Understanding Blood Cell Development in an Adult Fruitfly

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Certificate of Examination

This is to certify that the dissertation titled **"Understanding Blood Cell Development in Adult Fruit-fly"** submitted by Mr. Arashdeep Singh (Reg. No. MS08011) for the partial fulfilment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

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Dated:

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. Lolitika Mandal at the Indian Institute of Science Education and Research Mohali.

This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Mr. Arashdeep Singh

(Candidate)

Dated:

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. Lolitika Mandal

(Supervisor)

Acknowledgement

Ever since I started working in Developmental genetics Laboratory (DGL), I feel privileged to be working with some of the most fantastic people I have ever met. During this stay, I have gained qualities essential to continue working in science and I feel confident as well as motivated to pursue my career in science.

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List of Figures

Figure 1: Life cycle of *Drosophila Melaogaster* at 25 degree Celsius.

Figure 2: Schematic representation of the Gal4 UAS system.

Figure 3: *Drosophila* Lymph Gland.

Figure 4: Blood cell clusters in adult *Drosophila.*

Figure 5: ECM holding the Clusters.

Figure 6: Hml Positive cells of the Clusters

Figure 7: Pxn Positive cells of the Clusters

Figure 8: Crq Positive cells of the Clusters

Figure 9: Zcl2897 positive cells of the Clusters

Figure 10: Serrate positive cells in the Clusters.

List of Tables

Table 1: Comparison table for Vertebrate hemtopoiesis and *Drosophila* hematopoiesis.

Table 2: Larval Bleeding Vs. Adult Bleeding

Abstract:

Hematopoiesis is the process of origin and maintenance of blood cells and their progenitors. The term hematopoietic stem cell (HSC) is used to describe a cell which has the potential to divide into one or more type of blood cells. These HSCs take care of our entire hematopoietic requirement by giving rise to new cell types as and when required during development or during insults. During the last decade work on Drosophila hematopoiesis has established it as a great model system to understand the biology of hemocytes. Since there is a high degree of conservation in hematopoietic development with mammals, this model system is an obvious choice in understanding HSC biology too.

Though there are similarities reported in this biphasic process what is lacking is our understanding of hematopoiesis in adult fruit fly. The mammals are known to harbor the HSCs in the form of adult stem cells in their bone marrow. But no such analogous structure maintaining the adult HSCs in *Drosophila* is known till date. Here in this work, we intended to investigate this aspect and on doing so discovered blood cell clusters present in the abdominal area of the fly. My work was focussed in molecular characterization of the adult cluster in fruit fly.

Chapter 1

Introduction, Objectives and

Materials and Methods

1.1 Introduction

1.1.1 *Drosophila melanogaster:*

Drosophila melanogaster is a holometaboluos insect, commonly known as fruit fly, belongs to order Diptera in family Drosophilidae. *Drosophila* is one of the most exploited model organism in biological research where it has provided the unique insights in various biological phenomena ranging from genetics to physiology to life history evolution.

1.1.2 Physical appearance:

A wild type *Drosophila melanogaster* is about 2.5mm long segmented individual with three pairs of legs and a single pairs of wing. They exhibit sexual dimorphism with many important morphological differences between males and females that helps the biologists to identify the two sexes. These differences are listed below.

- Males are slightly smaller in size as compared to the females.
- Dorsal side of the abdomen in cases of males is darker when compared to females. This is because of the fusion of few posterior abdominal segments that results in darkly pigmented posterior end.
- Males have a row of dark bristles called sex comb on the first pair of their legs (tarsus) whereas females lack this structure.
- Presence of claspers surrounding the anal plate in the ventral side of the abdomen is quite conspicuous feature of a male.

1.1.3 Life cycle of *Drosophila:*

Life span of *Drosophila melanogaster* at 25^0 Celsius is of 45 days. Both the life span and rate of its development varies with temperature as it is an ectothermic species. At 25^0 Celsius, its developmental time is of 10 days and at 16 degree Celsius, it becomes 24 days.

Drosophila melanogaster is a polyandrous species. Females are receptive to males after the 8-12 hours of their emergence. A few hundred sperms are transferred to the female reproductive track during each copulation event. Female stores sperm in two mushroom

shaped structures called spermathecae and may reproduce multiple. The female lays about 400 eggs during its life time. Egg is 0.5 mm long and hatches after 24 hours of egg lying. The newly hatched larvae feed voraciously and grow for about 5 days. Within this period it molts twice to become 1st instars, 2nd instars and third Instar. This is followed by formation of a pupal-case, within which it undergoes complete metamorphosis and ecloses out as an adult fly.

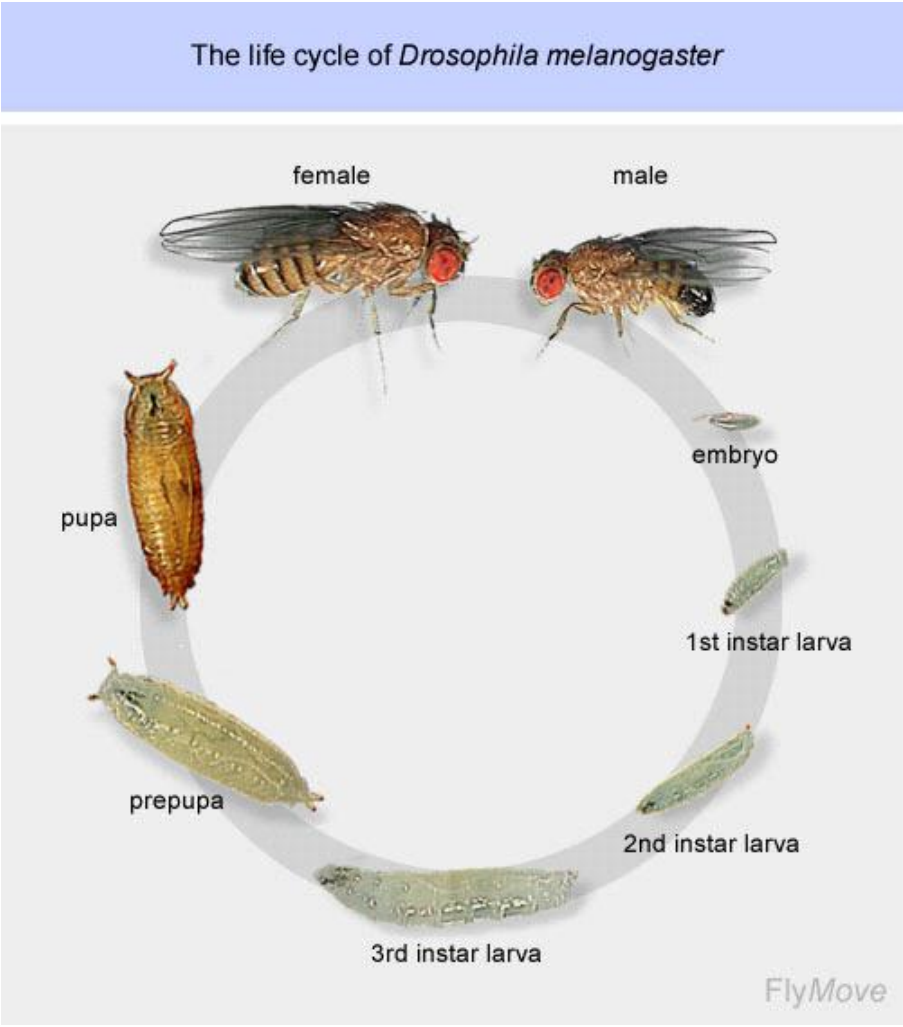

Figure 1: Life Cycle of a Drosophila. (flymove.uni-muenster.de)

1.1.4 *Drosophila* as a model system:

Past few decades of intense work has established *Drosophila* melanogaster as an excellent model system to study various biological processes. There are many features of *Drosophila* which make it a great model system to work with are listed below:

- Generation time of *Drosophila* is less (10 days) and it is easier to rear and handle in the laboratory. So it does not take too much to observe the effect of a specifically introduced mutation in *Drosophila*. On the other hand, due to high fecundity of the females, large number of eggs or progeny of desired genotype can be obtained and the results can be compared in large sample size.
- Its genome has been fully sequenced. This opens up the opportunity to manipulate almost any gene in *Drosophila* and to understand its role in normal development.
- There is large number of mutants an array of genetic tools available which makes it feasible to perform manipulations not only at the level of tissues but can be targeted to a cell type of interest.
- Gene knockouts or gene up regulation studies are feasible and help us to dissect intricate signaling pathways.

And above all, many of the molecular and signalling pathways involved in various biological processes are conserved in *Drosophila* and vertebrates. So the investigations made in *Drosophila* can be suitably extended to the vertebrate or more precisely human systems.

1.1.5 Genetic tools in *Drosophila*:

One of the most important genetic tools available in *Drosophila* is Gal4 UAS system. Gal4 UAS system is a bipartite approach of Brand and Perrimon where driver lines and responder lines can be reared separately until they are crossed with each other. Schematic representation of this bipartite approach is shown in Figure 2. Any gene which controls the expression of Gal4 is called Driver line and any gene which is placed under the control of UAS element is called responder or the reporter line.

Gal4 protein is a yeast transcriptional activator of UAS (Upstream Activating Sequence). Whenever Gal4 is transcribed, it goes and binds to UAS, and initiates the transcription of any gene which is the downstream of UAS. In *Drosophila*, many enhancer trap gal4 lines are

present where a specific gene controls the production of gal4. UAS line can be controlling the transcription of a particular gene which can be a reporter gene like GFP or RFP or a responder gene. The summary of how this system works is given in Figure2.

This system can be used in various ways ranging from reporting the gene expression or driving the ectopic gene expression. Further using RNAi of a specific gene under the control of UAS, this system can be used for down regulating a specific gene. Further, the functionality of this Gal4 UAS system can be combined with Gal80 protein, which can be used to inhibit Gal4 protein at 16 degree Celsius. But Gal 80 itself is inactive at 25^0 Celsius. So the combination of Gal4 and Gal80 can be used to perform the gene up regulation or gene down regulation at a specific time window during development by transferring the fly line suitably at higher or lower temperatures.

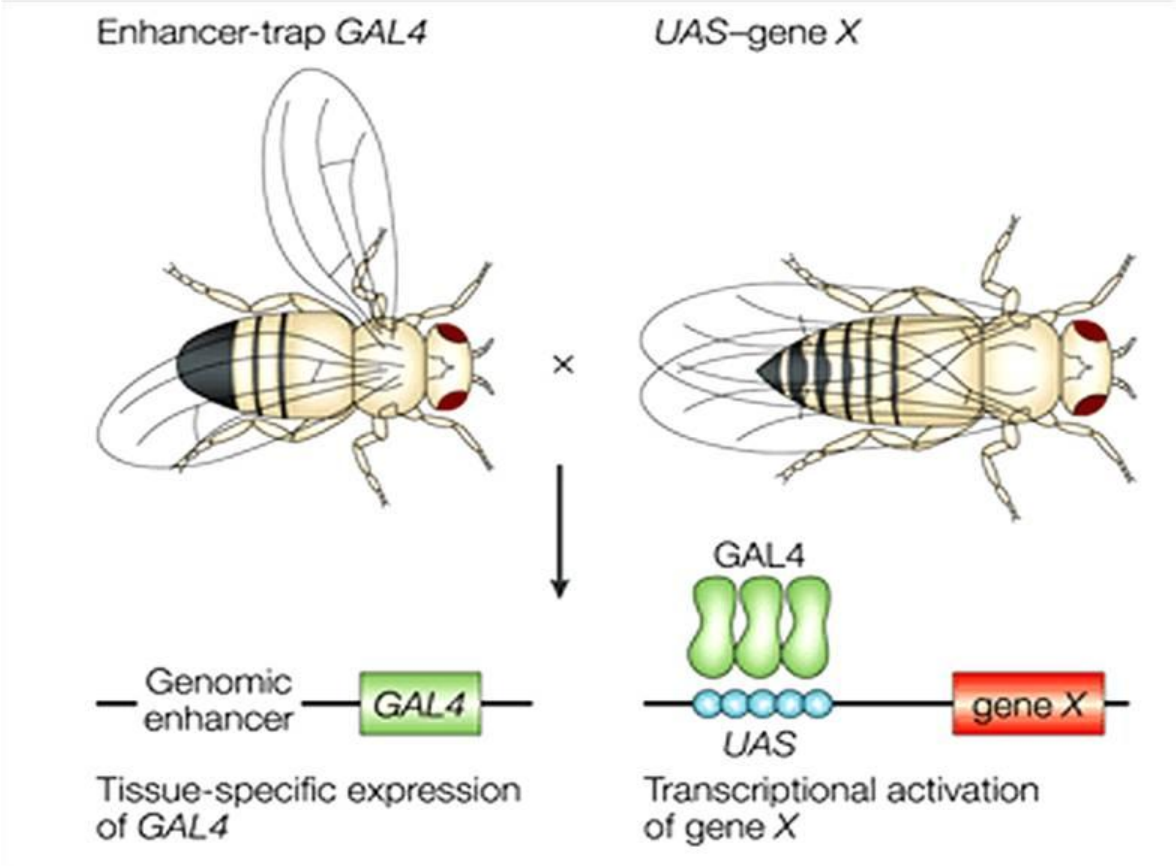

Figure 2: Schematic representation of the Gal4 UAS system. This system is used widely to express a gene in a tissue specific manner

1.1.6 Vertebrates Hematopoiesis:

Hematopoiesis is the process of blood cell genesis from their progenitor cells. In vertebrates hematopoiesis is biphasic and is separated spatially as well as temporally. The first or the primitive hematopoiesis occurs in embryonic yolk sac while the definitive is initiated in Aorta-Gonadal-Mesonephros (AGM) region which is the part of lateral plate mesoderm. Though some hematopoietic activity is found in foetal liver, spleen and placenta, the final hematopoietic site for adult stage is bone marrow where it creates foundation for adult hematopoiesis. Mammalian hematopoietic stem cell give rise to two types of progenitor cells Lymphoid and myeloid which give rise to B lymphocyte, T lymphocyte, dendritic cells as well as all type of phagocytic macrophages.

When we compare vertebrate hemtopoiesis with *Drosophila* hematopoiesis, we find that many of the transcription factor and signalling pathways involved in hematopoiesis are evolutionary conserved. Box 1 lists the conserved factors in hematopoiesis across these divergent taxa.

Box1: Comparison table for Vertebrate hemtopoiesis and *Drosophila* hematopoiesis

Transcription Factors and Cofactors	
Drosophila	Vertebrates
Serpent (Srp)	GATA
Lozenge (Lz)	Runx
Ushaped(Ush)	Friend of GATA (FOG)
Collier (Col)	Early B cell Factor (EBF)
Antennapedia (Antp)	HOX
Signalling Pathways	
Notch	Notch
Hedgehog	Hedgehog
Wingless	WNT
JAK STAT	JAK STAT
Pvf	PDGF/VEGF

1.1.7 *Drosophila* hematopoiesis:

Like vertebrate counterpart, *Drosophila* hematopoiesis is biphasic (Lebestky et al., 2000; Evans et al., 2003; Hartenstein, 2006). Moreover, the two phases**:** Primitive Hematopoiesis and Definitive Hematopoiesis are distinct both spatially and temporally.

Primitive hematopoiesis:

The primitive hematopoiesis occurs in mesoderm in the head region of *Drosophila*. The earliest known molecular signature which specifies the blood cell population is Serpent (srp), which is a zinc finger transcription factor of GATA family (Rehorn et al., 1996; Lebestky et al., 2000; Patient and McGhee, 2002). The cells expressing serpent are committed to blood cell lineage (Holz et al., 2003). GATA family of proteins is conserved from yeast to vertebrates and controls many of the developmental programs (Orkin et al., 1998; Lowry and Atchley, 2000; Cantor and Orkin, 2002; Maduro and Rothman, 2002; Patient and McGhee, 2002). There are evidences that show the involvement of murine GATA family in promoting hematopoietic development (Pevny et al., 1991; Tsai et al., 1994; Ting et al., 1996; Shivdasani et al., 1997; Pai et al., 2003; Rodrigues et al., 2005). Interestingly in mammals GATA family of proteins are the earliest hematopoietic determinants (Fujiwara et al., 2004; Ling et al., 2004). GATA family of proteins are found to interact with FOG (Friend of GATA) to promote hematopoiesis in vertebrates. The *Drosophila* homologue of FOG, pannier and U-shaped, has regulatory roles in limiting the blood vascular development (Haenlin et al., 1997; Fossett et al., 2000; Fossett et al., 2001, 2003). Furthermore Srp is found to interact with a Runx protein, Lozenge which specifies the crystal cell fate (Lebestky et al., 2000). Another important factor in specifying blood cell lineage is Tinman (Tin) which is *Drosophila* homolog of vertebrate Nkx2.5 (Tin; Azpiazu and Frasch, 1993; Bodmer, 1993). Initially Tin is expressed throughout the entire mesoderm of head and trunk. Then expression of buttonhead in the cylindrical domain of embryonic head activates Srp and represses Tin (Yin et al., 1997).

Definitive Hematopoiesis:

Definitive Hematopoiesis, which is the second phase of hematopoietic development in *Drosophila* occurs in a specialized organ called lymph gland which forms during the late

embryonic stage, becomes mature and functional during the larval stages and with the onset of pupation it ruptures to release hemocytes in circulation. The lymph gland arises from the cardiogenic mesoderm that is comparable to the lateral plate mesoderm that gives rise to definitive hematopoiesis in vertebrate. In Drosophila, this cardiogenic mesoderm gives rise to cardioblasts, pericardial cells and cells of lymph gland lineage. Strikingly, like mammals, where the blood vascular system is supposed to be derived from a common precursor cells called hemangioblast (Murray, 1932; Choi et al., 1998), there is evidence for the existence of hemangioblast like cells in case of *Drosophila* also ((Mandal et al., 2004).

This cardiogenic mesoderm is specified by the activity of Tin and a GATA factor Pannier (pnr). The Tin expression in this case is maintained by Decapentaplagic (dpp) and Heartless (htl) signalling pathways. Then activity of Wingless (Wg) signaling in the cardiogenic mesoderm is required to promote the all cell type fates. The Notch signalling plays role both during the specification of cardiogenic mesoderm as well as cardioblasts (Mandal et al., 2004). During the 12th stage of embryo, the Tin and Pnr expression in the cardiogenic mesoderm starts restricting to cardioblasts. Then a zinc finger protein Odd- Skipped is expressed in the cell clusters of T1 to A6 segment cardioblasts. The T1 to T3 segment become lymph gland progenitors whereas abdominal clusters form pericardial cells (Mandal et al., 2004). Then these lymph gland progenitor cells start expressing Srp to convert it to the true component of hematopoietic system. The lymph gland is restricted to the thoracic segments only because of expression of ultrabithorax (Ubx) in the abdominal segments (Mandal et al., 2004).

During larval stages, the lymph gland grows in size and by late second instar three distinct zones (Figure 3) harbouring different cell types comes into existence ((Jung et al., 2005)). The cortical zone (CZ) in the periphery of the lymph gland is populated by mature blood cells which is evident by markers like Hemolectin (Hml, Goto et al., 2003), Peroxidasin (Pxn, Nelson et al., 1994), and Lz (Lebestky et al., 2000). The inner core, medullary zone (MZ), expresses distinct cellular markers like *Drosophila* E Cadherin (DE- cadherin), domeless Gal4 (Bourbon et al., 2002) and unpaired3 gal4 (Agaisse et al., 2003) and ZCL 2897. The MZ consist of tightly packed cells, devoid of maturation markers and are slow cycling, label retaining and multipotent cells. The third zone, the posterior signaling center

(PSC) which acts as niche for the maintenance of progenitor populations (MZ). The PSC region expresses the Notch ligand Serrate (Ser, Lebestky et al., 2000) Antennapedia (Mandal et al., 2007), Collier (Crozatier et al., 2004) and can also be visualized by Dorothy-gal4 (Dot), and Upd3-gal4 (Jung et al., 2005).

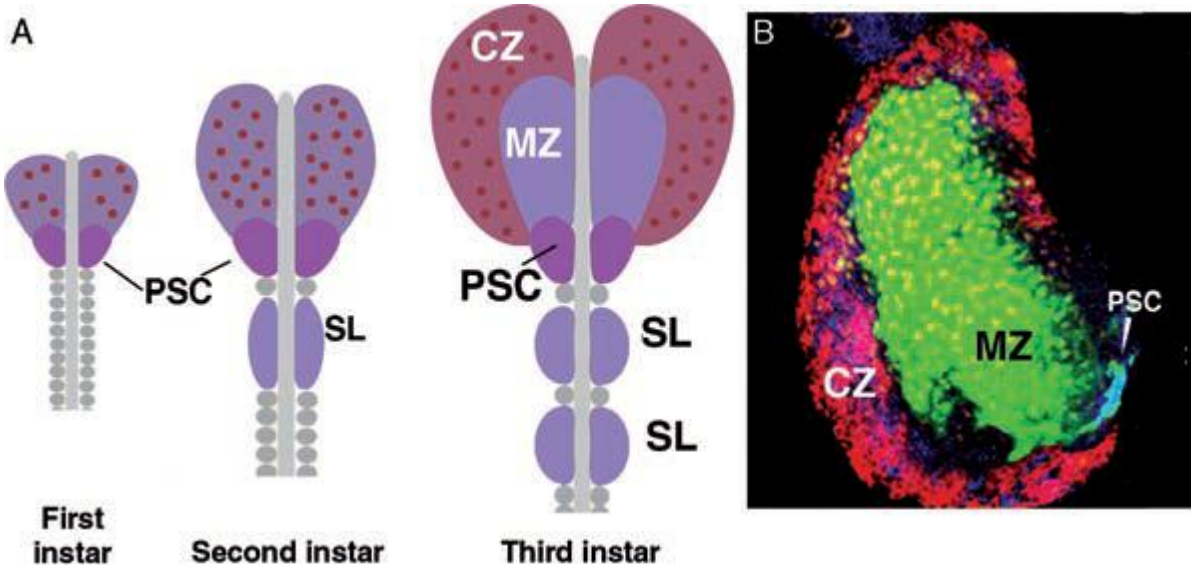

Figure 3: A Schematic representation of growth and development of lymph gland during the larval stages. B: the three zones in a mature third instar lymph gland. (*Evans et al* 2007)

1.1.8 Blood cell types in *Drosophila*: There are three different types of hemocytes present in *Drosophila*. These include 1) Plasmatocytes, 2)Crystal cells and 3)Lamellocytes.

Plasmatocytes are the most abundant cells present in *Drosophila* blood circulation (>90%, Rizki, 1956, 1978). Rest of the hemocytes are crystal cells with a few or no lamellocytes appearing in the healthy *Drosophila*. But immunological challenges such as wasp infection may trigger the differentiation of this cell type in large numbers (Rizki and Rizki, 1992; Lanot et al., 2001; Sorrentino et al., 2002).The hemocytes in *Drosophila* have roles in developmental context and in immunological context. In developmental context, they take

part in the clearing the apoptotic bodies and remodelling of larval tissue structures during pupation (Fessler et al., 1994; Murray et al., 1995; Yasothornsrikul et al., 1997; Franc, 2002). Many of the developmental abnormalities, such as disruption of embryonic ventral nerve cord are known to arise in the absence of plasmatocytes (Sears et al., 2003). Plasmatocytes clear out the pathogens by phagocytic engulfment and encapsulation of large pathogens. In contrast, crystal cells heal the physical wounds by the process of melanisation (an insect specific response) of damaged structures. The pathogens which are too large for the phagocytosis by plasmatocytes are taken care by lamellocytes which function during the encapsulation response.

1.1.9 Hematopoiesis in Adult *Drosophila*:

Although we see a high degree of conservation in the process of hematopoiesis when we compare *Drosophila* with vertebrates but vertebrates are known to harbour hematopoietic stem cells in their bone marrows, where hematopoiesis continues throughout the life of an individual. But there are no reports of hemtopoietic activity in adult *Drosophila* and it is believed that adult fly survives on the hemocytes produced during primitive and definitive hematopoieis throughout its life (*Holz et al*, 2002).

Preliminary observation from our laboratory revealed the presence of novel clusters of blood cells in the abdominal area of the fly (Figure 4). Though it was evident that they are blood cells but our interest was to find out the nature of cell types residing in this novel cluster.We therefore launched a comprehensive investigation in order to characterize this cluster and also assay its potential.

1.1.10 Objectives:

Our investigation towards the understanding of adult hematopoiesis was two prong approach. First we decided to see if hemocyte compositions of adult fly are any different from the larvae by presence of molecular markers. As the larval hematopoietic organ is known to harbour progenitor cells and the fact that it disintegrates during pupation led us to propose that maybe in adult circulation we will be able to see the presence of this progenitor that shows signature of lymph gland derived hemocytes. So for this purpose, we decided to analyse and compare the larval bleed with adult bleed.

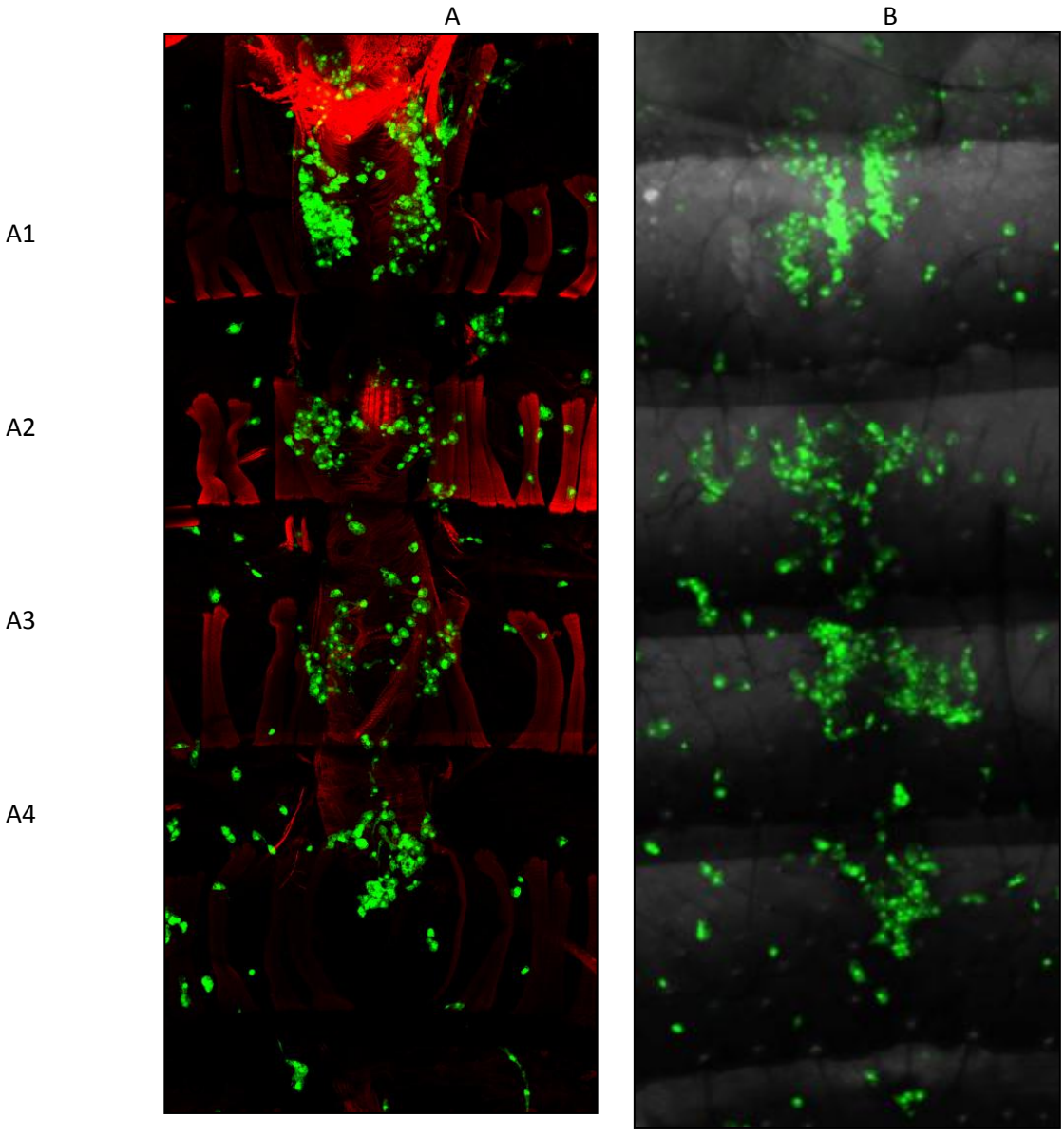

Figure 4. A. *Drosophila* hemocytes are marked in green expressing endogenous GFP under control of differentiated blood cell enhancer. Muscle is marked in red by phalloidin. B. Abdominal segment is shown in DIC image.

So, we started to do the molecular genetic characterization of the novel cluster to see if there are multiple cells types like differentiated hemocytes, stem like progenitor blood cells and niche cells are present in this cluster.

By analysing the results obtained from above two approaches, we can further follow the signalling pathways involved in the maintenance of these clusters.

1.2 Material and methods

1.2.1 *Drosophila* strains:

Fly stocks that were used in this study were obtained from different sources and Bloomington stock centre. Where ever necessary new genetic recombined lines were generated by me for detailed analysis.

Hml Gal4 UAS GFP and Pxn Gal4 UASGFP are bonafide marker of larval and embryonic differentiated hemocyte. Croquemort which is an ortholog of mammalian CD36 family of macrophage receptor is also bonafide differentiated hemocyte marker used in this study. Serrate Gal4 UAS GFP (Lebestky *et al* 2002), UAS GFPnls, UAS mCD8GFP, UAS Ds Red was used in reporter assay.

1.2.2 Immunostaining in adult fly:

Anaesthetised flies were dissected on PBS through fine scissor and fixed in 4% formaldehyde for 30min then, washed three times with PBS at 10 min interval each. Then sample was incubated on 0.3% PBT (PBS with 0.3% TritonX100) and washed with PBS three times. After that sample was blocked with 10% BSA from Sigma for 1hr at RT and incubated in primary antibody 4^0C overnight. Next, sample was given three washes with 0.3% PBT at 10 min interval and treated with 10%BSA for 1hr and incubated in secondary antibody for 4hr at room temperature. After that, sample was washed three times with PBS and either mounted in DAPI vector shield or incubated with Topro-3 (1:500) from Invitrogen. Then sample was washed one time in PBS and mounted in Vectashield from Vecta Laboratories. For muscle staining Rhodamine phalloidin (1:100) was used.

1.2.3 Antibodies :

Primary antibodies were used mouse Pericardin (1:3) from Developmental Studies Hybridoma Bank, mouse P1 (1:200) kind gift Prof. I. Ando, anti-GFP antibody: 1:100 (Sigma). Secondary antibodies were used anti-mouse Alexa 488, Alexa 568, Alexa 647 from Invitrogen.

1.2.4 Larval bleeding:

For bleeding experiments we standardized a protocol which will give appropriate fixation to the cells without inducing much of auto fluorescence in the components of bleed. The larva was washed with 1X PBS and dried over Whatman's filter paper. Then it was placed in 4 µl of 1X PBS over a glass slide and the anterior part of body was pulled out against posterior part with the help of fine needles. Then the larval bleed was collected for 5 seconds. Then it was left undisturbed for about 5 minutes to let the cells settle down over slide in 1X PBS. Then PBS was soaked with the help of Whatman's filter paper by slightly tilting the slide. Then a 4 µl drop of 4 % formaldehyde was used for fixation up to 5 minutes. Then fixative was removed in a similar way the 1X PBS was removed. Then a single wash of 4 µl 1X PBS was given in a similar way. Then a 4 µl drop of DAPI Vector shield was added and a cover slip was placed over it. Then the slides were observed under the simple fluorescence microscope.

1.2.5 Adult bleeding:

For bleeding of the adult flies, flies were anesthetized with 4 drops of ether for about 3 minutes. Then the fly was placed in 6 µl of 1X PBS and poked into thorax with the help of fine needles over a glass slide. Then the bleed was collected for 10 seconds. Earlier we tried to do the adult bleeding by puncturing in abdominal region of fly, but that gave a lot of debris and fat bodies in the bleed. Finally found the lateral side of thorax is a good place to obtain clear bleed. Rest of the protocol is same as in the case of larval bleedings.

1.2.6 Adult fly staging:

Egg were collected over a period of six hours with sufficient number of fly on *Drosophila* food containing Maize Powder, Yeast , agar and antifungal agent methyl paraben at 25^{o}C.

Batches of synchronized hatched 1st instar larvae were then reared to pupal stages in regulated number with utmost care. Flies that eclosed within a span of 1hour were collected in a fresh vial and aged for the timed experiments.

1.2.7 Imaging: All imaging was done with Zeiss LSM 780 confocal microscope using Zen 2010 imaging software and processed with imageJ software.

Chapter 2

Results and Discussion

2.1 Results

2.1.1 Results of the bleeding experiments

Following the standardization of the protocol given in 'Materials and Methods' section, we obtained the bleed samples from larvae and adults of the several transgenic reporter lines which we found relevant in context of hematopoiesis and then subjected them to fluorescence microscopy.

Name of Gene	**Larval bleed**	**Adult Fly bleed**
Hml (Hemolectin)	Present	Present
Crq (Croquemort)	Absent	Present
Pxn(peroxidasin)	Present	Present
Stat (Signal Transducer and activator of transcription)	Absent	Absent
Dot (Dorothy)	Absent	Present
Dome (Domeless)	Present	Present
How (Held out Wings)	Present	Absent
Upd3 (Unpaired3)	Absent	Absent
Hand	Absent	Absent

Box 2 : Larval Vs. Adult bleeding.

We found that Crq is one such gene which is not present in the larval bleed but it is present in the adult bleed. Crq is known to be expressed in the hemocytes of embryonic origin till the stage 13^{th} of the embryo and is absent from the lymph gland. It therefore suggest that the Crq positive cells in adult bleed are probably arising in the adult stages only and is suggestive of a hematopoietic activity that is going on during this stage. Furthermore, Dorothy (Dot) is another such gene which our result shows to be specifically present in the adult bleed only. Although Dot does not mark embryonic hemocytes, therefore it is not expected to be present in the bleed of a healthy larvae but it is present in the medullary zone of lymph gland where

it marks the blood cell progenitors. Therefore, the presence of Dot in the adult bleed can be explained in two different ways. Either it is released from the rupturing lymph gland which is less likely because there are reports which indicates that all the hemocytes from the lymph gland are differentiated before it ruptures (Gregorian *et al*, 2011). But it remains to be investigated whether the dot expression in the secondary lobes of the lymph gland is also eliminated by pupation. If it is present in these reserve pool of hemocytes than it can be derived from the secondary lobes of the lymph gland. Or the other possibility is that like Crq, Dot positive cells have also arisen from the de novo genesis in adult. In that case it proposes that the adult stages active hematopoiesis is on. Though studies (Holz, 2002) shows that the embryonic and larval hemocytes can be tracked in adult, our investigation reveals that there are few genes that are unique for each stage. Dot, Crq are adult specific whereas held *out wing* (How) expression by reporter assay was only detected in the larval bleed and interestingly was absent in the adult.

2.1.2 Results of the Genetic Characterization of clusters

Anatomical Location of the Cluster

First and one of the most important results that we obtained from the investigations of adult fly is the physical attachment of the cells of this cluster with the extracellular matrix (ECM). When the ECM was labelled by using immunostaining against Pericardin (prc), which is a *Drosophila* type 4 collagen and one of the major constituent of ECM, we found that the cells of the cluster are embedded in the prc network (Figure 4).

Genetic characterization of the cluster:

In an attempt to do the genetic characterization of the cluster, I used Gal4 UAS system to mark the gene expression as explained in the ‘introduction’ section. I dissected the abdomen of specific fly line to be characterized and mounted in DAPI vector shield or in Topro to mark the nucleus using the protocols described in the ‘Materials and Methods’ section. After mounting, the samples were photographed using confocal microscopy.

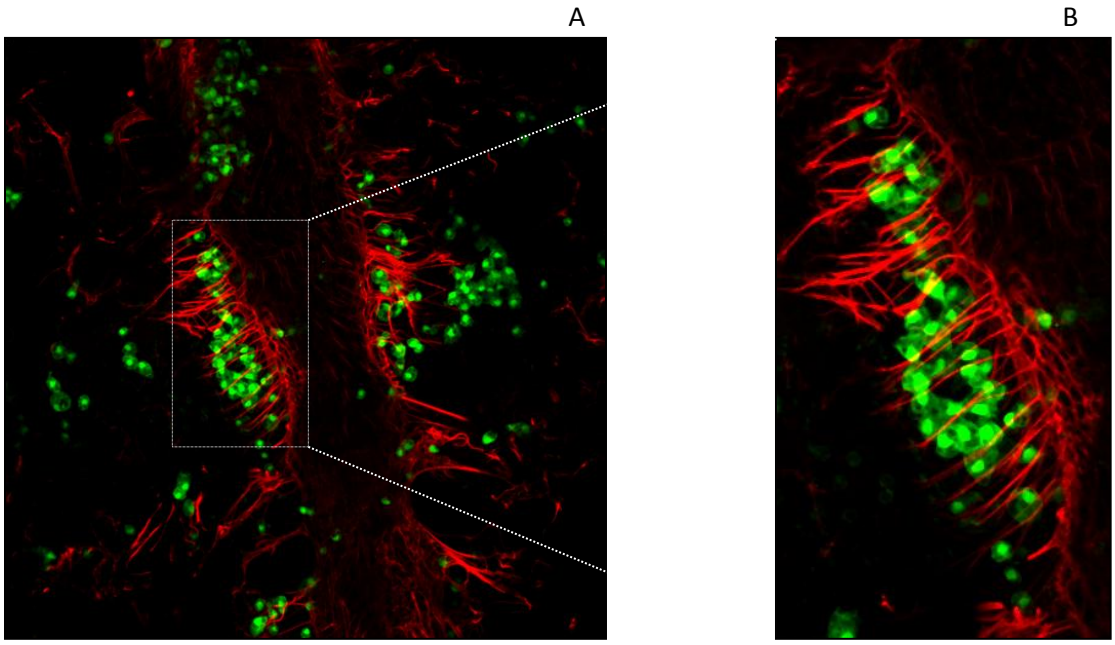

Figure 5. A. GFP expressing cells are differentiated cells marked by Hml Gal4 UAS GFP. ECM are shown in red by pericardin. B. A zoomed in view showing the details of intercalation between the hemocytes and its ECM.

Characterization of differentiated blood cells:

The first marker that we tested was Hemolectin (Hml). Hml marks the differentiated blood cell populations obtained from both primitive hematopoiesis as well as definitive hematopoieisis. In the case of clusters also, Hml+ve cells are present in all of the four clusters (Figure 6). Then we found that Peroxidasin, which is another marker for the differentiated blood cells of *Drosophila*, is also present in the cluster (Figure 7). Further we found that Croqumort, which marks the differentiated blood cells of the embryonic origin, is also present in the clusters (Figure 8).

Charaterization of progenitor blood cells:

In an attempt to characterize the progenitor blood cells in the clusters, I used medullary zone of lymph gland as reference and tested many of the markers which are known to express in this zone of blood cell progenitors. Though most of the assays showed negative results, we found ZCL2897, which is an uncharacterized gene from 'flytrap' and marks the medullary zone of lymph gland, is present all of the four clusters (Figure 9), indicating the existence of progenitor cells.

Characterization of niche specific markers:

Once we found that the clusters contain stem like progenitor blood cells, we raised the question about the existence of the niche in the cluster on the grounds that there should be a cellular or non-cellular niche to support the stem-ness of the stem like progenitor cells of the cluster. Again taking PSC region of lymph gland as our reference, we tried several genetic markers and found that Serrate, which marks the hematopoietic stem cells in lymph gland also, is present in this clusters (Figure10). When we compared this result with vertebrate system, Jagged, which is a mammalian orthologoue of Serrate, is expressed in the niche cells of the bone marrow. So it is highly probable that the clusters that we have discovered have a cellular niche to maintain the number of progenitor blood cells population of the novel clusters.

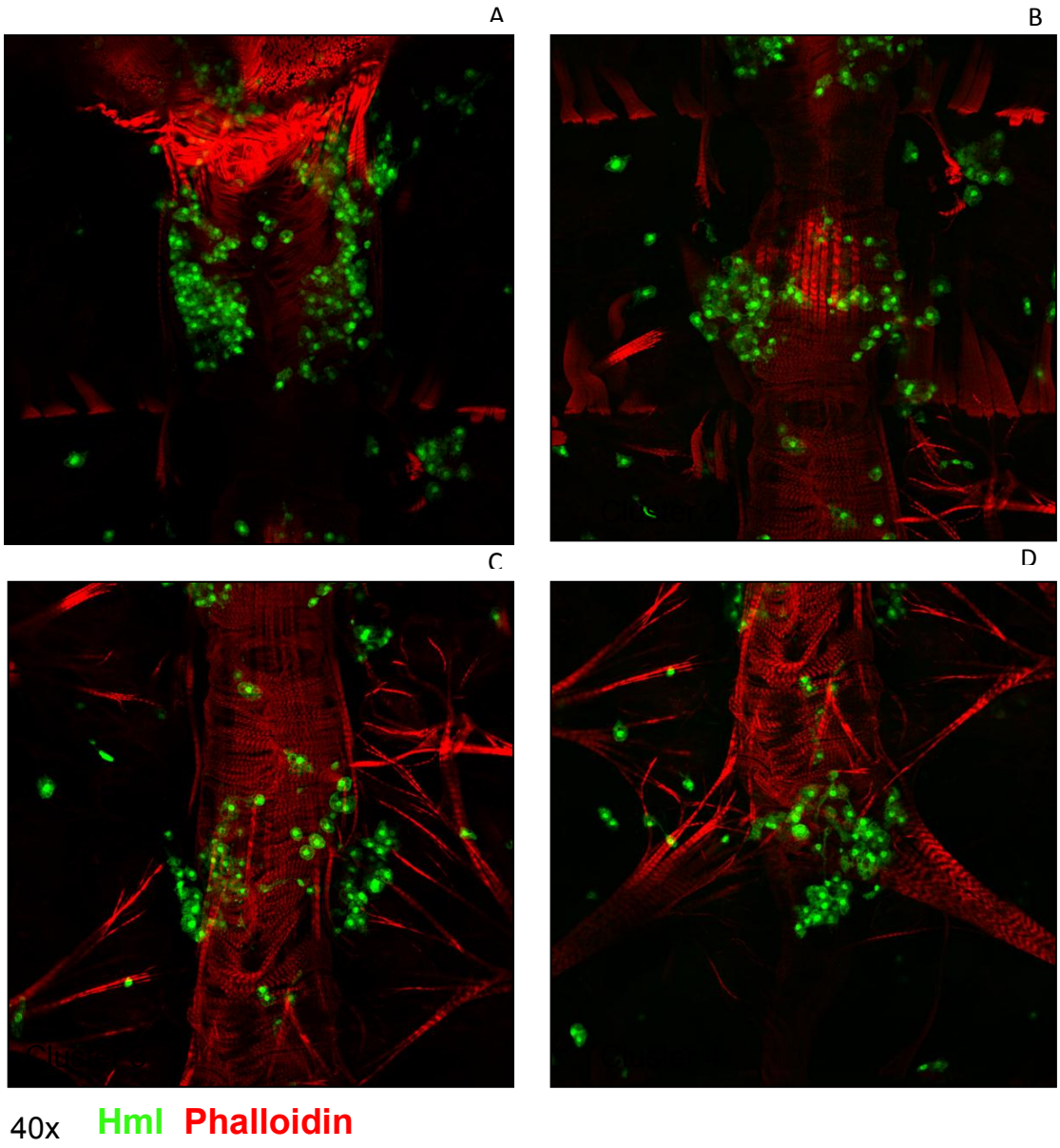

Figure6. GFP expressing cells are differentiated cells marked by Hml Gal4 UAS GFP. Muscles are marked in red by phalloidin. A-D is the four abdominal clusters of a single fly.

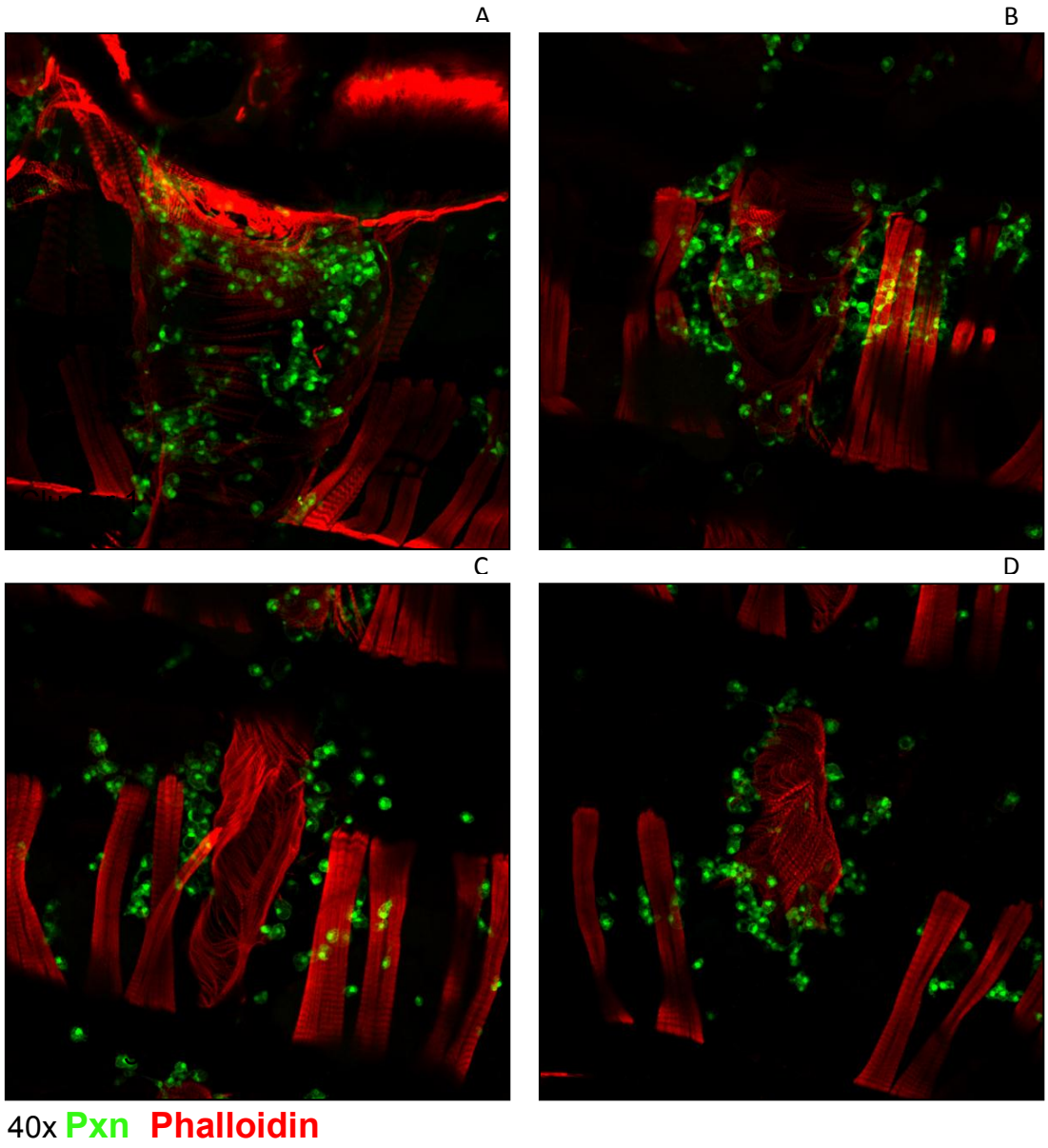

Figure 7. GFP expressing cells are differentiated cells marked by Pxn Gal4 UAS GFP. Muscle is marked in red by phalloidin. A-D is the four abdominal clusters of a single fly.

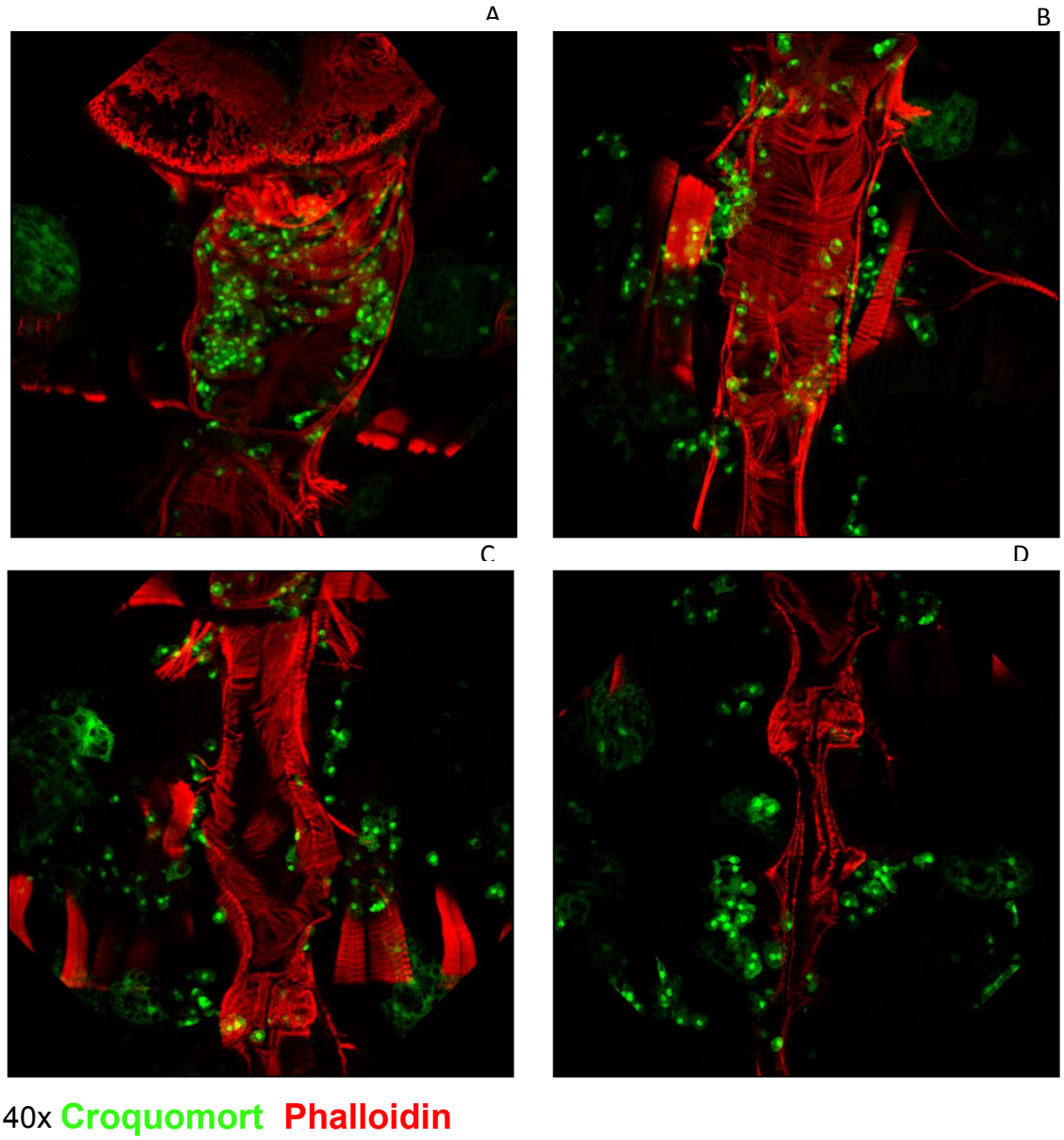

Figure 8. GFP expressing cells are differentiated cells marked by Crq Gal4 UAS GFP. Muscles are marked in red by phalloidin. A-D is the four abdominal clusters of a single fly.

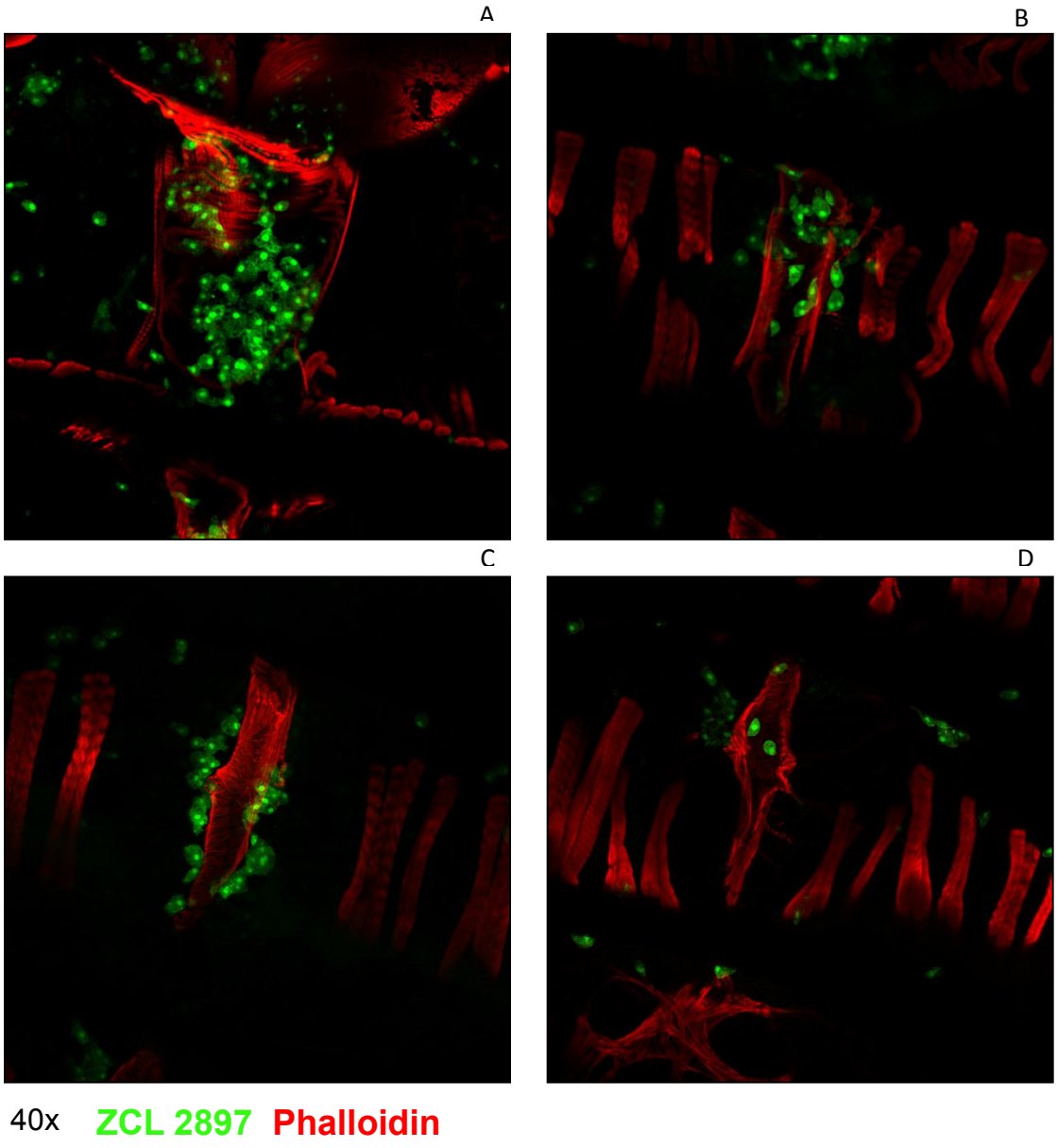

Figure 9. GFP expressing cells are stem like progenitor cells marked by ZCL2897. Muscles are marked in red by phalloidin. A-D is the four abdominal clusters of a single fly.

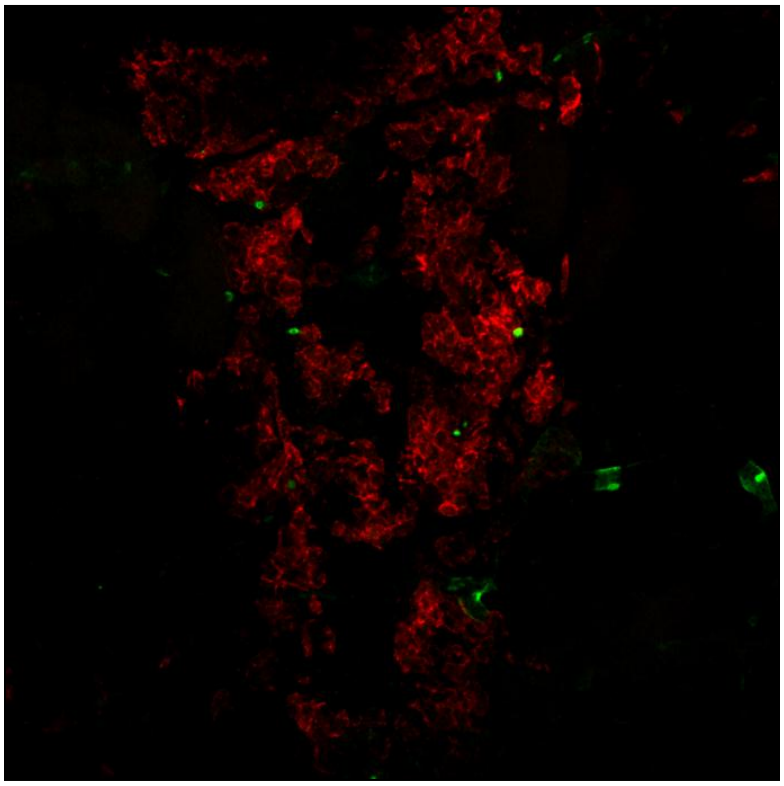

Figure10: Serrate GFP cells present in the cluster are proposed to be niche cells. These cells are salt and peppered within the cluster and are negative for the marker for differentiation (P1 in red).

2.1.3 Discussion

In this work, we proposed that the clusters present in the abdominal area of the fly are hematopoietic in nature. To test this idea, we did the genetic characterization of these clusters and supplemented these results by larval and adult bleeding experiments. So far our investigations have shown that the clusters are heterogeneous population of the blood cells which show the signs for the existence of stem like progenitor blood cells as well as for the existence of probable cellular niche. In the genetic characterization experiments, we found that Crq is present in this clusters. Our bleeding results in the larvae suggests that Crq is absent in the larval circulation, lymph gland but present in the adult circulation. This is very suggestive of the fact that the Crq which we observe in case of adult circulation might actually coming from the clusters. This in itself is a big clue for the presence of hematopoietic activity in the adult *Drosophila*. Additionally, the presence of ZCL 2897, which is a bonafide marker for the blood cell progenitors in lymph gland of *Drosophila*, in the clusters indicate that this cluster is having progenitor stem cells also. The presence of progenitors and differentiated cells cells in close proximity is indicative of an active site of hematopoiesis. The expression of niche marker like Serrate in the clusters is also very suggestive of the presence of cellular niche within this cluster.

Further there are reports of presence of sessile population of blood cell under the larval cuticle also, which are known to form various cluster following the neuronal cues (Markus *et al*, 2009), but our cluster is different from this sessile population, first because it has the signature of differentiated as well as progenitor blood cells whereas sessile population is not reported to contain progenitor blood cells. Secondly, adult cluster is embedded in the dense ECM of pericardin, whereas larval sessile population is a dynamic structure, where hemocytes keep on migrating and no ECM structure is known to support those sessile populations in the larvae.

2.2 Future prospects

As we have shown that this cluster expresses the specific markers for the stem like progenitor cells, in future we would like to see the effect of specifically altering the function of these particular markers using RNAi techniques. Also it would be interesting to see if we genetically ablate the progenitor cells by expressing reaper, will the cluster size be compromised? If we do the manipulation at the level of niche will that affect the progenitor?

These types of studies will establish the functionality of the domains and their relationship to each other. Also we can attempt to explore various signalling pathways that are involved in the maintenance or genesis of this cluster.

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