Mutant Analysis of Leukocyte Antigen Related-like (LAR) in *Drosophila* hematopoiesis

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CERTIFICATE OF EXAMINATION

This is to certify that the dissertation titled “Mutant Analysis of Leukocyte Antigen Related-like (LAR) in *Drosophila* hematopoiesis” submitted by Ashutosh Tiwari (MS12055) for the partial fulfillment of B.S.-M.S. dual degree program 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.

Prof. Anand K. Bachhawat Dr. Kavita Babu Dr.Lolitika Mandal
(**Supervisor)**

Dated:

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.

Ashutosh Tiwari
Dated:

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
(superviosor)

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ABSTRACT

LAR or Leukocyte Antigen Related Like is a trans-membrane receptor protein tyrosine phosphatase with multiple domains. It is widely known for its involvement in motor axon guidance but it's role in haematopoiesis is not been investigated yet. A genome-wide RNAi screening targetted to the niche was conducted to discover potential players of maintenence and functionality of niche. Dlar has emerged as a key player whose phenotype has given us the potential phenotype to unravel the physiological role of LAR in the hematopoiesis. We have used lymph gland- the larval hematopoietic organ of *Drosophila* melanogaster as a model system. In this study I tried to find out a classical loss of function mutant for LAR so that its role in hematopoiesis can be established with certainty.

CHAPTER 1

INTRODUCTION

1.1 *Drosophila melanogaster:* The Fruit Fly

Drosophila melanogaster, commonly known as the fruit fly is an extraordinary and widely used model system for studying the cellular and developmental processes in genetic and developmental fields. The short life cycle, ease of handling in laboratory condition, small fully sequenced small genome makes *Drosophila* an expedient model system for research purposes.

Drosophila melanogaster contains a pair of sex chromosome and three pairs of autosomes, constituting a total of four pairs of chromosomes. An interesting fact about the male fruit flies, is that they do not show any meiotic recombination. In fruit fly, the modified chromosomes known as balancer chromosomes play a very crucial role genetically as scientists can use them to control the *Drosophila genetics* in order to keep recessive lethal mutations in stock, preventing meiotic recombination. Balancer chromosomes consist of many multiple inverted sequences which carry recessive lethal mutations and dominant visible markers(Greenspan et al 2004).

1.2 Life cycle:

One of the biggest advantage of *Drosophila* melanogaster as a model organism is the shortened life cycle. The complete life cycle of *Drosophila* at room temperature (25°C) is about 10 days. This life-span can change at different temperatures as at at 25^0 C it is normally 45 days but at 29^0 C the life span reduced. *Drosophila* is a holometabolous insect of order Diptera, which means it have distinct developmental stages as- egg, larva, pupa and adult. A mature adult female lays eggs at a rate of 50-70 per day at room temperature, the eggs hatches after 24 hours after the egg laying and the first instar larva emerges out of it. The *Drosophila* larva has three larval stages which feeds on the provided food: first

instar (after 24 hours), a bigger second instar larva (after next 24 hours), the third instar larva (next 72 hours) which also feeds on food but later moves out of it to a relatively dry region to start pupation. The pupal stage lasts for 3-4 days during which the metamorphosis takes place by replacing most of the larval structures with adult structures. The adult fly which emerges from the pupal case has a life span of about 6 weeks.(Lawrence et. al.1992) (*Figure 1*)

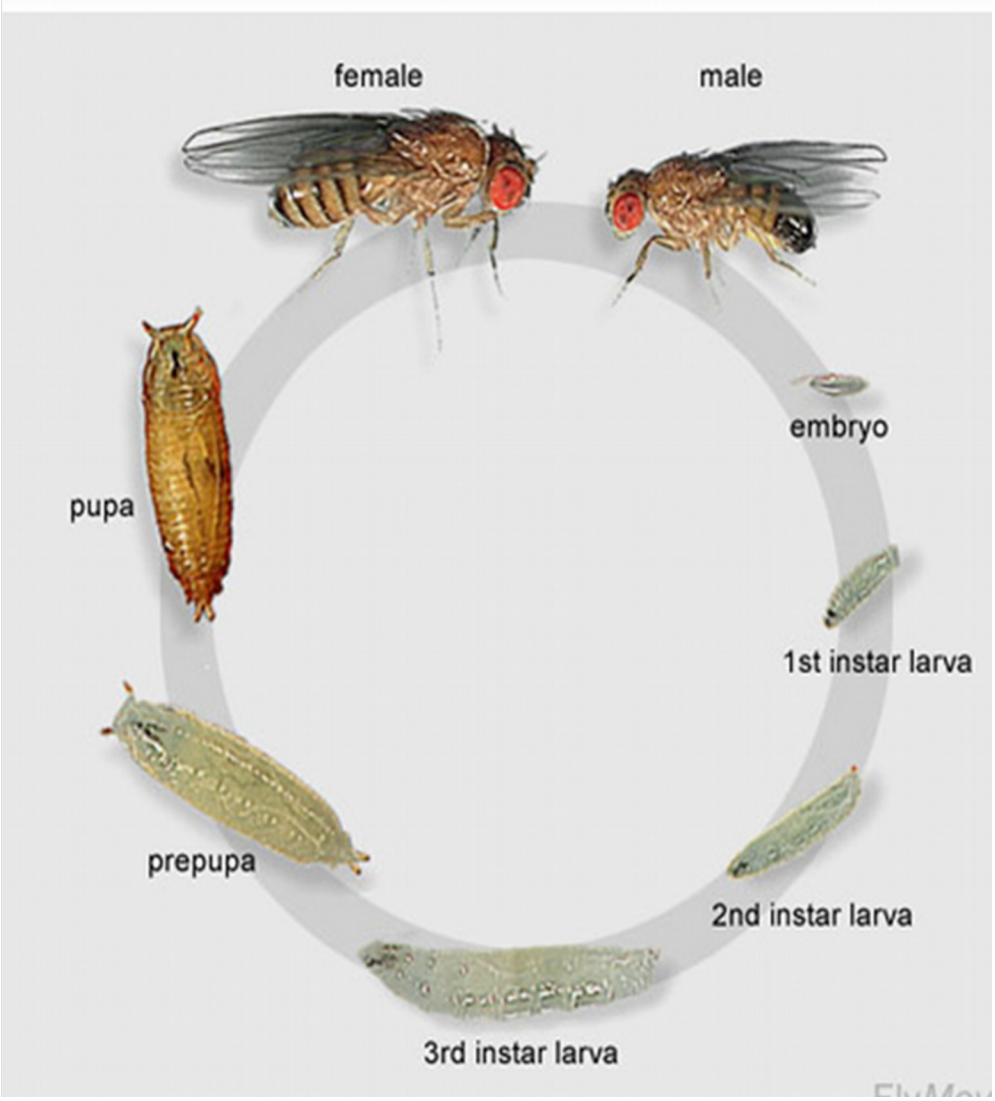


Figure (1.): *Drosophila* life cycle.(http://flymove.uni-muenster.de/)

1.3 *Drosophila melanogaster*: as a model organism

The fruit fly or *Drosophila* melanogaster has been broadly studied for decades as a model organism for different research purposes. There are a lot of characteristics which make it a perfect model organism to study different aspect of biology from developmental to neurobiology, from cancer research to muscles formation. Here are some factors which makes its such an important model organism:

1. A short life cycle of around 10 days makes it a convenient to handle and study the specially induced mutations.
2. The fully sequenced genome of *Drosophila* makes it possible to manipulate its genome as desired.
3. *Drosophila* genome shares around 60% homology with vertebrate which makes it ideal to study for the research purpose.
4. *Drosophila* do not contains or spread any disease or contamination.
5. *Drosophila* as a model organism provides genetics tools such as gal4uas system, fly-fucci which makes it easier to manipulate, regulated or down regulate the trans gene, hence giving us an extra layer of control over it.

1.4 Hematopoiesis:

Hematopoiesis is the process of blood formation ,a developmental process which is conserved across various taxas. *Drosophila* melanogaster has emerged as an effective invertebrate model system to investigate various characteristic of blood development. *Drosophila* hematopoiesis shares an overwhelming similarity to vertebrate hematopoiesis with respect to the origin of blood; phases of development, and involvement of transcription factors. Significant degree of conservation is present between *Drosophila* hematopoiesis and the vertebrate counterpart not only in terms of the transcription factors and signaling pathway molecules but also in terms of the phases of hematopoiesis that occurs during the development. (Table 1*).*

1.5 Hematopoiesis in vertebrates:

Hematopoiesis in mammals is biphasic, blood development occurs in two phases known as primitive and definitive. The Primitive hematopiesis occurs in embryonic yolk sac and definitive hematopiesis originates from AGM (Arota Gonad Mesonephros) region. There are several reports which suggest the hematopoietic activities are also found in fetal, spleen, liver and placenta.The site of hematopoiesis in adult vertebrates is primarily bone marrow. The mammalian hematopietic stem cell give produces two type of proginator cells: lymphoid and myeoloid.(Galloway and Zon, 2003)

1.6 Hematopiesis in *Drosophila* :

As in mammals, *Drosophila* hematopoiesis also happen in two waves- primitive and definitive (Figure 2) whcih is separated from each other spatially and temporally.

In *Drosophila* , the primitive wave occurs in the head mesoderm during embryonic stage .Serpent (srp), a zinc finger transcription factor which is also member of GATA family is the early molecular signature for specification of blood cells during primitive hematopiesis. Serpent is orthologous to the GATA factors involved in the blood cell specification of vertebrates.(Evans et al 2007)

The definitive hematopoiesis in *Drosophila* occurs during larval stages in the specific hematopietic organ: lymph gland which is similar to the mammalian definitive hematopoietic wave that arises in the Aorta-Gonad-Mesonephros (AGM).

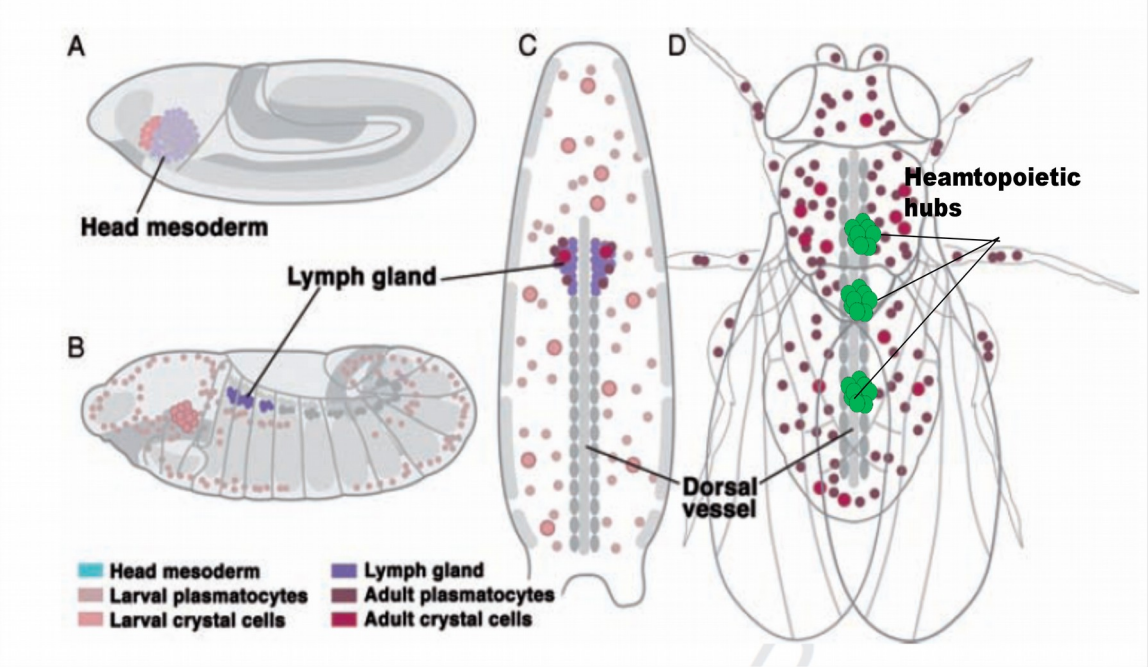


Figure 2: *Drosophila* hematopoiesis(modified from Evans et al 2007)

Drosophila	**Mammals**
Serpent	GATA
U shaped	Friend of GATA
Lozenge	Runx
Cut	Cux
Dorsal/Cactus	NFkB/IkB
Collier	Early B cell factor
Notch/Serrate	Notch/Jagged
Hop/Stat92E	JAK/STAT
Toll/18-Wheeler	Toll like receptors
Dpp	BMP4

Table 1.: Conservation of transcription factors and signaling molecules

1.7 *Drosophila* blood cell type:

In contrast to the large variety of blood cell types present in vertebrates, only three blood cell types are found in *Drosophila* . -

1. Plasmatocytes
2. Crystal cells and
3. Lamellocytes.

These blood cell type can been seen in the circulation of *Drosophila* with plasmatocytes representing more than 90% of total population. Plasmatocyte are the primarily differentiated blood cell in the circulation. The main function of the plasmatocyte is phagocytosis during various developmental stages including apoptotic clearance of cell debris in metamorphosis as a key mediator of cell immunity.

Most of the remaining hemocyte are the crystal cell, function is melanization. . The darkening and hardening of tissue is due to the local deposition of melanin, which is generated by the oxidation of phenols to quinones mediated by the Phenol oxides(PO).(Evans et al 2007)

The last cell type, Lamellocytes are the large cell which are the generally not seen in normal healthy individual but are induced in special immune challenged condition like parasitic wasp infestation to encapsulate the large wasp egg (Evans et al 2007).

1.8 Lymph gland-the larval hematopoietic organ

The lymph gland originates from the three thoracic segments(T1,T2 and T3) that express odd-skipped a zinc finger protein by stage 16 of embryo. Three odd skipped-expressing (odd skipped-a zinc finger transcription factor) clusters from these embryonic segments come together to form the primordial lymph gland having two primary lobes composed of approximately 20 primitive lymphocytes (Mandal et al 2004). By the end of second in star stage, the cells in primary lobe would proliferate to form about 200 cells to 2000 cells per primary lobe in a mature third in star lymph gland. This larval hematopoietic organ gets ruptures

during the first 12 hours of occupations and the lymphocytes are released into circulation. (Grigorian et al 2011)

Lymph Gland is multimedia structure, the primary lobes of the lymph gland which is basically a ball of cells are very well characterized. into three major regions-

1. the outer cortical zone (CZ),
2. the inner medullary zone (MZ) and
3. the Posterior signaling centre (PSC) (Figure.3).

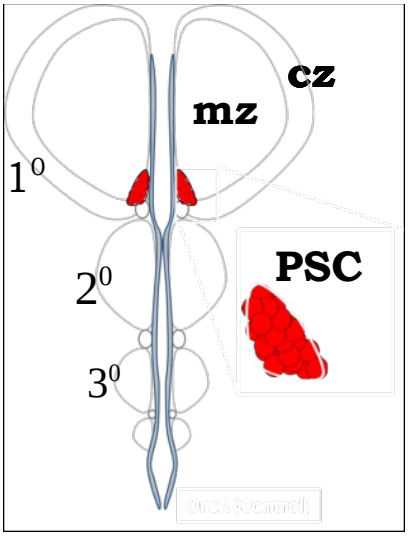


Figure 3: Lymph Gland

The outer cortical zone is primarily composed of the differentiated cells which includes cytoplasm, crystal cell(rarely and lymphocyte (only in case of immune response). The inner dullard zone is homed by the stem like progenitor cells which mainsail their emptiness by self-renewing themselves using the hedgehog and PVF signaling (Chon et al 2002)and also differentiating into blood cell and the posterior signaling centre which is composed of around 50 cells which functions as a enviornment for the maintenance of progenitor cells also called as niche of the lymph gland.(Evans et al 2007)

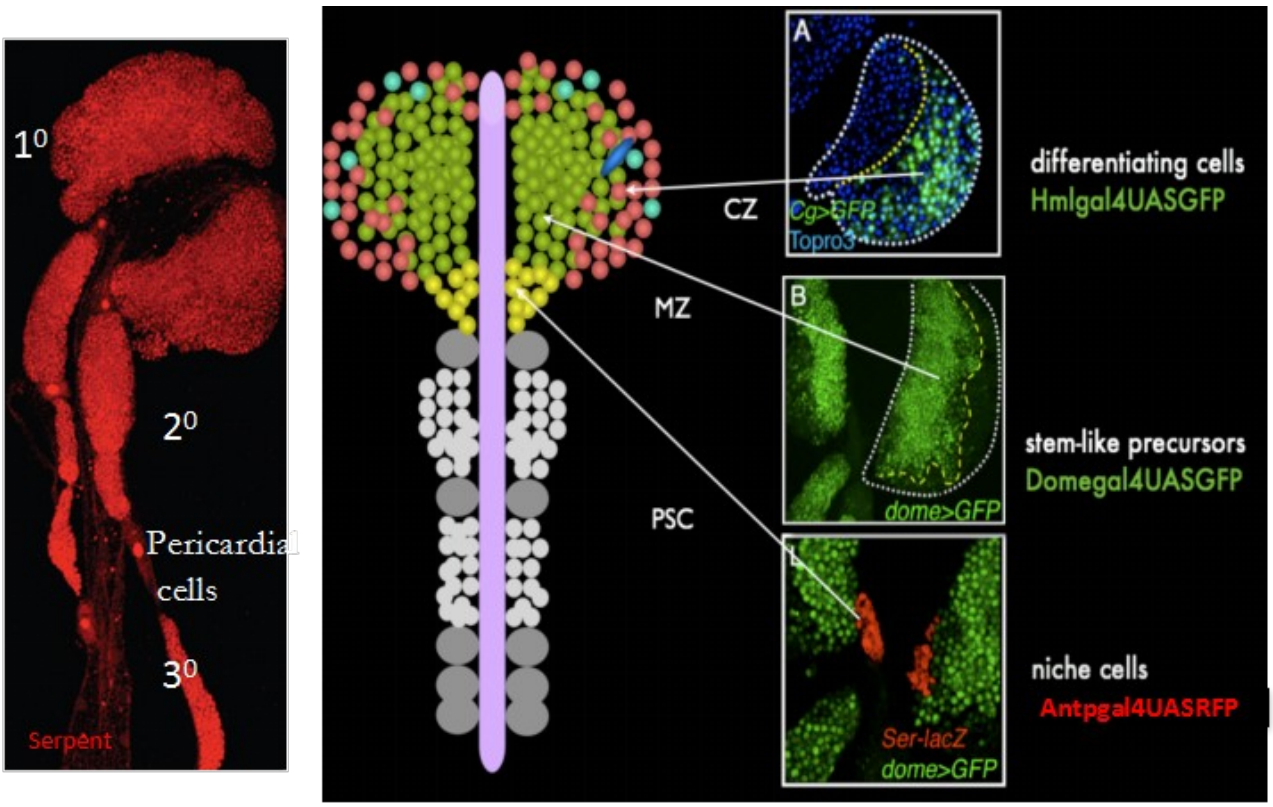


Figure 4: Lymph Gland (courtesy: Dr. Lolitika Mandal)

1.9 Genetic tools used in this study:

I.9.1 Gal4-UAS system

Gal4 –UAS system is one of the most elegant genetic tool that has been commonly used in fly system, giving us control over the spatial and temporal expression of our gene of interest, hence helping us to understand the various role of it in developmental context.(Duffy et al 2012) Gal4-UAS system is a bipartite system which basically relies on two components;

- Gal4, a transcriptional activator from yeast which is expressed in tissue specific manner; and
- A transgene under the control of upstream activation sequence(UAS) that is activated upon binding of GAL4.

A
B

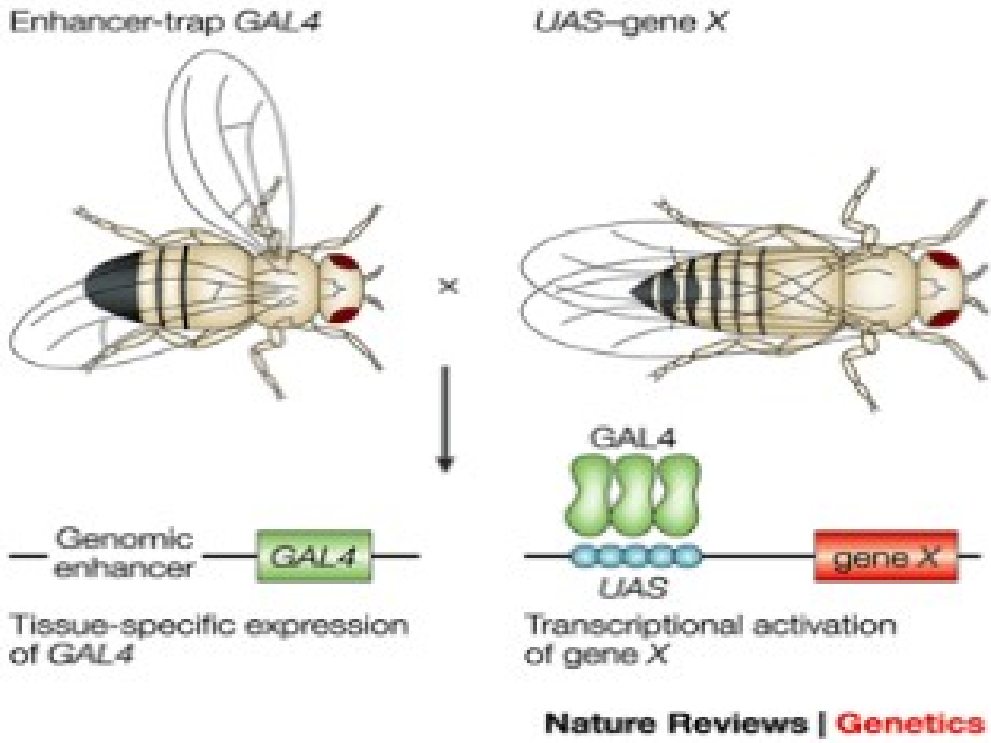


Figure 5: Gal4-UAS system (Johnson et al 2002)

Two components bought together in a simple genetic cross, two different transgenic parental lines are used- an activator and an effector line. In the activator line, yeast transcription factor Gal4 is inserted downstream to a tissue specific promoter. The effector line has our gene of interest fused next to the UAS sequence which is the binding site for the Gal4 protein. These two lines are crossed .In the F1 progeny, Gal4 protein is expressed in tissue specific manner. This Gal4 then binds the UAS sequence and express our gene of interest. Hence, our gene of interest also follows the similar tissue specific expression pattern . (Johnston et al 2002)

In order to achieve temporal control of the expression pattern of our gene of interest in real development time, we use tubgal80^{ts,} which provide a extra layer of temporal regulation over gal4 system. Gal80 has its maximum activity at 29^{0}c and lowest activity at 18^{0}c.

1.9.2 RNA interference (RNAi)

RNA interference is very useful genetic technique which was discovered by Fire and Mello in *Caenorhabditis* elegant. The double stranded RNA (ds RNA) is a source of sequence specific post transcriptional inhibition of gene expression. As a concept of central dogma, all gene has been translated into their respective proteins which execute their respective functions. This translation has a very sensitive step where mRNA from nucleus comes to cytoplasm which eventually goes to ribosomes for further translation process. Now, in this technique, we interfere with this process using small interfering RNA or siRNAs or short mRNAs which with the help of enzyme DICER and DROSHA form a complex RISC, which eventually led to degradation of the mRNAs. (Mello et al 1998)

In the fruit flies, the RNA interference have been broadly used a to generally knock down a gene of interest in a tissue specific manner along with the Gal4-UAS system. This system can be used to find out tissue specific role of different genes.

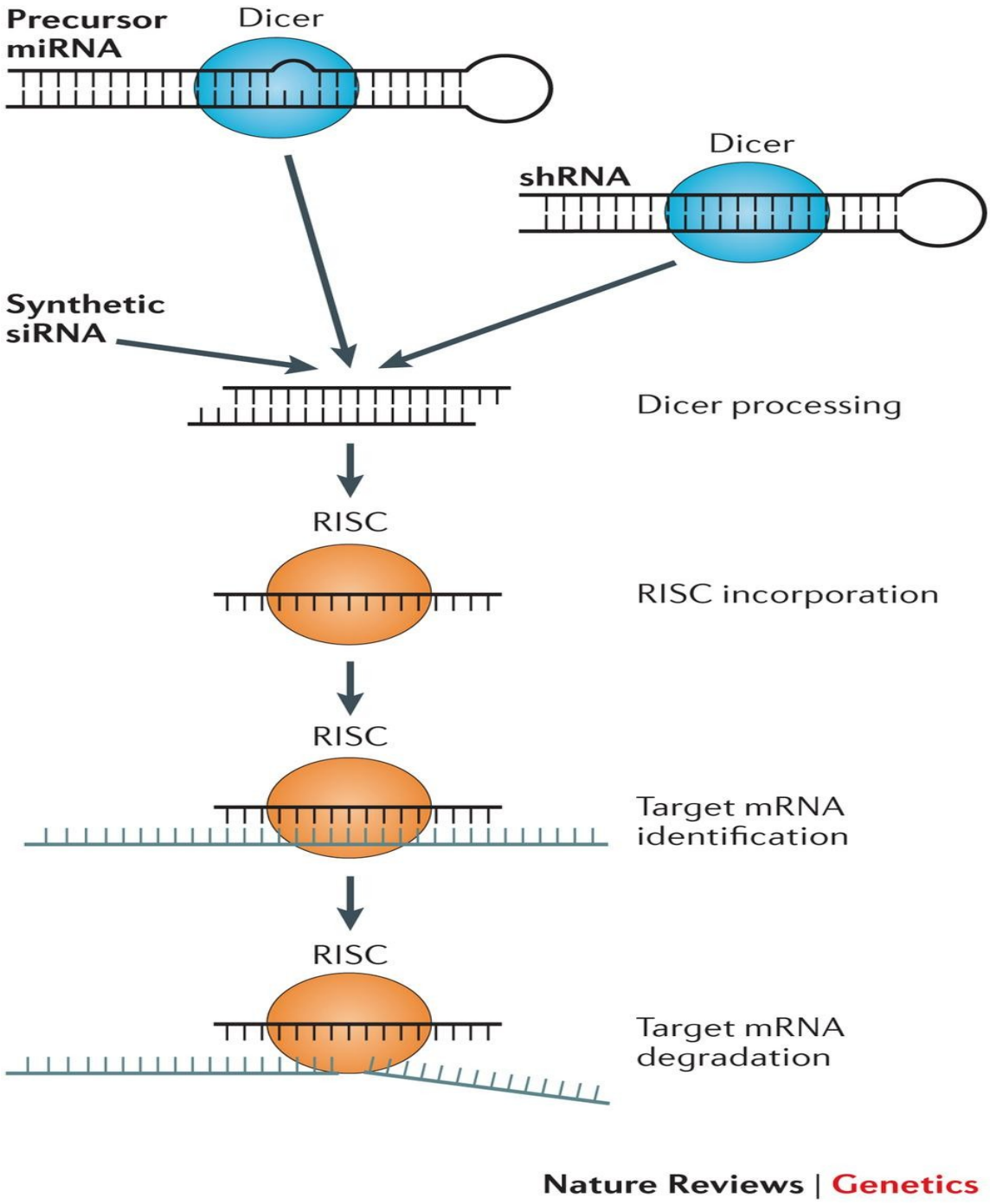


Figure 6: RNA interference pathway

(Anders Wittrup & Judy Lieberman 2015)

1.10 Leukocyte Antigen Related like Protein:

Leukocyte Antigen Related like Protein (lar) is transmembrane Receptor Protein Tyrosine Phosphatase. IT is a multidomain structure which contains three different kinds of domains:

1. Immunoglobin or Ig domain(total of 2 subdomains)
2. Fibronectin III Domains(total of 9 subdomains)
3. PTP phosphatse domains (total of 2 subdomain)

Out of these, first two domains are extracelluar and the PTP domains are cytoplasmic, divided by the transmembrane(TM).*Drosophila* LAR or Dlar is a protein coding gene from *Drosophila melanogaster*. It has been experimentally established that Dlar has its role in the various molecular process such as: protein tyrosine phosphatase activity; cellular component organization or biogenesis;; biological regulation and growth(Kruger et al 1989). It has been extensively worked out using the *Drosophila* brain as model organ and known to be involved in motor neuron axon guidance, neuronal development.(Kruger et al 1996)

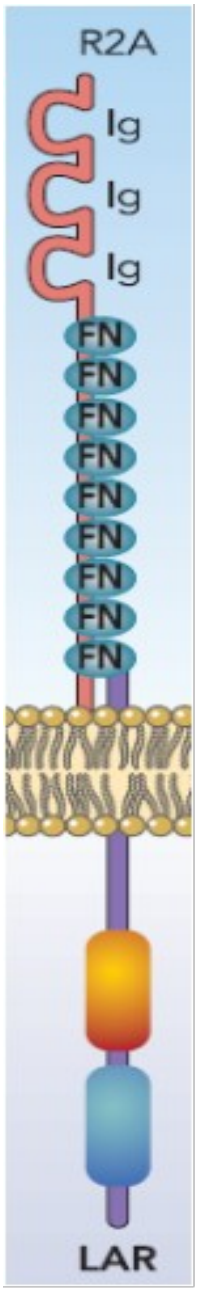


Figure 7: ***Leukocyte Antigen Related like Protein***

Soulsbey and Bennett., 2009

1.11 Objective:

The stem cell niche provides a micromanagement for the progenitor cell to maintain their emptiness and regulate the differentiation of these cells, hence maintaining the balance between these two cell types. This balance is necessary for the maintenance of the tissue morphology in an organism. In *Drosophila* Dlar has been proved to have an important role in hematopoiesis. Now it is very important and and crucial to establish a classical loss of function mutant of LAR .This mutant will not only strengthen the validity of pre-existing results but also will help in rescuing the phenotype which will add up another layer of endorsement in these results.

In this study, I tried to find out and subsequently validate a classical loss of mutant for LAR in hematopoiesis, using various combinations of available mutant lines.

CHAPTER 2

MATERIALS AND METHODS

2.1 Fly stocks

Flies were cultured and maintained at 25^0c for normal growth except for some experiments.Normal food consists of agar, sugar and corn flour yeast medium.For cross purpose, we have used 20 virgin females and 15 males. Some stocks were purchased from the Bloomington *Drosophila* Stock Centre .These lines were gifted from the following lab.

- Dlar 5.5/cyo, Dlar 13.1/cyo,Dlar OD 16/cyo , Dlar bypass/ cyo , Dlar 5.2/cyo from David Van Vector lab.

- w;Dlar 451/cyo; FRT40A/CyO, and w;Dlar 2127, FRT40A/CyO, from Margaret Fuller lab.

2.1.1Mutant lines:

(all line are in the background of cyo actin gfp e.g.. Dlar 13.2 is Dlar 13.2/cyo actin gfp ;for writing purpose they written as only by their numbers.)

No.	Dlar mutants	Function
1.	Dlar13.1	Behavior defective, late third instar larval lethal
2.	Dlar13.2 · Embryonic lethal · Point mutation	Neuroanatomy defective(with 5.5) ; neurophysiological defective((*Kaufmann et al., 2002*, *Desai et al., 1997*)); behavior defective Homozygotes show an ISNb bypass phenotype at moderate frequency (*Bateman et al., 2000*)
3.	Dlar5.1	Behavior defective, late third instar larval lethal
4.	Dlar5.2	Behavior defective, late third instar larval lethal
5.	Dlar5.5	neuroanatomy defective{(*Kaufmann et al., 2002*);with Lar13.2(*Fox and Zinn, 2005*); embryonic stage(*Weng et al.,*

	· Embryonic lethal	*2011*);| larval stage (with Lar13.2)(*Johnson et al., 2006*);neurophysiology defective
	· Point mutation	(*Desai et al., 1997*); Behavior defective Homozygotes show an ISNb bypass phenotype at moderate frequency (*Bateman et al., 2000)*
6.	Dlar451	optomotor response defective | somatic clone (*Clandinin et al., 2001*)
7.	Dlar2127	neuroanatomy defective(*Choe et al., 2006*, *Nern et al., 2008*); optomotor response defective | somatic clone (*Clandinin et al., 2001*)
	· Embryonic lethal	
	· Point mutation	
8.	DlarE55	neurophysiology defective; ISNb axons often show a "bypass" phenotype
	· Embryonic lethal	(*Desai et al., 1997*)
	· Chromosomal deficiency	
9.	DlarTW84	**NA**
10.	DlarOD16	neurophysiology defective; ISNb axons often show a "bypass" phenotype
	· 1	(*Desai et al., 1997*)
	· Chromosomal deficiency	

11.	DlarBYPASSS	neuroanatomy defective
	· Embryonic lethal · Deletion	(*Kaufmann et al., 2002*); "bypass" phenotype (failing to enter the normal muscle target domain just outside the ventral nerve cord and instead following the intersegmental nerve towards dorsal targets) in homozygous embryos.

Table 2: Mutant of Dlar and their function

2.2 Immunocytochemistry assay

Fresh late third instar larvae has been taken for dissection. Dissection has done in freshly prepared PBS which provides a osmotic environment to the cell to avoid the degradation. After dissection ,tissues are fixed in 6% freshly prepared PFA for 55 minutes.Tissues were then washed with 0.3% PBT for 4 times with 15 min. interval between them.After washing, tissues were put in block (10% NGS) for 1 hours, this was followed by incubation of the tissues with primary antibody for 18- 20 hours at 4^0c. Post primary incubating tissues were washed with PBT for 3-4 times and reblocked (10% NGS) before incubating the tissues with secondary antibody for 18 hours at 4^0c. Post secondary incubation, tissues were washed again before DAPI staining for 1 hour at room temperature.Finally tissues were mounted in vecta-shield medium.

2.3 Western Blotting

Western Blotting is molecular method to separate two protein based on their sizes using gel electrophoresis and then using antibody staining we separate out our protein of interest in the mixture of proteins.It consist of two process:

1. gel electrophoresis to separate out protein based on its size

2. immunotagging or blotting using specific antibody to separate out protein of interest.

Here are protocol which I had follow for the western Blotting:

1. Sample preparation:

Take 10 larvae from the fresh culture,crush it in1% PBT,100 µl.then centrifuge it for 15 min at 4^0C at 12000rpm.take the supernatent an mix it in laemmli buffer at 1:1 ratio.boils the mixture for 5 min.at 95^0C.settle the temperature to come down.

Laemmli buffer contains beta mercaptoethanol to break disuphide bonds,glycerol to increase viscosity,SDS to denature the protein Tris HCl for charging and bromophenol blue as dye.

2. Quantitation of Protein sample:

Using Bradford assay(5 times dilution in stock), to quantitate the optical density of each sample n order to know ho much sample we need to load in the gel electrophoresis.

3. Gel electrophoresis:

    1. Running buffer:

to prepare 1X running buffer we need glycine (14.4gm), Tris base (3gm.).SDS (1gm.),Water (1L) and pH to be maintained at 8.3.

    2. Ammonium PerSulphate(APS), which along side with timid polymerise the gel, should be 10 % in 500µl.

    3. Resolving Gel for 12 %

Acryl/bisacryl or sol A =3.6ml

1.5M Tris or Sol B =2.25 ml

water =3.05ml

APS =100µl

TEMED =8µl

    4. Stacking Gel 4.5%

Acryl/bisacryl or sol A =450µl

1.5M Tris or Sol C =750µl

water =1.08ml

APS =20µl

TEMED =10µl

5. After assembling the setup, first prepare and pour resolving gel leaving 2 inches space. After polymerization, pour the freshly prepared stacking gel along side with comb.Then remove the comb after polymerization, clean the well with water and load the protein samples. Run the gel at 80 V for 5 hours.

6. After protein has been resolved in the remove the gel carefully and prepare for the blotting..we using PVDF membrane according to dimension of gel using filter paper we blot the gel into membrane.this whole process occurs in thetransfer buffer as it is wet blotting.

2.4 Antibody used;

Different primary antibodies used in this study are listed below

Antibody	**Developed in**	**Details**	**Source**
Anti-antennapedia	Mouse	Working dilutuion 1:10	DHSB
Anti –L1	Mouse	Working dilution 1:500	Gifted by Dr. Istvan Ando
Anti-P1	Mouse	Working dilution 1:40	Gifted by Dr. Istvan Ando

Anti-βPS	mouse	Working dilution 1:5	DHSB

Table 3: List of primary antibodies.

Secondary antibodies for immunostaining

Different secondary antibodies used in this study are listed below.

Secondary antibody	Details
Cy™3-Conjugated affinipure goat anti mouseIgG(H+L)	● conjugated with cyanine Cy™3 dye (absorption maxima/ emission maxima is 550 nm/ 570 nm) ● working dilution: 1:700 ● Detect primary antibodies raised in rabbit.

Table 4: List of secondary antibodies used.

2.5 Stains used

DAPI (4',6-diamidino-2-phenylindole dihydrochloride,) is a blue fluorescent dye that binds to A-T rich region in dsDNA.(Kapuscinski et a l 1995) It is mostly used to stain nuclei in live as well as fixed tissues. The working dilution is 1µg/ml.Tissue after the secondary antibody incubation were washed with 0.3% PBT for four time and then put in DAPI for nucleus staining for 1 hours at room temperature

2.6 Imaging and Image Processing

Images of the mounted samples were taken by using confocal microscope(Zeiss LSM780).The images were further processes by using Image J(NIH software)

Magnification: (40xoptical magnification)x(0.9digital zoom).

zoomed image (63x optical magnification)x(1.3digital zoom)

CHAPTER 3

RESULTS AND DISCUSSION

In our laboratory, a genome wide RNAi based screen was launched to unravel the genes that are involved in the regulation of niche cell maintenance. Using Gal4 UAS system, individual genes were knock down specifically from the niche specific population employing a niche specific driver and the resultant niche cell number was counted based on the GFP expression .

3.1 The Phenotype of LAR knockdown:

When I joined the lab, *Drosophila* Leukocyte Antigen related like or Dlar was already recognized as potential gene candidate for the regulation and maintenance of the niche. Harleen Kaur, A Ph.D. student from this lab has established the phenotype of this gene.

Since the total loss of function of Dlar was embryonic lethal, the challenge was to find out the heteroallelic combination of classical loss of function knock out which can phenocopy the RNAi mediated knockdown result.

3.2 Mutant hunting and line making

Drosophila LAR is trans membrane RPTP(Receptor Protein Tyrosine Phosphatases) located at 38A on the left arm of the second chromosomes (Kruger et al 1996).There are various loss-of function line has been generated in the past two decades ,out which these 11 mutation line which we had used in our experiments. these loss of function lines contains various origins of mutations: some are point mutations, some are chromosomal deficiency and some are hypomorphic mutation .these mutation ,their type and their function have been listed as below:

Since we are entering into the details of various mutation line it is very important to know where exactly in the Dlar protein, these mutations are residing .Dlar is a transmembranal protein with 3 major domains(Ig domain,Fibronectin-III domain and PTPase domain) (Kruger et al 2003).These depiction of the Dlar protein will explains the position of their point mutation site in the protein and in the chromosomes. Generally , most of the point mutation namely Dlar 5.5, Dlar 13.2 and Dlar 2127 are in FN-III domain while deletion of a 4 bp which is called as

Dlar bypass has been originated in between two PTPase domain. All the deficiency which basically means a part of chromosomes has been deleted which also include the part which is responsible or the occurance our gene of interest.

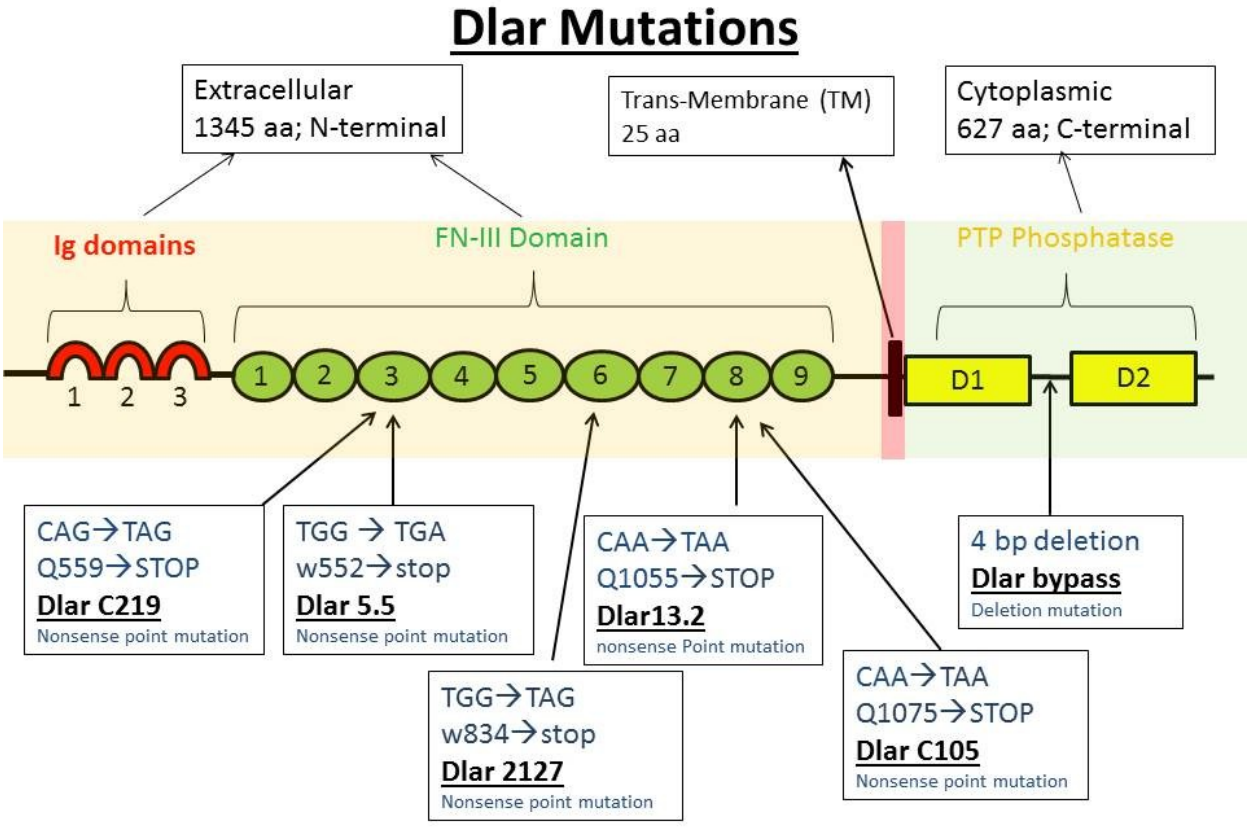


Figure 8. Mutations of Dlar gene(modified from Kruger et al 1996)

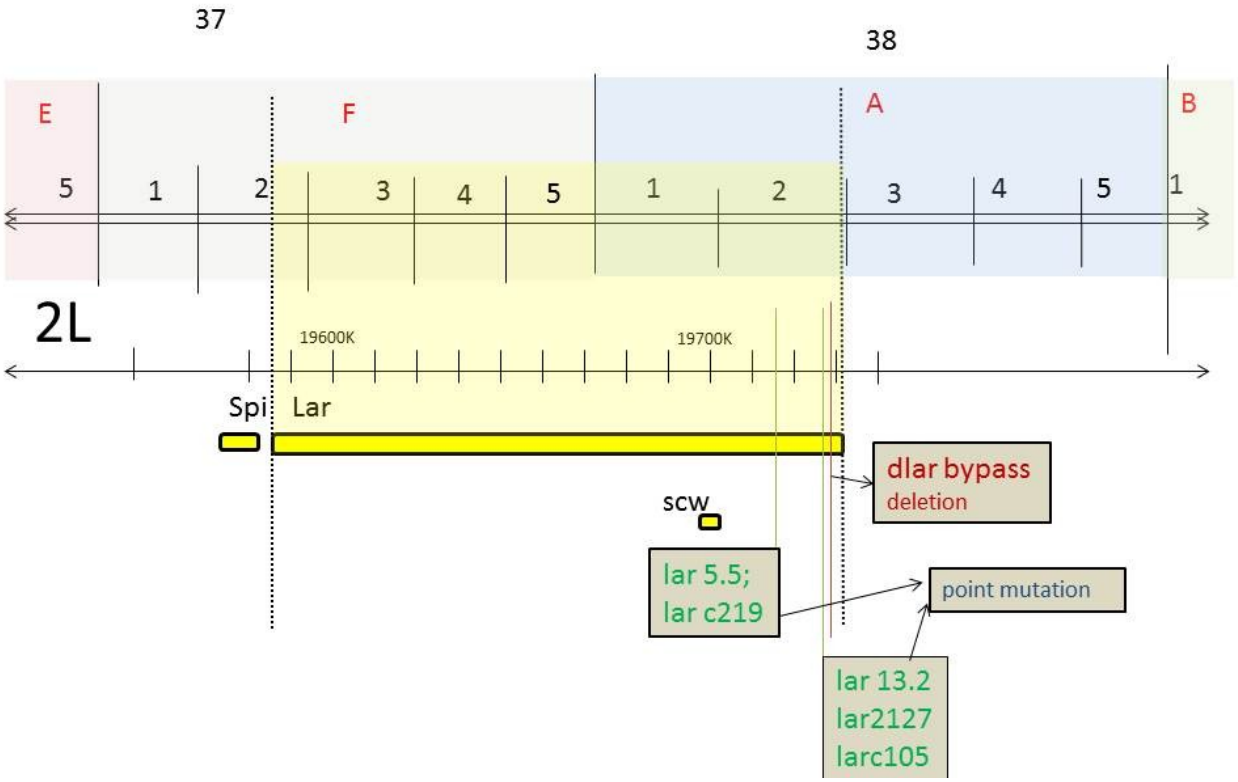


Figure 9. Dlar locus in *Drosophila* chromosomes (modified from Kruger et al 1996)

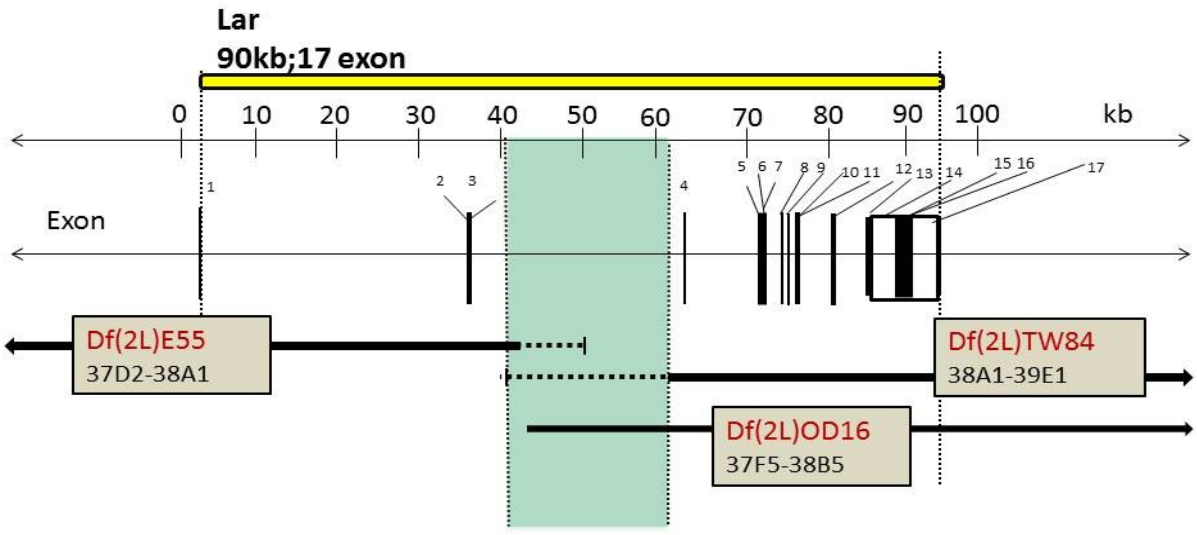
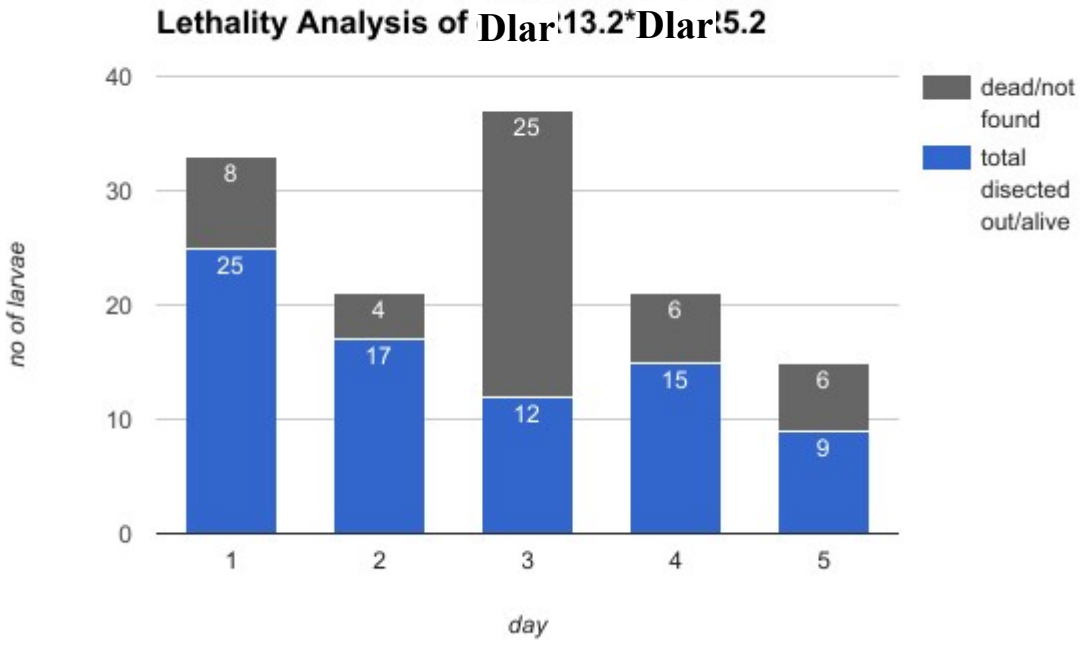


Figure 10.Chromosomal deficiencies in the Dlar **gene (modified from Kruger et al 1996)**

3.3 Hetroallelic Combination of mutants

All of these mutant lines are in the background of a second chromosome balancer known as curly O or cyo (whose phenotype is the curly wings). In order to facilitate selection of both homoallelic and heteroallelic combination, I bought GFP tagged actin with cyo balancers in the back ground of these mutants.

There is a serious problem with survival of these mutations which is mostly the homoallelic combination of these mutation has been reported to lethal at embryonic stages and some combination do have escapers but mostly they do not survive till adult stages.(Kruger et al 1996 and 2003) Since we are investigating the definitive hematopoiesis which occurs during larval stage, we have to find a mutant which will survive not only till larval stage but also gives us the phenotype we required.



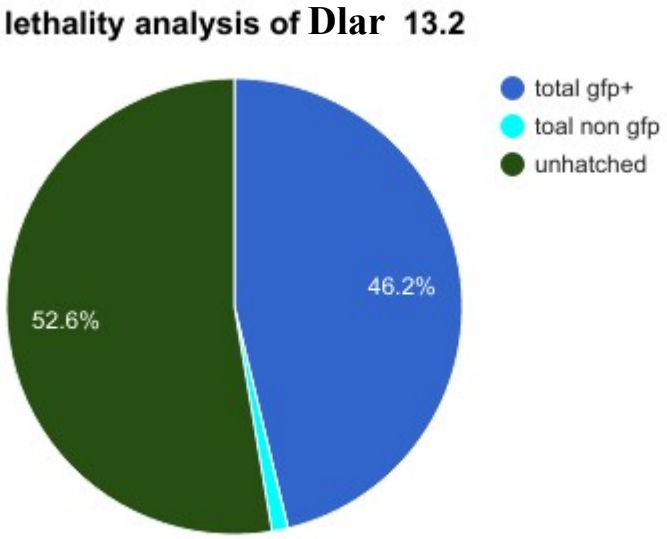


Figure 11&12. Mutant Analysis of homoallelic combination

3.4 Mutant analysis

Homoallelic combinations as it has been reported above, are mostly embryonic lethal. To validate this results we have done the mutant analysis of the these homoallelic combinations. From the healthy culture of the mutant line (with background of cyo actin gfp),we have pre-selected the first instar larvae for non-gfp as a signature for homoallelic combination.

We have observed the development of these selected larve till 3rd instar larvae to find out exactly at what point during the development, these homoallelic combination suffers lethality. As per observation, each mutant have different time-point of lethality e.g.Dlar 13.2 and Dlar TW84 suffers embryonic lethality while Dlar 5.2 suffers late 2nd star lethality.(table 2)

We have try to look for the our mutant of interest in the heteroallelic combination. Since these combination has not been reported anywhere that's why we have to create these combination one of which will be a potential desired mutant.

There are 11 Dlar mutant line are available on Bloomington stock center out which we posses 8 lines. Total no. of combination of Dlar mutations upon pairing is 36. These were then dissected, immunostained and processed for documentation. Till now we have finish 21 of these combination and most of these combination has given us the negative results.

Table 5. Mutants analysis of different combination and their results.

Dlar 13.2	Dlar 13.2	lethal		Dlar 2127	No phenotype
	Dlar 5.2	No phenotype		Dlar E55	
	Dlar 5.5			Dlar TW84	
	Dlar 13.1	No phenotype	Dlar 13.1	Dlar 13.1	No phenotype
	Dlar 451	No phenotype		Dlar 451	
	Dlar 2127	phenotype		Dlar 2127	No phenotype
	Dlar E55	No phenotype		Dlar E55	No phenotype
	Dlar TW84	No phenotype		Dlar TW84	No phenotype
Dlar 5.2	Dlar 5.2	lethal	Dlar 451	Dlar 451	No phenotype
	Dlar 5.5			Dlar 2127	
	Dlar 13.1			Dlar E55	
	Dlar 451			Dlar TW84	No phenotype
	Dlar 2127	No phenotype	Dlar 3127	Dlar 2127	No phenotype
	Dlar E55			Dlar E55	No phenotype
	Dlar TW84			Dlar TW84	No phenotype
Dlar 5.5	Dlar 5.5	No phenotype	Dlar E55	Dlar E55	No phenotype
	Dlar 13.1			Dlar TW84	No phenotype
	Dlar 451		Dlar TW84	Dlar TW84	lethal

3.5 Dlar mutant

The one heteroallelic combination that served as classical loss of mutant for Dlar in hematopoiesis was a hetroallelic combination of 2 mutant line Dlar 2127 and Dlar 13.2. Both of these mutation are point nonsense mutation which occurred on the sixth and eighth sub domain of fibronectin domain of LAR protein.

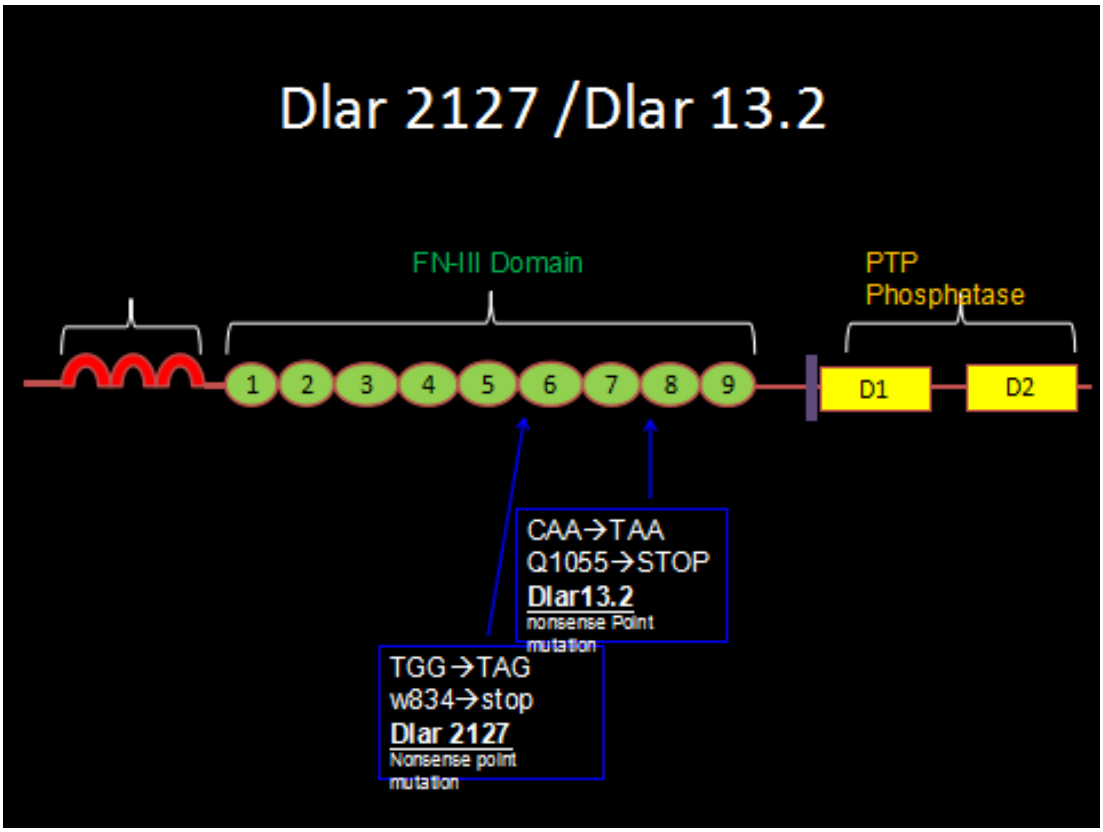
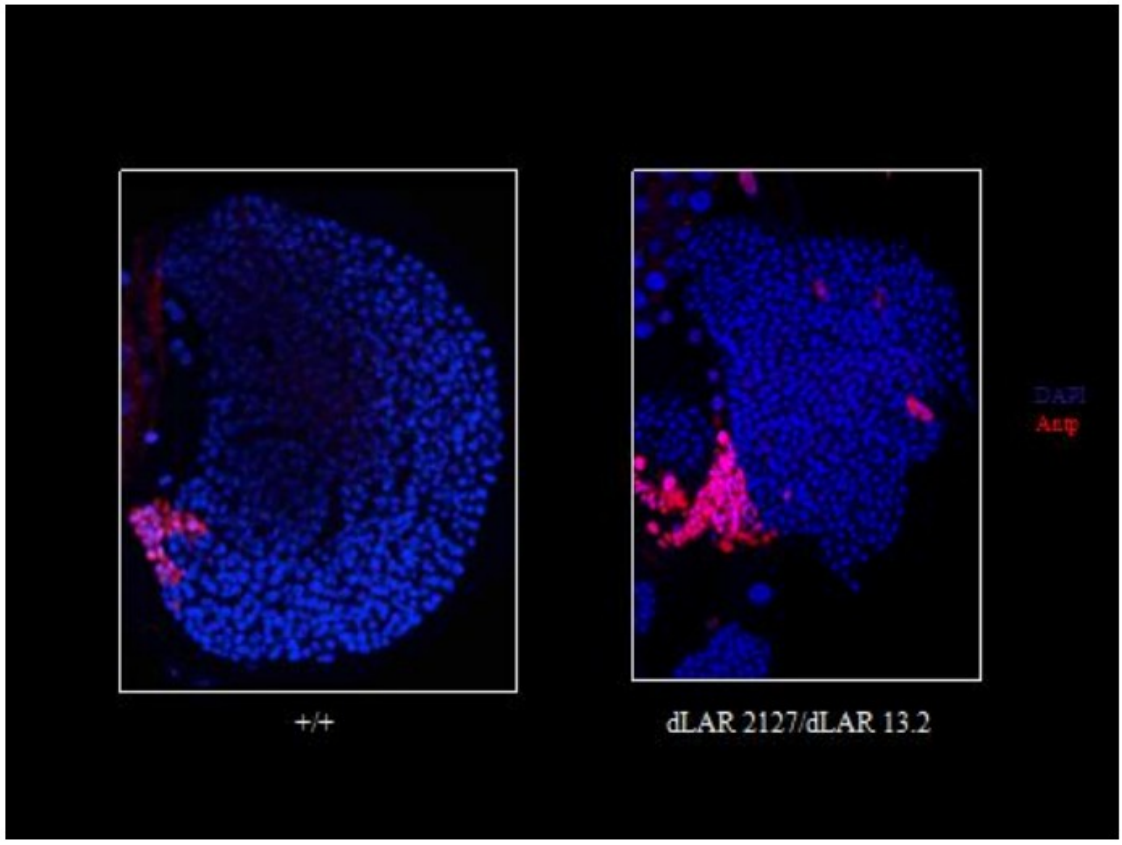


Figure 13. Dlar mutations: Dlar 2127 and Dlar13.2

3.6 Immunostaining

To validate the results, we have dissected out the late 3rd instar larvae of the healthy culture of the combination and immunostained with the Antp antibody, which has reported to mark niche cells and our results have shown an increment in the niche cells.(figure 14) Although the increment is not as robust as it was in lar knock down but was enough (approximate 3-fold or 150 cells compare to 50 cells of wild type) to strengthen its candidacy for potential mutant. Furthermore, the population of differentiated cell in cortical zone has been checked using the P1 antibody which primarily mark the plasmatocytes and the increment can be observed (Figure 15) This clearly indicates an increase in CZ cells and a concomitant decrease in MZ cells.

Another interesting phenotype we got was presence of immune response which was evident by the presence of lamellocytes. Presence of lamellocytes can be seen using beta-PS antibody. (figure 16)



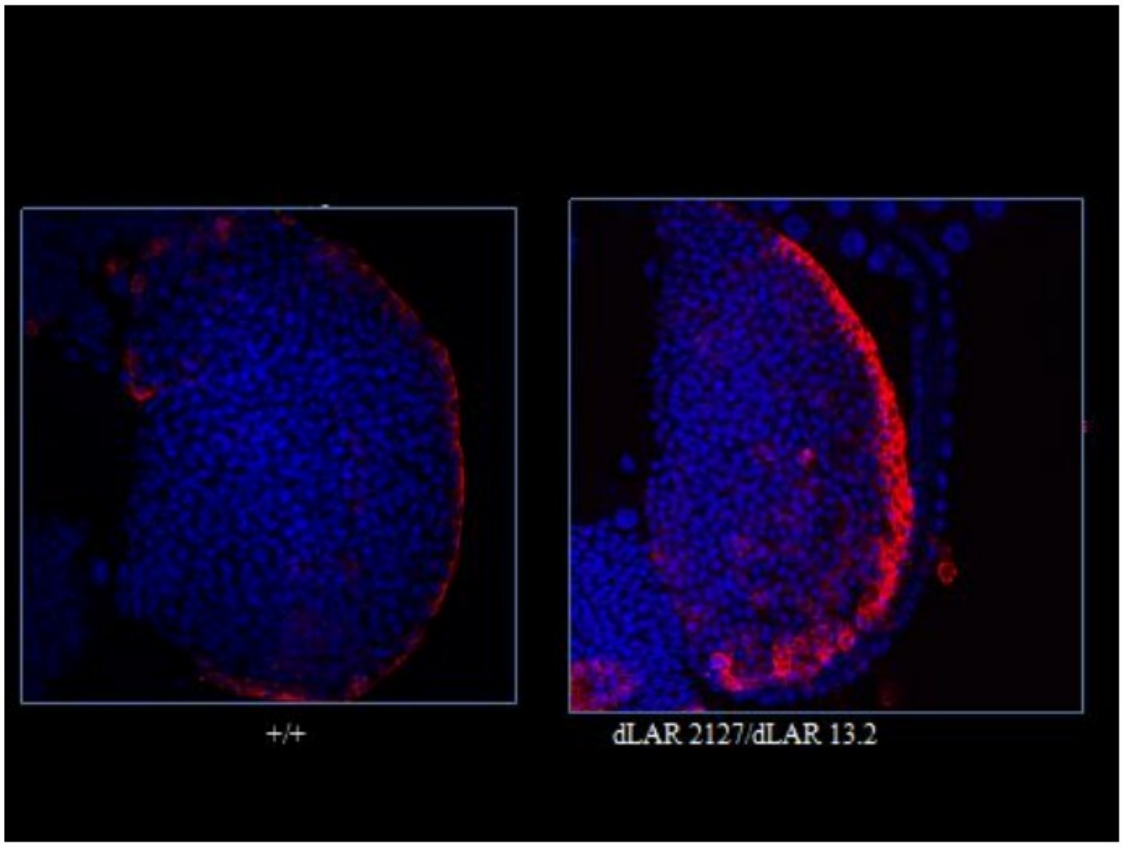


Figure 14&15.Antp and P1 staining of the larval lymph gland

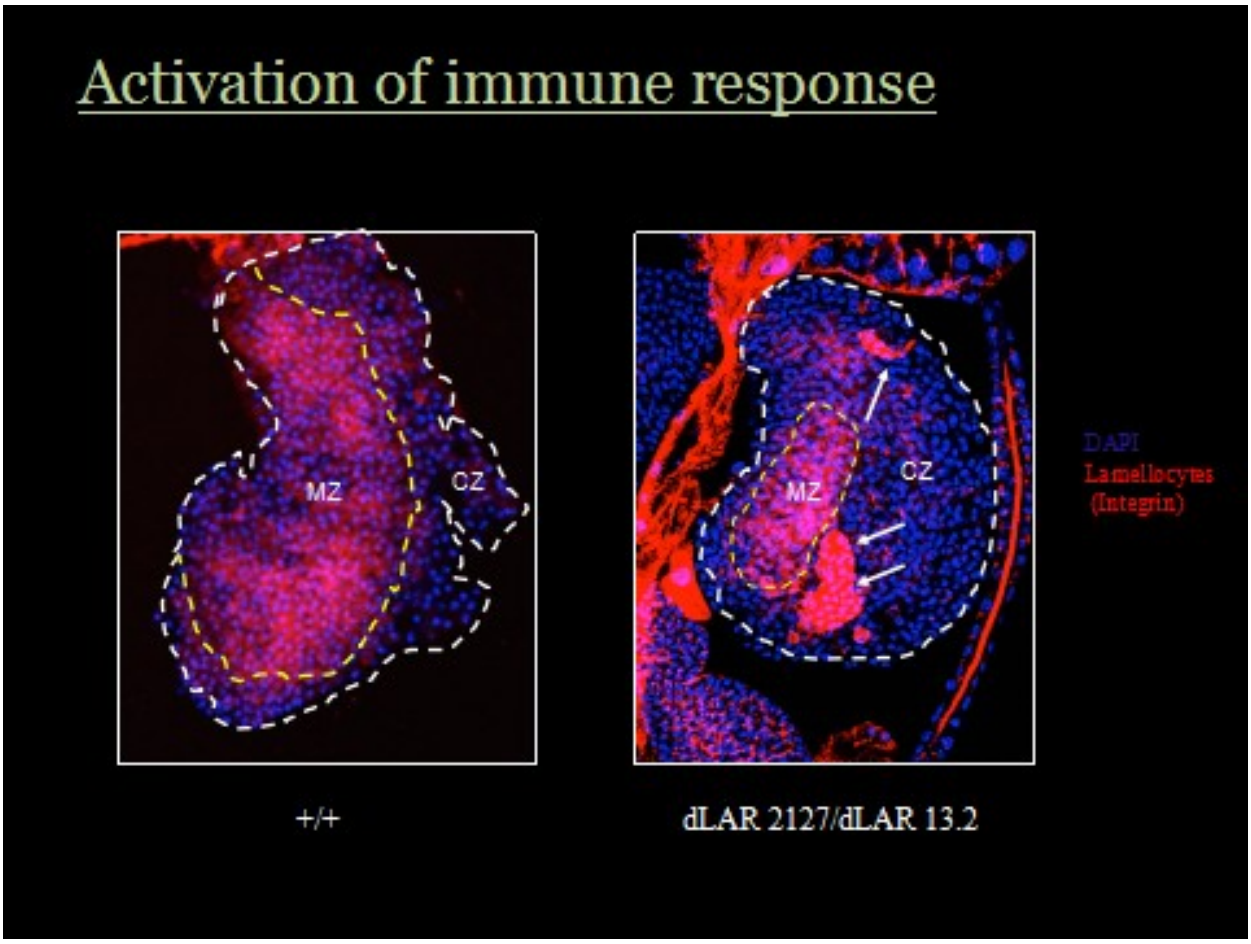


Figure 16. β-PS staining of the larval lymph gland

3.7 Rescue

The rescue for this classical loss of function mutant is planned next and genetic lines are to be prepared for the same.

3.8 Western blotting

To validate our classical loss of function mutant we aspired to do western blot and check whether there is actual loss of Dlar protein in our mutants as compared to wild type. Unfortunately the DSHB antibody is reported not to work in western blotting for Dlar (Weng_et_al-1999). Dlar is known to undergo translational modification and loose its extra cellular domain and the antibody is against extra cellular domain.

3.9 Discussion

Drosophila hematopoiesis provides an excellent platform to study and investigate how the niche cell maintain and regulate themselves in order to control the self renewal and differentiation of progenitor cell. A lot of molecular and cellular activities are responsible for it.

The niche or micro-environment are the group of cell which primarily maintain the progenitor cell via Hedgehog signaling and backward signaling (PVF signaling) (Mandal et al 2007). However it remains a mystery that how this niche maintain and regulate itself.

Leukocyte Antigen related like or Dlar is Receptor Protein Tyrosine Phosphatases which is known to have many functions but best known for its roles in embryonic motor axon guidance and its target recognition by axon of R7 photoreceptor. (Cameron et al 2013) It has been extensively been worked out in neurobiological field but its role in hematopoiesis is still a challenging mystery. To unravel this mystery Harleen, a senior from my lab has established that Dlar has a very important role in the *Drosophila* hematopoiesis. Since Dlar has been shown to be a very important gene of interest it is crucial to validate its results and also to find out what function the different domains of Dlar gene holds? This could help us to understand better the mechanism by which Dlar gene operates in the niche.

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CHAPTER 4

CONCLUSION AND FUTURE PERSPECTIVE

4.1 Conclusion

Through this study, we have established combination of Dlar 2127 and Dlar13.2 as the desired classical loss of function knockout mutant for the Dlar gene.

4.2 Future perspective

Using this mutant, we can further validate the results by rescuing the LAR knockdown phenotype in background of overexpression of LAR. Once we establish this, we will tend to analyse the functions of different domains of LAR protein individually. We believe that each domain has a unique function which can play a crucial role *Drosophila* hematopoiesis.

Bibliography

1. Mark D.Adams et al (2000) The Genome Sequence of Drosophila melanogaster. Science, 287(5461): 2185-2195.
2. Gerald M. Rubin et al (2000) Comparative Genomics of the Eukaryotes. Science, 287(5461): 2204-2215
3. L. Mandal, U. Banerjee, V. Hartenstein (2004)Evidence for a fruit fly hemangioblast and similarities between lymph-gland hematopoiesis in fruit fly and mammal aorta-gonadal-mesonephros mesoderm.Nat Genet, 36 (2004): 1019–1023
4. K.P. Rehorn, H. Thelen, A.M. Michelson, R. Reuter(1996)A molecular aspect of hematopoiesis and endoderm development common to vertebrates and Drosophila Development, 122: 4023–4031
5. Jung SH, Evans CJ, Uemura C, Banerjee U. (2005)The Drosophila lymph gland as a developmental model of hematopoiesis. Development.;32:2521–2533.
6. Krzemien J, Dubois L, Makki R, Meister M, Vincent A, Crozatier M. (2007)Control of blood cell homeostasis in Drosophila larvae by the posterior signalling centre. Nature; 446:325–328.
7. Greenspan, Ralph J. "Fly pushing: the theory and practice of Drosophila genetics." (2004).
8. Lawrence, Peter Anthony. The making of a fly: the genetics of animal design. Blackwell Scientific Publications Ltd, 1992.
9. Mandal L, Martinez-Agosto JA, Evans CJ, Hartenstein V, Banerjee U. (2007)A Hedgehog- and Antennapedia-dependent niche maintains Drosophila haematopoietic precursors. Nature;446: 320–324.
10. Krueger, Neil X., et al. "The transmembrane tyrosine phosphatase Dlar controls motor axon guidance in Drosophila ." Cell 84.4 (1996): 611-622.
11. Krueger, Neil X., M. Streuli, and H. Saito. "Structural diversity and evolution of human receptor-like protein tyrosine phosphatases." The EMBO Journal 9.10 (1990): 3241.
12. Desai, Chand J., et al. "Competition and cooperation among receptor tyrosine phosphatases control motoneuron growth cone guidance in Drosophila ." Development 124.10 (1997): 1941-1952.

13. St Johnston, Daniel. "The art and design of genetic screens: Drosophila melanogaster." Nature reviews genetics 3.3 (2002): 176-188.
14. http://flybase.org/reports/FBgn0000464.html
15. http://www.sdbonline.org/sites/fly/newgene/Dlar.htm
16. Duffy, Joseph B. "GAL4 system in Drosophila : a fly geneticist's Swiss army knife." genesis 34.1-2 (2002): 1-15.
17. St Johnston, Daniel. "The art and design of genetic screens: Drosophila melanogaster." Nature reviews genetics 3.3 (2002): 176-188.
18. Tools for studying and using small RNAs: from pathways to functions to therapies' and Animation on RNA interference by nature genetics poster
19. Kapuscinski J (September 1995). "DAPI: a DNA-specific fluorescent probe". Biotech Histochem. 70 (5): 220–33. doi:10.3109/10520299509108199. PMID 8580206.
20. Cameron, Scott, et al. "Visual circuit assembly requires fine tuning of the novel Ig transmembrane protein Borderless." Journal of Neuroscience 33.44 (2013): 17413-17421.
21. Galloway, Jenna L., and Leonard I. Zon. "3 ontogeny of hematopoiesis: examining the emergence of hematopoietic cells in the vertebrate embryo." Current topics in developmental biology 53 (2003): 139-158.
22. Cho, N.K., Keyes, L., Johnson, E., Heller, J., Ryner, L., Karim, F., Krasnow, M.A. (2002). Developmental control of blood cell migration by the Drosophila VEGF pathway. Cell 108(6): 865--876.
23. *Fire A., Xu S.Q., Montgomery M.K., Kostas S.A., Driver S.E., Mello C.C. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 1998; 391:806-811.*