Characterization of previously unexplored progenitors in the posterior lobes of larval lymph gland in *Drosophila melanogaster*.

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Pranav Vijay Joshi

(MS15044)

Dedicated to my family

Certificate of Examination

This is to certify that the dissertation titled “Characterization of previously unexplored progenitors in the posterior lobes of larval lymph gland in *Drosophila melanogaster*” submitted by Pranav Vijay Joshi (Reg. No. MS15044) for the partial fulfillment of B.S.-M.S. dual degree programme of the Institute, has been examined by the thesis committee duly appointed by Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Lolitika Mandal

(Supervisor)

Declaration

The work presented in this dissertation has been carried out by me under the supervision 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 institution. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgment 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.

Pranav Vijay Joshi

(Candidate)

In my capacity as a 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)

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Abbreviation Table

Abbreviation	Full Form
PSC	Posterior signalling centre
MZ	Medullary zone
CZ	Cortical Zone
PL	Posterior lobes
LG	Lymph Gland
Hml	Hemolectin
Hnt	Hindsight
FUCCI	Fluorescence ubiquitination cell cycle indicator
EdU	5-ethynyl-2´-deoxyuridine
PH3	Phospho-histone H3
Antp	Antennapedia
Kn	Knot
AEH	After Egg Hatching
BL	Bloomington

Table 1 :- Abbreviation used in this thesis.

Table of content

Chapter 1:- Abstract

Drosophila melanogaster is used as a model organism for genetics since the last century. Various phenomenon have been studied using this model organism such as neurodegenerative disorders, metabolism, behavioural studies as well as immunity. In this thesis, we have analysed the process of developmental hematopoiesis or blood cell formation.

Hematopoiesis of vertebrate and *Drosophila* are quite similar. In *Drosophila*, there are two waves of hematopoiesis. Larval lymph gland (larval hematopoietic organ) generates hemocytes in definite hematopoiesis. The lymph gland is a bilobed tissue that flanks the dorsal vessel. The primary lobes of the lymph gland are extensively studied, but posterior lobes(PL) that houses progenitor cells remain unexplored. In this study, we tried to understand PL hematopoiesis in detail.

It has been reported that PL constitutes of a homogeneous population of progenitor cells distributed in secondary and tertiary lobes. However, when we tried bonafide blood progenitor markers such as TepIV or Knot (Kn), we found that both of them was not uniformly expressed in the reserve progenitor pool. Interestingly, the TepIV expressing domain and Kn expressing domain were mutually exclusive. Our characterization to date indicates that the Knot domain in the tertiary lobe is actually "prospective niche," which emanates maintenance signal for progenitors in PL, just like PSC/niche of the primary lobe.

The development of the lymph gland is a dynamic process. Hence it was important to know, at what exact time point, this heterogeneity in the PL arises. For this purpose, we performed time-kinetics of the lymph gland at different developmental time points.

Further, to understand the cell cycle status of the newly identified domains: TepIV domain and Kn domain, we employed fly FUCCI construct. The results reinforce our hypothesis that the blood progenitors of PL resembles the progenitors of the primary lobe and except that they are developmentally lagging in time. As the progenitors of primary lobe differentiate, the progenitors in the PL initiates their differentiation program .Thus, for differentiation, reserve progenitors of PL seem to "tailgate" the progenitors of the primary lobe during development.

Chapter 2:- Introduction

2.1) *Drosophila melanogaster* as genetic model

Scientific classification of *Drosophila melanogaster* is as follows.

Kingdom	Animalia
Phylum	Arthropoda
Class	Insecta
Order	Diptera
Family	Drosophilidae
Genus	*Drosophila*
Subgenus	*Sophophora*
Species	*D. melanogaster*

Table 2 :- Scientific classification of *Drosophila melanogaster*

Drosophila melanogaster is a useful model organism that has been extensively studied for more than 100 years. Scientists have used this model organism to study a very diverse phenomenon. It has been used to study diverse process that includes but not limited to inheritance, developmental processes, neurodegenerative disorders, metabolic pathways, behavioural mechanism. Although William Castle's group was the first to use the *Drosophila* melanogaster in the laboratory at Harvard University in 1901, Thomas Hunt Morgan is recognised as the "Father" of *Drosophila* research for his work1. Morgan used this model organism to explain many phenomena such as sex-linked inheritance, heredity, gene epistasis, gene mapping etc[2].

Drosophila melanogaster contains four pairs of chromosomes in its nuclear genome. Because of its wide use as a model organism, its entire genome has been sequenced, mapped, and published[3]. When *Drosophila melanogaster*'s genome is compared with the human genome, scientists have found that 60% of the genes have been conserved between these two species4. Researchers have found that 714 distinct genes that have been involved in various diseases in humans (accounts for nearly 77% of disease gene searched in humans) were matching to the

548 unique genes of the *Drosophila*. Hence these genes are amenable to study using *Drosophila* as model organism[5].

Drosophila melanogaster is easy and economical to culture and maintain. They are easy to work with and not known as carriers of any diseases that can infect humans. They have a relatively short life cycle of around ten days at an optimal temperature of 25^{o}C. Their lifespan is on an average of 45 days. Thus, many generations can be produced in a minimal amount of time. It has relatively fewer chromosomes (only four).

Further, the last chromosome is the dot chromosome, which contains very few base pairs and genes. *Drosophila* females can lay a large number of eggs up to 100 eggs/day, thus having a high fecundity rate. This model organism has a broad genetic toolkit such as the GAL4-UAS system, *UAS-DualFUCCI, UAS-Gtrace, FLP-FRT system, CRISPR-Cas system*, etc., and thus has amenability for genetic manipulation[6]. Hence *Drosophila melanogaster* is proved to be an excellent testable model organism.

2.2) Life cycle of *Drosophila melanogaster*.

The insects which are holometabolous (which show complete metamorphosis) have four stages of development in their life: Egg, Larva, Pupae, and Adult (fig 1).The life cycle starts with the fertilised egg, which lasts around 24 hours before the hatching (25^{o}C). In this period, the embryo goes through the 16 stages of development[7]. After the egg hatching, the larval period starts. The larva of *Drosophila* is a worm-shaped, segmented and white. It contains a pair of spiracles at anterior and posterior both ends for tracheal breathing. Two molting events occur in the larval stage of development in which larva shed off their cuticle. The larval stage can be divided into three parts based on the molting event. First instar starts after the egg hatching and ends at first molting. The second instar lasts during the first and second molt. Furthermore, the third instar stages starts after the second molt and lasts till the beginning of the pupal stage. The first and second instar lasts for about 24 hours, but third instar can last for 72 hours at 25^{o}C. A particular instar stage of the larva can be identified by observing the size (increases from first to the third instar) and the shape of the mouth hook. At the end of the third instar, larva comes out of the food medium, stops moving and prepares itself for the pupal stage of the development[8].

When the pupation process starts, the cuticle secretes many proteins, which facilitates the the transformation to a puparium. Early on, puparium is soft and white, but later on, it becomes hard and black. The larva inside goes under another molting process, which results in detachment of the larva from the puparium. Pupation is an extremely dynamic process. Larvae undergo a metamorphosis that results in the emergence of adult body structures. When larva turns into a pupa, a large amount of cell death and tissue breakdown occurs (histolysis) in the process of metamorphosis. The imaginal discs present in the larvae are the thick sac of epithelial cells, which transforms into adult structures like an eye, antenna, haltere, genitalia, wings, etc. After the completion of the pupal stage, adult fly ecloses out of the pupa [8,9,10].

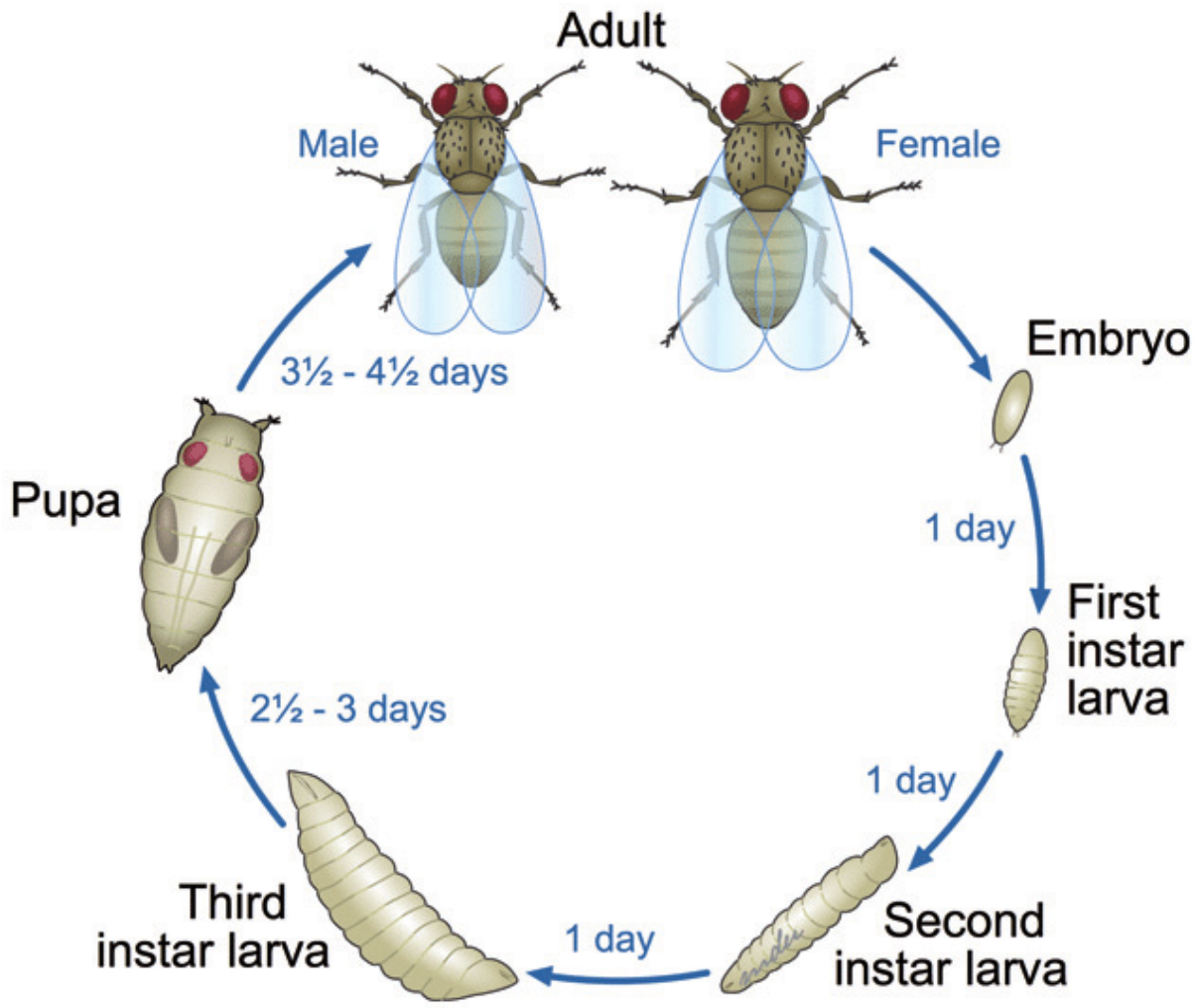


Figure 1. This diagram represents the life cycle of *Drosophila melanogaster* at 25^{o}C [11].Picture credit :- Cynthia ong et. al.

2.3) Genetic toolkit of *Drosophila melanogaster*

Following genetic tools were used in this study:

A) Gal4 - UAS System

The Gal4-UAS system was one of the first genetic manipulations in *Drosophila melanogaster*.GAL4 gene, which is endogenous to the *Saccharomyces cerevisiae*, produces Gal4 protein of 881 amino acids. This protein is a transcription regulator protein that binds to the Upstream Activation Sequence (UAS). Gal4 protein is not endogenous to the *Drosophila melanogaster*. The UAS controls the expression of downstream genes. This system uses the bipartite approach[12]. One parental line contains the Gal4 gene downstream to the enhancer/ promoter of the required gene. This parental line is called the "driver" line. The other parental line contains the UAS sequence upstream of the responder gene is called the "responder" line. When these two lines are crossed, the progeny would have both the Gal4 protein and the UAS. Thus, responder gene will get expressed in in those tissue or cells where the driver line is active. This enables us to get spatial control (cell type specific) over the expression pattern of the responder gene. By incorporating the "Gal80" gene in either driver or responder line, temporal control can also be achieved. Gal80 gene is a negative regulator of the Gal4 gene. Gal80 binds to the Gal4 protein and inhibits its transcription activity. Gal80 gene gets activated at a lower temperature typically at 18^{o}C and gets deactivated at temperature typically at 25^{o}C . Hence by shifting the temperature surrounding the flies, temporal control over the expression of the responder gene is achieved.

Spatio-temporal control over the expression pattern of the required gene has great advantages. This system was first introduced by Andrea H. Brand and Norbert Perrimon in 1993[13].

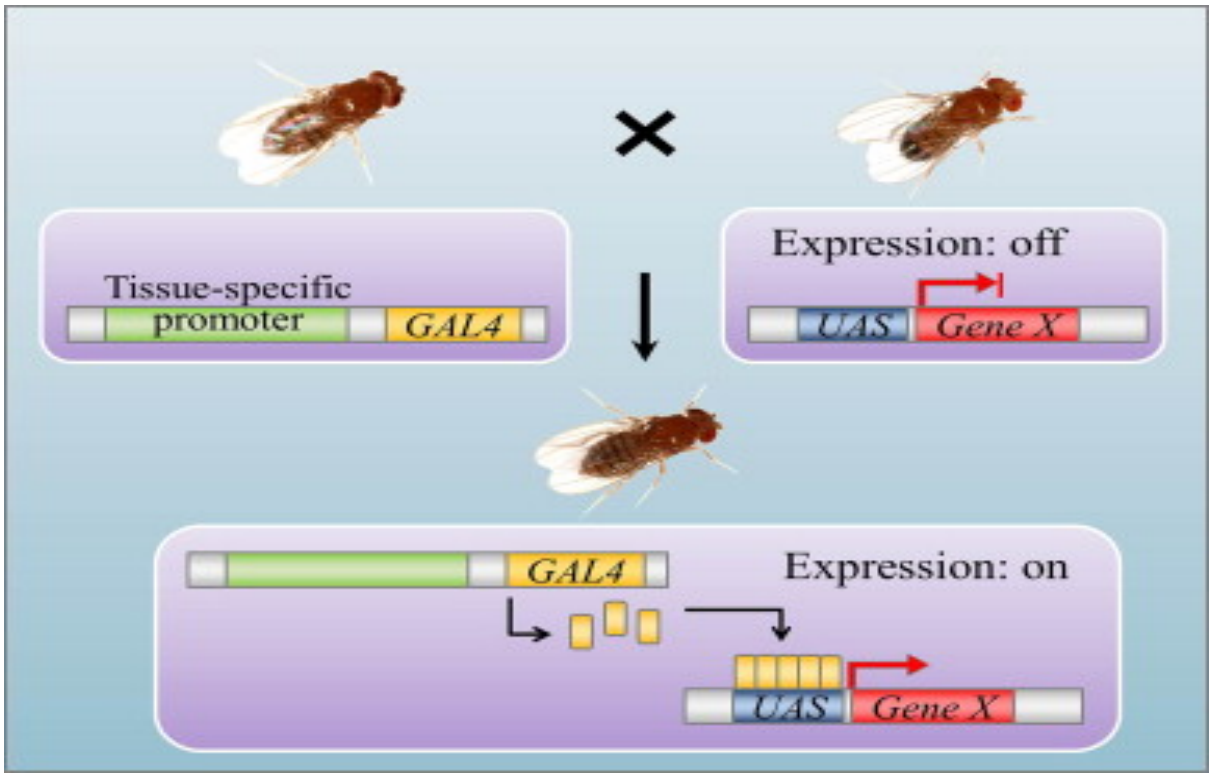


Figure 2. The Gal4-UAS system using bipartite approach. In the F1 generation tissue specific promoter initiates the GAL4 protein expression. This GAL4 protein in turn, binds to the UAS sequence and turns on the expression of downstream gene, in this case gene X[13]. (Picture Reference:-Brand and Perrimon 1993.)

B) Fly FUCCI (Fluorescent Ubiquitination Based Cell Cycle Indicator).

By knowing the cell cycle status in a particular tissue can give us great insight into tissue's characteristics. Dividing cell goes through the five phases of the cell cycle. G0, G1, S, G2, M. In eukaryotic cell several proteins regulate the cell cycle such as cyclin-dependent kinases.

Cyclin B and E2F1 are degraded by ubiquitin E3-ligase APC/C and $CRL4^{cdt2}$, respectively. From mid mitosis till the end of G1 phase, Cyclin B is degraded by ubiquitin ligase APC/C. And similarly in S phase of the cell cycle, $CRL4^{cdt2}$ degrades the E2F1 protein. In fly FUCCI technique, this information is manipulated. Herein, Cyclin B is tagged to mRFP1 fluorochrome, and E2F1 is tagged with GFP fluorochrome. Thus, from mid-mitosis to the ending of the G1 phase, only GFP gets expressed in the cells. Furthermore, in the S phase,

only mRFP1 is expressed. In the G2-M phase of the cell cycle, neither mRFP1 nor GFP gets degraded, and the cell appears to be yellow in colour[14,15].

By using the Gal4-UAS technique, we can express Fly FUCCI construct in the tissue/cells of our interest at a specific time point.

By using the Phospho-histone H3 antibody, we can further differentiate between G2 and M phases. In the M phase of the cell cycle, chromosomes are highly phosphorylated at the onset of metaphase. For mitotic chromosome segregation and condensation, phosphorylation of H3 at serine residue 10 and 28 in critical. PH3 antibody binds to the phosphorylated H3, and thus we can distinguish cells that are in M phase.

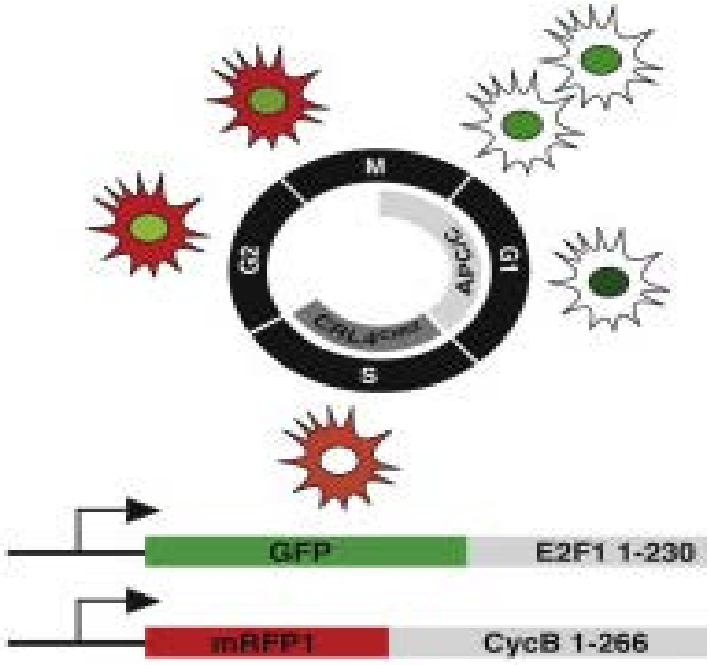


Figure 3. From mid mitosis till the end of G1 phase, cyclin B is degraded by ubiquitin ligase APC/C. Likewise in S phase, $CRL4^{CDT2}$ degrades the E2F1 protein. In fly FUCCI cyclinB is tagged by RFP and E2F1 is tagged by GFP. Hence the cells which are in G1 phase expresses only GFP, and those are in S phase only expresses mRFP1. $E2F1_{1-230}$ and $CycB_{1-266}$ are the respective degrons[16]. (Picture reference :-Norman Zielke et.al.)

2.4) *Drosophila melanogaster* hematopoiesis

The formation of blood cells from its mesodermal precursors is called hematopoiesis. The vertebrate and *Drosophila melanogaster* hematopoiesis has been conserved. In vertebrate, there are two waves of hematopoiesis. In primitive hematopoietic wave, which occurs during embryogenesis, extra-embryonic yolk sac gives rise to blood precursors. These blood precursors are basically erythroid in nature[17]. The definitive hematopoiesis gives rise to blood cells that will seed in to the future hematopoietic sites such as fetal liver and bone marrow in mammals. This second wave occurs in the aorta/ gonad/ mesonephros region of the embryo proper [18].There are two types of blood cell lineages in vertebrate myeloid lineage and lymphoid lineage[19,20].

Similarly, in *Drosophila melanogaster*, hematopoiesis occurs in two waves. The first wave, originates in embryonic head mesoderm and is referred as primitive hematopoiesis. It generates hemocytes (*Drosophila* blood cells)for all the four stages of life cycle[21,22]. The second wave is called the definitive hematopoiesis. It takes place in the blood forming organ: the Lymph Gland (LG)[23,24]. Eventually the hemocytes generated by these two waves contributes to the hemocytes in the adult stage.

Drosophila blood cells (Hemocytes) have two essential functions. They are required for the removal of the debris and dead cells from the hemolymph. The hemocytes also secrete and remodels extracellular matrix, which is crucial for morphogenesis[25,26,27]. The hemocytes also play a role in humoral immunity. They are capable of engulfing foreign particles and thus act as sentinels in the hemolymph monitoring the presence of a pathogen. They also regulate the signalling pathway to the larval fat body, which has the capacity to produce large amount of antimicrobial peptides[27,28,29]. The adult hemocyte population is a contribution of both the lymph gland and cells derived from head mesoderm[24].

The number of hemocytes increases from the embryonic stage to the adult stage. In the embryonic stage, there are a few hundreds of hemocytes[21]. As in the pupal stage, metamorphosis begins, the demand for hemocytes increases, because lot of debris and dead cells are generated by the process of tissue remodelling in metamorphosis. In pupal stage, there are more than 5000 hemocytes[30,31].While in the adults there are around 1000 to 2000 hemocytes[30].

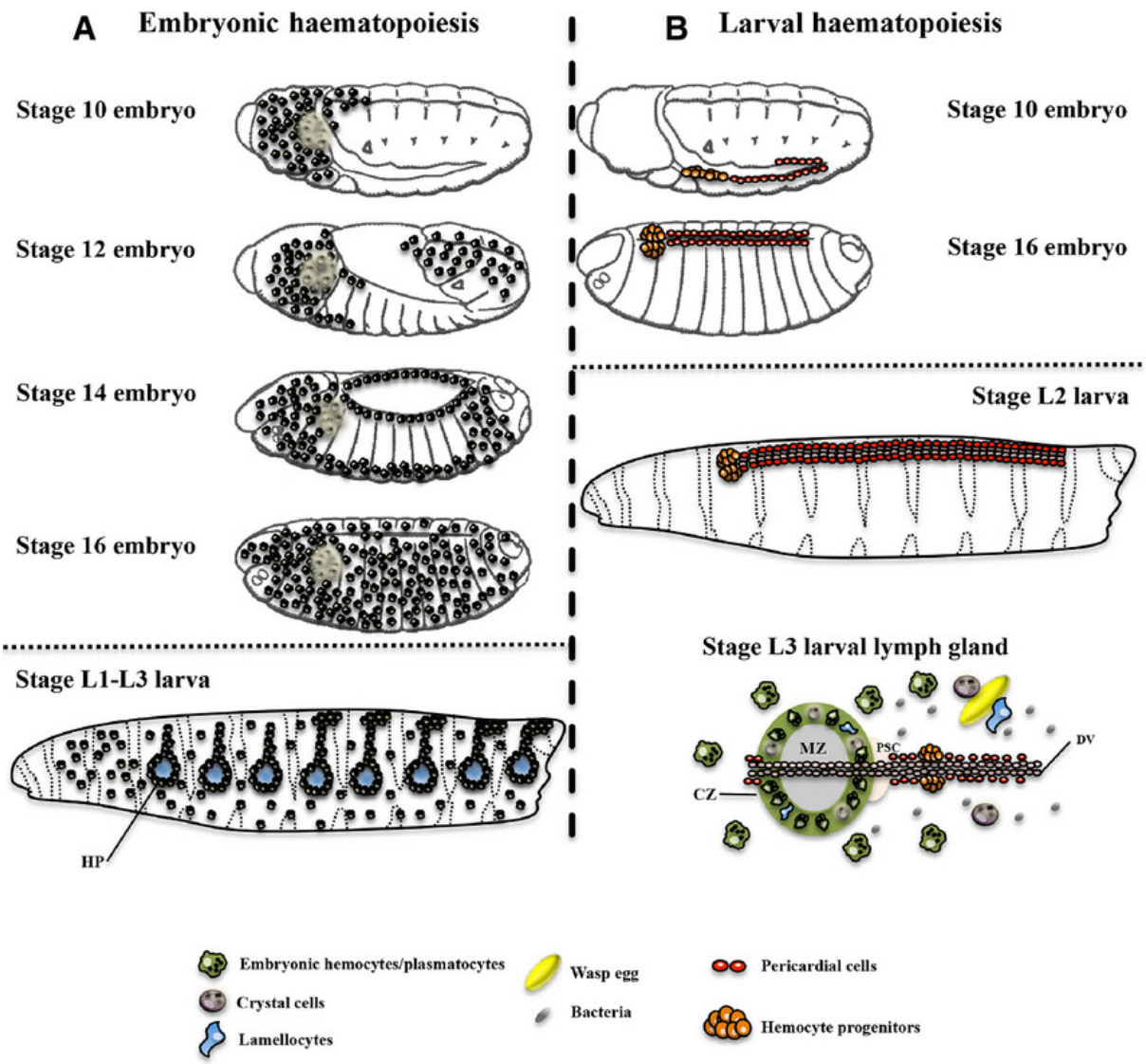


Figure 4. Two waves of hematopoiesis in *Drosophila*[31].(Picture Reference :- Kalpana Makhijani et.al.)

2.5) Larval hematopoietic tissue: Lymph Gland (LG)

The dorso-lateral mesoderm generated from anterior trunk segment[22]gives rise to the small population of cells during embryogenesis which form the lymph gland precursors. The lymph gland at the end of embryogenesis is placed around the heart which is located at dorsal side. There are several similarities and differences between prohemocytes derived from the head mesoderm and lateral mesoderm. Both types of prohemocytes express Srp, which is a GATA factor[35].However, prohemocytes generated by head mesoderm differentiate in the embryonic stages, while lymph gland prohemocytes starts proliferating during early larval stages. The anatomy of the lymph gland is unique. It consists of at least three pairs of cell clusters/ lobes. The lymph gland is a dynamic tissue, which undergoes continuous changes in cell number, the expression pattern of different genes etc, throughout the larval stage.

The lobes of the lymph gland are categorised in two parts. First, is the anterior-most lobe, which is also called as primary lobe, and the rest are collectively called as posterior lobes (PL).All these lobes are thought to be separated by pericardial cell. The primary lobe has been extensively studied. Several studies have shown that the primary lobe is not a homogenous population of the cells. It is divided into different zones based on gene expression patterns and morphological characteristics[36]. There are three zones in the primary lobe. Cortical Zone which consists of differentiated hemocytes, is at the periphery of the lobe. The differentiated hemocytes are mainly of two types: Plasmatocytes and Crystal cells[33,34]. Upon immune challenge, the third type of differentiated hemocyte appears, which is called Lamellocyte[34]. Medullary Zone is at the core of the lobe and is filled with progenitor cells. The group of cells posteriorly located in the gland are referred as the Posterior signalling centre (PSC) that acts as a niche for the maintenance of the progenitors. i.e. it maintains the stemness of the progenitor cells. This subdivision of the primary lobe indicates there is spatio-temporal regulation of hemocytes in the larval stage[37,38,39]. The several morphological characteristics and differential gene expression patterns distinguish these zones from one another(see table 1).

Region	Cell type	Genetic markers
PSC	Niche cells	Antennapedia, Hedgehog, Knot[37,39,44]
MZ	Progenitors	Dome, unpaired3, wingless, TepIV[36,38,40]
CZ	Plasmatocytes	Hemolectin (Hml), Peroxidasin (Pxn), Nimrod C1/P1 antigen[33,34,36,43]
	Crystal cells	Lozenge, Hindsight, prophenoloxidases1[33,34,41]
	Lamellocytes	Atilla, βPS[34,42]

Table 3 :- All markers mentioned above are reported in LG which is at 96 hours after egg hatching (AEH) developmental stage.

Some important markers :-

Hemolectin (Hml) :- Early differential marker, specific to plasmatocytes.

Hindsight (Hnt) :- Crystal cell marker.

Knot (Kn) :- It is also known as collier (col). It is required for wing disc patterning. PSC marker

Thio-ester containing protein 4 (TepIV) :- It has endopeptidase activity(Stat Response). Progenitor marker.

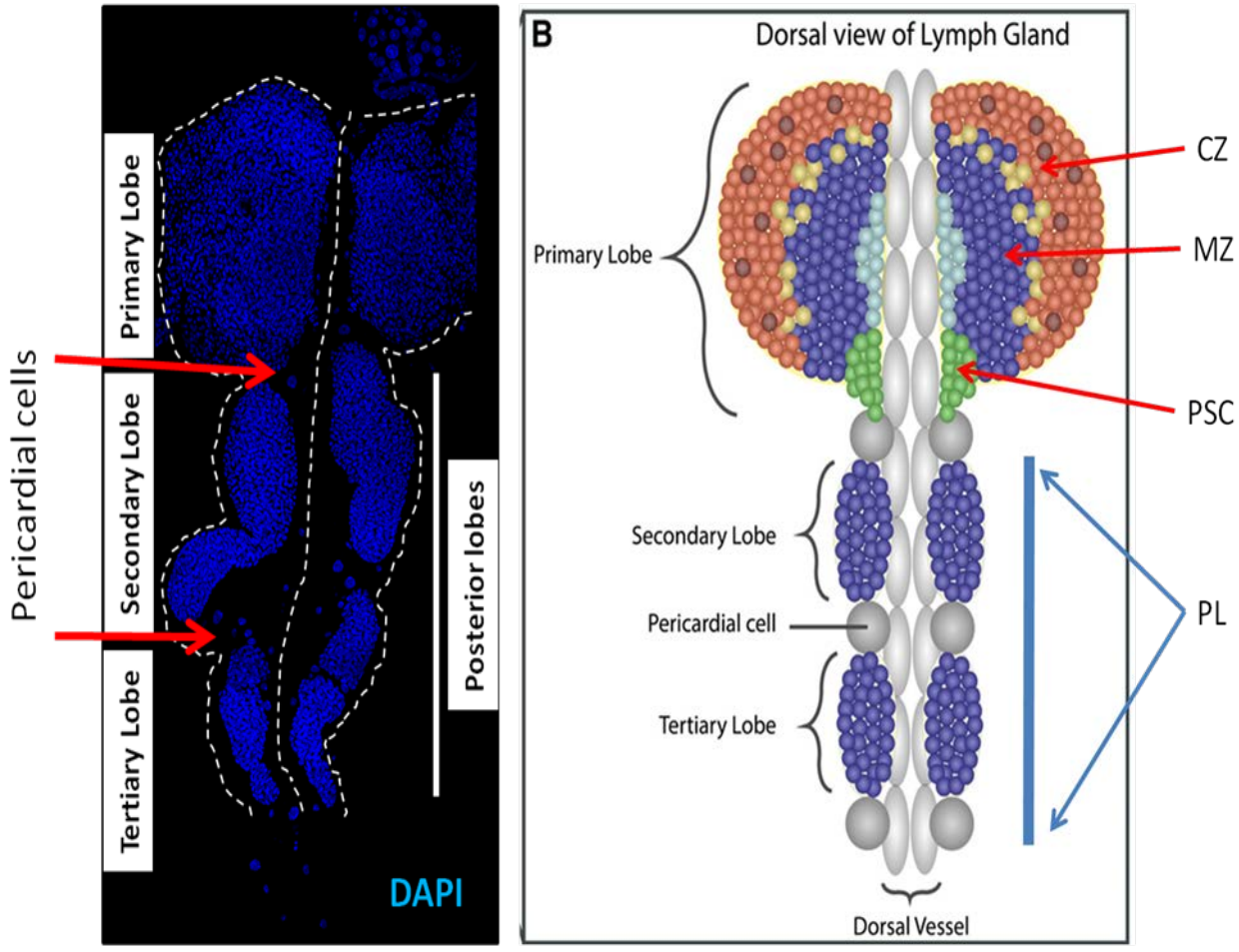
2.6) Posterior Lobes (PL)

The slender posterior lobes (PL: secondary and tertiary lobes) are located to the posterior end of the primary lobe. These lobes becomes conspicuous during later stages of larval development. There is a great deal of similarity between the MZ and PL. The PLs contain tightly packed progenitor cells and are enriched in express E-cadherin, Dome and Collier [36]. The progenitors of PL remain undifferentiated throughout larval life until they experience any form of stress. It is reported that upon immune challenge such as wasp infection, the progenitors in the PL differentiate prematurely[45,46].

Throughout the larval stage, the PL lack differentiation markers such as Hml, Pxn, P1 antigen. Only at the prepupal stages progenitors in the PL starts differentiating[47]. The PL hemocytes populate the adult hematopoietic hubs[24]and is thus believed to have a role in *Drosophila* adult hematopoiesis.

2.7) EdU (5-ethynyl-2´-deoxyuridine) assay

Measuring the ability of cell proliferation in particular tissue can give an insight in tissue dynamics. Cell proliferation can be measured by detecting DNA synthesis which occurs in S phase of the cell cycle. EdU gets incorporated into the newly synthesize DNA because it is a nucleoside analog thymidine. The incorporated EdU can be detected using click reaction, which based on, copper mediated covalent reaction between azide and alkyne. EdU has alkyne in it, and we provide azide in the form of "Picolyl azide" to the cells. This assay does not require to melt DNA strands for the detection of EdU, like in BrdU assay.



(A) (B)

Figure 5 :- (A) Confocal microscopy image of 96 AEH LG. (B) Schematic diagram of LG[48].(Picture Reference :- Banerjee U. et.al.)

3) Objective

To characterize previously unexplored progenitors in the posterior lobes of larval lymph gland.

4) Materials and methods

4.1) Regular fly maintenance.

All the stock fly lines were at 25^{o}C. The food was prepared by using Corn powder, Agar and sugar solution. The stock flies *were* regularly flipped. Fruit plates were prepared for collection of eggs and further synchronised during hatching.

Genotype	**Source**
+/+ ; TepIV-Gal4/cyo;+/+	DGRC 105442
+/+ ; +/+; Kn-Gal4/Kn-Gal4	BL49248
+/+ ; Hml-dsRed/cyo; +/+	Katja bruckner
+/+; +/+ ; Antp-Gal4/TM3	University of Copenhagen, Denmark. S.cohen
+/+ ; UAS-DualFUCCI/cyo ; MKRS/Tb	BL55121
+/+ ; Trio-Gal4/cyo ; +/+	BL 48798
+/+ ; +/+ ; UAS-mCD8RFP/TM3	BL27399

*BL = Bloomington

4.2) Analysis Softwares and Databases.

ImageJ software:- It is a publically available image editing software created by NIH. It is used to process images taken from confocal microscopes, by selecting appropriate Z stacks of raw image. The counting of cells was also done by this software.

Flybase :- It is a online database which provides information about *Drosophila* genetics and molecular biology.

Bloomington Drosophila Stock Centre (BDSC) :- The website" https://bdsc.indiana.edu/ " has a list of stock fly lines. Each line has been given a specific number. It also states the function of respective Gene/Promoter/protein and mentions source from which the stock centre has received the respective line.

Vienna Drosophila Resource Centre (VDRC) and Kyoto Drosophila Genetic Resource Centre (DGRC) are also stock centres similar to the BDSC.

4.3) Preparation protocols of different chemicals used.

a) 1X PBS (Phosphate buffer saline)

- Weigh 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4.
- Dissolve the above chemicals in 1 L of distilled water.
- Adjust the pH of solution to 7.20-7.25, by adding small droplets of concentrated HCl.
- Put it in autoclave machine for 45 minutes (121^oC , 22 psi)

b) 10 % PFA (Para-Formaldehyde)

- Weigh 0.2 g of PFA powder in 2ml eppendorf. Add 2 ml of 1X PBS.
- Shake it vigorously and place the eppendorf in 65^oC Water bath till the PFA dissolves completely
- Precaution:- PFA is a potential carcinogenic and it is stored in cold temperature.

c) 0.3% PBT (PBS- TritonX100)

- Add 120 µl of TritonX100 (Detergent) in 40 ml of 1XPBS.
- Keep it on rotator for 30 minutes.

d) 5% BSA block (Bovine Serum Albumin)

- Weigh 0.1 g of BSA and dissolve it in 0.3% PBT.

e) 2.5 % NDS block (Normal Donkey Serum)

- Add 25 µl of 100% NDS in 975 µl of 0.3% PBT.

f) Antibodies used

Antibody	Dilution factor	Raised in
PH3	1:200	Rabbit
Hnt	1:10	Mouse
Secondary antibody 647	1:500	Mouse

g) DAPI

DAPI binds to the AT rich region on the DNA. It is cell permeable and used to mark nucleus of cell.

4.4) Immuno-fluorescence protocol.

- Make required number of pullouts in PBS and place them in cavity block filled with PBS. Remove the PBS and add 5% PFA to the pullouts. Keep the cavity block on rotator for 1 hour(RT, 60 rpm)
- Take out PFA and add 0.3% PBT. Give 3 washes of 0.3% PBT for 15 minutes each on rotator. Then add 5% BSA block solution in cavity block. Keep it on shaker for hour (RT, 60 rpm).
- Carefully take out the pullouts, with the help of needles, and dip them in the solution of primary antibody. Wells of Knuck plate have 9-10 µl primary antibody. Keep the Knuck plate at 4^{o}C, for 18 hours.
- Take out the pullouts from Knuck plate and put them in the cavity block filled with 0.3% PBT. Give 3 washes of PBT. Then add 5% BSA block solution in cavity block. Keep it on shaker for hour (RT, 60 rpm).
- Add secondary antibody to the cavity block. And keep it at 4^{o}C for 18 hours.
- Remove secondary antibody. Add 1X DAPI solution. Keep it on rotator (RT, 60 rpm).
- Give two washes of PBS. Add 10µl of vecta shield on glass slide. Carefully place the pullouts in vecta shield
- Separate out the lymph gland from the pullouts and rearrange them. Put a cover slip.

4.5) EdU assay protocol (Click-iT plus)

- Dissect the larva at room temperature.
- Dip the pullouts in Component A and place it on the shaker for 45 minutes. Component A contains 1000X concentration of EdU. Therefore, dilute Component A (1:1000) with PBS.
- Remove Component A and add 5% PFA. Shaking 1 hour.
- Remove the fixative and give 3 washes of 0.3% PBT of 10 minutes each.
- Add 5% BSA block for 30 minutes.
- Make working solution(1X) of "Click-iT reaction buffer" from the stock(10X).
- Prepare EdU working solution

Reagents	50µl EdU solution	200µl EdU solution
1XClick-iT reaction buffer	4.4 µl	17.6 µl
Copper protectant	1 µl	4 µl
Alexa Fluor picolyl azide	0.12 µl	0.48 µl
Reaction buffer additive	0.5 µl	2 µl
H_2O	43.98 µl	179.92 µl

Table 4 :- EdU working solution

- Remove the block solution, and incubate the pullouts in EdU working solution for 30 minutes with slight shaking (20rpm).
- Give 3 washes of 3% BSA for 15 minutes each. Followed by one 15 mitutes wash with PBS.
- Incubate in DAPI for 45 minutes on shaker. Followed by 2 PBS wash of 10 minutes each.
- Mount the samples for documentation.

4.6) Imaging

The mounted samples were imaged in a confocal microscope (LeicaSP8: IISER Mohali confocal facility).

4.7) Fruit plate preparation protocol

- Weigh 10 g Agar and 8 g sugar in a beaker. Add 100 ml of distilled water to the beaker. Shake vigorously.
- Add 100 ml of fruit juice to the above solution. Heat it with microwave (Approximately 3 minutes). Make sure that the solution is homogenised.
- Pour appropriate amount of solution in the Petri Plates. Allow them to cool (for 5 Minutes).

5) Results

5.1) Progenitors present in the Posterior lobes(PL) are not homogeneous population.

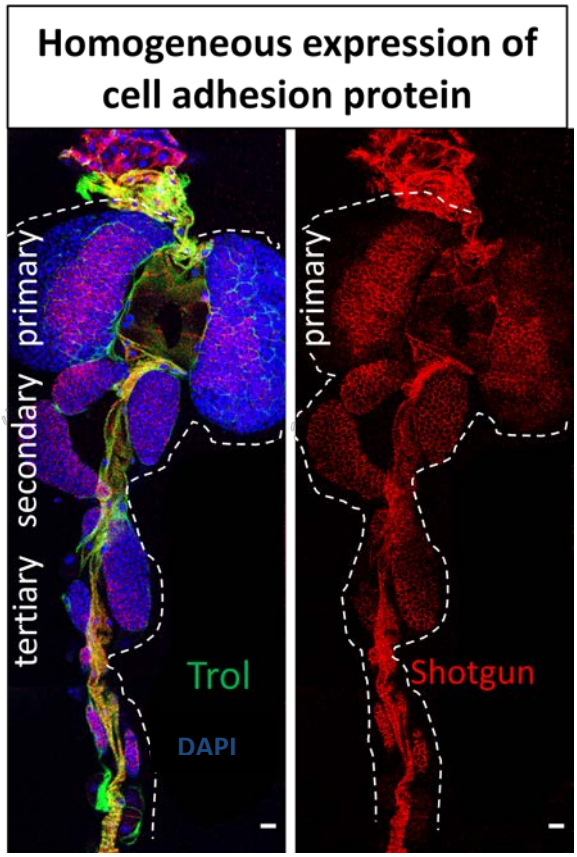


Figure 6 (LG 96 hrs AEH):- Trol is expressed as part of the extracellular matrix (ECM). Shotgun is expressed homogeneously in the PL.

The Drosophila E-cadherin: Shotgun is a progenitor marker in lymph gland[36]. Presence of Shotgun in the PL indicated that progenitors therein are also homogeneous population. To further endorse this claim, we checked the expression of another progenitor marker such as TepIV[49]. To our surprise, we found that it was expressing heterogeneously in the PL (Figure 7).

While screening for the heterogeneous markers in the PL we found that Knot (Kn) was also expressing heterogeneously in the PL. Interestingly, TepIV and Kn are expressed in mutually exclusive zones of the PL. That is TepIV domain and Kn domain never overlapped with each other. Kn is reported to be expressed on the niche/ PSC[37]. Hence, we are hypothesizing that Kn domain is an actually prospective niche in the tertiary lobe. Nevertheless, this claim demanded further validation.

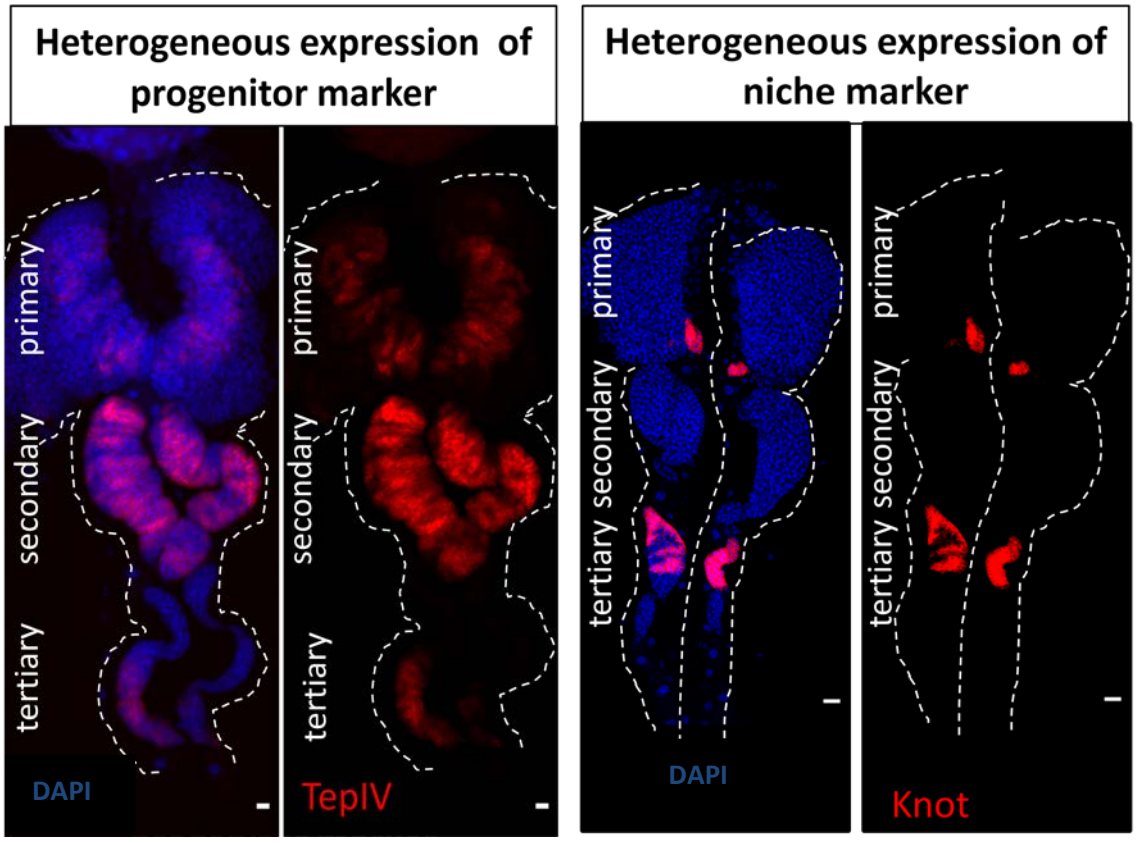


Figure 7 (LG 96 hrs AEH) :- TepIV and Knot expression domain are antagonist to each other. This suggest just like primary lobe[48], there might be zonation of PL as well.

*Scale bar indicates 20 µm for all the diagrams.

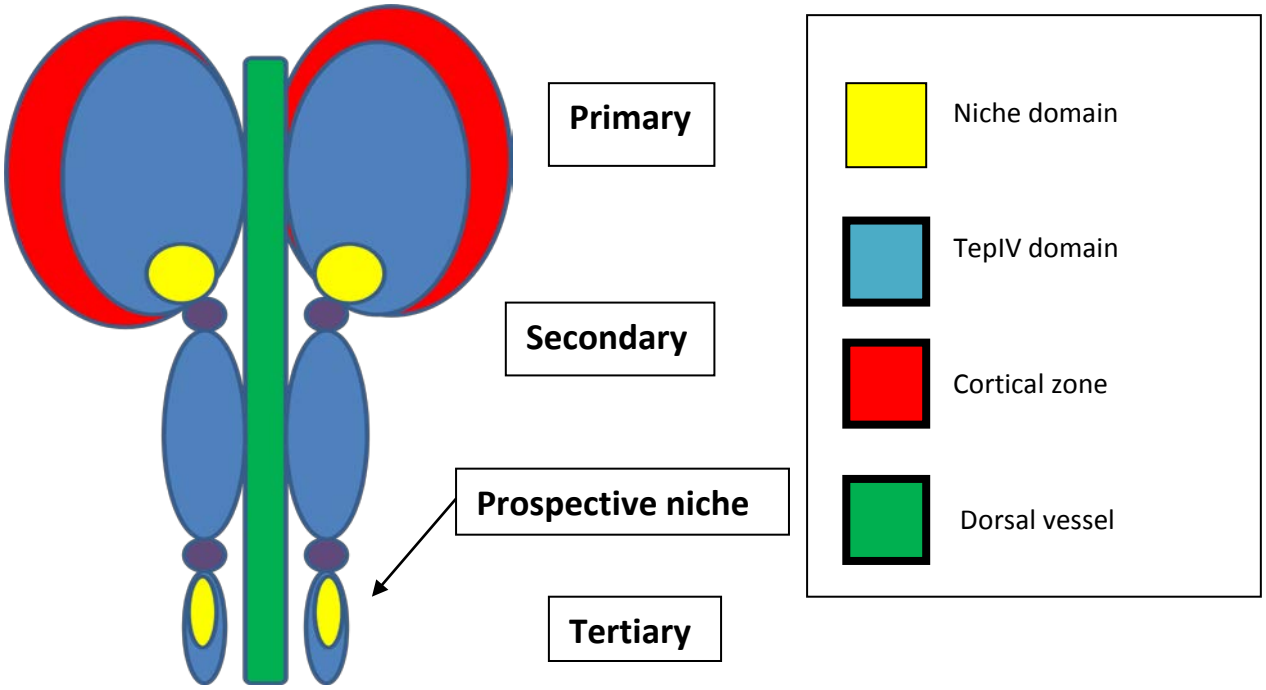


Figure 8 (Third instar LG) : New model of the LG. We are hypothesizing the Kn domain in the tertiary lobe as the " Prospective niche".

5.2) Time series analysis

The development of lymph gland is a very dynamic process. Many markers in lymph gland change their expression over the developmental time. PL also shows dramatic changes on developmental timeline. In the first instar larva (24 hours AEH) there are very few cells in PL comparing to the mature, proliferated lobes at third instar (96 hours AEH).(Figure 9)

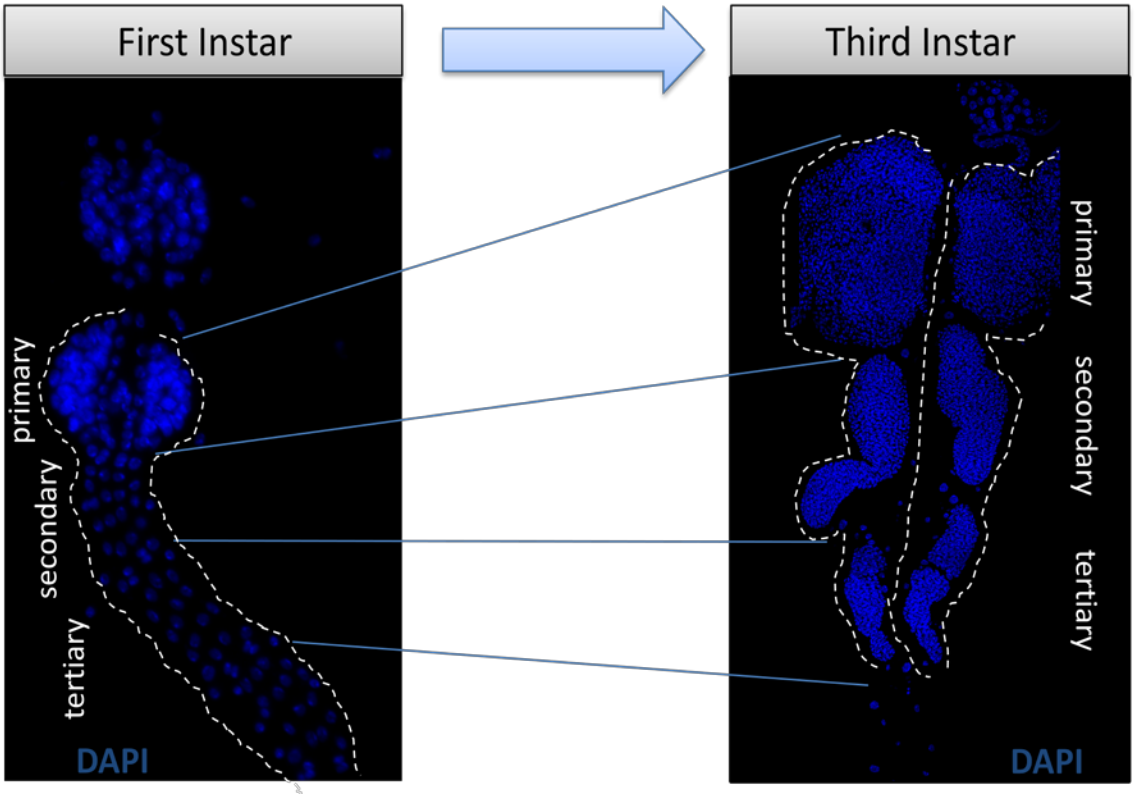


Figure 9:- Increase in number of cell from first instar to the third instar is quite evident.

Therefore, it is important to know at what developmental stage this heterogeneity in the PL is arising. To address this, we did time-series analysis. In time-series experiment we analysed the lymph gland at various developmental time period (24 hours AEH, 48 hours AEH, 72hours AEH,96hours AEH, 110hours AEH) of various genotypes. The genotypes include

a) TepIV-Gal4 >UAS-mCD8RFP

b) Kn-Gal4 >UAS-mCD8RFP

c) Antp-Gal4 >UAS-mCD8RFP

d) Hml-dsRed with Hnt 647 antibody

a) TepIV-Gal4 >UAS-mCD8RFP

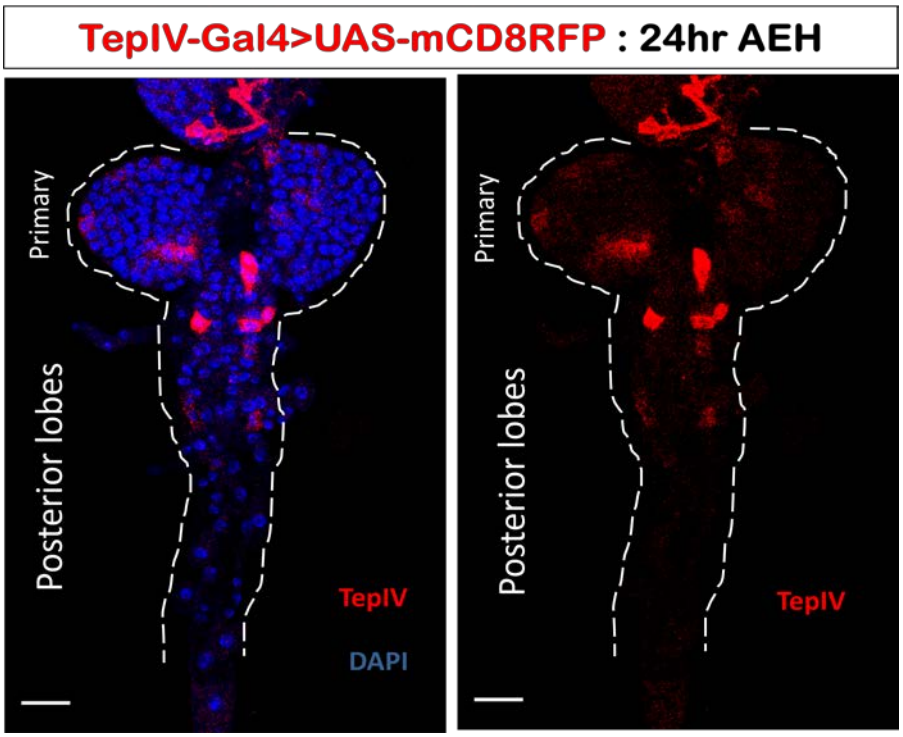


Figure 10 :- Very less expression of TepIV in PL

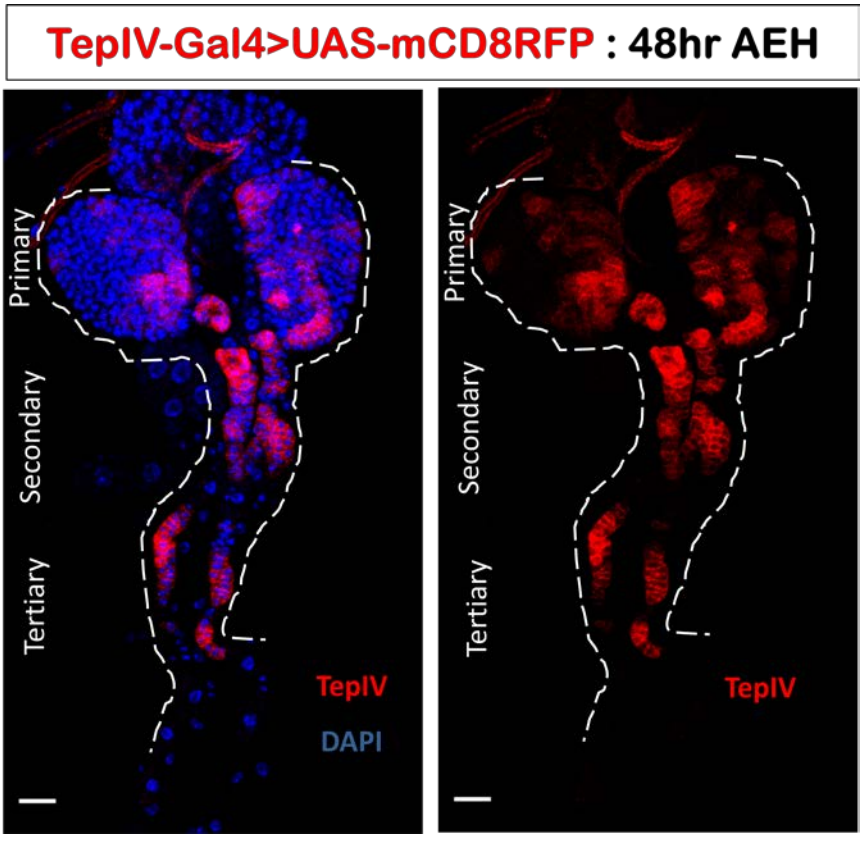


Figure 11 :- TepIV is expressed in entire PL.

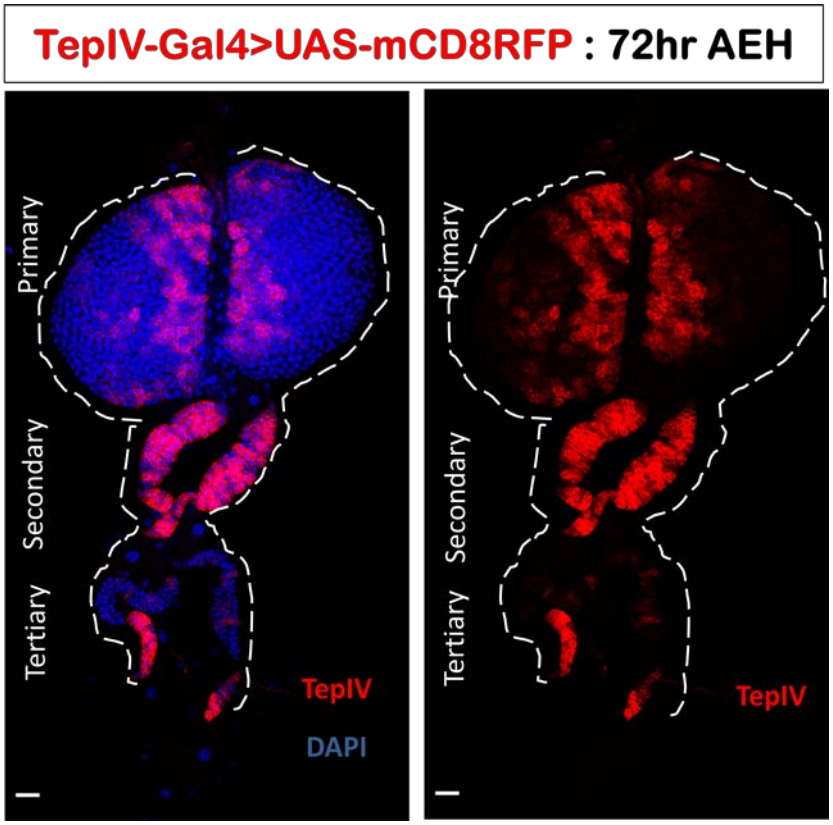


Figure 12 :- Expression of TepIV starts regressing in tertiary lobe.

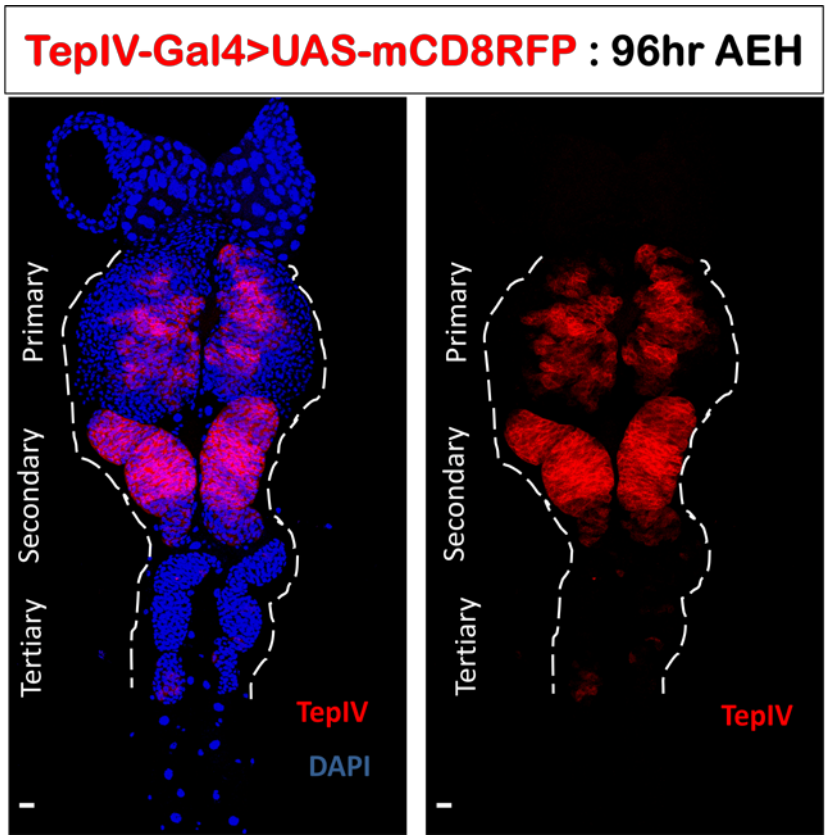


Figure 13 :- TepIV expression regresses further.

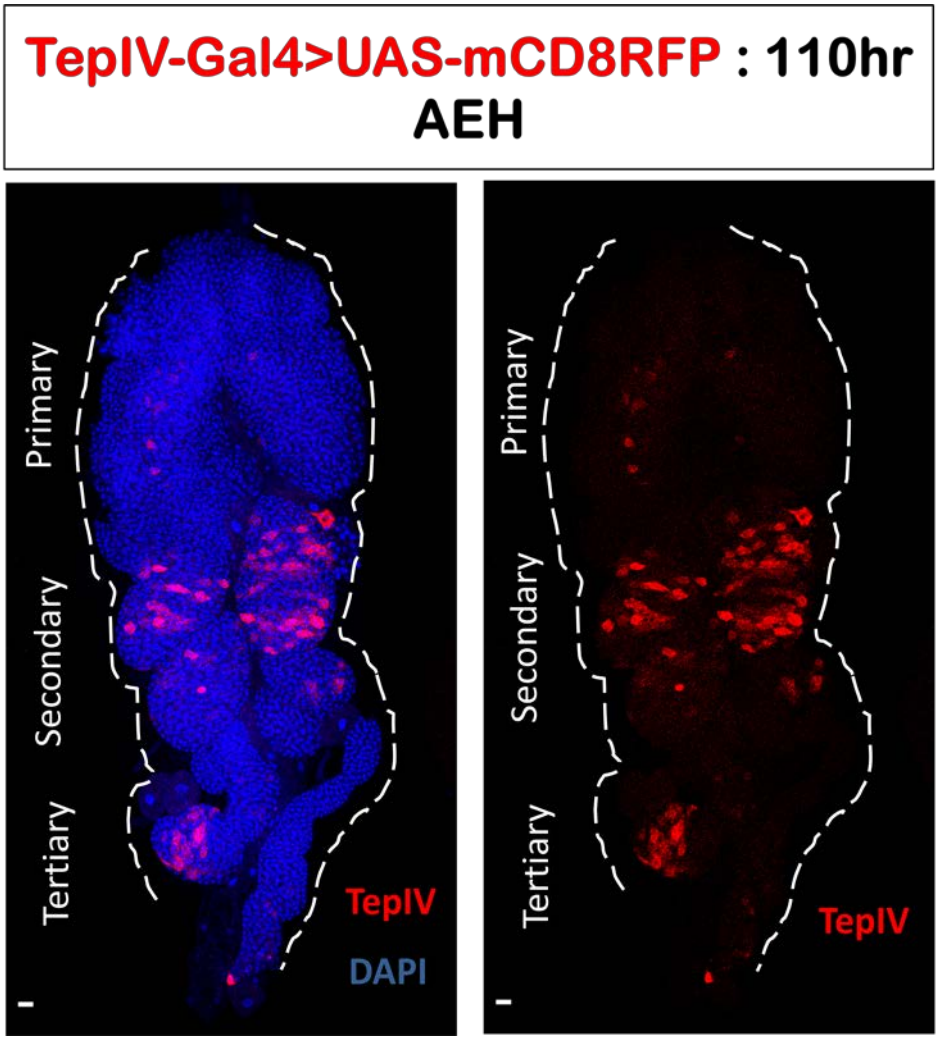


Figure 14 :- The level of expression decreases further in the PL.

The time series of TepIV expression performed suggests that, the TepIV domain (mentioned in figure 8) is not detectable in early lymph gland. One can hypothesize that it might be created by dynamic cell-cell interactions, and different developmental cues during the larval stages. To have a insight into the exact mechanism of this dynamic expression of TepIV further studies are required.

b) Kn-Gal4 >UAS-mCD8RFP

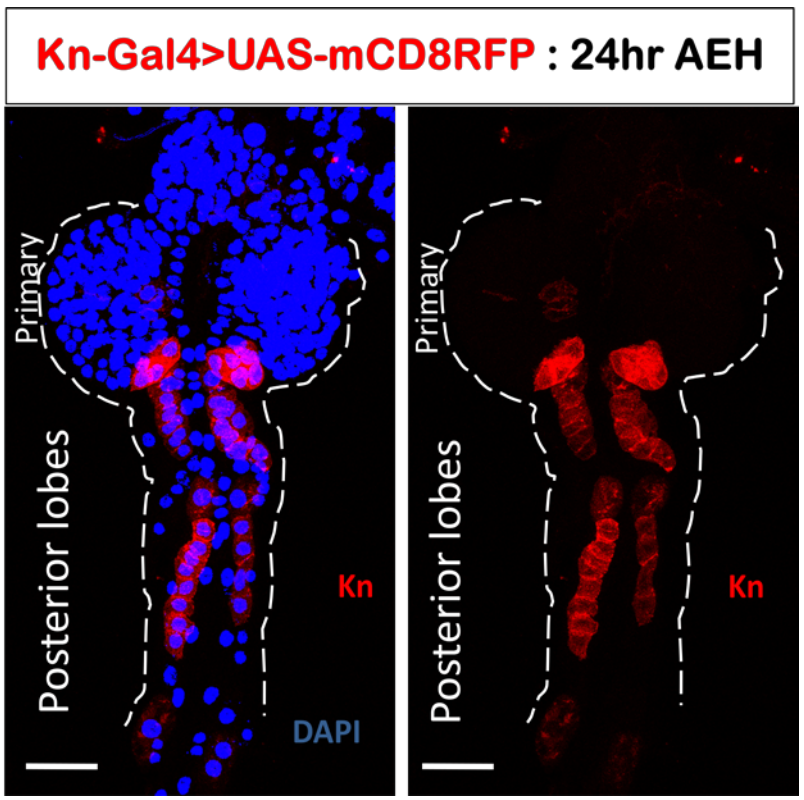


Figure 15 :- Knot is expressed in the entire PL and PSC.

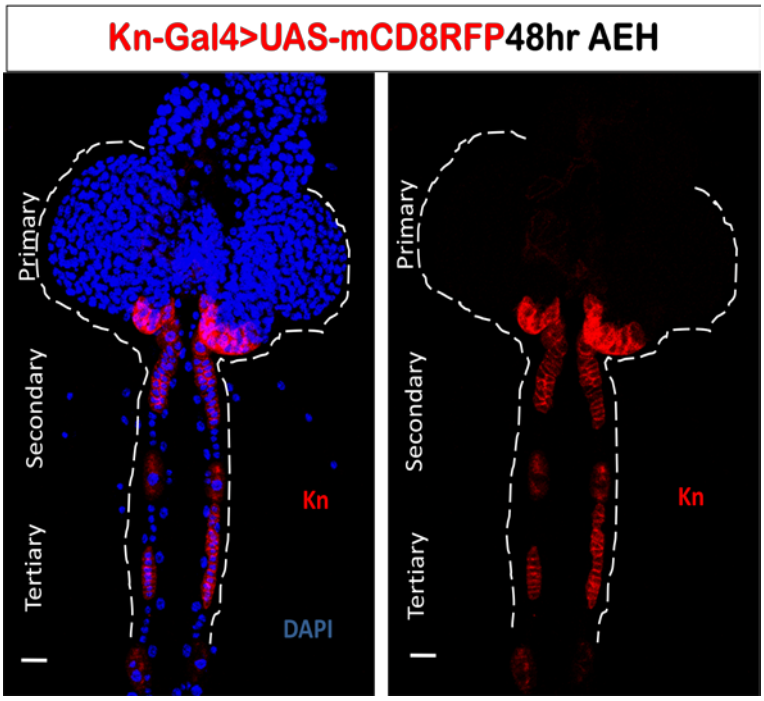


Figure 16 :- Knot is expressed in the entire PL and PSC.

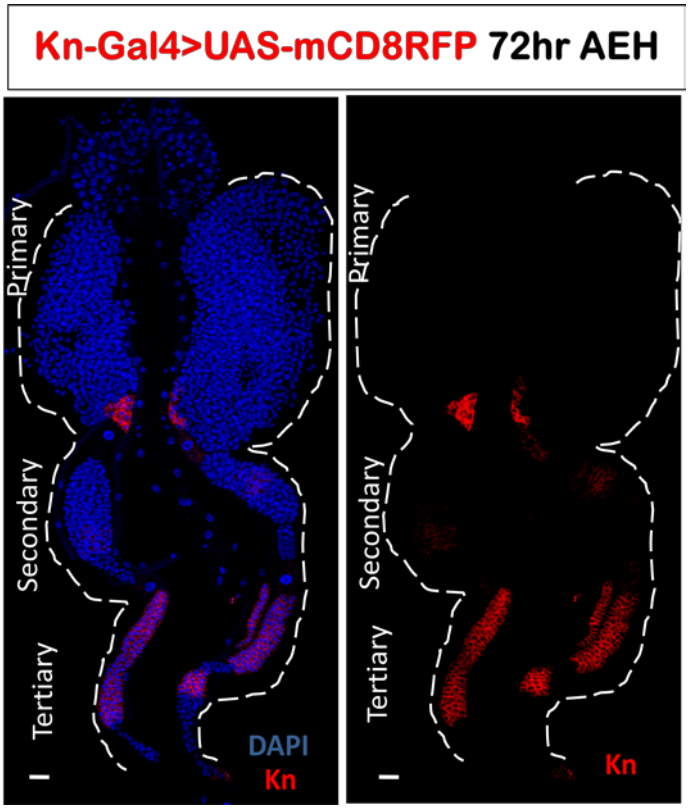


Figure 17 :- Expression of Knot regresses to the Knot domain and PSC.

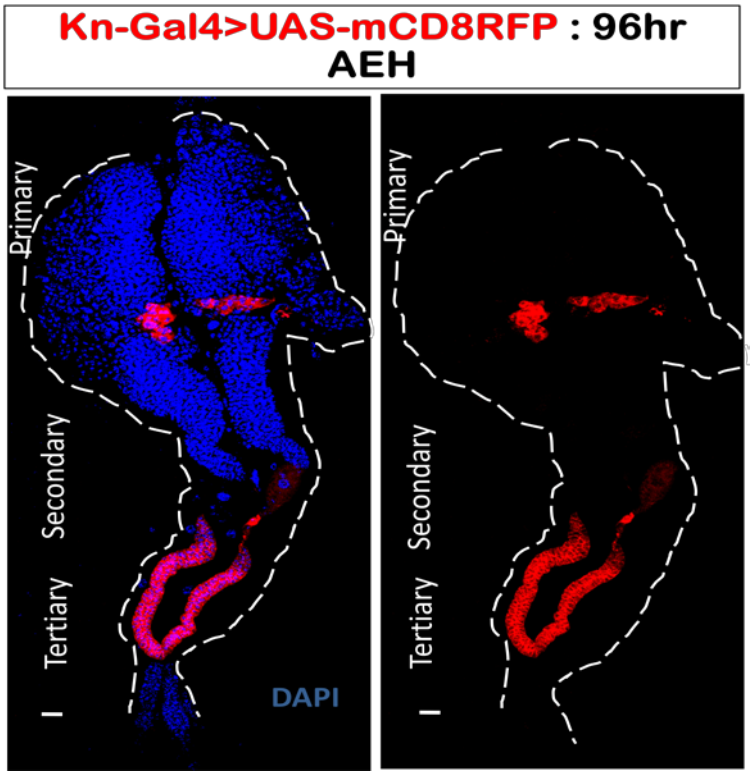


Figure 18 :- Knot is expressed in PSC and perspective niche.

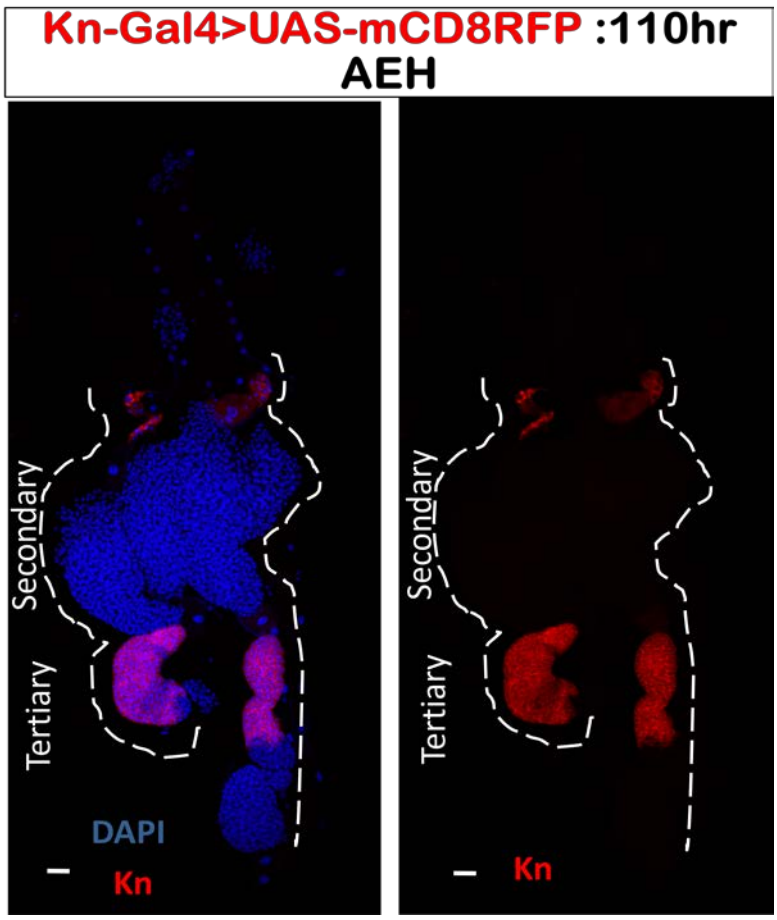


Figure 19 :- Primary lobe is dissociated, except PSC. Knot is expressed in the prospective niche and PSC.

Similar to the expression of TepIV, Knot domain is not defined in the early lymph gland. From the 72 hours AEH onwards Knot expression is restricted to the PSC of the primary lobe and the prospective niche of the tertiary lobe.

c) Antp-Gal4> UAS-mCD8RFP

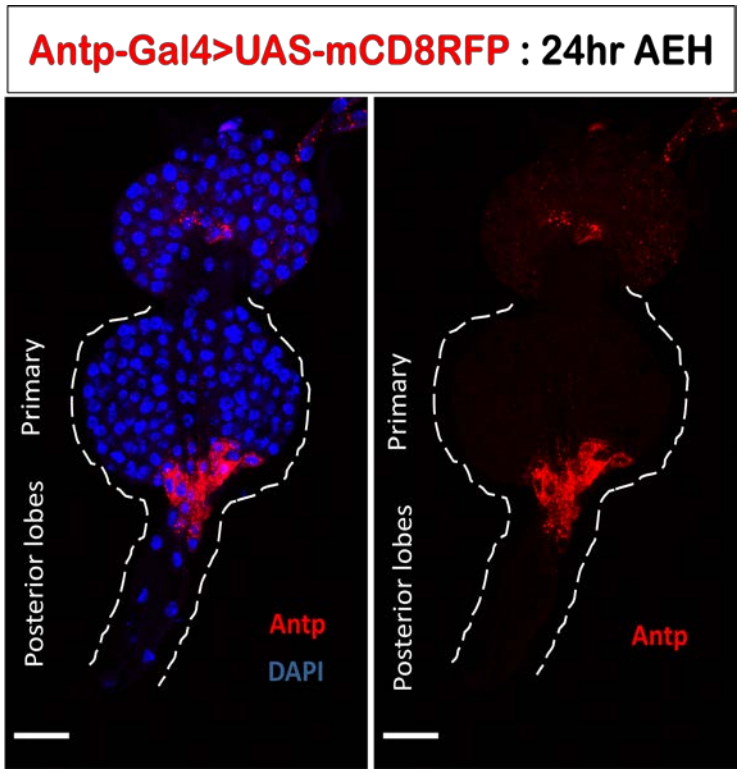


Figure 20 :- Antennapedia is expressed only in PSC

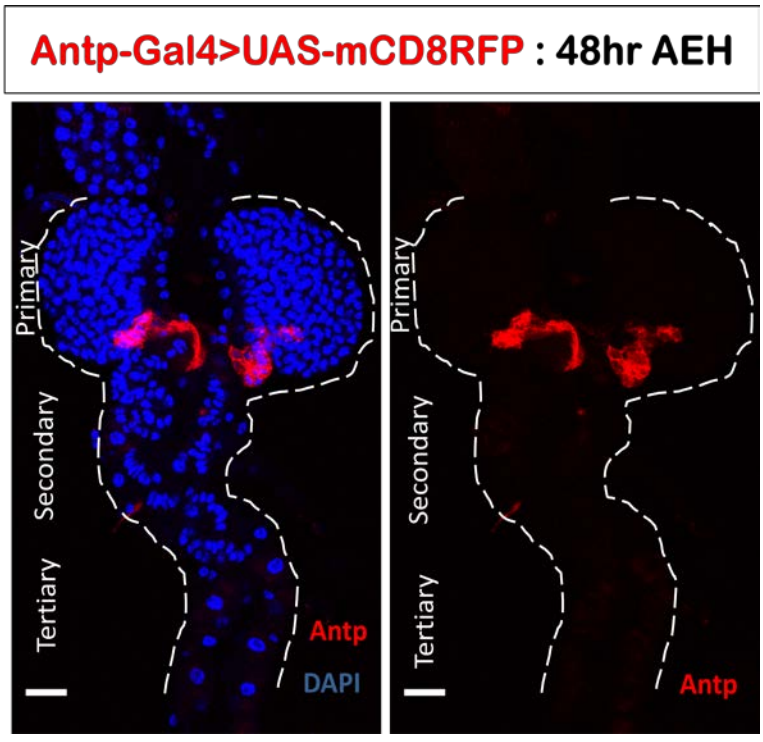


Figure 21 :- Antennapedia is expressed only in PSC

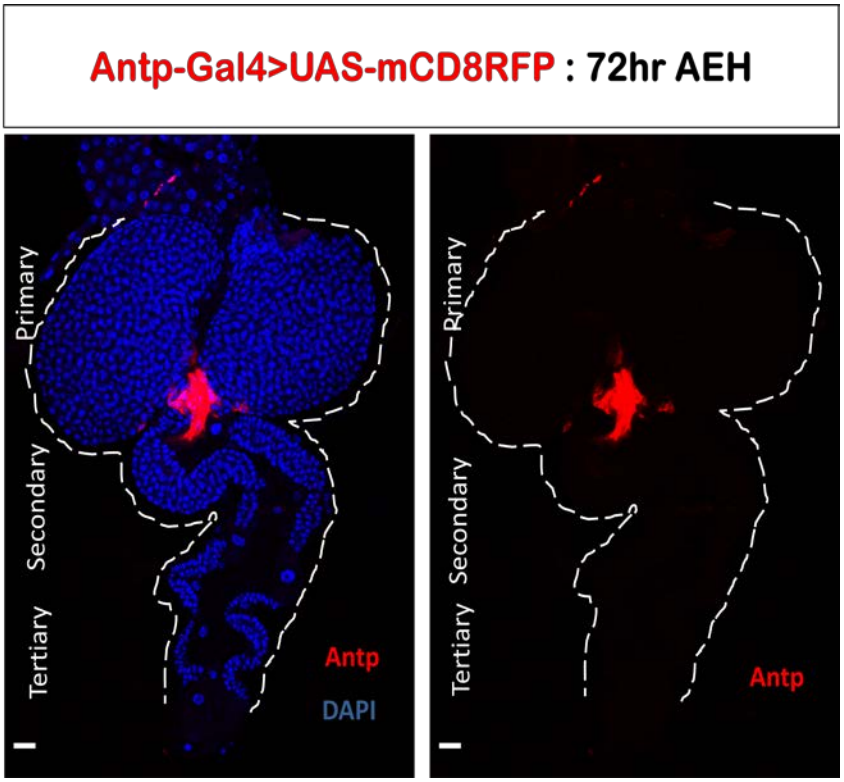


Figure 22 :- Antennapedia is expressed only in PSC

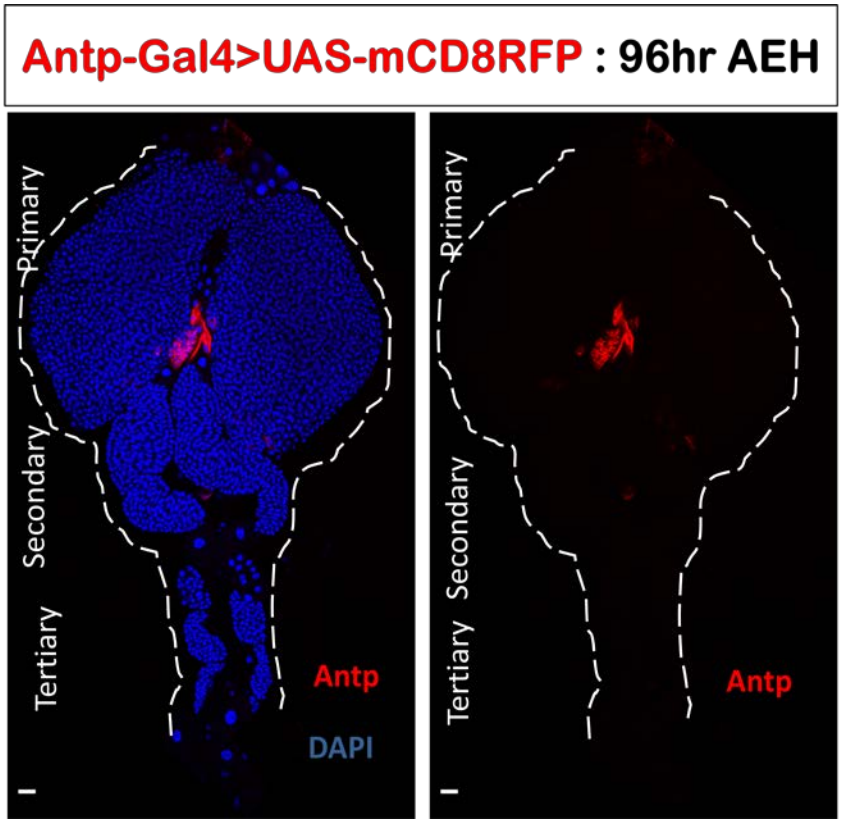


Figure 23 :- Antennapedia is expressed only in PSC

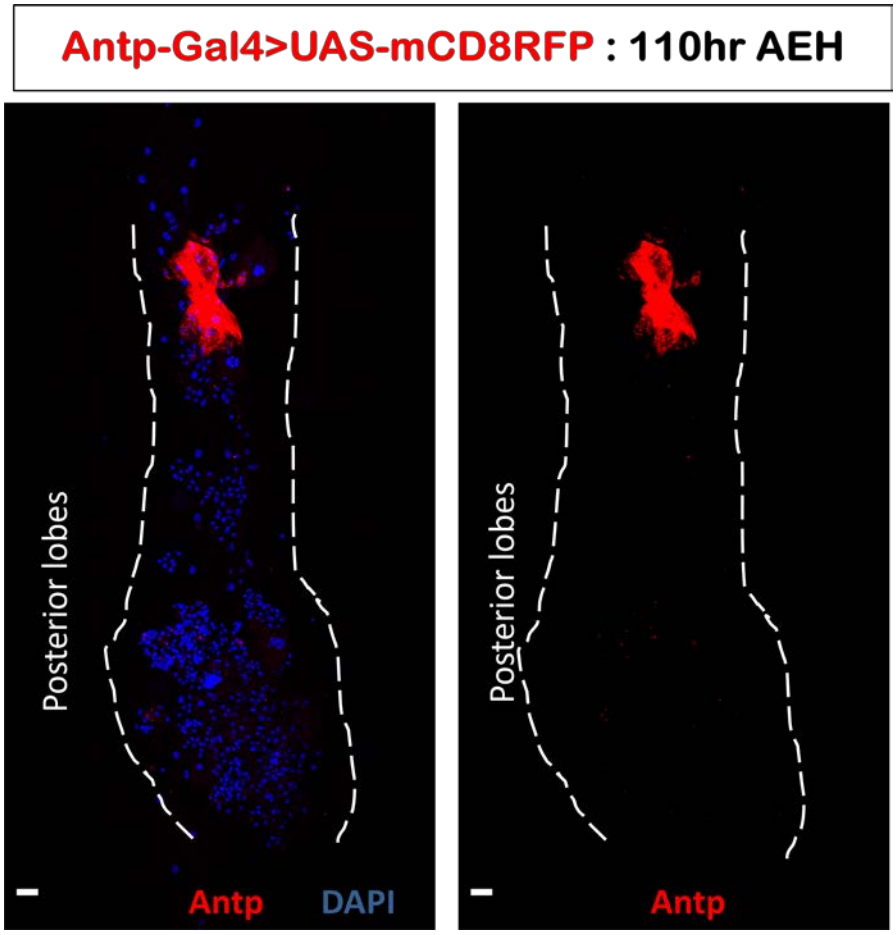


Figure 24 :- Antennapedia is expressed only in PSC. Primary lobe has been dissociated at this time point, except PSC/niche can be detected.

As mentioned earlier, Hox gene Antennapedia expresses in the anterior lobe niche/PSC[39]. We wanted to explore if the Antennapedia expresses in the prospective niche as well. But this time series indicates that, Antennapedia does not get expressed in the prospective niche at any time period of developmental stage. Further experiments are required to check whether any other Hox gene (e.g. Ultrabithorax or Ubx) is expressed in the prospective niche.

d) Hml-dsRed with Hnt antibody

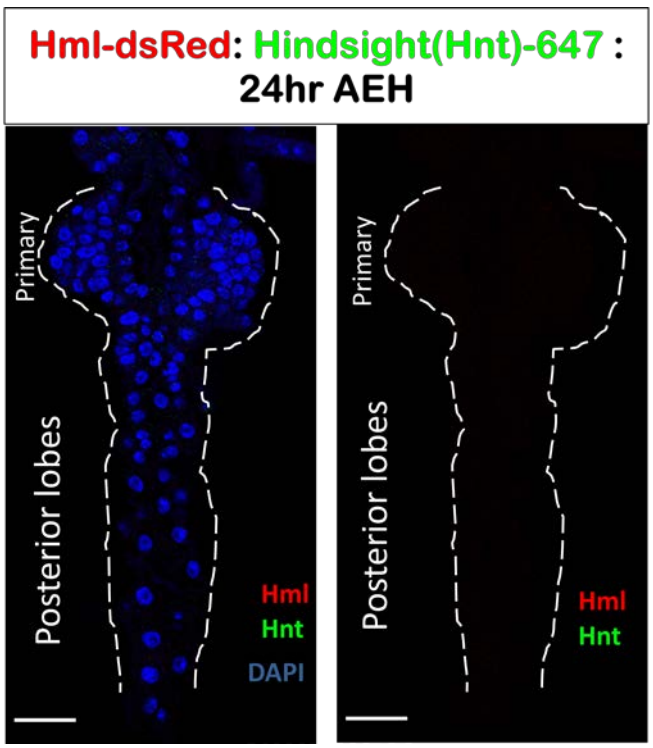


Figure 25:- No expression of Hml or Hnt

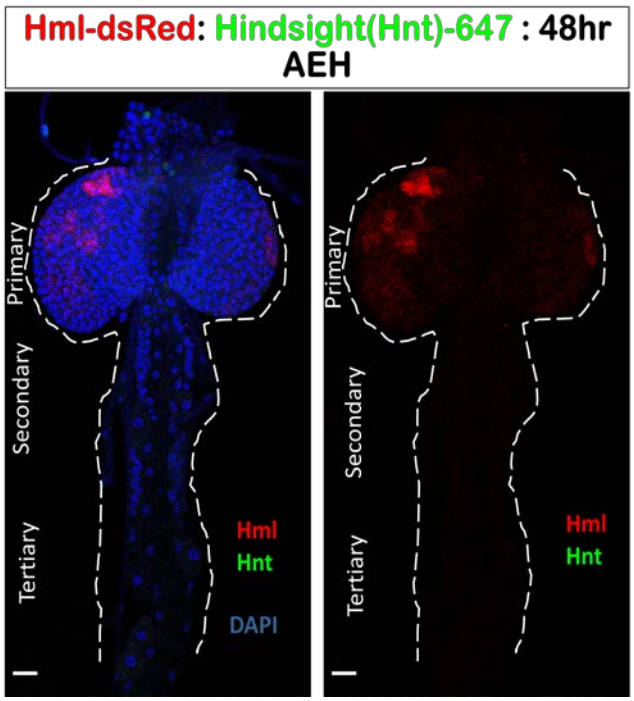


Figure 26:- No expression of Hnt. Mild expression of Hml in the CZ. No expression of both markers in the PL

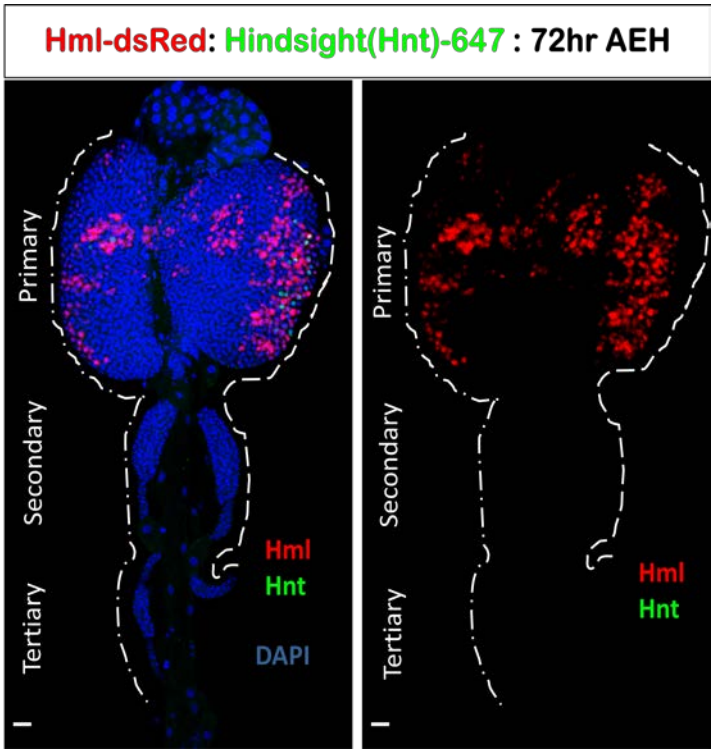


Figure 27:- No expression of Hnt. Increased expression of Hml in the CZ. No expression of both markers in the PL

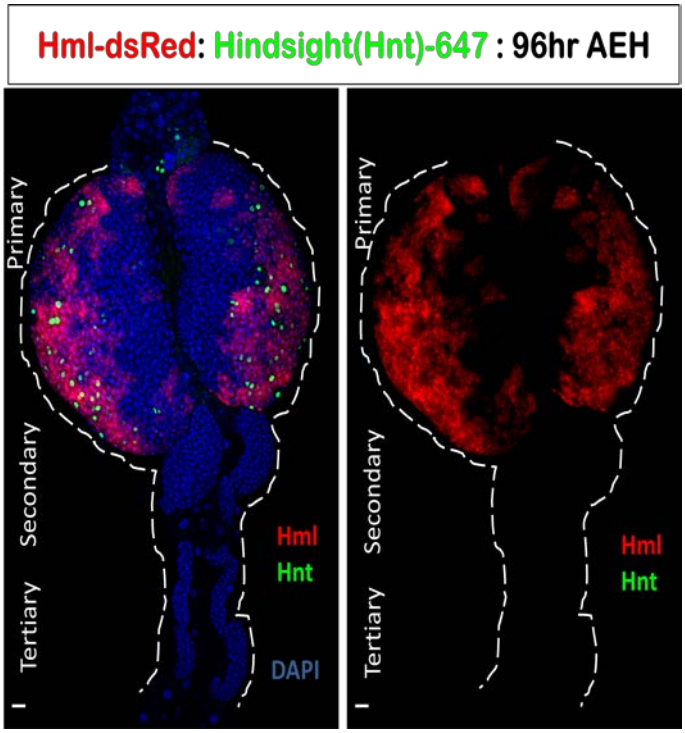


Figure 28:- High expression of Hml and ectopic expression of Hnt in CZ. Neither marker are expressed in the PL.

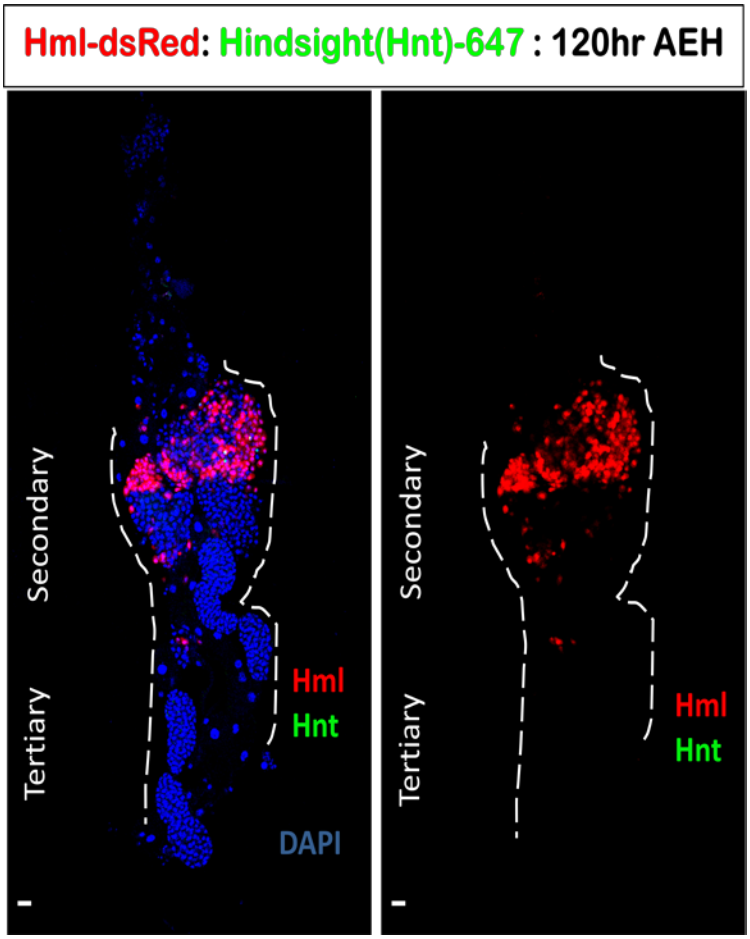
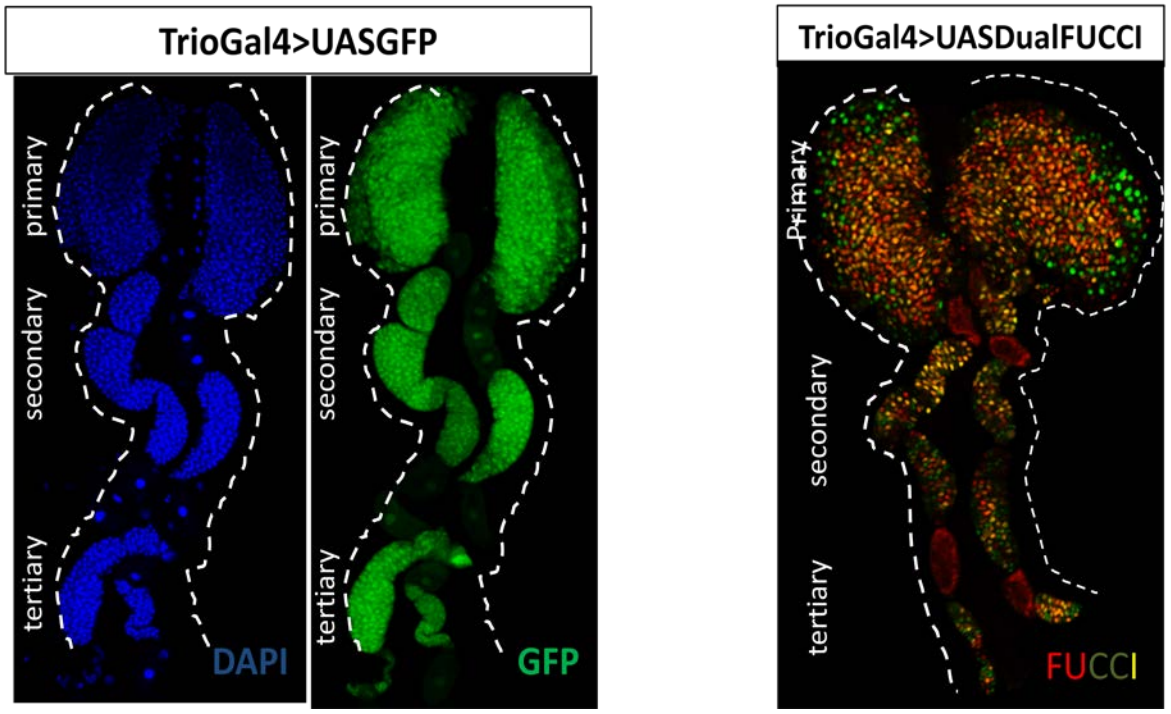


Figure 29 :- Primary lobe has been dissociated. Hml is expressed in the secondary lobe. No expression of Hnt is observed.

Both Hemolectin (Hml) and Hindsight (Hnt) are the differentiation markers of Plasmatocytes and Crystal cells respectively. Hml is an early differentiation marker. It does not get expressed in the PL until 120 AEH. This means the progenitors of the PL are maintained till the late third instar. Interestingly, crystal cells (Hnt) is not present in the PL during development further endorsing the progenitor status of PL cells.

5.3) The cell cycle status PL.

To achieve an insight into the cell cycle status of PL hemocyte progenitors, we used fly FUCCI technique. To confirm this FUCCI construct actually works in the lymph gland, we used *TrioGal4* to drive UAS-FUCCI. Trio is expressed in the entire lymph gland. It is a guanine exchange factor which regulates actin remodulation[50].



(A) (B) (C)

Figure 30 :- Diagram (C) clearly shows that "Trio-Gal4 > UAS-DualFUCCI" indeed work in Lymph Gland.

To check cell cycle status in mutually exclusive domains of the PL, we used TepIV-Gal4 and Kn-Gal4 to drive UAS-DualFUCCI.

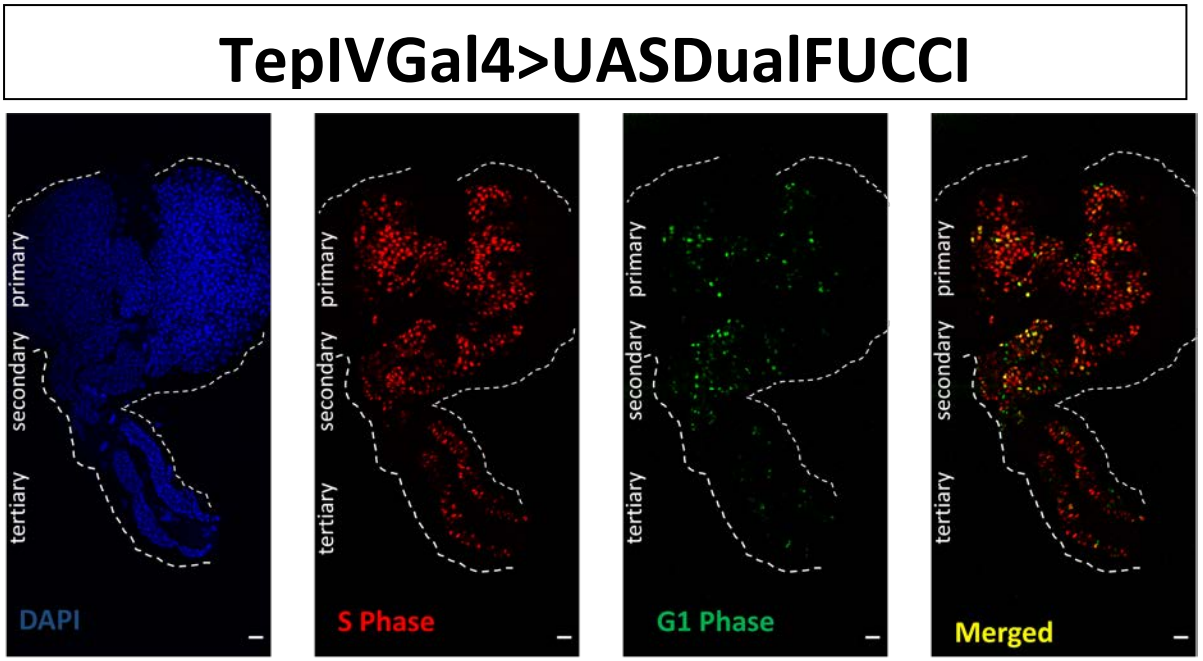


Figure 31 :- Representative diagram of TepIV-Gal4>UAS-DualFUCCI.

We counted the cells in each phase using imageJ software.

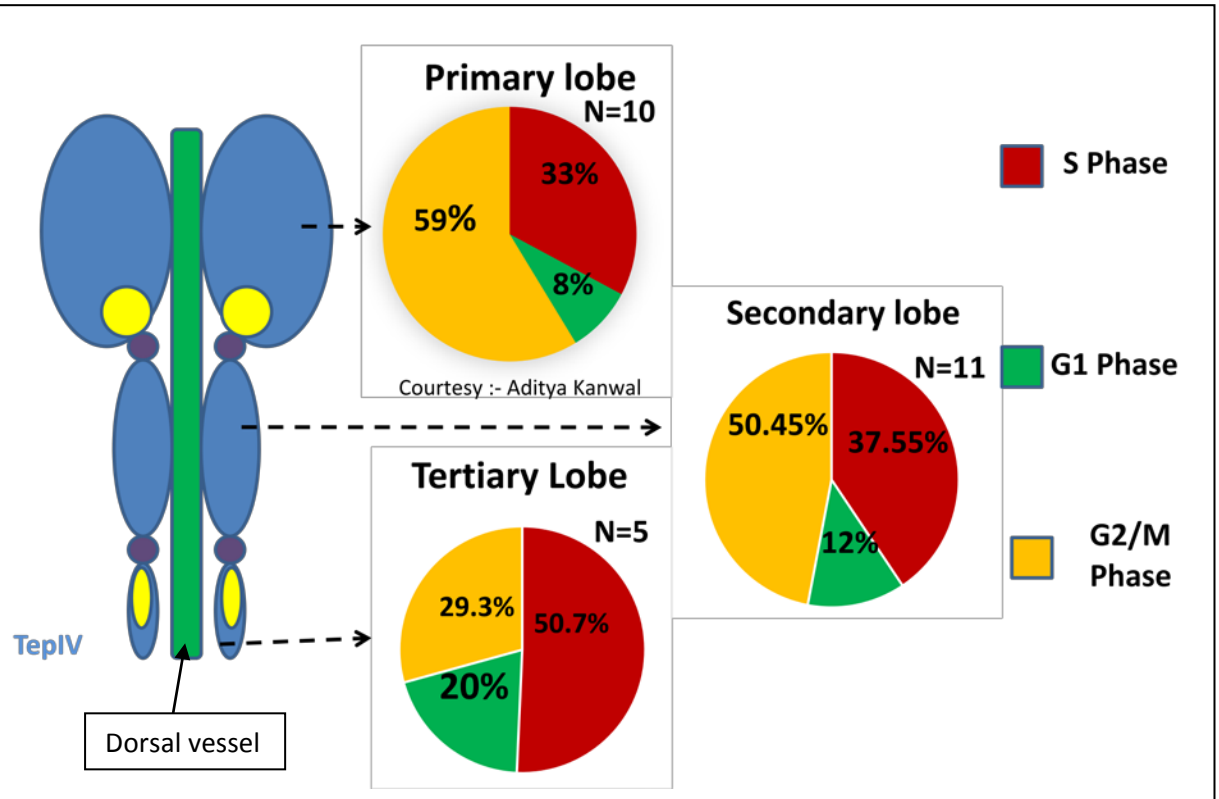


Figure 32 :- The quantification of cell cycle status of the hemocytes in TepIV domain. It is evident from the data that as we move from primary to secondary and then to tertiary there is an increase in number of cells in S phase, G1 phase and decrease in the number of cells which are in G2-M phase.

Similar analysis of cell cycle status was performed in Knot expressing domains, using KnGal4 as driver line.

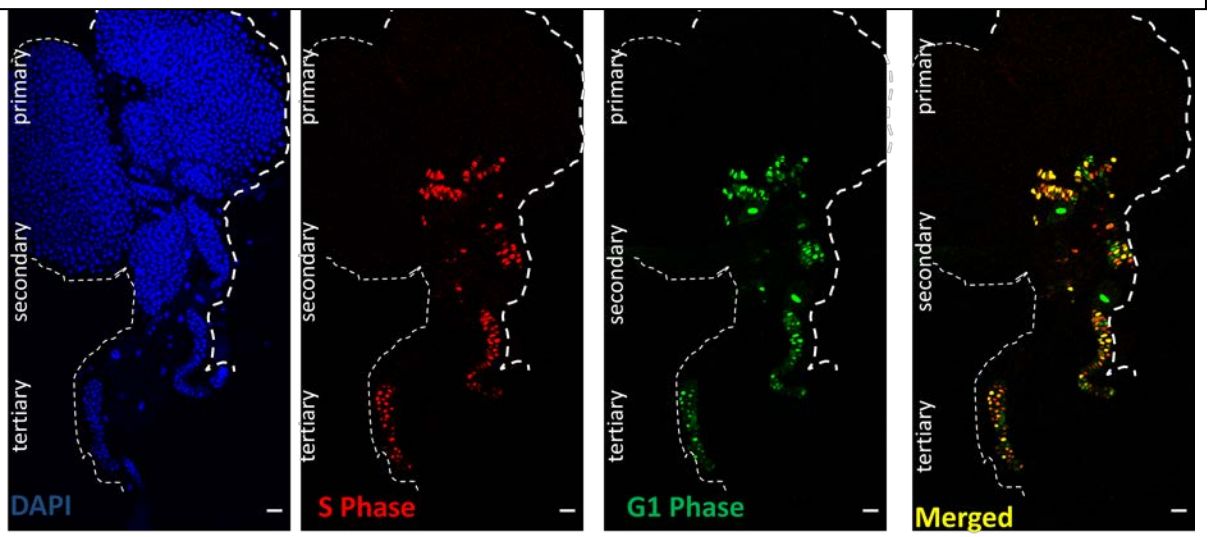


Figure 33 :- Representative diagram of the Kn>FUCCI

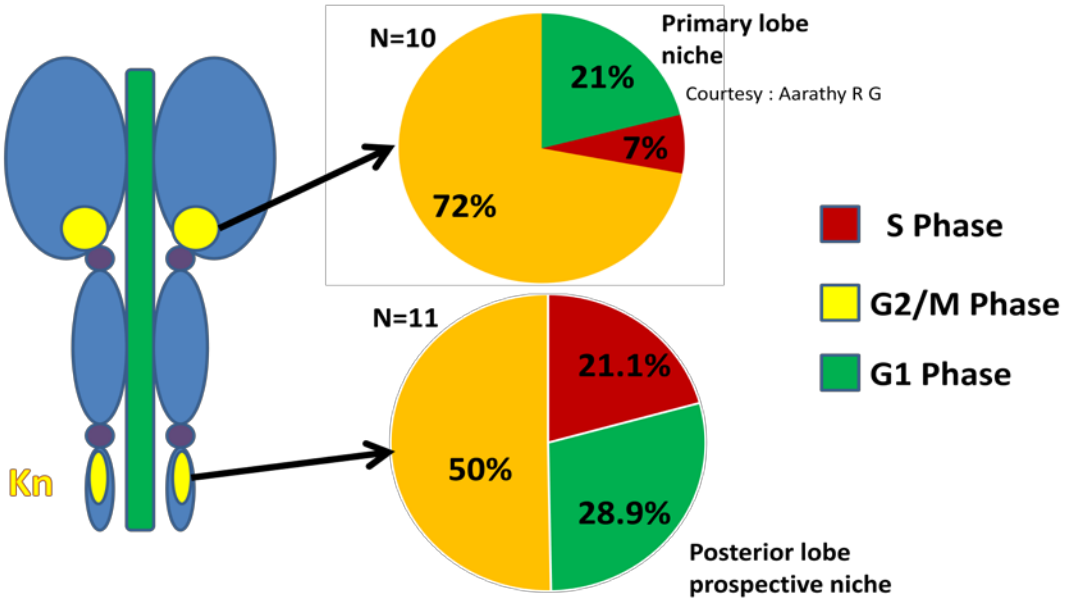


Figure 34 :- The quantification analysis of cell cycle status in Knot domain. It is evident from the data that as we move from primary to secondary and then to tertiary there is an increase in S phase, G1 phase and decrease in number of cells which are in G2-M phase,.

Therefore, going from primary to PL, percentage of cells in S and G1 phase increase and G2-M phase decreases.

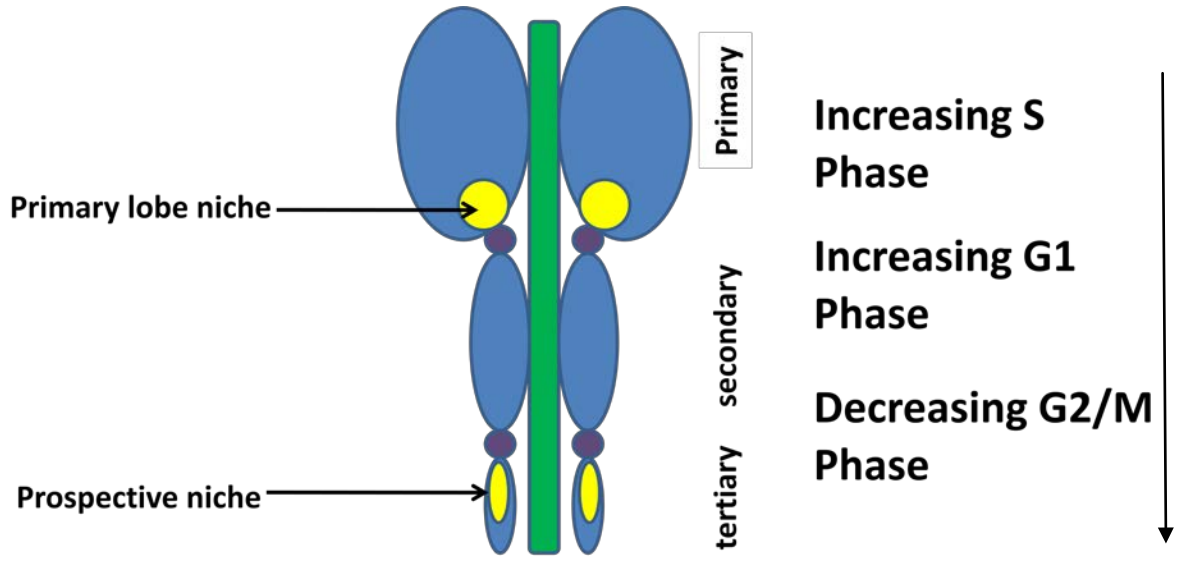


Figure 35 :- Model of cell cycle status of Lymph Gland.

To further differentiate between G2 and M phase, we used pH3 antibody to mark cells, in both TepIV and Kn domain, which are in M phase.

Kn-Gal4 > UAS-DualFUCCI

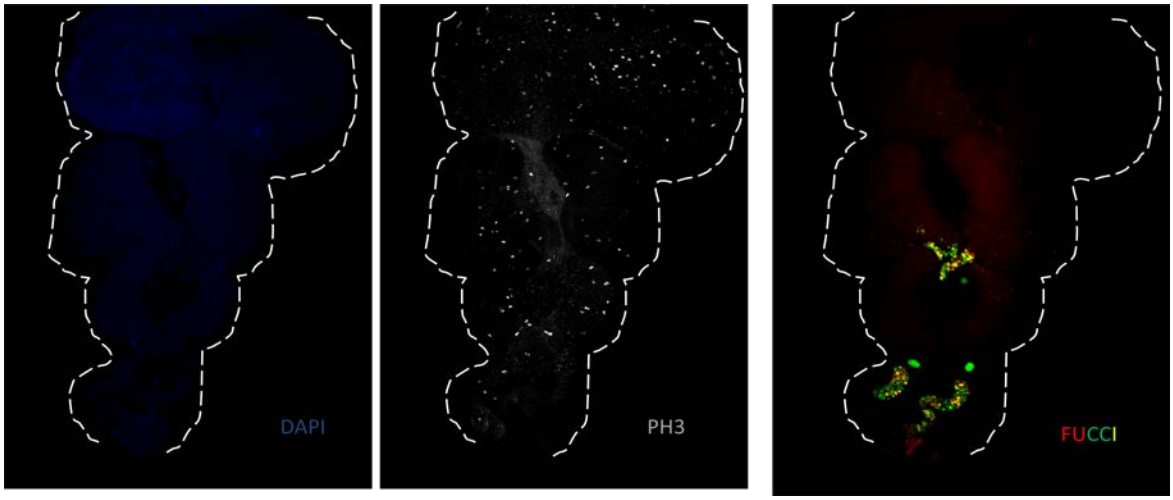
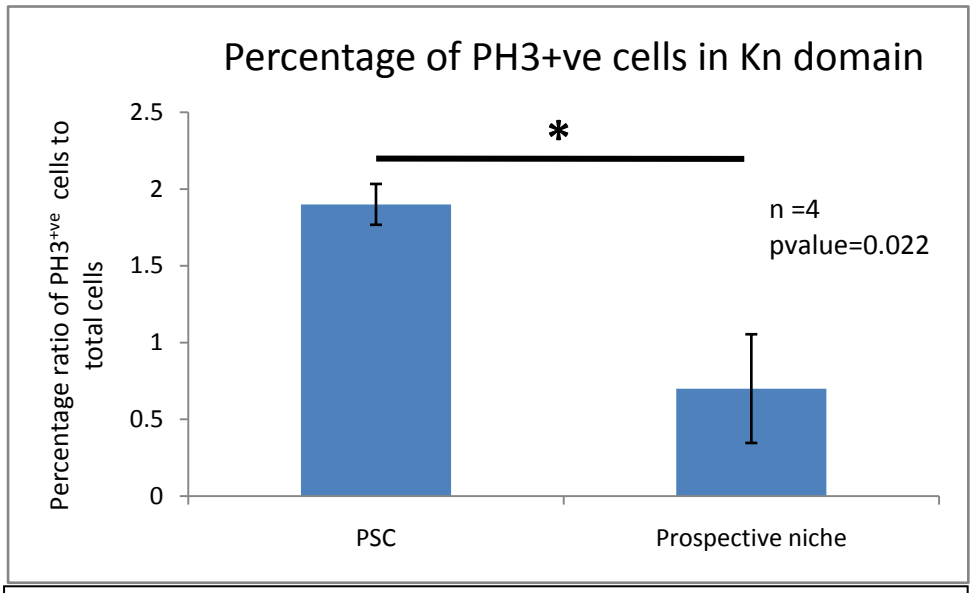


Figure 36 :- Representative diagram Kn-Gal4>UAS-FUCCI with PH3 antibody. 96 Hours AEH

Cells which are in M phase in Knot domain

Region of Knot domain	Mean percentage of $PH3^{+ve}$ cells	Standard deviation of samples
PSC/niche of primary	1.89 %	0.133
Prospective niche	0.7 %	0.354



Graph 1:- The percentage of $PH3^{+ve}$ cells in Prospective niche is significantly lower than PSC.

As we have showed earlier in our FUCCI result, the number of cells in G2-M phase in Kn domain, decreases from PSC to the prospective niche. And this result highlights the subsequent decrease in M phase cell from PSC to prospective niche.

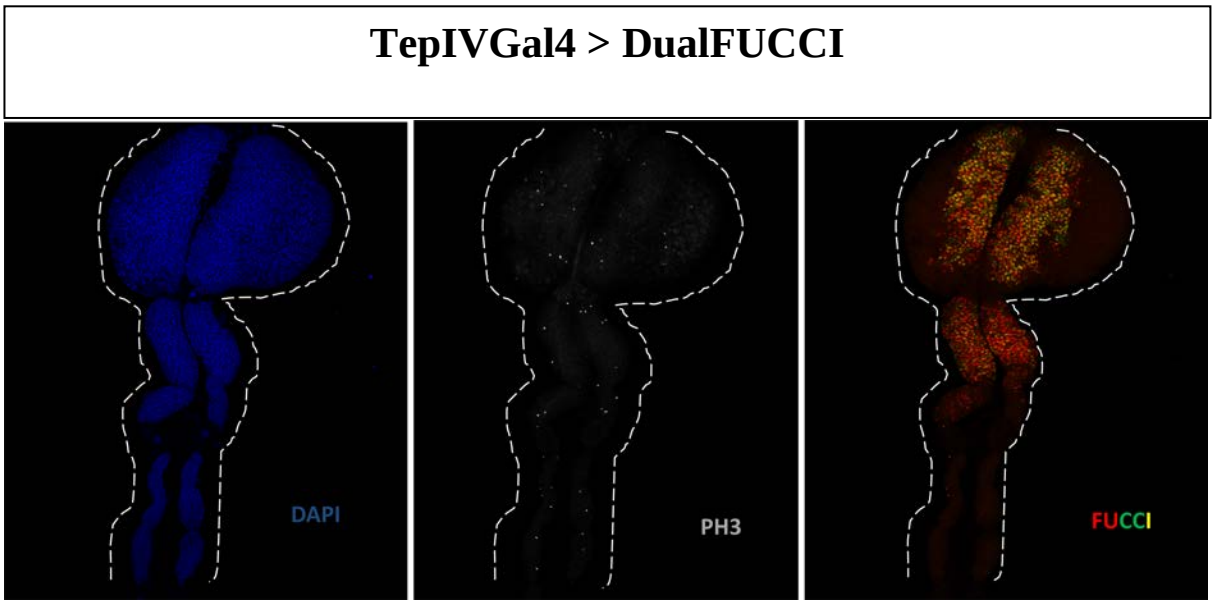
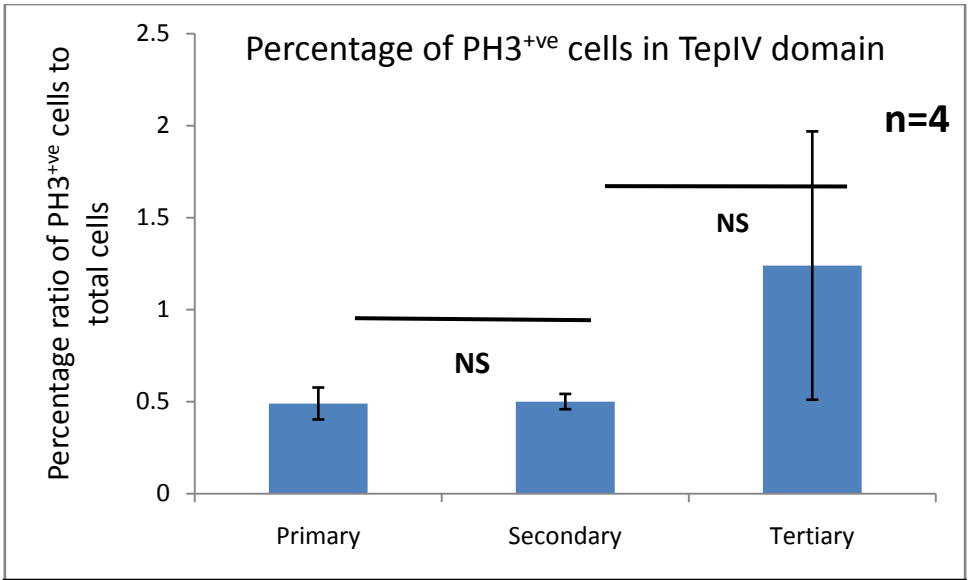


Figure 37 :- Representative diagram Tep-IV>FUCCI with PH3 antibody. 96 hours AEH.

Region of TepIV domain	Mean percentage of $PH3^{+ve}$ cells	Standard deviation of samples	P value
Primary lobe	0.490036 %	0.086	(1^{o}-2^{o}) 0.826
Secondary lobe	0.5%	0.041	(2^{o}-3^{o}) 0.089
Tertiary lobe	1.23 %	0.729	(1^{o}-3^{o}) 0.087



Graph 2:- Percentage of M phase cells in TepIV domain. There is no significant difference of $PH3^{+ve}$ cell population between primary, secondary and tertiary lobe.

The change in $PH3^{+ve}$ cells in TepIV domain is not significant. Sample number should be increased for any conclusive result.

5.4) EdU incorporation assay

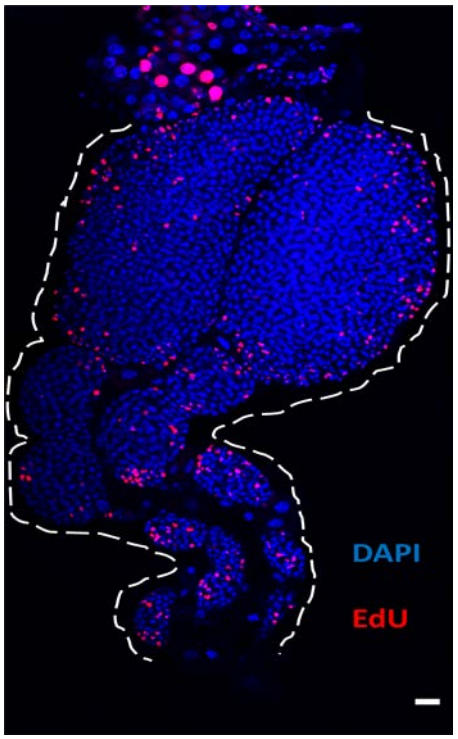
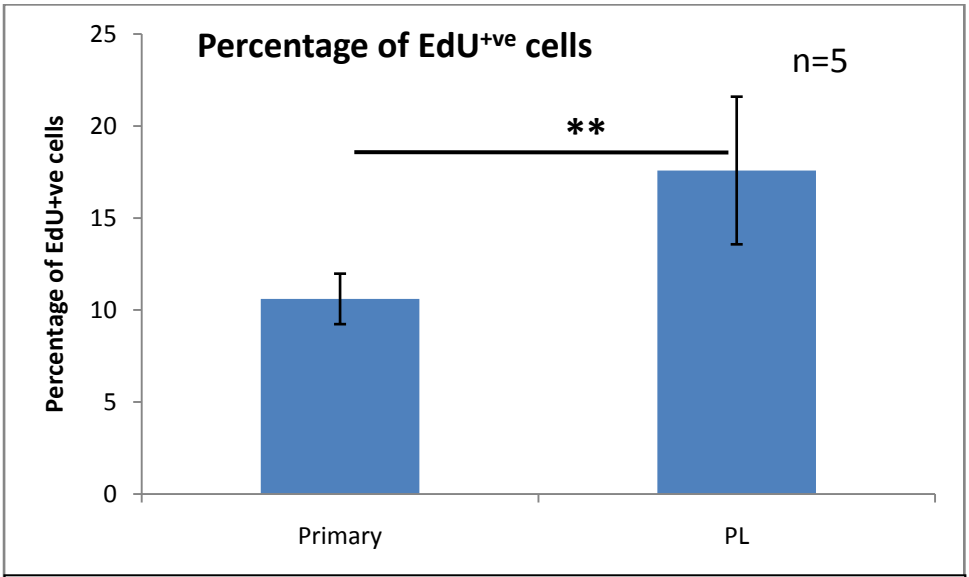


Figure 38 :- Representative diagram of EdU assay.

To further validate the FUCCI data, we performed EdU assay to find out cell proliferation status in the PL.

We quantified the number of cells which are in S phase i.e. positive for EdU and normalised with total number of cells.

Region	Mean percentage of EdU $^{+ve}$ cells	Standard deviation of samples	P value
Primary lobe	10.6%	1.37	0.006
PL	17.58%	4.01	



Graph 3 :- Percentage of EdU positive cells is significantly more in PL than in Primary lobe

The cells which are EdU positive are in proliferation state. The PL has higher percentage of EdU cells than the primary lobe further endorses ourFUCCI data, which shows percentage of cells which are in S phase is higher in PL than in primary lobe.

6) Discussion

Two waves of hematopoiesis have been reported in *Drosophila melanogaster*[30,47]. However, work from our lab has shown that hematopoiesis also occurs in the adult, and Posterior Lobes contribute to it[24]. Hence the PL needs to proliferate and remain undifferentiated in a progenitor-like state throughout the larval stage of development. This has been demonstrated by the time series of the expression pattern of differentiation markers Hml and Hnt. We showed that indeed the PL remains undifferentiated until very late third instar. For this purpose, similar to well explored primary lobe[39], one would expect that there must a signalling centre, which relays molecular cues in order to maintain this progenitor-like state. This line of thought strongly indicates that the prospective niche in the tertiary lobe is might be a "niche" for PL.

The primary lobe actively contributes to the larval hematopoiesis while PL is reserved for the Adult hematopoiesis. Proliferation and cell cycle assays further validate the hypotheses that, PL follow the same developmental fate as the primary lobe but at a later stage of the development. That means PL is "tailgating" to the primary lobe on the developmental time line. The progenitors in the S phase and G1 phase of the cell cycle are more in the tertiary lobe followed by the secondary lobe, and the primary lobe. Concomitantly the progenitors in the G2-M phase decrease from the primary to tertiary lobe because primary lobe has well defined MZ. An increase in the S phase percentage also suggests that the progenitors in the PL are proliferating. Similar results can be found in PSC and niche like domain in tertiary lobe. The time series analysis of TepIV and Kn illustrates, the domains in which these markers are expressing are changing over the developmental timeline. These domains were overlapping in the PL at 48 hours AEH, and then onwards, they start to express in mutually exclusive zones in PL. This indicates the zonation of PL is an ongoing dynamic process at early instar.

Further time series analysis reveals that the PSC is always marked by the Hox gene Antennapedia. On the other hand, the prospective niche in the PL lacks its expression. This observation leads to two possible notions. One is prospective niche lacks the expression of any Hox gene, or following body plan other Hox genes such as Ultrabithorax (Ubx) might express there. Further experimentation is required to check these both possibilities.

This also necessitates that PL act as an independent unit. The heterogeneous expression of progenitor and niche cells markers help in making PL an independent unit. There are no studies that have investigated the role of PSC in the maintenance of the PL. Having a niche like domain in the PL gives it the opportunity for independent regulation. This study however, at this time point, doesn't rule out the role of primary lobe PSC/niche in PL maintenance.

7) Future prospects

The posterior lobes of the lymph gland (PL) remain largely unexplored. This study has raised many interesting questions. To validate that the prospective niche is indeed a niche, many experiments designed to compare and contrast PSC and prospective niche, need to be performed. PSC secretes maintenance signal to the progenitors of MZ e.g., Hedgehog[39].It would be interesting to investigate whether the prospective niche secretes such a maintenance signal in PL?

When the functioning of PSC is disturbed either by killing the cells in a niche or by downregulating the signalling molecule released by PSC, then ectopic differentiation of the progenitors is observed[39]. It remains to be seen whether similar manipulations in the prospective niche yield identical phenotype in the PL .affecting the function of prospective niche.

If entire PL is eliminated from the lymph gland, how will it affect the adult hematopoiesis is still unknown. How significant PL is for the adult hematopoiesis during immune challenge or aging is unclear.

The PSC is defined by the expression of Hox gene Antennapedia[39]. It would be interesting to know whether any Hox gene gets expressed in the prospective niche.

In future investigating the role of primary lobe PSC/niche if any in PL maintenance and proliferation will give us an insight to composite niche interactions.

Answers to these questions will shed more light on the precise role of the reserve progenitors of the posterior in *Drosophila melanogaster* hematopoiesis and immune challenges.

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