation had been described to disrupt hemocyte clusters. A misexpression screen identified some genes that overexpressed in hemocytes can disrupt hemocyte clusters (Stofanko et al. 2008). However, we did not use these lines to test for crystal cell/plasmatocyte ratios alterations because it would be difficult to discern between the effect of not having the clusters and the effect of gene up-regulation in hemocytes. One other way to disrupt the hemocyte clusters is to physically manipulate the larvae (Makhijani et al. 2011). After hemocyte disruption clusters start to be formed again gradually (Figure 2.5A). To maintain hemocytes in circulation for long periods of time we disrupted hemocytes clusters in larvae every 1h30m. After the treatment, we disrupted all hemocyte clusters in control and treatment groups to sample the majority of cells and measured the proportion of $Hml^{+}Lz^{+}/Hml^{+}Lz^{-}$ cells by flow cytometry. The proportion of $Hml^{+}Lz^{+}/Hml^{+}Lz^{-}$ decreases in the treatment group (Figure 2.5B). This indicates that the number of $Hml^{+}Lz^{+}$ induced cells is diminished when the clusters are disrupted.

However, the cluster disruption treatment could also disrupt the lymph gland or change the rate of apoptosis differentially between $Hml^{+}Lz^{+}$ and $Hml^{+}Lz^{-}$ cells. We tested if the rate of apoptosis changes when we disrupt the clusters with PI exclusion assay (see methods). There is no significant difference between control and treatment groups (Table Supplementary). To check if cluster disruption also disrupts lymph glands we made use of DotGAL4 that is expressed in lymph gland hemocytes and not in haemocoelic hemocytes (Honti et al. 2010). Performing lineage tracing with Dot-GAL4 driver (see methods) we can check cells that are derived from the lymph gland. If we check pupal hemocytes or wasp infected larvae it is possible to see lymph gland derived hemocytes in circulation by flow cytometry

(Figure 2.5C). When the same technique is used in cluster disrupted larvae there is no detectable lymph gland derived hemocyte (Table Supplementary). Altogether these results show that clusters are necessary for the proper development of $Hml^{+}Lz^{+}$ hemocytes and to reach the correct proportion of this cell type.

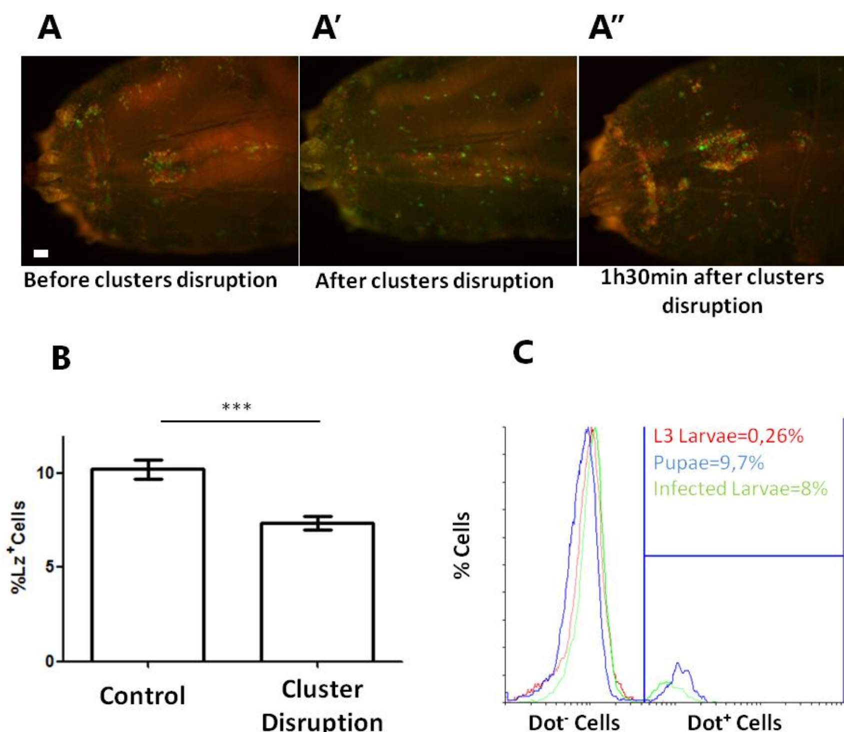


**Figure 2.5 – Clusters are necessary for the proper development $Hml^{+}Lz^{+}/Hml^{+}Lz^{-}$ ratios. (A)** Physical manipulation can disrupt the clusters **(A')** but after 1h30m most larvae have the clusters reconstituted **(A'')**. **(B)** Continued cluster disruption during 10h in early third instar larvae leads to a reduction in the proportion of $Lz^{+}$ cells (n=10) **(C)** DotGAL4 cell lineage tracing marks cells in the lymph gland but they can only be collected from hemolymph in pupae and parasitoid infested larvae. Error bars represent SEM *** = p-value <0.0001

### 2.4.6 Crystal cells originate from transdifferentiation of plasmatocytes

Only a small proportion of $Hml^{+}Lz^{-}$ cells are induced into $Hml^{+}Lz^{+}$ throughout development. It is possible that any $Hml^{+}Lz^{-}$ cell has the capacity to transform into a $Hml^{+}Lz^{+}$ cell or that there is a subpopulation of $Hml^{+}Lz^{-}$ cells with this potential. A good candidate to mark such a subpopulation is the Eater-GAL4 driver. Eater is a membrane receptor that recognizes bacteria (Kocks et al. 2005). The expression of this driver starts in second instar larva and in late third instar larvae only plasmatocytes express the Eater-GAL4 driver (Kroeger et al. 2011). There are two different fly stocks carrying the Eater-GAL4 driver, one on the X chromosome and another on the second chromosome. We measured Eater expression throughout third larval instar with flow cytometry. The dynamics of Eater expression is different between constructs and between male and female (Figure 2.6A-B; Supplementary Figure 2.6B). We wanted to test if $Eater^{+}$ cells are important contributors for crystal cell number increase or only $Eater^{-}$ cells are capable to differentiate into crystal cell. To test this we used the two Eater-GAL4 stocks to drive *Notch*$^{RNAi}$. For the two drivers the number of crystal cells is reduced in males and not in females (Figure 2.6C; Supplementary Figure 2.6A). One possibility to explain this difference is the higher percentage of hemocytes that express $Eater^{-}$GAL4 in males compared to females (Figure 2.6A-B). In fact, when we combine the two drivers, *Notch* knockdown decreases the number of crystal cells both in males and females (Figure 2.6D). This suggests that $Eater^{+}$ cells contribute for crystal cell population.

Another marker that distinguishes plasmatocytes from crystal cell is the antibody P1 that recognizes an epitope of the phagocytic

receptor *NimrodC1* (Kurucz, Márkus, et al. 2007). Some *Drosophila* laboratory strains have a truncation in *NimrodC1* locus that impairs the antibody staining (Honti et al. 2013). Both stocks used in this work that have the driver Lz-GAL4 are P1 negative. To circumvent this problem we crossed this stock with the Oregon R reference strain to check if we could find $Lz^{+}$ cells that are P1 positive. Morphologically distinguishable crystal cells are P1 negative. Noticeably, some Lz+ cells are positive for the P1 cell marker (arrows in Figure 2.6E).

$Eater^{+}$ cells also transdifferentiate into lamellocytes (Honti et al. 2010). In this case cells that are phagocytically active become $non^{-}$ phagocytic and start to express lamellocyte markers. Because mature crystal cells are non-phagocytic cells we checked if the same phenomenon happens during crystal cell maturation. To test phagocytosis we used *E. coli* particles marked with pHrodo fluorescence marker. At a neutral pH the fluorescence of pHrodo is too dim but it increases dramatically when the pH decreases, as it happens inside phagosomes. When we inject these particles in larvae and analyze phagocytosis one hour later we can estimate that ~92% of $Lz^{-}$ and ~30% of $Lz^{+}$ hemocytes cell have at least one pHrodo particle. Strikingly, there is a higher ratio of GFP low expressing cells with phagocytosis when compared with GFP high expressing cells that are virtually non-phagocytic (Figure 2.6F). This indicates that induced $Lz^{+}$ cells are phagocytically active plasmatocytes that loose the phagocytic capacity as they mature. Collectively, these results show that mature plasmatocytes, that are phagocytically active, have the capacity to transdifferentiate into a $non^{-}$phagocytic crystal cell.

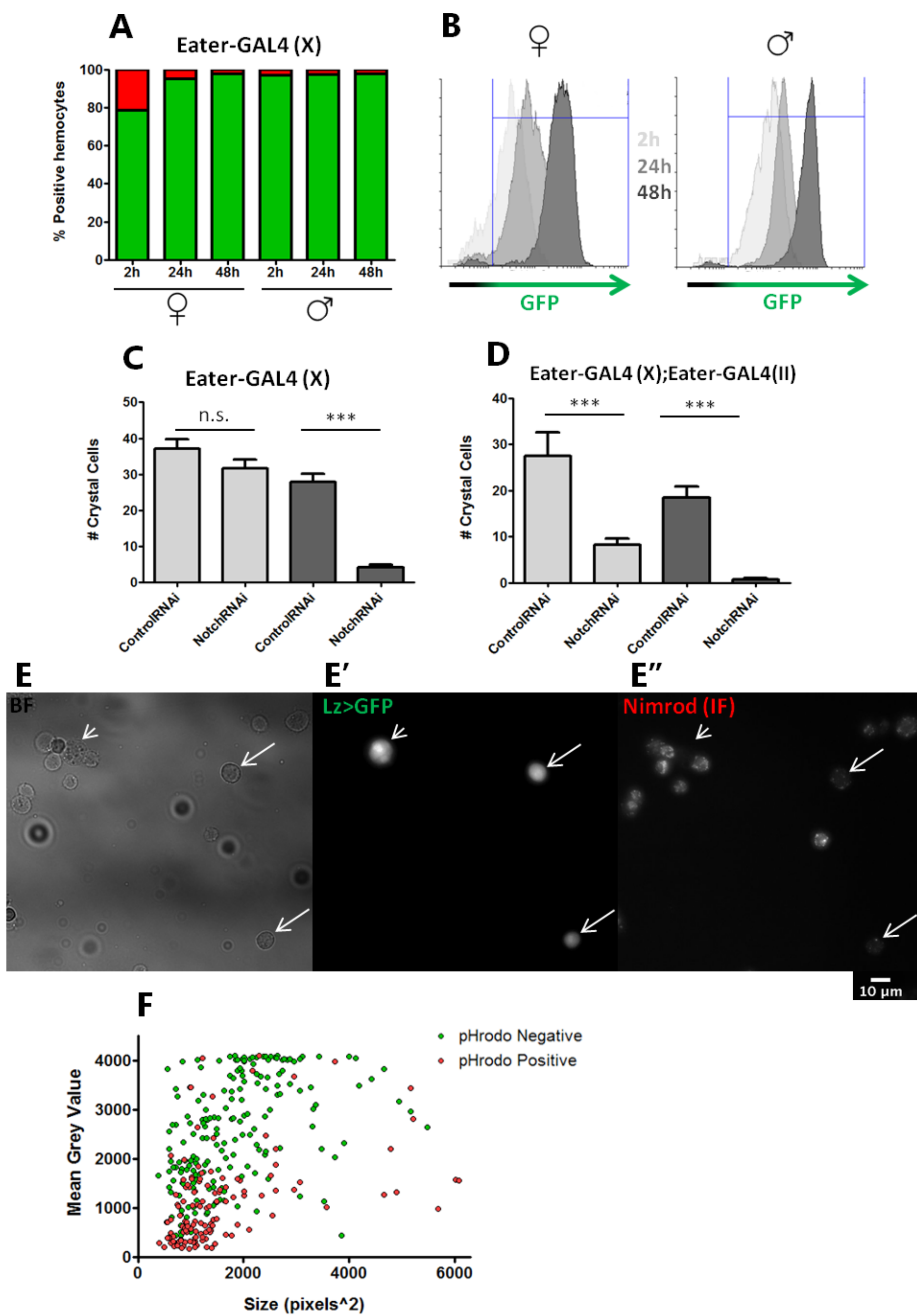
A
Eater-GAL4 (X)
% Positive hemocytes
100
80
60
40
20
0
2h
24h
48h
2h
24h
48h
♀
♂
B
♀
♂
2h
24h
48h
GFP
GFP
C
Eater-GAL4 (X)
# Crystal Cells
n.s.
***
ControlRNAi
NotchRNAi
ControlRNAi
NotchRNAi
D
Eater-GAL4 (X);Eater-GAL4(II)
# Crystal Cells
***
***
ControlRNAi
NotchRNAi
ControlRNAi
NotchRNAi
E
E'
E''
BF
Lz>GFP
Nimrod (IF)
10 µm
F
pHrodo Negative
pHrodo Positive
Mean Grey Value
Size (pixels^2)
0
2000
4000
6000

**Figure 2.6 (*preceding page*) – Crystal cells transdifferentiate from mature plasmatocytes. (A)** Eater-GAL4(X) drives expression of GFP in the majority of Hml+ hemocytes measured by flow cytometry. **(B)** The intensity of GFP driven by Eater-GAL4 (X) is always higher in males and increases throughout time. **(C)** Notch$^{RNAi}$ driven by Eater-GAL4(X) reduces the number of crystal cells in males but not in females. **(D)** Notch$^{RNAi}$ driven by Eater-GAL4(X) and Eater-GAL4(II) reduces the number of crystal cells both in males and females. **(E)** Majority of Lz$^{+}$ hemocytes are NimroC1 negative (arrow head) but some are still positive (arrows). **(F)** Low GFP expressing hemocytes are still phagocytically active and GFP high expressing hemocytes are virtually non-phagocytic. Error bars represent SEM n.s. = non-significant p-values *** = p-value <0.0001

### *2.4.7 lozenge expression induction can occur in G1 phase of the cell cycle*

The fact that plasmatocytes are in proliferation creates a heterogeneous population of cells. This heterogeneity could influence the capacity of cells to respond to Notch activation and hence explain why only a portion of cells induce *Lz* expression. During *C. elegans* vulval development, cell cycle asynchrony is essential for the proper development of cell fates (Nusser-Stein et al. 2012). The mechanism is dependent on Notch with cyclins playing a role in Notch degradation and stabilization. In *Drosophila,* during bristle development, *Notch* expression is enhanced in the S phase of the cell cycle (Remaud et al. 2008). Inspired by these discoveries we asked if Lz$^{+}$ cells are induced only during the S phase of the cell cycle. With that intent, we performed a cell cycle analysis by flow cytometry that can distinguish G1/S/G2 phases of the cell cycle based on DNA content (Figure 2.7A and see methods). The majority of

plasmatocytes from wandering larval stage are in G1 phase (~85%) (Figure 2.7B). Similarly, the majority of $Lz^+$ cells are in G1 phase (~64%). This indicates that $Lz^+$ cells are induced in G1 phase since after they become $Lz^+$ there is no division. A greater proportion of $Lz^+$ cells have higher DNA content than $Lz^-$ cells (Figure 2.7B). This could happen due to a higher propensity of S/G2/M cells to respond to Notch signaling but also can reflect the endoreplication process that occurs in mature crystal cells (Terriente-Felix et al. 2013).

With these results we can affirm that *lozenge* can be induced in G1 phase of the cell cycle but we cannot conclude if the same is true for the other phases. It is possible that they are only differentiated in G1 and they continue with DNA replication or maybe they are induced when the DNA content is already higher than 2n. Further tests are needed to elucidate the dependence of *lozenge* induction on cell cycle. For example, none of the dividing cells in our movie analysis induced *lozenge* expression. It would be important to continue movies with more time points but in our conditions the larva would enter starvation, which could affect cell differentiation.

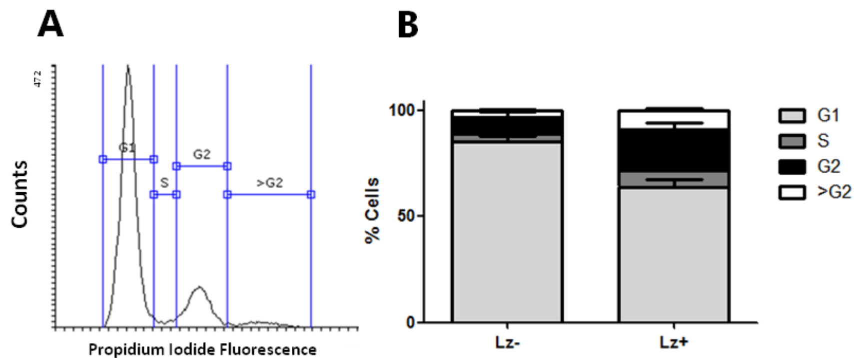


**Figure 2.7 – *lozenge* expression can be induced in G1 hemocytes. (A)** PI DNA staining intensity correlates with DNA content of a cell, which permits to distinguish the different phases of the cell cycle. **(B)** In 110h old larvae (larval third instar) the majority of haemocoelic hemocytes will be in G1 whether they are $Lz^+$ or $Lz^-$.

### *2.4.8 Notch promoter reporters are expressed in crystal cells and in a subset of plasmatocytes*

It is possible that the ability to transdifferentiate into crystal cells is restricted to a sub-population of plasmatocytes. Necessarily, plasmatocytes that differentiate into crystal cells need *Notch* expression. To test for *Notch* expression in hemocytes we used the set of GAL4 drivers (FlyLight) that span the majority of intergenic and introns of the *Notch* locus (Jenett et al. 2012) (Figure 2.8A). Only two of these stocks drive UAS-GFP in HmlΔ-nuclearDsRed cells: GMR30A01 and GMR30C06 (Figure 2.8B). It is possible to see GFP expression in plasmatocytes and in all morphologically distinguishable crystal cells (arrow in Figure 2.8C). Noticeably, both $GFP^+$ and $GFP^-$ cells divide in the clusters when we image these cells

for periods of three hours (Supplementary movie 2). With these results we were motivated to test the hypothesis that these two drivers are expressed in a subpopulation of plasmatocytes with the ability to transdifferentiate into crystal cells *via* Notch. With that intent we counted the number of crystal cells of each driver driving *Notch*$^{RNAi}$. Blackened crystal cells by heat shock treatment were counted in the most posterior dorsal hemocytes cluster. Unexpectedly, this treatment does not lead to a reduction in crystal cell numbers (Supplementary Figure 2.7).

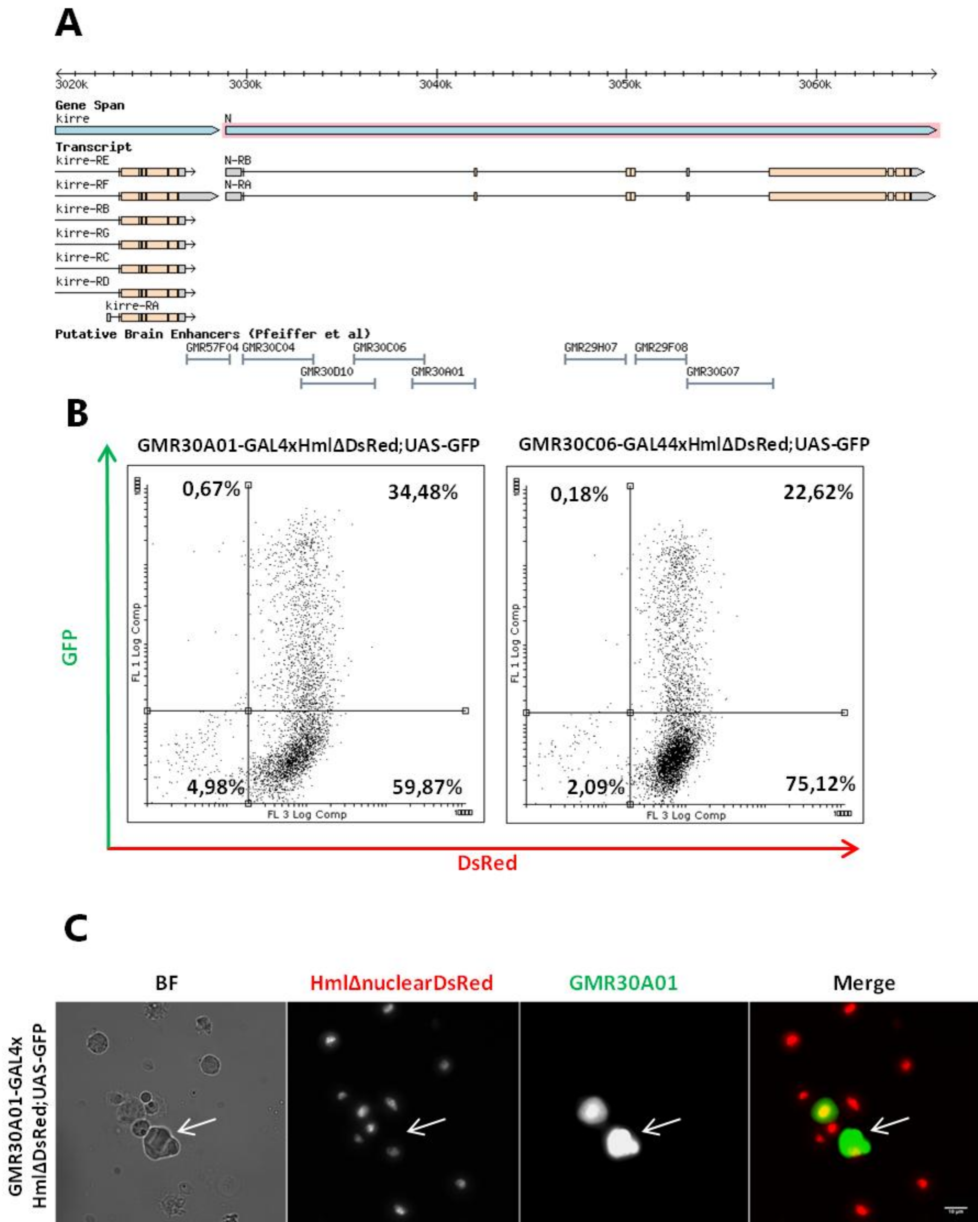


**Figure 2.8 – Notch reporters expression in hemocytes (A)** FlyLight project transgenics related to Notch. **(B)** Two of these transgenics are expressed in HmlΔ-nuclearDsRed cells. **(C)** GMR30A01-GAL4 driver expresses GFP in mature crystal cells (Arrow).

## 2.5 Discussion

Our motivation to carry out this work was to resolve an apparently paradoxical observation: although only plasmatocytes can proliferate, both plasmatocytes and crystal cells increase in number, maintaining roughly the same proportion during larval development (Rizki 1957; Lanot et al. 2001). Since the larva possesses a hematopoietic organ it was tempting to postulate that the new crystal cells are differentiated in there (Duvic et al. 2002). However, the work of Holz and colleagues demonstrates that no cell from the lymph gland enters circulation in homeostatic development (Holz et al. 2003). This was confirmed using cell lineage tracing techniques (Honti et al. 2010). Taking together these observations, we can propose three models to explain haemocoelic crystal cell numbers increase throughout larval development: i) A population of replicating pro-hemocytes exists in haemocoelic hemocytes that have the ability to differentiate into crystal cells. ii) A population of pro-crystal cells exists in haemocoelic hemocytes and its role is to proliferate and mature into crystal cells. iii) Plasmatocytes, that are mitotically active, have the capacity to transdifferentiate into crystal cells.

A population of prohemocytes was proposed to exist in circulation by Lanot and colleagues (Lanot et al. 2001). The authors observe that hemocytes did not phagocyte India ink particles injected 2h before the proliferation assay. This could mean that a population of prohemocytes is dedicated to proliferate and differentiate into plasmatocytes and crystal cells. However, as the same authors state, it could be that cells entering mitosis are simply less able to phagocyte (Lanot et al. 2001). In another investigation Makhijani and colleagues inject beads in larvae and wait for a longer period of time

(3-4h) before assaying for DNA replication (Makhijani et al. 2011). In this case the cells that replicated DNA do phagocytose at the same rate as the ones that did not replicate DNA. This shows that one of the functions that distinguish plasmatocytes from crystal cells and lamellocytes, phagocytosis, is performed by dividing cells in circulation and in sessile tissues. Moreover, dividing cells express Pxn-GAL4, a genetic marker that is characteristic of differentiated hemocytes (Makhijani et al. 2011). With this set of evidence the authors conclude that the replicating haemocoelic hemocytes are mature plasmatocytes (Makhijani et al. 2011). There is always the possibility that a yet undescribed lineage marker distinguishes plasmatocytes with the capacity to divide. However, with our current knowledge, there is no functional evidence to show that a population of prohemocytes is dedicated to proliferation. Instead it seems that mature plasmatocytes, with the capacity to phagocytose, are the hemocytes in proliferation within the haemocoelic compartment (Makhijani et al. 2011).

One other possibility to explain crystal cell number increase is that a population of pro-crystal cells exists. This population would proliferate in the larva before cell maturation or exist in sufficient numbers at the beginning of larval development to mature into crystal cells throughout development. The first known upregulated gene in crystal cell development is the transcription factor *lozenge* (*Lz*) (Lebestky et al. 2000). Hence, a cell will be $Lz^+$ before it matures into a crystal cell, maintaining its expression afterwards (Lebestky et al. 2000). When we measured the number of $Lz^+$ cells in the sessile population of hemocytes throughout third instar development we could show that their number increases. The proportion of $Lz^+$ hemocytes to $Lz^-$ even increases in the first 24h of third instar

development. This observation excludes the possibility that a population of $Lz^+$ precursors exists in fixed number and matures into crystal cell. When we use *string*$^{RNAi}$ to inhibit cell entry into mitosis in $Lz^+$ cells it does not affect the number of differentiated crystal cells. Additionally, we do not see $Lz^+$ cell division in our movie analysis. Altogether, these results show that a replicating population of pro-crystal cells $Lz^+$ does not exist. Yet, it is still possible that a population of $Lz^-$ pro-crystal cells exists amongst sessile hemocytes. Further molecular characterization of plasmatocytes is needed to understand to each extent they form a homogeneous population or instead several subpopulations co-exist. For example, in our analysis only a subpopulation of plasmatocytes expresses the *Notch* reporter drivers that are also expressed in mature crystal cells.

The activation of *lozenge* in hemocytes is Notch dependent with Serrate acting as the ligand (Duvic et al. 2002; Lebestky et al. 2003). In the lymph gland *Serrate* is expressed in the Posterior Signaling Center (PSC) and in scattered cells inside the lymph gland (Lebestky et al. 2003; Crozatier et al. 2004). The scattered cells inside the lymph gland are the ones responsible for inducing crystal cells (Crozatier et al. 2004) and most probably this happens in the cortical zone where *lozenge* is first expressed (Jung et al. 2005). When we ablate *serrate* expression only in hemocytes using the HmlΔ-GAL4 driver the number of differentiated crystal cells is severely reduced in sessile hemocyte population. This indicates that haemocoelic hemocytes are the cells responsible for crystal cell induction. Moreover the hemocytes inducing crystal cell development are themselves $Lz^-$ because *serrate*$^{RNAi}$ driven by Lz-GAL4 driver does not change the number of induced crystal cells. For Notch to be activated it requires that a *serrate* expressing cell is in contact for a

certain period of time (Guruharsha et al. 2012). If hemocytes are inducing other hemocytes to crystal cell fate they need to be in contact. Hemocyte clusters found in close contact with epidermis create the opportunity for such contacts. In fact we see that Hml$^{+}$Lz$^{+}$ cells are induced in these hemocyte clusters from Hml$^{+}$Lz$^{-}$ cells. This creates a parallel with haemocoelic crystal cell development and lymph gland crystal cell development. In both cases the precursor is an Hml$^{+}$Lz$^{-}$ hemocyte (Mukherjee et al. 2011). However, there is a fundamental difference in cells where *lozenge* starts to be expressed in the two situations. Although hemocytes only activate *lozenge* expression in the cortical zone of the lymph gland (Lebestky et al. 2000), the work of Krzemien and colleagues suggest that in the lymph gland, cells are already committed to be transformed into crystal cells in late 2$^{nd}$ instar in the medullary zone (Krzemien, Oyallon, et al. 2010). Notch activation is only required later in development in the third larval instar. This suggests that medullary zone cells migrating to the cortical zone can be already considered pro-crystal cells. Our analysis of crystal cell differentiation in hemocyte clusters suggest that *lozenge* induction occurs in mature plasmatocytes. Firstly, crystal cells in the haemocoelic compartment derive from a cell that expressed *croquemort* during embryonic stages (Honti et al. 2010), *croquemort* being a phagocytic receptor specific for plasmatocytes (Franc et al. 1999). Lebestky and colleagues consider that a small portion of Lz-GAL4 positive cells gives rise to plasmatocytes distinguished by morphology and *croquemort* expression (Lebestky et al. 2000). In the light of our results we propose that something else is happening. The plasmatocyte-like cells expressing *lozenge* are plasmatocytes where *lozenge* was induced and are in route to be transformed into a crystal cell. Supporting this idea, when we ablate *Notch* expression with the Eater-GAL4 driver there is a reduction in

the number of differentiated crystal cells. The Eater-GAL4 driver in late stages of larva development is restricted to plasmatocytes but there is an overlap of *eater* and *lozenge* expression at earlier stages (Kroeger et al. 2011). Another line of evidence is that $Lz^+$ low GFP expressing cells also can function as phagocyte while $Lz^+$ high GFP cells do not engage in phagocytosis. $Lz^+$ low GFP cells are, according to our movie analysis, the first stages of crystal cell differentiation. Throughout time these cells increase their GFP expression and we never saw a $Lz^+$ cell becoming $Lz^-$ which suggests that $Lz^+$ state is irreversible. As a whole, our results suggest that mature plasmatocytes can differentiate into crystal cells. Interestingly, in *Bombyx mori*, oenocytoids seem to derive not from prohemocytes but from mature plasmatocytes (Nakahara et al. 2010). We call to the attention that *B. mori* oenocytoid is the functional equivalent to the *Drosophila* crystal cell and the *B. mori* plasmatocyte corresponds to the *Drosophila* lamellocyte.

With the results and interpretation exposed in here, we propose that hemocyte clusters work as a hematopoietic tissue. Importantly, the presence of hemocyte clusters is necessary for the proper establishment of $Hml^+Lz^+/Hml^+Lz^-$ proportions during larva development. The hemocytes in clusters are in dynamic relation with circulating hemocytes (Babcock et al. 2008; Welman et al. 2010; Makhijani et al. 2011). Cells can enter circulation from the patches and circulating hemocytes can become sessile. This behavior is observed in our movies were we can detect cells suddenly entering circulation and cells that adhere to the clusters. This dynamic exchange between sessile and circulating cells gives the opportunity for differentiated crystal cells in clusters to enter circulation. Sessile plasmatocytes have a higher division rate than circulating ones

(Makhijani et al. 2011). This could happen because there is a different molecular ‘environment’ in hemocyte clusters or only because when a cell is sessile this enhances the probability of entering cell division. We think that these two characteristics are sufficient to consider the hemocyte clusters as hematopoietic tissues. They enhance hemocyte proliferation and cell contacts between cells influences cell fate choices. It is possible that, contrary to the lymph gland, the hemocyte clusters are not regionalized (Honti et al. 2014). Nevertheless the establishment of cell contacts between hemocytes is essential for the correct development of different hemocyte types.

We consider that the results here presented, open new interesting biological questions. An important one is to investigate what determines a certain proportion of plasmatocytes to differentiate into crystal cells. The hemocyte clusters may have different mechanisms to control for cell proliferation and differentiation than in the lymph gland. In support of this notion, misexpression of some genes in hemocytes can disrupt hemocyte clusters without affecting lymph gland morphology (Stofanko et al. 2008). In another perspective it would be interesting to investigate the transcriptional landscape of a plasmatocyte transforming into a crystal cell. It is now evident that plasmatocytes are very plastic cells and may represent a rare case of mature cells transdifferentiating into other cell types: lamellocytes (Honti et al. 2010) and crystal cells. Transdifferentiation, the process where a cell changes its cell fate without passing through a less differentiated state, is used recurrently in cell culture assays but rarely seen *in vivo* (Jopling et al. 2011). Yet, a recent report shows that transdifferentiation of parathyroid cells into cervical thymi promotes atypical T-cell development, contributing to cell diversity in

the immune system (Li et al. 2013). It is important to understand how recurrent this mechanism is in animal development.

## 2.6 Author's Contributions

Alexandre Leitão and Élio Sucena designed the experiments. Alexandre Leitão performed the experiments.

## 2.7 Acknowledgments

We thank Marc Dionne, Robert A. Schultz and István Andó for fly lines and/or reagents, the VDRC for the RNAi fly lines and the Hybridoma Bank (Iowa, US) for monoclonal antibodies. Also, we like to acknowledge the Unit of Imaging and Cytometry at IGC for their great effort in maintaining all the machines running properly.

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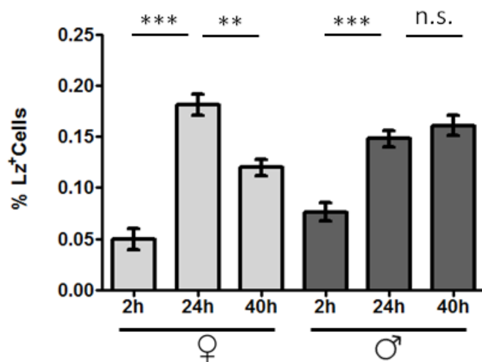
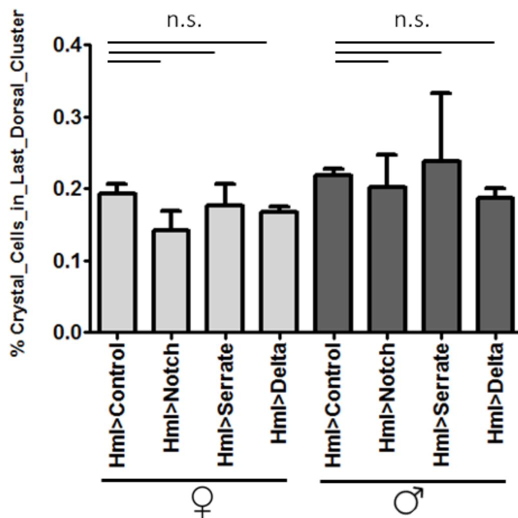
## 2.8 Supplemental Material



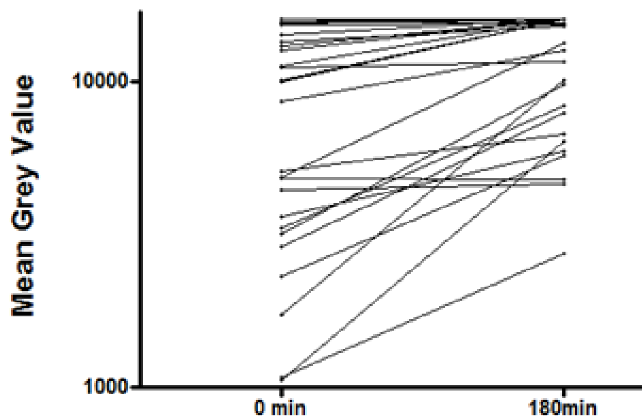

**Supplementary Figure 2.1** – During the first 24h of 3rd instar larval development the proportion of Hml+Lz+/Hml+Lz- cells increases. In females this proportion decreases in the last 16h of larval development. Error bars represent SEM n.s. = non-significant p-values ** = p-value <0.001 *** = p-value <0.0001



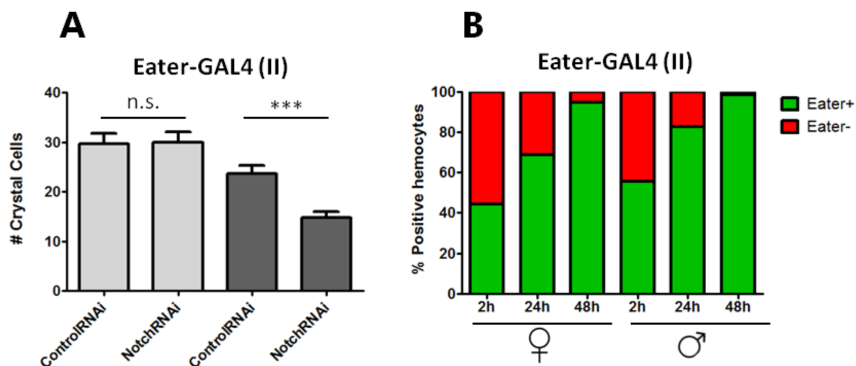

**Supplementary Figure 2.2 –** Notch pathway manipulation by RNAi does not changes the ratio of crystal cells in the A7 dorsal cluster. Error bars represent SEM n.s. = non-significant p-values

**Supplementary Figure 2.3 –** Notch knockdown leads to a reduction in the number of larval crystal cells only when manipulated during larval stages. Crq driver is only expressed in embryonic hemocytes and Pxn and Trio are expressed in the majority of larval hemocytes. Females (light grey) and males (dark grey). Error bars represent SEM n.s. = non-significant p-values

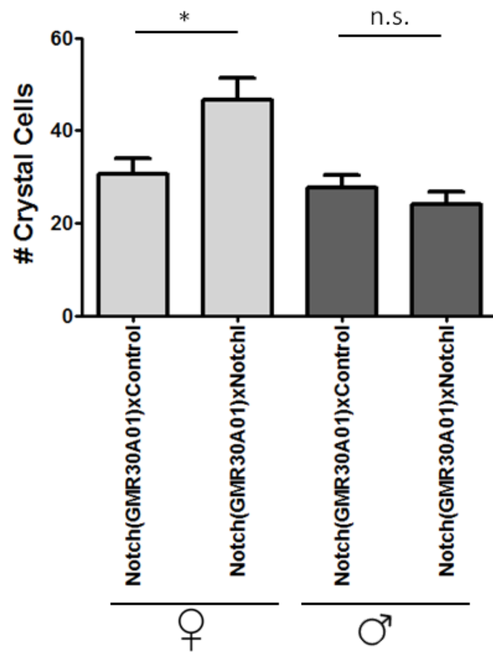
**Supplementary Figure 2.4 – Notch knockdown in Lz+ cells does not reduce the number of larval crystal cells. (A)** Notch knockdown by RNAi using the Lz-GAL4 driver does not reduce the number of crystal cells in clusters. **(B)** Notch knockdown using the Lz-GAL4 driver does not leads to a bursting phenotype. Error bars represent SEM n.s. = non-significant p-values * = p-value <0.01




**Supplementary Figure 2.5** – Intensity of GFP, as measured by Mean Grey value increases in Lz+ cells during the 3 hours time lapse imaging.




**Supplementary Figure 2.6 – Eater-GAL4 (II) is expressed differently in males and females** (A) Notch knockdown using the Eater-GAL4 (II) driver does not reduce the number of crystal cells in females but decreases in males. (B) The proportion of hemocytes that express the Eater-GAL4 driver increases during 3rd instar development and is always higher in males (hours post 3$^{rd}$ larval instar) . Error bars represent SEM n.s. = non-significant p-values *** = p-value <0.0001




**Supplementary Figure 2.7** – Notch knockdown by RNAi using the Notch driver reporters does not reduce the number of crystal cells. Error bars represent SEM n.s. = non-significant p-values * = p-value <0.01

**Supplementary table 1 – Flow cytometry analysis of cluster disrupted larvae for cell viability and Dot-GAL4 lineage tracing –** Disrupting the clusters does not increase the percentage of dying hemocytes and do not induce the release of hemocytes from the lymph gland. Numbers represent means ±SEM.

| Treatment | % PI positive cells | % Dot lineage positive cells | n |
|---|---|---|---|
| **Control** | 10,31±3,78 | 0,11±0,21 | 4 |
| **Cluster Disruption** | 7,16±1,24 | 0±0 | 4 |

**Supplementary movie 1 – *lozenge* expression induction in hemocyte clusters-** HmlΔ-nuclearDsRed; Lz>EGFP hemocytes in a dorsal cluster. In the movie it is possible to see Hml+Lz- hemocytes in division (examples are highlighted by green circles) and Hml+Lz- hemocytes differentiating into Hml+Lz+ hemocytes (example highlighted by a red circle). Scale bar = 10μm

**Supplementary movie 2 –** Notch reporter marks cells with proliferation activity – HmlΔ-nuclearDsRed; Notch(GMR30A01)>EGFP In the movie it is possible to see negative and positive cells for the Notch reporter in division.

# Gprk1 *is involved in the control of hemocyte loads*

## 3.1 Summary

How animals control the number of blood cells in circulation is a fascinating developmental question. To answer it we need to understand how the hematopoietic organs control cell proliferation and differentiation. In the *Drosophila melanogaster* larva, hemocytes in circulation are derived from a pool of hemocytes produced during embryogenesis (Holz et al. 2003). Two circulating cell type are distinguishable in homeostasis, plasmatocytes and crystal cells. These embryonic derived hemocytes aggregate in clusters along the body axis and are in contact with the epidermis and neurons (Lanot et al. 2001; Makhijani et al. 2011). In these clusters plasmatocytes proliferate with a higher rate when compared with circulating ones (Makhijani et al. 2011). Also, as we have shown in chapter 2, the clusters are essential to differentiate crystal cells. Since the two hemocyte compartments change cells between it is important to study the number control of hemocytes in these hemocyte clusters. In this study we measured the number of crystal cell in the biggest of these clusters in a panel of isogenic lines fully sequenced with great genetic variability between them (Drosophila Genetic Reference Panel) (Mackay et al. 2012). The results show that the number of produced crystal cells in these clusters varies greatly between lines. The Genome Association Study indicates that the *G protein-coupled receptor kinase 1* (*Gprk1*) is essential to achieve the correct load of hemocytes in larva. Interestingly, the vertebrate ortholog *GRK2* plays a similar role in innate immune cells (Otten et al. 2013).

## 3.2 Introduction

Organ size control is a complex process that involves coordination between different cell types and genetic cascades. The final organ size will ultimately depend on cell size and cell numbers. The size control of the hematopoietic system involves another layer of complexity because blood cells are formed in one location, disperse through circulation and colonize new sites where they can have hematopoietic activity. How blood cell numbers are controlled is thus a difficult question to tackle. The *Drosophila* larval hematopoietic tissue is a good model to address this problem because relatively few differentiated hemocytes are produced and they may be categorized in only three types: plasmatocyte, crystal cell and lamellocyte. Plasmatocytes are phagocytic cells and constitute the majority of hemocytes in the larva (up to 95%) (Lanot et al. 2001). Crystal cells are non-phagocytic cells that produce proPhenoloxidase (PPO), a essential enzyme in the melanization cascade (Binggeli et al. 2014). Lamellocytes are long flattened cells that do not phagocyte but rather encapsulate large foreign bodies.

All three hemocyte types intervene in one of the most prevalent immune threats to *Drosophila* larvae in nature: infection by parasitoid wasps (Fleury et al. 2004). If parasitoid development is successful inside the larva, it will consume the internal body tissues and kill the larva. To avoid wasp embryonic development, *Drosophila* larvae mount a cellular immune response that involves the production of lamellocytes, the hemocyte type that is rarely seen in healthy larvae (Sorrentino et al. 2002). This cellular immune response involves the two big compartments of hemocytes in the larva, the lymph gland and the haemocoelic hemocytes that consist of

circulating cells and sessile cells in epidermis-associated clusters (Lanot et al. 2001; Meister & Ferrandon 2012). The lymph gland plays an important role in parasitoid resistance. Wasp infection dramatically changes hematopoiesis in the lymph gland, which start to differentiate lamellocytes and is disrupted within a few hours to release the lamellocytes into circulation (Sorrentino et al. 2002).

Haemocoelic plasmatocytes have a crucial role in the beginning of the immune response. They first recognize the parasitoid egg and possibly signal to the lymph gland to start the production of lamellocytes (Crozatier et al. 2004). Moreover, plasmatocytes adhere to the wasp egg and form tight junction with one another, thus producing the first layer of cells around the capsule (Carton et al. 2008). After this, lamellocytes adhere to the first layer of plasmatocytes and form the capsule that ultimately will become melanized. Also crystal cells, the only source of PPO1 and PPO2, seem to play a role in this response since the capsule is not melanized in PPO1/PPO2 double mutants (Binggeli et al. 2014).

Part of the success in defeating a parasitoid wasp infection appears to rely on the number of haemocoelic hemocytes that a larva has prior to wasp infection (Sorrentino et al. 2004). There is a positive correlation between the number of hemocytes and the successful resistance to the parasitoid in different *Drosophila* species (Eslin & Prevost 1998). Also, when *Drosophila melanogaster* outbred populations are selected in the lab for parasitoid resistance, the evolved populations produce higher number of hemocytes in the absence of parasitism (Kraaijeveld et al. 2001). Hence, the control of hemocyte loads appears to be an essential mechanism for the efficacy of the response against parasitoids.

Such an important fitness related phenotype should have a great genetic diversity. This seems to be the case when one compares hemocyte loads in different lab genetic backgrounds or new *Drosophila* lines derived from wild-caught females (Gerritsma et al. 2012). The control of haemocoelic hemocyte numbers is, to some degree, independent from the control of hemocyte numbers in the lymph gland. For example, misexpression of specific genes in hemocytes changes the numbers within the haemocoelic compartment without changing the overall morphology of the lymph gland (Stofanko et al. 2008). Importantly, plasmatocytes have a higher division rate in clusters (Makhijani et al. 2011) and crystal cells are formed in the clusters, as previously shown in this work. What are the genetic cascades that control the numbers of different hemocyte types produced in these locations and their relation with the circulating pool remains an open question.

In this chapter we use a Genome Wide Association Study (GWAS) approach to determine genetic components controlling crystal cell numbers in hemocyte clusters.

## 3.3 Material and Methods

### *3.3.1 Fly stocks maintenance and egglays*

All fly stocks were maintained in standard fly food at room temperature. Experiments were performed at 25°C except for RNAi experiments, performed at 29ºC. The following stocks with stock numbers in brackets were obtained from the Bloomington Stock Center: Lz-GAL4, UAS-mCD8GFP (X) (6314); HmlΔ-GAL4 UAS-2xEGFP(II) (30140); UAS-White$^{RNAi}$ (33613); UAS-*Gprk1*$^{RNAi}$ /TM3,Sb (28354); e/TM3-ActinGFP (7408). The line UAS-White$^{RNAi}$ was used

as control for RNAi experiments. A subset of 87 DGRP lines was used. For the crystal cell numbers screen experiments 10 females were allowed to lay eggs in standard food vials. To avoid larval over-crowding tubes were checked every 2h and eggs counted. Females were taken from tubes once the number of eggs reached 40.

### *3.3.2 Parasitoids maintenance and infection*

Leptopilina boulardi strain G486 females were allowed to lay eggs in second instar *Drosophila Dif* mutants at room temperature. Adult parasitoids were maintained in fly food vials with a drop of honey. For infections experiments 30 late L2 larvae were selected and transferred to fly food vials with 3 replicates per sample. 5 *L.boulardi* females were added to the tube and allowed to infect larvae for 6h, before they were taken from the tubes. Adult flies emerged from infections were scored for melanized eggs inside the body. Every adult with melanized eggs inside the body was scored as resistant and the number of flies without eggs was excluded from the analysis.

### *3.3.3 Total hemocyte loads counts and crystal cells counts*

To estimate hemocyte concentration in the hemolymph six wandering male or female larvae were selected, briefly washed in Ringer's solution and dried in filter paper. The six larvae were pooled in a glass well and bled by rupturing the cuticle in the ventral side to avoid disturbance of sessile hemocytes in the dorsal part where they are more abundant. All the hemolymph was collected and pooled in a 0,5ml microcentrifuge tube. 1µl of hemolymph was diluted in 9µl of Ringer's solution. 9,5 µl of hemolymph dilution was loaded into a Neubauer chamber and hemocytes counted in all squares of 1mm$^2$

area. This way the hemocyte concentration can be estimated by the formula: [number of counted cells]*$10^5$ cells/ml. To count crystal cells in larva we performed a 70°C heat shock treatment for 10min. With this treatment mature crystal cells auto-melanize and it is possible to visualize crystal cell numbers through the cuticle. Pictures were taken for every larva and crystal cells counted in the dorsal part of the seventh abdominal segment. We used two independent samples for every line and each sample had 8 to 10 males and females.

### *3.3.4 Phenoloxidase activity*

12 larvae were washed in Ringer's solution and dried on a filter paper. They were bled in a glass well. All the hemolymph was collected and pooled in a 0,5ml microcentrifuge tube. 3µl of hemolymph was diluted in 9µl of Ringers solution and kept on ice before the assay. 3 samples were prepared for every stock. The hemolymph samples were diluted in 90µl L-DOPA (2mg/ml phosphate buffer pH 6.6) in a 96 well plate and optical density was measured for 1 hour at 490nm. The results of the measurements were ploted against time in Prism. The region where the increase of absorbance was linear was selected to calculate the maximum absorvance velocity: MaxAbs/min= $(Abs_{t+1}-Abs_t)/(Time_{t+1} - Time_t)$.

### *3.3.5 Hemocyte immunohistochemistry*

6 larvae were bled in 20µl Ringer's solution. Hemocytes were allowed to settle to slide glass in a humid chamber for 10min and fixed with 4% formaldehyde solution for 20 min. After fixation cells were washed three times with PBS and blocked with PBT (PBS + 0,1% TritonX, +1% normal goat serum) for 30 min. After washing the cells with PBS anti-*lozenge* antibody was added (1:100 dilution,

Developmental Studies Hybridoma Bank) and cells incubated overnight at 4°C. Cells were then washed 3 times with PBS for 15 min and the secondary antibody Alexa Fluor 488 (1:1000 dilution, Life Technologies) was added. Cells were incubated at 4ºC overnight with the secondary antibody. The secondary antibody was washed three times with PBS. DAPI was added and incubated for 3 min followed by 3 washes with PBS. Slides were mounted with 80% glycerol solution and kept at 4°C before image acquisition.

### *3.3.6 Statistical analysis*

Mean crystal cell number was used to test for the effect of sex and *Wolbachia* status with ANOVA. The GWAS was performed in the DGRP website (http://dgrp.gnets.ncsu.edu/). ANOVAS were performed when several comparisons were necessary and Dunnett's multiple comparison tests were applied to test differences between pairs of treatments. Statistical test and graphics were performed in Prism v5.01 (GraphPad Software) and R v2.15.2

## 3.4 Results

### 3.4.1 Crystal cell numbers vary across DGRP lines

To tackle the genetic basis of hemocyte number control we phenotyped the number of crystal cells in the lines of the *Drosophila melanogaster* Genetic Reference Panel (DGRP) (Mackay et al. 2012) This panel consists of 168 fully sequenced inbred *D. melanogaster* lines that were derived from an outbred population (DGRP Freeze 1.0) (Mackay et al. 2012). More recently, the number of sequenced lined was expanded to 205 lines (DGRP Freeze 2.0) (Huang et al. 2014). The sequencing of the lines permitted to distinguish ~5 million Single Nucleotide Polymorphisms (SNP). Upon phenotypic characterization of the lines, it is possible to statistically test the association between phenotype and each SNP.

Here we sought SNPs that somehow affected hematopoiesis in larval hemocyte clusters. To this aim, we counted the number of crystal cells in the dorsal cluster of the seventh abdominal segment (A7). In this cluster there is a great concentration of hemocytes and, as we have shown before (Fig 2.2 A), the total number of crystal cells correlates with the number of crystal cells in this cluster. Also, counting the number of crystal cells is a very straightforward procedure through a simple heat-shock treatment and subsequent imaging (Rizki 1957; Neyen et al. 2014) (see methods). This method makes it easy to count crystal cells underneath the cuticle because they become melanized.

We have characterized 87 randomly chosen Freeze 1.0 DGRP lines. We have found a great variation in crystal cell numbers between lines (Figure 3.1). Variation spans across two orders of

magnitude. On one end of the spectrum we find an extreme case in which it is not possible to see any melanized crystal cells. On the opposite side of the distribution, the line with higher crystal cell density has a mean of 83 crystal cells in females and 77 in males (Figure 3.1). The number of crystal cells tends to be higher in females and sex has statistically significant effect in the ANOVA (mean ± SEM; females= 33.04 ± 0.61 and males= 25.37 ± 0.51; p-value<$2.2*10^{-6}$) (Figure 3.1A and 3.1B). This result is probably reflecting the fact that females are bigger than males (Ashburner et al. 2005).

Roughly half of the DGRP lines are infected with *Wolbachia pipientis*, an intracellular endosymbiont bacterium found in many insect species (Huang et al. 2014). Because *Wolbachia* presence can influence immune related traits (Teixeira et al. 2008), we tested if it affects the number of crystal cells. Indeed, there is a significant effect of *Wolbachia* infection status in crystal cell numbers (mean ± SEM; *Wolbachia*$^{+}$ =28.25 ± 0.61 *Wolbachia*$^{-}$ = 29.69 ± 0.54; p-value=0.01225) (Figure 3.1A).

Overall, we can conclude that there is a large variation in crystal cell numbers within *Drosophila* that is mostly determined by the genetic make-up of the flies though a small effect of the symbiont *Wolbachia* can be detected. This characterization establishes a level of phenotypic variation for this trait, which holds the promise of a successful association analysis.

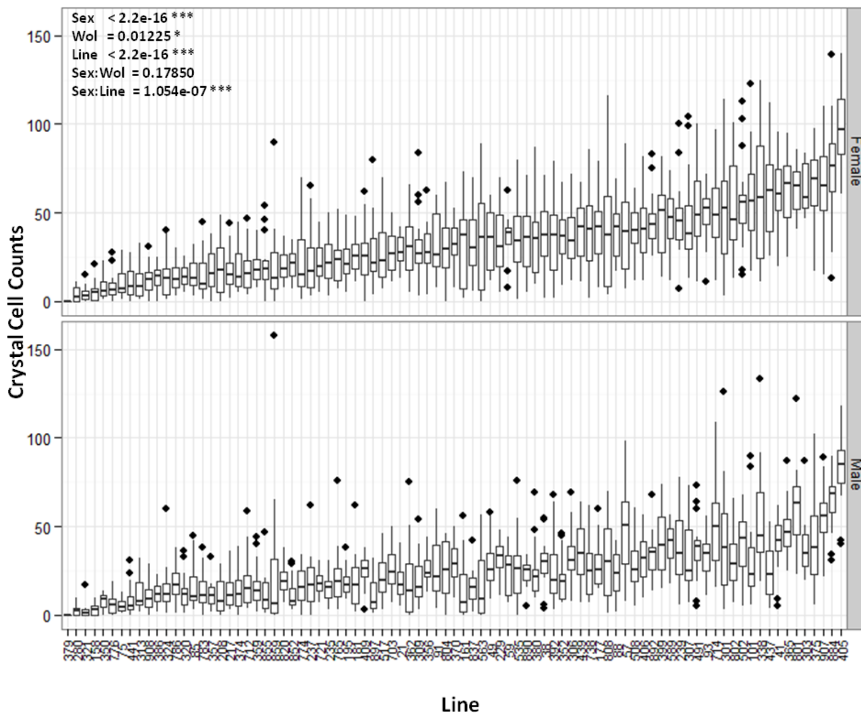



**Figure 3.1 – Crystal cell counts in DGRP lines.** Box-and-Whisker plots of crystal cell counts in the dorsal hemocyte cluster of the A7 abominal segment. Crystal cells were blackened by a heat shock treatment for 10 min at 70ºC. Lines are ordered according to mean values in females.

### *3.4.2 No correlation is found between parasitoid resistance and crystal cell numbers*

As mentioned above, hemocyte loads correlate positively with parasitoid resistance across different *Drosophila* species (Eslin & Prevost 1998; Kacsoh & Schlenke 2012). Yet, the importance of crystal cell numbers in parasitoid resistance is not clear. Nonetheless, we can expect that crystal cells play a role in parasitoid resistance since they are the only source of pro-Phenoloxidase 1 (PPO1) and pro-Phenoloxidase 2 (PPO2) (Binggeli et al. 2014). Without these

proteins the capsule formed around the parasitoid egg does not melanize (Binggeli et al. 2014). Hence, we tested for a correlation between crystal cell numbers and parasitoid resistance.

To this purpose, we infected third instar larvae with *Leptopilina boulardi* females, strain G486 (Russo et al. 1996) (see methods). As observed for crystal cell number (see above), we could measure a large range of resistance to parasitoid infection in these lines (Figure 3.2A). Indeed, we can find a spectrum of survival that spans from completely susceptible lines (100% mortality) to 80% survival. Interestingly, we find that the majority of the DGRP lines is poorly resistant to the infection in spite of the fact that we used a parasitoid strain characterized as susceptible to *Drosophila* immune reaction (Russo et al. 1996) (Figure 3.2A). Because *Wolbachia* infection has been reported to have a small effect in *D. melanogaster* larvae resistance to *L. heterotoma* infection (Xie et al. 2013) we measured the interaction between these two parameters. *Wolbachia* infection status, in our experiment, influences parasitoid resistance (mean ± SEM; *Wolbachia*$^-$ =9.45% ± 1.52 *Wolbachia*$^+$ = 15.25% ± 1.74; p-value=$7.29 \times 10^{-5}$). Notably, when we test the correlation between crystal cell numbers and resistance there is no significant effect (Figure 3.2B).

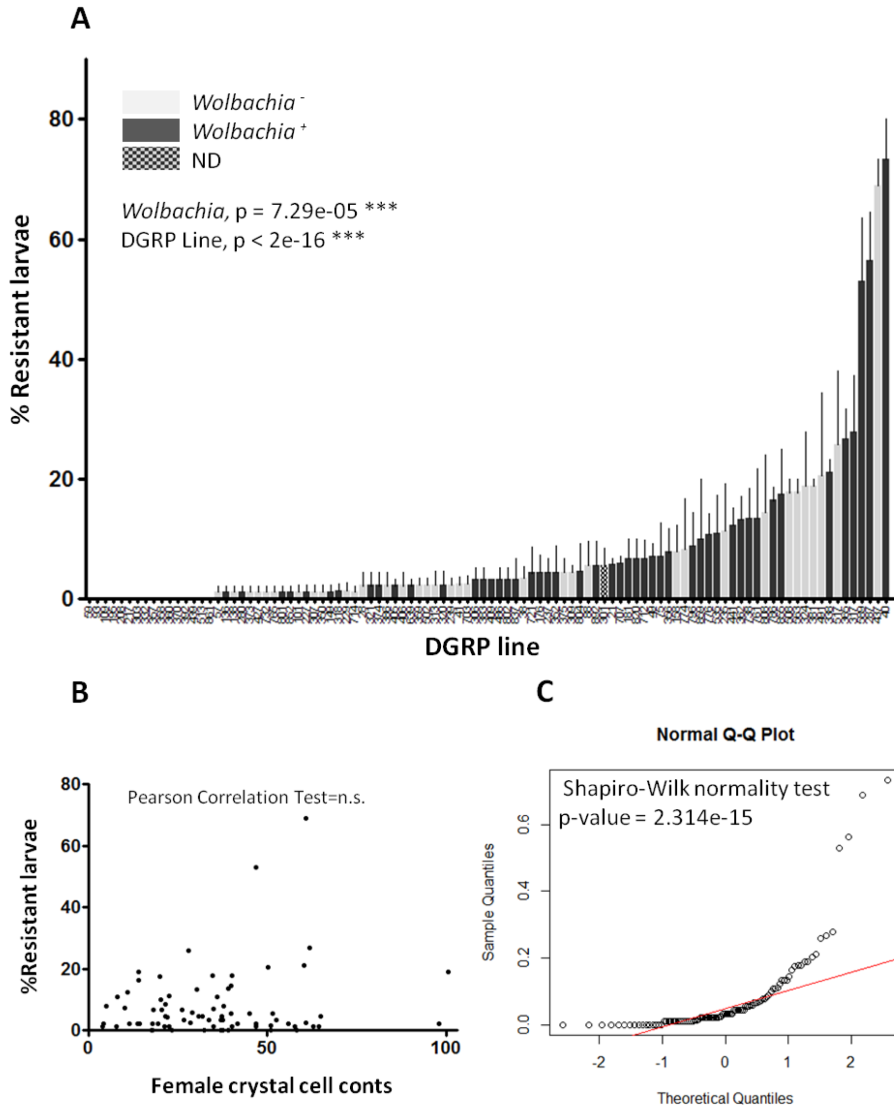



**Figure 3.2 – *L. boulardi* resistance in DGRP lines. (A)** Percentage of resistant larvae to *L. boulardi* parasitoid infection, *i.e.*, percentage of parasitized larvae that metamorphosed into adults. **(B)** There is no correlation between crystal cell numbers counted in the A7 dorsal cluster and *L. boulardi* resistance. **(C)** The data of *L. boulardi* infection deviates from normality.

### *3.4.3 Further characterization of the 10 top and 10 bottom DGRP lines*

With the objective of characterizing better the differences between lines with high and low crystal cell numbers, we picked the 10 lines on each extreme of the distribution. When we retest these lines for crystal cell numbers we still could separate them into two groups (Figure 3.3A). Importantly, we could replicate the results from the first round of phenotyping. Namely, melanized crystal cells were absent in line 379 and, line 405 displayed the highest number of crystal cells. But, the difference in crystal cell numbers could be explained by differences in total hemocyte counts. To check if this would explain the results, we estimated the hemocyte concentration in these lines. There is no trend between the two groups (Figure 3.3B). Noticeably, the two lines with less crystal cell numbers are the ones with higher hemocyte loads in circulation (Figure 3.3B). This could mean that these lines do not form clusters and all the cells are in circulation. However, this is not the case because we could see in all lines, under bright-field microscopy, hemocytes in the A7 segment clusters (data not shown).

One other possibility to explain the differences in crystal cell numbers is that the percentage of crystal cells is drastically different between lines. To percentage of crystal cells in circulating hemocytes we used the anti-*lozenge* (Lz) antibody in hemolymph smears. Using antibody staining we could be more confident in crystal cell characterization than with morphological criteria. Note that with this methodology we will underestimate the number of crystal cells because some of them will burst during the process. When we compared *lozenge*$^{+}$ cells between groups we could not confirm that

lines at the top of the distribution have higher percentage (Figure 3.3C).

Since the heating leads to spontaneous activation of PPO in crystal cells it is reasonable to assume that the differences found in our screen would be correlated with the melanization capacity of the larvae. To test for this correlation, we measured PO activity with a DOPA quantitative colorimetric assay (Neyen et al. 2014) (see methods). Unexpectedly, there is no correlation between PO activity and crystal cell counts (Figure 3.3D). It is worth noticing that, although we could not find a correlation between PO activity and crystal cell numbers, there is a great variation in measured PO activity. Strikingly, one of the DGRP lines (908) consistently shows no signs of melanization in our assay (Figure 3.3D).

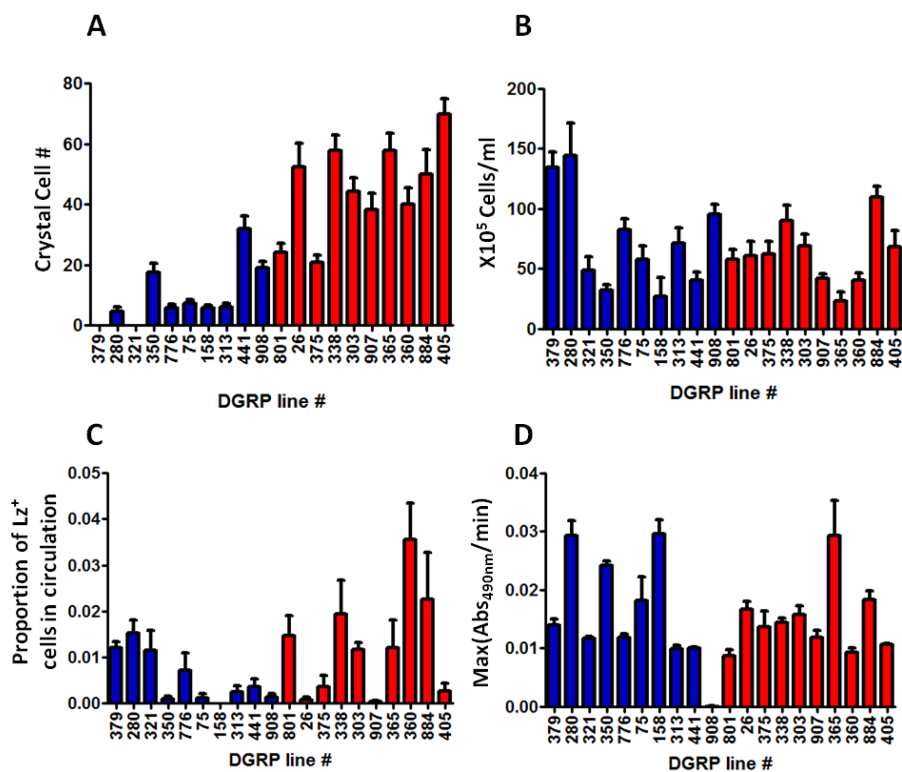


**Figure 3.3 (*preceding page*) – Characterization of the 10 top and 10 bottom DGRP lines.** All analysis were performed in females **(A)** Recounts of crystal cells in the 10 top and bottom DGRP screened lines forms two groups of lines, high (red bars) and low (blue bars) crystal cells numbers. **(B)** There is no correlation between crystal cells counts in clusters and concentration of cells in circulation. **(C)** There is no correlation between crystal cells counts in clusters and Lz+ cells in circulation. Hemocytes were stained with Lz antibody **(D)** There is no correlation between crystal cells counts in clusters and Phenoloxidase activity. Phenoloxidase activity was inferred by measuring by the absorbance increase at 490nm, correlated with the oxidation products of L-DOPA by phenoloxidase.

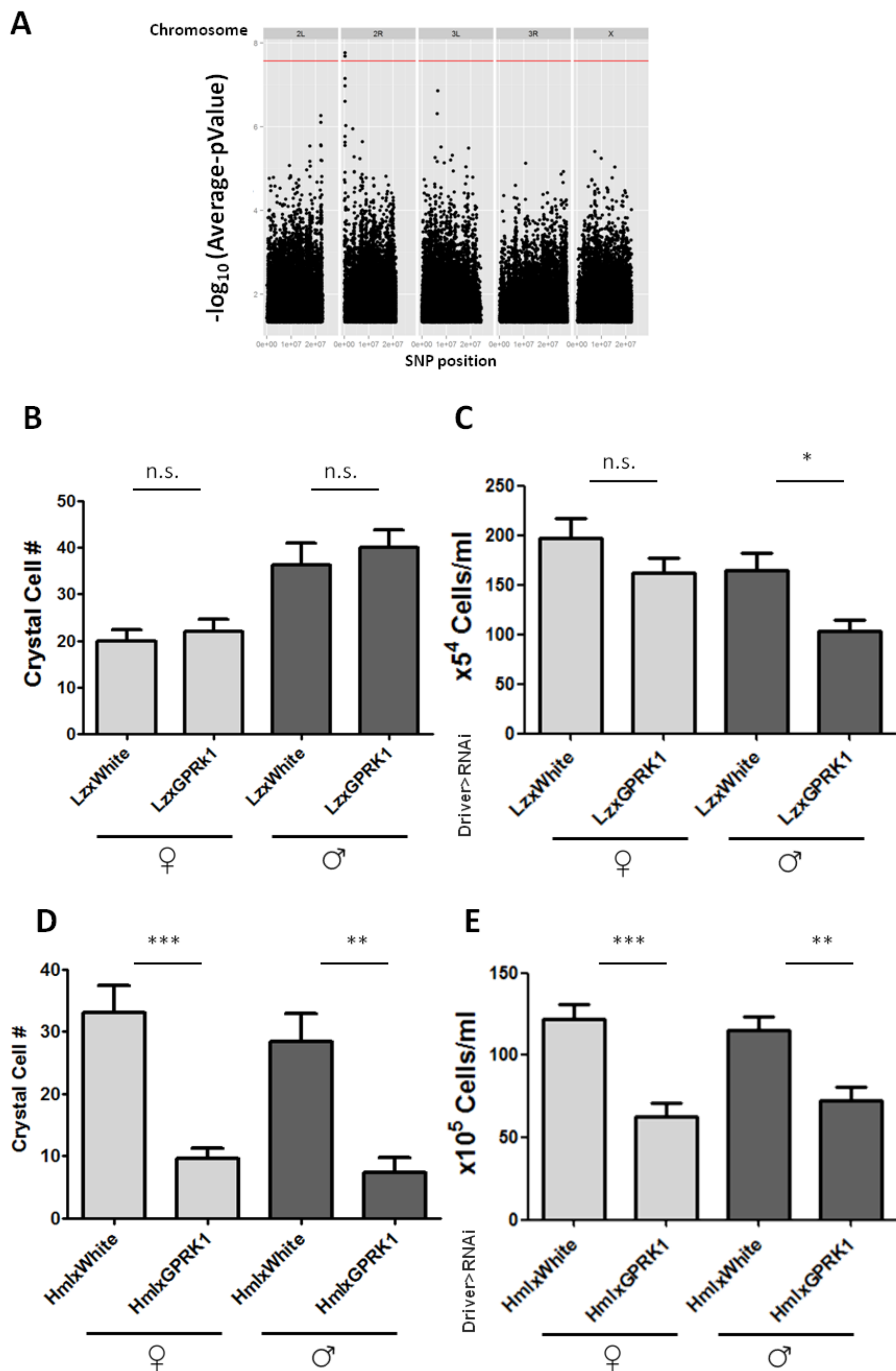
### *3.4.4 GWAS for crystal cell numbers*

In an attempt to dissect the genetic basis for differences in crystal cell numbers, we performed a Genome-Wide Association Study (GWAS). To perform this analysis, we made use of the web-based pipeline provided by the Mackay laboratory (Mackay et al. 2012) (http://dgrp.gnets.ncsu.edu/). The first version of this webtool runs an ANOVA for every SNP that is present in at least 5% of the lines. The statistical analysis results in p-values for every SNP with regard to its association with the phenotype under study. The results are presented with a $-\log_{10}$ transformation of the data for better visualization (Figure 3.4A). Since with our results we did 1,894,937 comparisons we performed a Bonferroni correction to pick the p-values that are significant, $-\log_{10}(0.05/1894937)=7.58$. Using this stringent cutoff only two SNPs are statistically significant (Figure 3.4A). These SNPs form, in fact, a haplotype with two consecutive base changes in the fifth intron of the gene *G protein-coupled receptor kinase 1* (*Gprk1*). In *Drosophila,* this protein functions as the kinase of major Rhodopsin *Rh1* (Lee et al. 2004).

The fact that a SNP is significant in a GWAS can mean that the SNP itself is the cause of the phenotype or that it is in close linkage with a SNP that is responsible for the phenotype (Mackay et al. 2012). The two SNPs distinguish two haplotypes present in DGRP lines. When we test the sequence ± 10 nucleotides surrounding the two SNPs in a transcription factor binding site database (JASPER), an interesting difference emerges. Of the two haplotypes only the major one is recognized by the heterodimer of *runt*/*Big brother* (Meng et al. 2005). Runt recognizes the DNA binding sequence through a conserved Runt domain that is also present in Lozenge transcription factor (see below).

More recently, the DGRP panel was updated and SNPs were reassessed (Freeze 2.0) (Huang et al. 2014). In this new pipeline, phenotypes are adjusted for *Wolbachia* and relatedness between lines. When we run our analysis in this new version there is no longer any statistically significant SNP. In short, the positive SNPs in our first analysis did not pass the quality filter of the second round. Nevertheless, we considered that there were enough reasons to test if *Gprk1* plays a role in crystal cell number control (see below).

As for the wasp infection trait, we did not perform a GWAS with *L. boulardi* resistant phenotype data because the means distribution deviates greatly from normality (Figure 3.2C). This type of results would interfere greatly with the statistical analysis and produce highly unreliable data (Mackay et al. 2012).


A
Chromosome
2L
2R
3L
3R
X
-log10 (Average-pValue)
SNP position
B
n.s.
n.s.
Crystal Cell #
LzxWhite
LzxGPRK1
LzxWhite
LzxGPRK1
♀
♂
C
n.s.
*
x5^4 Cells/ml
Driver>RNAi
LzxWhite
LzxGPRK1
LzxWhite
LzxGPRK1
♀
♂
D
***
**
Crystal Cell #
HmlxWhite
HmlxGPRK1
HmlxWhite
HmlxGPRK1
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♂
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***
**
x10^5 Cells/ml
Driver>RNAi
HmlxWhite
HmlxGPRK1
HmlxWhite
HmlxGPRK1
♀
♂

**Figure 3.4 (*preceding page*) – GWAS suggests that *Gprk1* is important for hemocyte numbers development**. **(A)** two SNPs show a significant association with crystal cell numbers. The red line represents the Bonferroni corrected 0.05 p-value threshold **(B)** When driven with Lz-GAL4 *GRPK1*$^{RNAi}$ the number of crystal cells is not reduced. **(C)** The concentration of hemocytes is slightly reduced in males when *Gprk1*$^{RNAi}$ is driven with Lz-GAL4 **(D)** HmlΔ-GAL4 driving *Gprk1*$^{RNAi}$ leads to a reduction in crystal cells. **(E)** The concentration of hemocytes is reduced in males and females in HmlΔ>*Gprk1*$^{RNAi}$. Error bars represent SEM n.s. = non-significant p-values * = p-value<0.05 ** = p-value<0.01 *** = p-value <0.001

### *3.4.5 Gprk1 is essential to control hemocyte loads*

To test the role of *Gprk1* in hemocyte number control, we first tested if it is essential in *lozenge*$^{+}$ cells to maintain the correct number of crystal cells. If we drive *Gprk1*$^{RNAi}$ with the Lz-GAL4 driver, there is no difference in the number of crystal cells in clusters (Figure 3.4B). A small reduction in circulating hemocytes is measured in the same treatment but the effect is only visible in males (Figure 3.4C). With *Gprk1*$^{RNAi}$ driven in all hemocytes making use of the HmlΔ-GAL4 driver, the number of crystal cells is reduced (Figure 3.4D). Again, this result could be due to smaller proportion of crystal cells under this condition or because the total number of hemocytes is reduced. To discern between these two alternatives, we measured the total hemocyte concentration when *Gprk1*$^{RNAi}$ is driven in all hemocytes. In this case there is also a reduction in the number of total hemocytes both in males and females (Figure 3.4E). Importantly, hemocyte clusters are present in *Gprk1* knockdown conditions and we cannot distinguish morphological differences in the lymph gland when compared to controls. Altogether, these results show that *Gprk1* plays a role in the proliferation and/or maintenance of hemocytes.

## 3.5 Discussion

Studies that compare hemocyte loads in different *Drosophila* species and parasitoid resistance show that there is a positive correlation between the two phenotypes (Eslin & Prevost 1998; Kacsoh & Schlenke 2012). Thus, it is reasonable to assume that it is vital to achieve a certain hemocyte concentration in circulation to mount a proper immune response against parasitoid wasps. Although the lymph gland plays an important role in parasitoid infection resistance it does not influence the number of circulating hemocytes prior to infection (Holz et al. 2003; Honti et al. 2010). The number of haemocoelic hemocytes must be controlled in hemocyte clusters and within circulating hemocytes. These are not two distinct populations of cells but a dynamic population where circulating hemocytes adhere to the clusters and cells in the clusters enter circulation (Babcock et al. 2008; Welman et al. 2010). Importantly, hemocytes behave differently in the two compartments. When plasmatocytes are in clusters they have a higher division rate than when they are in circulation (Makhijani et al. 2011) and crystal cells, that do not proliferate, are differentiated in clusters from plasmatocytes (see chapter II). Hence, total hemocyte concentration in circulation will be determined by the balance of plasmatocyte division with circulation entrance/exit and crystal cell differentiation. What determines this balance is open to investigation.

To search for genetic players in the control of haemocoelic hemocyte numbers we made use of DGRP lines. Since hemocyte loads are so important in host-parasite interaction, genetic diversity for this trait must exist. When we count crystal cells in these lines, we observe a great diversity in numbers. This diversity is somewhat

confounded by a gender effect because females tend to have higher numbers of crystal cells in hemocyte patches than males. Yet, we think that this observation is rather trivial and is a mere reflection of the fact that females are bigger than males. Of more interest is the observation that one of the lines (DGRP line 379) did not have melanized cells upon heat treatment. Nevertheless, crystal cells are induced in this line because it is possible to stain a proportion of circulating cells with anti-*lozenge* antibody. One compelling possibility is that crystal cells do not mature in this line. However, we tend not to favor this hypothesis because in our PO activity assay we can measure normal levels of melanization and this would not be possible without mature crystal cells (Binggeli et al. 2014). Another possibility to explain the absence of melanized crystal cells in line 379 is a mutation in the *PPO2* locus. A recent study shows that *PPO2* mutants, the only PPO stored in crystals, shows no melanization of crystal cells upon heat-shock treatment but *PPO1* mutants do become melanized (Binggeli et al. 2014). Hence, it is possible that in this line, crystal cells are differentiated but are deficient for PPO2 or a component essential to form the crystals. In contrast to line 379, all other tested lines show melanized crystal cells upon heat-shock treatment and their numbers vary greatly between lines.

These differences can be explained by differences in hemocyte loads and/or proportions of crystal cells in these lines. Thus, we measured hemocyte concentrations in hemolymph and the proportion of *lozenge* positive cells for the top and bottom 10 lines. None of these phenotypes can explain the crystal cell number differences encountered in the A7 dorsal patch. This result suggests that a great variability exists between lines regarding the proportion of hemocytes in clusters and in circulation. Although they are

interchangeable cell populations (Babcock et al. 2008; Welman et al. 2010), several genetic pathways must be regulating the dynamics between the two compartments (Stofanko et al. 2008). In this light, it would have been important to check total hemocyte counts in clusters and crystal cell proportions. Although we can visualize hemocytes through the cuticle in bright field microscopy it is hard to image and count total hemocytes. The alternative of using indian ink particles to enhance plasmatocytes visualization (Lanot et al. 2001) 2001 is also discarded because the protocol affects to great extent the number of hemocytes in clusters since they disperse upon sterile wounding (Márkus et al. 2005). Unfortunately, we could not develop a method without genetic manipulation to count the number of total hemocytes in the clusters. Still, our results show a great variation in the total number of crystal cells found in clusters. These differences must influence animal physiology in situations where Phenoloxidase activity is required.

Unexpectedly, our measurements of Phenoloxidase activity did not show differences between lines with high and low crystal cells numbers. We are confident that the assay is measuring the activity of hemolymph phenoloxidase activity because one of the lines (DGRP line 908) consistently showed no melanization in our assay. One possibility to explain these results is the difference between the PPO1 and PPO2. Although both PPOs are expressed in crystal cells only PPO2 is present in crystals necessary for auto-melanization upon heat-shock treatment (Binggeli et al. 2014). A confounding factor in these experiments may be the rate of PPO1 production and release into the hemolymph. Although crystal cells are the only source of PPO1 and PPO2, it is difficult to find PPO1 inside these cells by antibody immunohistochemistry (Binggeli et al. 2014). It is

conceivable that PPO1 is released from crystal cells as soon as is produced (Binggeli et al. 2014). If this is the case, a compensatory regulation mechanism may be occurring for optimal PPO1 concentration in hemolymph and PPO1 production in crystal cells. A mechanism like this could reconcile our disparate observations: a great variation in the number of crystal cells and relatively low variation in melanization capacity. Also, and despite the fact that crystal cells are necessary for parasitoid egg melanization, we did not find a correlation between crystal cell numbers and resistance to *L. boulardi* infection. Once again, the discrepancy between crystal cell numbers and melanization capacity may explain this result. At the same time, other factors must be influencing parasitoid resistance including the total number of circulating hemocytes (Eslin & Prevost 1998; Kacsoh et al. 2014). Also, the lymph gland response and the proportion of produced cell types are certainly influencing larval survival upon parasitation (Gerritsma et al. 2012). Interestingly, we find an effect of *Wolbachia* infection status in parasitoid resistance. *Wolbachia*$^{+}$ lines have a higher tendency to resist to *L. boulardi* infection. In contrast with our results, a recent study comparing *Wolbachia* infection status and parasitoid species infections, show that *Wolbachia* only confers resistance against *L. heterotoma* infection and not to *L. boulardi* infection (Xie et al. 2013). In other host-parasitoid species pairs the result is different, *Wolbachia* presence in *D. simulans* reduces the resistance capacity to *L. heterotoma* infection (Fytrou et al. 2006). Both these referenced studies only use one strain of *Drosophila*. This shows the importance of studying an effect with variable genetic backgrounds, especially when studying organisms that are co-evolving (Martins et al. 2014). Most probably, *Wolbachia* confers protection in certain genetic backgrounds and not in others. It would be interesting to explore if the

increase in resistance in the presence of *Wolbachia* is somehow correlated with the observation that *Wolbachia* infection status alters slightly the number of crystal cells. Nevertheless, the effect of *Wolbachia* presence on crystal cells numbers is minimal when compared with the influence of genetic background. Thus, it was of our interest to characterize what genetic players are involved in determining the number of differentiated crystal cells.

The GWAS statistical analysis shows that only two adjacent SNPS are significantly different when we test for crystal cell numbers effect. The SNPs are inside the fifth intron of *Gprk1* gene. This gene encodes a kinase that phosphorylates G protein-coupled receptors (GPRC). Vertebrates have 7 different G protein-coupled receptor kinases (GRKs or GPRKs). GRK1 and GRK7 phophorylate Rhodopsin and GRK2 and GRK3 phosphorylate the β-adrenergic receptor (Lee et al. 2004). Although Gprk1 has been shown to phosphorylate the major rhodopsin (Rh1), it is more closely related to vertebrate non-visual GRK2 (Lee et al. 2004). This protein is involved in several steps of hematopoiesis and blood cell physiology (Arnon et al. 2011; Otten et al. 2013). Interestingly, *GRK2*$^{+/-}$ mice have fewer innate immunity cells (Otten et al. 2013). With this, we were motivated to test the role of *Gprk1* in hemocyte numbers. From the sequence data (available at http://dgrp.gnets.ncsu.edu/) we can see that the two significantly different SNPs in our analysis always segregate together and, hence, form one haplotype in DGRP lines. When we run the two haplotypes (SNPs ±10 nucleotides) in the JASPER database (http://jaspar.binf.ku.dk/) the algorithm only finds a difference between the two. The major allele has a Runt:Big brother heterodimer binding site (Meng et al. 2005), which is absent from the minor allele. The Runt domain is a conserved 128 amino-acid long DNA binding

domain present in several proteins including the transcription factors Runt and Lozenge (Kaminker et al. 2001; Waltzer et al. 2010). The DNA binding of the Runt domain is enhanced by dimerisation with CBFβ family proteins, Brother (Bro) and Big Brother (Bgb) (Waltzer et al. 2010). We do not know if *bro* or *bgd* play a role in *Drosophila* hematopoiesis (Waltzer et al. 2010) but since Lozenge has a Runt domain and one of the DGRP haplotypes lost the binding site for Runt:Bgd we considered the possibility that the loss/reduction of transcription *Gprk1* in $Lz^+$ cells would lead to their decrease in numbers.

When we downregulate *Gprk1* in $Lz^+$ cells, the number of crystal cells is not affected. This suggests that the gene is not required for crystal cell maturation/maintenance. One other Runx factor, *RunxB*, has been implicated in cell growth and viability in two embryonic hemocyte cell lines (Boutros et al. 2004). Although we do not know if *RunxB* has a hematopoietic role *in vivo* it is possible that other Runx proteins are important for hemocyte differentiation or proliferation/viability (Waltzer et al. 2010). This means that *Gprk1* may be regulated in other plasmatocytes by Runx factors. In fact, we find an effect of *Gprk1* down-regulation when we use the pan-hemocyte driver HmlΔ-GAL4. Under these conditions, the number of circulating hemocytes decreases. The effect of *Gprk1* downregulation is most probably affecting hemocyte proliferation because we do not find differences in hemocyte morphology, but further tests are needed. As expected, the number of crystal cells in clusters is also reduced in these conditions.

Overall, these results indicate that *Gprk1* is an essential gene in the proper development of the *Drosophila* larva hematopoietic system. In the future it will be important to find what is the protein that

*Gprk1* is phosphorylating as well as the intricacies of its own transcriptional regulation to better understand the mechanisms by which hemocytes numbers are regulated.

A better characterization of *Gprk1* function in *Drosophila* hematopoiesis is important to understand if it has conserved hematopoietic roles during animal evolution. The *Gprk1* vertebrate gene ortholog, *GRK2*, plays distinct functions in the hematopoietic system. Heterozygous mutants for *GRK2* alter distinctively several blood cell types. For example, monocytes in circulation decrease but granulocyte numbers increase (Otten et al. 2013). These effects are due to hematopoietic defects in the bone marrow (Otten et al. 2013). In another cell type, the lymphocyte, GRK2 is essential to desensitize the S1PR1 receptor and overcome their attraction to blood (Arnon et al. 2011). Without GRK2, lymphocytes do not move into lymph nodes from blood (Arnon et al. 2011). These two phenotypes fall in two distinct categories, hematopoietic defects and blood cell behavior. It is important to discern between the two in the *Drosophila* hematopoietic system.

The results presented here show that a great variation in hemocyte numbers exists between different *Drosophila melanogaster* genetic backgrounds. Although hemocyte load differences correlated with immune responses have been attributed to species differences (Eslin & Prevost 1996), it is important to note that the same phenomenon can occur in a genetically diverse population. This diversity, in association with the new sequencing methods, can be used to find the genetic basis of those traits. Moreover, we can greatly improve our knowledge on the structure of the genetic variability that is used by natural selection. Finally, our results suggest

that a segregating mutation in *Gprk1* locus can be influencing the number of circulating hemocytes in natural populations.

## 3.6 Author's Contributions

Alexandre Leitão and Élio Sucena designed the experiments. Alexandre Leitão performed the experiments.

## 3.7 Acknowledgments

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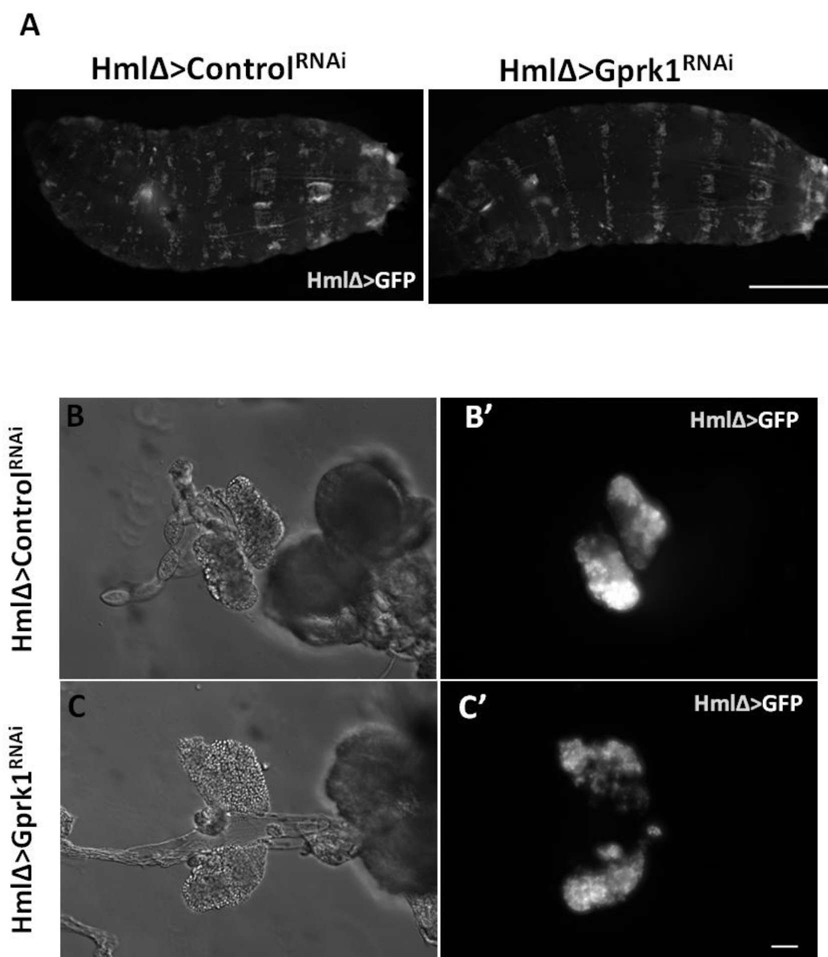
# 3.8 Supplemental Material




**Supplementary Figure 3.1 –Gprk1 knockdown does not disrupt the lymph gland nor hemocyte clusters. (A)** Hemocyte clusters are present in $Control^{RNAi}$ larvae and in $Gprk1^{RNAi}$ larvae. Scale = 1mm **(B)** Lymph gland of a control larva and the HmlΔ-GAL4 driver GFP expression. **(C)** When we knockdown Gprk1 with RNAi driven by the HmlΔ-GAL4 driver it is still possible to find intact lymph glands without changed morphology. Scale = 50µm

# 4 General discussion

In this work we set out to advance the description of hematopoiesis during larval stages of *Drosophila melanogaster*. The cellular immune system of the *Drosophila* larva is, arguably, the best studied cellular immune system of an invertebrate species. The study of cellular physiology and differentiation of *Drosophila* hemocytes gives us the opportunity to understand key concepts in cell biology including: phagocytosis, cell migration, chemoattraction and intracellular signaling (Wood & Jacinto 2007). The best characterized cellular immune response in *Drosophila* is the encapsulation response against parasitoid wasp eggs (Carton et al. 2008). This interaction embodies the notion that ecological interactions between parasites and hosts are amongst the strongest driving forces in evolution (Schmid-Hempel 2001). Yet, to fully understand interesting evolutionary and ecological patterns such as the intraspecies geographical diversity in larval parasitoid resistance, fundamental mechanistic elements of this phenomenon need to be deciphered. For example, it is essential to understand how different hemocyte types differentiate in *Drosophila* larvae to understand its differential regulation.

The lymph gland has proven to be a prime model to understand hemocyte development and to allow for a great deal of valid extrapolation onto vertebrate hematopoiesis (Evans et al. 2003). But the lymph gland of *Drosophila* has a particularity that is not found, for example, in Lepidopteran hematopoietic organs: it does not release the produced hemocytes during homeostatic larval development (Holz et al. 2003). Hence, to fully understand how the cellular immune system develops during larval development we need

to also understand in the development of haemocoelic hemocytes, the hemocytes that are produced during embryogenesis and persist in larval stages as circulating and sessile cells. This thesis constitutes an effort towards filling this gap in our knowledge of the mechanisms regulating larval hematopoiesis.

## *4.1 Haemocoelic hemocytes compartments*

The haemocoelic hemocytes form a dynamic population of cells oscillating between circulation and sessile patches found underneath the epidermis (Babcock et al. 2008; Makhijani et al. 2011). The sessile population of hemocytes is not randomly dispersed; it forms clusters of densely packed cells (Lanot et al. 2001). The cells can move in these patches during larval development but the movements are very restricted (Babcock et al. 2008). Only when the larva pupariates will hemocytes disperse from the clusters and start to scavenge for dead cells (Regan et al. 2013). Even in wounded larvae, sessile hemocytes do not seem to be attracted to the wound as observed in the embryo (Babcock et al. 2008; Evans & Wood 2014). Instead, cells that are in circulation are sequestered to the wound (Babcock et al. 2008). Nevertheless, the hemocytes inside clusters are active cells. For example, they can phagocyte bacteria (Lanot et al. 2001). Importantly, they can be recruited to circulation upon parasitoid infection (Zettervall et al. 2004). This recruitment of sessile cells into circulation increases dramatically the number of cells in circulation and possibly is essential for the initiation of the encapsulation response by plasmatocytes.

Hemocyte clusters are present in every segment of the larva but their size is different along the body axis. In the posterior

segments clusters are larger but this distribution may be simply resulting from the fluid dynamics of larval hemolymph. Due to the fact that hemocytes are clustered in higher numbers in the last two abdominal segments it was proposed that they act as hematopoietic tissue (Kurucz, Váczi, et al. 2007). This definition implies that hemocyte cluster architecture influences cell differentiation and/or proliferation. If the cells behave in the same manner when clustered or isolated, the clusters do not function as hematopoietic tissue.

The first attempt to characterize hemocyte clusters as hematopoietic organs was done by Markus and colleagues (Márkus et al. 2009). In this report the authors take cells from the posterior clusters and transfer them into another larva. The transferred cells differentiated into lamellocytes (Márkus et al. 2009). This was an important finding to understand the contribution of different hemocyte compartments to the encapsulation response against parasitoid eggs. The authors also conclude that a pool of undifferentiated hemocytes exists in the posterior hemocyte clusters. However, the necessary complementary experiment where cells would be transferred from circulation into another larva is not reported. Only if circulating cells do not differentiate into lamellocytes would we have an indication that the clusters are necessary to induce/maintain these lamellocytes precursors.

The work of Makhijani and colleagues takes another step in the characterization of hemocyte clusters as hematopoietic tissue (Makhijani et al. 2011). Their innovative work shows that neurons support hemocyte homing to form the clusters and, importantly, provide unknown trophic signals for their maintenance (Makhijani et al. 2011). This dependence on neurons for hemocyte survival is the first indication that the architecture of the clusters is necessary for the

correct development of the cellular immune system. In the same report, it was shown that sessile hemocytes have a higher proliferation rate than circulating ones (Makhijani et al. 2011). So far we do not know if this difference is a simple property of sessile/circulation state or if cell signaling between different cell types in clusters is important for the proliferation control.

## *4.2 Hematopoietic cell fate decision in hemocyte clusters*

If the clusters are functioning as a hematopoietic tissue they are the ideal candidates to study the formation of haemocoelic crystal cells. Unlike the plasmatocytes, crystal cells are post-mitotic cells. Nevertheless, their number increases during larval development (Rizki 1957; Lanot et al. 2001). Lebestky and colleagues propose that $Lz^+$ hemocytes differentiated during embryogenesis give rise to all crystal cells during larval stages (Lebestky et al. 2003). Since crystal cells increase in number during larval stages this would indicate that $Lz^+$ cells proliferate during larval stages. In this scenario, $Lz^+$ cells would behave as pro-crystal cells and once fully mature they would enter a post-mitotic state. Our results do not support this hypothesis. We do not observe $Lz^+$ cells dividing and the proportion of $Lz^+$ induced cells in clusters is sufficient to explain their increase throughout development. Hence, there is no $Lz^+$ lineage that is formed in the embryo and gives rise to larval haemocoelic crystal cells. Instead, the precursors of crystal cells, Lz+ cells, are induced in hemocyte clusters during larval development. Noticeably, we also show that *Serrate* expression in Lz- hemocytes is necessary to differentiate crystal cells. This result implies that hemocyte clustering is necessary for crystal cell formation because Notch activation by Serrate requires cell contact (Fiúza & Arias 2007). We challenged this

necessity by disrupting hemocyte clusters during third instar larva development. Our results indicate that, indeed, when hemocyte clusters are disrupted the proportion of $Lz^+$ is reduced. The dependence of *lozenge* induction on other hemocytes is confirmed further by the correlation between the number of Lz+ cells in a given cluster and the number of contacting cells. Altogether, these results show for the first time that hemocyte clusters structure is necessary for hematopoietic cell fate choice decisions. One other line of evidence to support this notion comes from an unexpected but extremely interesting finding. In *Eater* mutants, a plasmatocyte specific receptor, plasmatocytes are only found in circulation (Bruno Lemaitre, personal communication). In agreement with our results, sessile crystal cells are absent in this mutant.

It is still possible that crystal cells originate from a crystal cell precursor population that is not $Lz^+$. However, our results do not support this hypothesis. In our analysis we can discern several stages of crystal cell development. They start with a small size and low GFP expression driven by the *lozenge*-GAL4 driver. During the differentiation process they increase the GFP intensity and increase in size. In the first stages of crystal cell differentiation cells are still phagocytic and express plasmatocyte makers. These results suggest that *lozenge* expression is initiated in mature plasmatocytes that eventually transdifferentiate into crystal cells. Together with the observation that plasmatocytes can differentiate into lamellocytes upon immune challenge (Honti et al. 2010), we can appreciate the fact that plasmatocytes are highly developmentally plastic cells.

Functionally, plasmatocytes are often compared to vertebrate macrophages. Both cells types are phagocytic cells with the ability to proliferate. The differentiation of crystal cells from plasmatocytes

within sessile clusters creates, also, an interesting parallel with macrophage development in vertebrates. In homeostatic conditions, part of the tissue macrophages and dendritic cells are differentiated from circulating monocytes that colonize different tissues (Shi & Pamer 2011). Macrophages are the most plastic cells in the vertebrate's hematopoietic tissue and their specialization *in vivo* depends on the local microenvironment provided by the tissue they colonize (Wynm et al. 2013; Ostuni & Natoli 2011). Similarly, here we show that the microenvironment provided by hemocyte clusters is necessary to induce crystal cell differentiation from plasmatocytes. The differentiation of new crystal cells is dependent on *Serrate* expressed in plasmatocytes. Interestingly, in vertebrates, Notch activation in monocytes is also important for cell fate choice decisions between macrophage and dendritic cell fates (Ohishi et al. 2001).

During larval development, only a fraction of plasmatocytes differentiates into crystal cells. How this process is regulated is now open to investigation. The cells that express the *Notch* ligand *Serrate* are plasmatocytes but we do not know what is the proportion of plasmatocytes expressing it. It is important to understand if the proportion of *Serrate* expressing plasmatocytes and the number of cell contacts involving these cells is sufficient to explain the induction of the correct proportion of crystal cells. Another important factor to elucidate is the proportion of Notch expressing plasmatocytes. Unfortunately, our staining with Notch antibody did not allow to clearly discern between positive and negative plasmatocytes for the Notch protein. GAL4 reporters for Notch expression that mark crystal cells suggest that plasmatocytes are formed by a heterogeneous population of cells. Less than 50% of plasmatocytes express the reporter and unlike $Lz^+$ cells they can divide. An interesting

hypothesis is that these reporters mark a subpopulation of plasmatocytes that are prone to differentiate into crystal cells.

Another characteristic of cell differentiation that may play a role in the proportion of differentiated cells is cell sensitivity, i.e., the ability of a cell to respond to a signal. Since plasmatocytes actively divide in the clusters (Makhijani et al. 2011), the composition of cell cycle kinases is different from cell to cell. This creates a heterogeneous population of cells that can change their sensitivity for Notch activation. For example, during bristle formation cells are more sensitive to Notch signaling during the S phase (Remaud et al. 2008). However, in crystal cell development the majority of Lz+ cells are in G1 phase, which shows that they can be induced at this cell cycle phase. We still need to test whether Lz+ cells can be induced at the S and G2 phases or if they can only differentiate in G1.

All these experiments and conjectures constitutes our effort to understand an essential problem in development: how the correct proportion of different cell types is established? With this perspective it is also important to compare how the ratios of crystal cells/plasmatocyte are set in hemocyte clusters and the lymph gland. Although both tissues share a common mechanism, Notch activation in Hml$^{+}$ cells by the ligand *Serrate*, some differences must occur in the two systems. Firstly, the lymph gland is a well structured organ and cell differentiation is influenced by a Posterior Signaling Center (PSC) (Lebestky et al. 2003; Crozatier et al. 2004). It is hard to conceive such a signaling center in the diffuse hemocyte clusters (Honti et al. 2014). However, other tissues may contribute to the molecular microenvironment in hemocyte clusters that regulate proliferation/differentiation. PNS neurons seem to play such a role in hemocyte survival (Makhijani et al. 2011) but muscle and epidermal

cells may also contribute to this regulation. More striking, is the apparent absence of prohemocytes in hemocyte clusters thus precluding this cell type from a role in this "peripheral" hematopoiesis. Although some reports suggest the presence of prohemocytes in the haemocoelic compartment (Sinenko et al. 2010), the great majority of hemocyte expansion is achieved by mature plasmatocyte proliferation (Makhijani et al. 2011) and plasmatocyte transdifferentiation to crystal cells. Contrarily, in the lymph gland, crystal cells differentiate from a progenitor that originates from a prohemocyte (Krzemien, Oyallon, et al. 2010). It will be interesting to explore the genetic differences of crystal cell differentiation in the two hematopoietic tissues/organs. Particularly, how the system evolved to maintain the relatively same proportion between crystal cells/plasmatocytes in species with different total hemocyte numbers (Eslin & Prevost 1998; Havard et al. 2009).

Hemocyte clusters are also observed in the larger fly species *Musca domestica* (Nappi & Stoffolano Jr. 1972). Even in mosquito larvae, although not so well characterized, larval hemocytes have been suggested to be more abundant in the posterior segments and to from clusters (Hall & Avery 2014). We currently do not know how widespread across Diptera larvae the typical distribution of hemocytes described in *Drosophila* is. Also, there is very little information on the presence of a lymph gland in other Diptera species. In a large fly species, *Calliphora erythrocephala*, hematopoiesis occurs in the hematopoietic organ located in the posterior part of the dorsal vessel (Zachary & Hoffmann 1973). In this case the organ does not seem to be gland like and hemocytes are released from the organ throughout larval development. Maybe in this fly species there is no necessity for plasmatocyte division or crystal

cells differentiation in clusters but further characterization is required to be able to compare hematopoiesis in this species with *Drosophila melanogaster*. The results from such type of study would help us to understand how common the colonization of tissues by hemocytes to regulate hematopoiesis is.

## *4.3 Control of hemocyte numbers*

The concentration of different hemocyte types in circulation depends greatly on hemocyte clusters. The survival of hemocytes depends on trophic signals secreted by neurons in clusters and their proliferation is enhanced within clusters relative to the division rate observed in circulation (Makhijani et al. 2011). In this work, we also demonstrate that crystal cells differentiate in hemocyte clusters. Hence, to understand how larval development generates the appropriate cellular immune system to respond to parasitoid infections, we need to characterize how cell numbers are regulated in clusters. Interestingly, the control of hemocyte numbers in clusters is dependent, in part, on different genetic cascades than in lymph gland (Stofanko et al. 2008).

Our analysis of crystal cell numbers in clusters shows a great variability for this trait between isogenic lines. Certainly several characteristics are affecting crystal cell numbers in clusters including total hemocyte numbers and crystal cell differentiation rate. The GWAS we have performed only identified two statistically significant SNPs to explain the differences in crystal cell numbers. Both SNPs hit the *G protein-coupled receptor kinase 1* (*GPRK1*) and form a single haplotype. Downregulation of GPRK1 by RNAi in hemocytes leads to a reduction in total hemocyte numbers. The gene does not seem to be required for crystal cell formation and maintenance. Most likely

*GPRK1* is regulating proliferation in hemocytes. *GPRK1* phosphorylates G Protein-Coupled Receptors (GPCR), which form a very diverse family of genes. In *Drosophila* there are around 200 GPCRs (Brody & Cravchik 2000). *GPRK1* could be affecting activity of any of these receptors expressed in hemocytes or even playing a GPCR-independent role (Watari et al. 2014). *GPRK1* shares high homology with vertebrate *GRK2*. Studies in *Mus musculus* show that *GRK2* is essential to reach wild type numbers of monocytes (Otten et al. 2013). It is possible that *GPRK1*/*GRK2* is another example of the extraordinary conservation of genetic cascades involved in hematopoiesis between *Drosophila* and vertebrates (Evans et al. 2003; Waltzer et al. 2010). GRK2 is also essential in lymphocytes to regulate the sensitivity of these cells to blood/lymph nodes (Arnon et al. 2011). This influences the concentration of circulating lymphocytes in blood. Certainly this is a type of mechanism to explore in hemocytes. We are only beginning to explore how the dynamics between sessile and circulating hemocytes is controlled (Stofanko et al. 2008).

## *4.4 Open questions for future enquire*

During the course of our experiments we defined the larval hemocyte clusters as a functional hematopoietic tissue. Due to the transparency of the larval cuticle and the use of live fluorescence genetic markers it is possible to analyze cell proliferation and differentiation *in vivo*. Certainly, the conjugation of different genetic tools and the imaging techniques applied in this work allow for the further investigation of cell types differentiation/proliferation in this simple hematopoietic system. All these technical features make the differentiation of crystal cells in hemocyte clusters a good model to

study the Notch pathway in a dynamic system where cells are proliferating at the same time.

According to our analysis, crystal cells in hemocyte clusters differentiate from mature plasmatocytes. These two cells types are dramatically different. Crystal cells are non-phagocytic and have crystalline inclusion of PPO2 (Binggeli et al. 2014). The differentiation of plasmatocyte into crystal cell is, very likely, an example of transdifferentiation, the differentiation of a mature cell type into another cell type (Jopling et al. 2011). Transdifferentiation is recurrently used in cell culture systems but few examples of transdifferentiation *in vivo* are known (Jopling et al. 2011; Tosh & Slack 2002). Possibly, our fascination with stem cells leads us to underestimate the prevalence of transdifferentiation mechanisms during normal development. In this plasmatocyte to crystal cell differentiation it is possible to select consecutive steps of differentiation based only in Lz marker expression. Coupled with cell sorting techniques and transcriptional/epigenetic analyses we can hope to comprehend better how a cell transits from one differentiated state into another.

## *4.5 Concluding remarks*

We started this work to answer a simple question that was latent in a 1957 paper: how do post-mitotic *Drosophila melanogaster* crystal cells increase in number during development (Rizki 1957)? The author explicitly states that he assumes crystal cells differentiate from the division product of plasmatocytes but cannot exclude the possibility that crystal cells are differentiating in other parts of the larval body. When reading the literature and realizing that the lymph gland does not release hemocytes into circulation we understood that

the question was still to be answered (Holz et al. 2003; Honti et al. 2010). While thinking on this problem we were inspired by the suggestion that hemocyte clusters function as hematopoietic tissue (Kurucz, Váczi, et al. 2007; Márkus et al. 2009). Trying to answer to Rizki's question we ended up to confirm his two hypotheses: plasmatocytes give rise to crystal cells and this happens in another part of the larval body. As rewarding as having answered Rizki's questions has been, to have more questions arising from our conclusions in this quest to understand the fascinating regulation of cell differentiation truly captures the beauty of our journey.

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