Exploring the novel role of IMD pathway in *Drosophila* hematopoiesis

Parvathy Ramesh

A dissertation submitted for the partial fulfillment of B.S.-M.S. dual degree in science

INDIAN INSTITUTE OF SCIENCE EDUCATION AND RESEARCH
MOHALI

Department of Biological Sciences

April 2015

Dedicated to my mother

ACKNOWLEDGEMENTS

I am grateful to my family for the support, critical guidance and love they have given me .My parents have provided me with the best education available and so lucky to have them in my life.

I am thankful to my thesis advisor Dr. Lolitika Mandal, who has played a majorrole in molding me into an enthusiastic research oriented student. Morethan a thesis advisor,she was my mentor who helped me in building up scientific temper within me.I am highly grateful to her for introducing me to one of the excellent model organism, which has revolutionized the field of developmental biology, *Drosophila melanogaster*.

I am thankful to Dr. Sudip Mandal for support, advice, encouragement, helpful comments I received from him .I thank all the members of the Developmental Genetics Laboratory and Molecular Cell and Developmental Laboratory, namely Nidhi Dey, Satish Tiwari, Saikat Ghosh, ShivKumar, Harleen Kaur, Poonam Aggarwal, Ashish Toshniwal, Swati Sharma, Sakshi Gupta, Geeta Sharungbum, Jayati Gera, Adhyasha Nayak, Surrender and Aditya for their constant support and help.

I am thankful to Nidhi Dey for the love, support and encouragement that I have received from her. She gave enough confidence and courage to move forward during my bad days when things were not working for me.

I want to thank INSPIRE, Department of Science and Technology, Government of India for supporting me during the entire duration of my course.

Finally, I want to thank Indian Institute of Science Education and Research Mohali for providing me a great platform for learning, understanding and appreciating science.

Parvathy Ramesh

CERTIFICATE OF EXAMINATION

This is to certify that the dissertation titled"Exploring the novel role of IMD pathway in *Drosophila* hematopoiesis" submitted by Parvathy Ramesh,MS10077for the thesis committee has examined the partial fulfillment of B.S.-M.S. Dual Degree Programme of the Institute, duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr.Sudip Mandal Dr.Rachna Chaba Dr.Lolitika Mandal

(Supervisor)

DECLARATION

I have carried out the work presented in the dissertation 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 other university or institution. 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.

Parvathy Ramesh

Dated: 24-04-2015

In my capacity as a supervisor of the candidates project work, I certifythat the above statements by the candidate are true to the best of my knowledge.

Dr.Lolitika Mandal

(Supervisor)

ABSTRACT

Toll and IMD pathway are two humoral immune response pathways present in *Drosophila melanogaster*.Although the developmental role of Toll pathway has been studied since decades, IMD pathway in context of development remained less explored. Here,using the hematopoietic organ, lymph gland of *Drosophila* larvae, we are able to unravel an unknown role of IMD pathway in hematopoietic niche maintenance.We conducted a loss of function analysis of the IMD pathway components and could demonstrate that functionality of niche is highly compromised in these genotypes. As a consequence there is a huge increment of differentiated hemocytes at the cost of progenitors. Thus, our results clearly establish the developmental role of IMD pathway in stem cell niche maintenance which was previously unknown. Since aberrant non-functional niches are characteristic feature of many hematopoietic malignancies, we foresee that this study can also shed light to our understanding of the basic differences in terms of molecular events happening at cellular level in functional and non-functional niches.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

Chapter 1

INTRODUCTION

I *Drosophila melanogaster*-a powerful model system

Drosophila melanogaster generally known as the common fruit fly is an excellent model organism that has been used inscientific research field for over a century.EventhoughWilliam Ernest Castle (1867–1962), a professor at Harvard University was the first person to bring the fly species belonging to the class Drosophilidae from its ancestral home in Africa to work benches, it was the seminal discoveries made by Thomas Hunt Morgan(1866-1945) and his students at Columbia university made the scientific world understand and appreciate the strength of the model system per se. (1)

Drosophila has short life cycle where new generation of adult flies arise in every two weeks. The features like small size, easy rearing in the laboratory conditions, low maintenance cost and fully sequenced small genome makes *Drosophila melanogaster* one of the favorite model systems for researches.With the advent of powerful genetic tools like UAS GAL4 system (spatio-temporal regulation over gene expression), P element mutagenesis (targeted gene mutations) etc. the life of Drosophila researchers has been much more simplified (2)

Drosophila melanogaster, a holometabolous insect, has the life cycle spaced in to definite stages like egg, larvae, pupae and adult(figure 1).Soon after the internal fertilization, eggs, which are around one and half mm in length, are laid by females at rate 50-70 eggs per day. At 25^{o}Celsius the egg hatches after 24 hours and the tiny larvae that emerges is called the first instar.It will feed on the food provided and after 24 hours of egg hatching, the first instar moltsinto a larger forms called the second instar. The second instar continues to feed on the food and after another 24 hours it will molt again to form the largest of all larval form known as the third instar larvae.At this stage the larvae climb upward and ultimately become sedentary and start preparing for the pupal stage. They develop hard outer case inside which the larvae undergo metamorphic changes including lysis of most of the larval structures and formation of adult structures(3). The pupal stage, which lasts about 4 days,ends with an adult fly eclosing out from the case. The adult fly has a life span of around 6 weeks(Figure 1).

With the help of extensive genome sequencing and annotation of the Genome Projects researchers have shown that 60% of *Drosophila* proteins share sequence similarity with human

proteins reason *Drosophila* is regarded by many as a superb model organism for the analysis of animal development. Advent of powerful genetic techniques has allowed *Drosophila* scientists to do precise spatiotemporal manipulations of gene expression for cell labeling, gene-function analysis or cell-lineage tracing. With such a high degree of evolutionary conservation among genes that control basic developmental processes, scientists are now establishing *Drosophila* as a model system for modeling human diseases including developmental disorders, neurological diseases or cancer,which they basically do by expressing the protein product associated with a particular disease state in the fly and then trying to establish whether the changes at genotypic as well as phenotypic level associated with the disease are mimicked in this organism. Eventhough the flies and vertebrates shared common ancestor 700 million years ago, *Drosophila melanogaster* provides an excellent in vivo model system to investigate and understand the development of higher order vertebrates.(4)

I.I UAS GAL4 system

UAS GAL4 system is a bipartite genetic tool which enables the users to have spatio-temporal control over the expression of their gene of interest.(Figure 2)Since 1993 when Robert Perimon first introduced this excellent system, researches have been using the same construct as well as its variants for tissue specific gene expression, gene knockdown, lineage tracing and so on. The Gal4 gene used owes its origin to *Saccharomyces cerevisiae*, upon activation the Gal4 protein binds to UAS or upstream activator sequence. (5)In *Drosophila melanogaster*,one parent will have Gal4 sequence downstream to a tissue specific promoter and the second parent will have UAS sequence upstream to the gene of interest that has to be expressed in a tissue specific manner. In the F1 progeny, upon activation of Gal4, its protein will bind to UAS sequence thereby turning on the ectopic expression of the gene of interest that is downstream to the UAS sequence.Often in order to gain a temporal control on this otherwise spatial regulation, a temperature sensitive variant of gal80protein is used. This potentially binds to the Gal4 activating domain present in Gal4 protein and thus prevents it from binding to the UAS sequence under restricted temperature conditions(18 ^{0}Celsius). The Gal4 protein shows maximum activity at 29^{0}Celsius(6)

I.II RNAi-gene knockdown using GAL4-UAS system

RNA interference(RNAi), one of the efficient ways through which a gene can be silenced was discovered in 1998 by Andrew Fire and Craig Mello.In their paper,through a series of experiments done in *Caeneorhabditis elegans*, they shows how introducing dsRNA can more efficiently block the translation of mRNA than ssRNA (7). Subsequent studies revealed the simple yet highly orchestrated pathway of RNA interference that is highly conserved across divergent taxa.As given in the (Figure 3), the nuclease III family member Dicer cleaves the dsRNA into small uniformly sized fragments known as siRNA.The siRNA will later on get unwound and one of the strands (guide strand) will get incorporated into RNA induced silencing complex (RISC) and the other strand will get degraded. The guide strand will then form complementary base pairing with the target mRNA which ultimately results mRNA degradation or translational inhibition. (8)

Unlike *Canerohabditis elegans*, which are fed with bacteria carrying dsRNA, dsRNA is expressed in the target cells in the form of short hairpin RNA (shRNA) using UAS GAL4 system. Instead of the coding sequence of the gene of interest in RNAi, UAS is followed by sequence coding sequence for shRNA.In F1 progeny thus has both the Gal4 and UASalong with its downstream RNAi sequence of the gene of interest together, the transcription of shRNA is initiated as soon as the GAL4 protein bind to UAS sequence. The shRNA synthesized will then get further processed to form siRNA and RNAi pathway is initiated.Vienna *Drosophila* RNAicenter (VDRC), Austria and National Institute of Genetics (NIH) Japan are the two major independently generated collections of *Drosophila* RNAi inducible strains. VDRC collection contains RNAistrains that can technically cover 88.2% of the whole genome of the fly whereas NIH strains covers48.8% of the genome. Few other comprehensive efforts to generate UAS RNAi lines includes Transgenic RNAiproject (TRiP) and Harward medical school, USA. (9)

I.III Hematopoiesis in *Drosophila melanogaster*

Hematopoiesis, the process through which blood cellular components are formed have been extensively studied in various model organisms. Keeping apart some of the small variations, the process of hematopoiesis is conserved in between vertebrates. There are two waves of

hematopoiesis in vertebrates: the primitive hematopoiesis which takes place during early embryonic development which give rise to erythrocytes and macrophages from common erythroid progenitor and the definitive hematopoiesis which happens later during the development where HSC's are involved .The HSC's born in aorta-gonadal-mesonephros(AGM) region of the embryo will later migrate to fetal liver and then to the bone marrow during definitive wave. (10)(Figure 4)

Drosophila hematopoiesis shows significant conservation to its vertebrate counterpart, not only with respect to the various transcription factors involved in the whole process(Table 1), but also to the two waves of hematopoiesis; primitive and definitive that differ with regard to time and location. In *Drosophila* the primitive hematopoiesis initiatesin the head mesoderm with the specification of blood cells(11). These cells are distinguished at embryonic development stage 5 by the expression ofa member of GATA family of zinc finger transcription factor, Serpent (srp). Evidences from several mutational studies in which researchers tried to abolish serpent function has revealed that serpent expression is important for the specification of blood cells. (12)

The definitive hematopoiesis or the larval hematopoiesis takes place in a specialized organ called the lymph gland. In *Drosophila* the lateral mesoderm give rise to dorsal mesoderm, which then give rise to cardiogenic mesoderm. With the help of various signals the cardiogenic mesodermis specified to three major cell types: cardioblasts (forms the dorsal vessel), pericardial cells (nephrocytic in function) and lymph gland anlagen (give rise to differentiated blood cell types). Several studies has shown that the ontogeny of blood and vascular cells are so closely related, similar to that of vertebrates and hence proposed the existence of hemangioblast in *Drosophila* that can potentially give rise to blood cells as well as endothelial cells. (11)

The lymph gland originates from the three thoracic segments (T1, T2 and T3) that expresses Odd skipped a zinc finger protein. The rest 6 abdominal segments (A1 to A6)that express Odd skipped will forms the pericardial nephrocytes.The cardiogenic mesoderm that forms a part of dorsal mesoderm requires the expression of homeobox protein Tin and GATA factor pnr for its specification. The specification of 3 cell types- lymph gland progenitors, cardioblasts and pericardial cells at cardiogenic mesoderm takes place at 8-10 hours after fertilization. Serpent expression is extremely critical for specifying as well as providing functional identity to lymph gland cells. (13)

I.IV Larval hematopoietic organ: ‘The lymph gland’.

By stage 16 of embryonic development, the cells clustered from 3 thoracic odd skipped expressing segments will form the so called primary lobes bilateral relative to the dorsal midline with 20 blood cells each .By the second instar stage few bilateral pairs of blood cells clusters will form which are known as secondary lobes and by this time, the cell number in the primary lobes rise up to 200 cells per lobe (14). (Figure 5)

Primary lobe of third instar larval lymph gland consists of three zones: the outermost cortical zone containing differentiated cells which expresses maturation markers like Hemolectin(hml),Peroxidasin(pxn) and Lozenge(Lz),inner medullary zone containing prohemocytes or progenitor cells which expresses reporters like domeless gal4,unpaired3 gal4 and GFP trapline ZCL2897 and the PSC(posterior signaling centre or the niche, positioned at the posterior tip of the primary lobe which expresses reporters like Antp gal4,Dororthy gal4 and upd3 gal4.There are around 50 cells in the lymph gland of wildtype third instar larvae and these cells are extremely important for the proper homeostasis of the lymph gland.(11)The PSC cells are involved in the maintenance of the progenitor cells. The major signaling involved in this process is Hedgehog (hh)signaling. The hh protein synthesized by the PSC cells is sensed by its receptor Patched (Ptc) present in the progenitor cells.JAK STAT signaling is one of the other signaling cascade which seems to be used by the PSC cells to communicate with the MZ cells there by maintaining them in the prohemocyte state. (15)The differentiated cells also plays a major role in maintaining the progenitor population through an extrinsic backward signaling mediated byAdenosine deaminase growth factor-A (Adgf-A) (16)

I.V Fly immunity

Since the system is devoid of adaptive immune immunity, immune responses against microbial infections and other insults in *Drosophila* are basically mediated by innate immune responses.The innate immunity itself comprises of cellular and humoral immunity. The3 terminally differentiated blood cell types including Plasmatocytes, Crystal cells and Lamellocytes, which are circulating through the hemolymph, the mammalian equivalent of blood, basically mediate the cellular responses.Plasmatocytes are the most abundant hemocytes in circulation and they are mainly involved in phagocytosis, encapsulation and the production of

antimicrobial peptides. The second most abundant blood cell type is Crystal cells and they have the ability to rupture and release components of the phenol oxidase cascade, and hence are involved in the melanisation of invading organisms as well as for wound repair and for coagulation. The third type, called Lamellocytes, are involved in phagocytosis of those objects that are way too big for plasmatocytes to engulf and they are rarely seen in healthy larvae(17)

Two independent pathways, Toll pathway and IMD pathway, which have their vertebrate counterpart TLR pathway and TNF-R pathway respectively, mediate the humoral immunity. When Toll pathway regulates the responses to Gram-positive bacterial and fungal infections, IMD pathway defends against Gram-negative bacterial infections. Activation of both of these pathways ultimately leads to the production of antimicrobial peptides from fat body, which is the functional homologue of mammalian liver (18,19)

I.VI Immune deficiency pathway

The IMD (Immune deficiency) pathway signaling plays a very important role in *Drosophila* defense against Gram-negative bacteria.The major components of the pathway includes receptors like PGRP-LC and PGRP-LE (long PGRPs which are membrane spanning and intracellular respectively), adaptor proteins like IMD and dFADD,DREDD which is a caspase,*Drosophila* IAP2 (inhibitor of apoptosis 2) which is an ubiquitin ligase,TGF activated kinase 1,TAK1 and its regulatory partner TAB2(TGF beta activated kinase 1), Drosophilla IKB kinase complex DmlIKβ (IRD5, catalytic subunit) and DmlIKγ (Kenny) regulatory subunit) and finally Relish(NF-KB),which is a transcriptional activator protein(Figure6).

The pathway is activated when Diaminopimelic acid type peptidoglycan(DAP type PGN) is recognized by PGRP-LC and PGRP –LE.This binding triggers the clustering of the receptors and the signal is transmitted to the adaptor protein IMD.IMD has characteristic death domain through which it interacts with *Drosophila* homologue of FADD,dFADD. dFADD recruits DREDD which is a mammalian caspase8 homologue via death effector domain interaction (DED).DREDD will further cleave IMD thereby exposing E3 ubiquitin ligase DIAP2 binding site. DIAP2 along with other conjugate enzymes Uev1a, Bendless (Ubc13) and Effete (Ubc5) drives IMD K63 polyubiqitination.It has been also reported that DIAP2 is also involved in activation of DREDD through DIAP2 mediated K63 polyubiquitination.Studies have

demonstrated that *Drosophila* IAP2 also auto-ubiquitinates once the immune pathway is activated.The polyubiqutin chain of IMD is recognized by TAB2 through their NZF(Npl24 zinc finger domain) domain, which results in the activation of TAK1 kinase through oligomerisation and autophosphorylation. TAB2 along with TAK1 is recruited into the place and TAK1, which is MAPKKK, phosphorylates IKK complex. Activated DmIKK complex can then phosphorylate Relish. It has been shown that Relish specifically interacts with DmIKK. DREDD is also required for the cleavage of the NF-kB precursor. Relish consists of both an N-terminal Rel homology (and NF-kB) domain and a C-terminal ankyrin-repeat/IkB-like domain. The N-terminal transcription factor domain (Rel N or Rel68) is released by endoproteolyticcleavage. This will further translocate into nucleus and activate target genes. The IkB region (Rel49) remains in the cytoplasm. It has been shown that the phosphorylation occurs at serine residues 528and 529 of Relish.(20)

I.VII Objectives

IMD pathway has been implicated in humoral immune responses since decades but its role in developmental context remained unexplored. Relish was amongst the several candidate genes that were picked up by a genome wide RNAi screen targeted in identifying the signals required for the maintenance of the hematopoietic niche in Drosophila. It was fascinating to see that the loss of *relish* transcripts from the niche drastically increases the size of the niche. We were excited to encounter a situation that will enable us to dissect out the role of *relish* in developmental context. The objectives of the project are as follows:

1.To find out whether attenuation of the function of other IMD pathway components from the hematopoietic niche similarly affects the niche cell number,similar to that of relish knockdown.

2. To check whether the cells of the hematopoietic niche, which are in quiescence in a late third, instar larvae has re-entered into proliferation mode in absence of Relish.

3.To assay the functionality of the ‘huge niche’ in relish loss background.We intend to analyze whether the increment in cell number has compromised its primary function of maintaining the progenitor population.

4.To check whether the classical loss of function mutants are mimicking the RNAi phenotype and thereby validating RNAi.

Exploring these questions will further provide us information regarding the critical molecules and signaling cascades involved in *Drosophila* hematopoietic niche maintenance and how IMD pathway, which was studied in context of humoral immunity, fits into one such signaling cascade.

Chapter II

MATERIALS AND METHODS

II.I Fly stocks

Flies were raised on standard agar-corn flour yeast medium and they were kept in 25 degree Celsius.The egg laying vials of crosses were kept in 29^{o}Csince the Gal4 shows maximum activity at 29^{o}C. Around 20 females and 15 males were used for crosses in vials.All the RNAi's and little classical loss of function mutants were obtained from different stock centers.Rest of the stocks was generously gifted. The list of fly stocks used for the project is given below;

1. UAS PGRP-LC RNAi-This line was procured from BDSC and was generated by Transgenic RNAiproject (TRiP) of Harward University, USA. It expresses dsRNA for RNAi of PGRP-LC under UAS control, and the insertion is in 3rd chromosome.

2. UAS IMD RNAi- This line was procured from BDSC and was generated by Transgenic RNAi project (TRiP) of Harward University, USA. It expresses dsRNA for RNAi of IMD under UAS control, and the insertion is in 3rd chromosome.

3. UAS DREDD RNAi- This line was procured from BDSC and was generated by Transgenic RNAi project (TRiP) of Harward University, USA. It expresses dsRNA for RNAi of DREDD under UAS control, and the insertion is in 3rd chromosome.

4. UAS TAK1 RNAi- This line was procured from BDSC and was generated by Transgenic RNAi project (TRiP) of Harward University, USA. It expresses dsRNA for RNAi of Tak1 under UAS control, and the insertion is in 3rd chromosome.

5. UAS RELISH RNAi- This line was procured from BDSC and was generated by Transgenic RNAi project (TRiP) of Harward University, USA. It expresses dsRNA for RNAi of RELISH under UAS control, and the insertion is in 3rd chromosome.

6.W*; tubGAL80ts/TM2;Antp GAL4 UAS GFP- Tublin GAL80ts line procured from BDSC was recombined with Antp GAL4 UAS GFP in the laboratory.The insertion is on 3rd chromosome and restrictive temp is 30 degree Celsius.

7. *pgrplc$^{\Delta 5}$*-This a loss of function allele of pgrplc,generated through p element mediated mutagenesis, procured from BDSC .The chromosomes affected are 1 and 3 and the allele is donated by Christine Kocks.

8. *imdshadok*-This is a loss of function allele generously gifted by Prof. Neal Silverman. The allele is generated through Pelement mediated mutagenesis and chromosome affected is 2nd.

9. *y^{1}w* dredd B118*-This is an amorphic allele generated by EMS mutagenesis, generously gifted by Prof. Bruno Lemaitre. Chromosome affected is 1st chromosome.

10. *y^{1}w* Tak1^{2-}*This is a null allele procured from BDSC,generated through EMS mutagenesis, affecting 1st chromosome donated by Ursula Weber &MarekMlodzik

11. *Relish$^{e20\ -}$* This is a loss of function allele generously gifted by Prof.Bruno Lemaitre. The mutagen is Delta2 3 transposase affecting 2nd chromosome.

12. *W^{1118}* –This is a loss of function allele, affecting 1stchromosome. It is procured from BDSC,whereit was donated by Michael Ashburner.

13. *W^{1118}*; Relish GFP/sm5-This is an enhancer trap line expressing GFP tagged Relish protein, procured from BDSC.The insertion is on 2nd chromosome and is donated by Rebecca Spokony& Kevin White.

II.II Immunocytochemistry assay

Late third instar larvae is dissected in ice followed by fixation of the tissues in 4% formaldehydefor 50 minutes at room temperature on shaker. After fixation tissues are washed three times in 0.3% PBT. This is followed by blocking in 10% NGS(Normal Goat Serum) for 1 hour. Once blocking is done, samples were incubated in primary antibody prepared overnight at 4 degree. After washing the incubated samples 4 times, 15 minutes each using 0.3%PBT and blocking using 10% NGS, secondary antibody (Alexa,1:400dilutions) was added and the samples were incubated overnight at 4 degree. After secondary incubation, the samples are washed using 0.3% PBT for 3 times each lasting 10 minutes and they were incubated with DAPI solution. After DAPI treatment samples were mounted in Vectashield (Vector Labs).

II.III Antibodies used

Antennapedia(1:5,mouse)was obtained from Developmental Studies Hybridoma Bank (DSHB),PH3(1:100,rabbit) from Cell signaling Inc., P1 (1:40,mouse), gifted by Prof. Ando and Relish (1:100,mouse) was gifted by Prof Neal Silverman.

II.IV Imaging

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

Chapter III

RESULTS AND DISCUSSION

III.I Knockdown of IMD pathway components caused drastic increment in niche cell number

To check whether all other upstream partners of relish in IMD pathway is giving us asimilar phenotype(i.e. huge increment in niche cell number) on removal of their function from the hematopoietic niche, we resorted in knocking them down one at a time from the niche by employing the Gal4-UAS technique. Flies line harboring AntpGal4UASGFP, a niche specific driver was crossed to the responder fly which had the UAS upstream sequence proximal to the RNAi of our gene of interest, whose function we wanted to attenuating the hematopoietic niche.(Figure7). The crosses was maintained in a vial at 25^{o}Cand only after egg laying the vials was shifted to 29^{o}C and reared till dissection. Using Antennapedia protein, we looked at the number of niche cells in the late third instar larval lymph gland, and found that compared to the control there is a huge increment in the niche cell number when there a loss of IMD pathway components from the niche. We manually counted the niche cells and found that the increment compared to the control is highly significant (Figure8). The maximum increase was found when relish is removed from the niche.

Relish knockdown kicked in proliferation in hematopoietic niche which otherwiseremains quiescent.

Niche cells of late third instar larval lymph gland remains quiescent in the absence of any external insult. Hence the next thing we checked was the proliferation status of niche cells in a relish loss background. We used an antibody against phosphorylated histone 3 and immunostained the control and experimental (relish loss) late third instar larval lymph gland. We detected several PH3 positive niche cells in *relish* loss of function indicating that the otherwise quiescent cells have re-entered into cell proliferation. As evident from the graph (Figure 9), compared to the control niche 5 times more cells are picking up PH3 in absence of a relish function. This result clearly suggested that the niche cells are no longer quiescent and they are undergoing accelerated proliferation when relish is lost from the niche.

III.II Loss of IMD components from niche affects the niche functionality

The blood cell types represented in *Drosophila*are three in kind.They are plasmatocytes, crystal cells and lamellocytes and these cells arise from the multipotent progenitor population present in the medullary zone. Furthermore, the distribution of these differentiating cells isrestricted to the peripheral region that normally constitutes the cortical zone(15). When we knocked down IMD pathway components from the niche,there was drastic increase in the number of differentiated blood cell type in the lymph gland. We stained the control and the experimental lymph glands using antibody against P1 antigen, which specifically marks plasmatocytes. P1 antigen (Nimrod) is a putative phagocytosis receptor with EGF repeats present in the cell surface of plasmatocytes. (20) Our analysis establishes that indeed the progenitor cells are lost and there is a tremendous increment in the number of plasmatocytes when the function of IMD pathway components are removed from the niche compared to the control. (Figure 10)

The above observation clearly demonstrates that the 'big niche' in IMD pathway components knockdown from the niche is non functional,because failure of the maintenance of progenitors have evoked ectopic differentiation.

III.III Relish expression in the niche

To check the expression of Relish in the niche, we used an enhancer trap fly line, Relish GFP which expresses GFP tagged relish protein. We did a time series assay to check the window during which Relish starts expressing in the niche. We dissected the lymph gland at different developmental stage and co-stained it with Antennapedia to identify whether the niche cells are expressing Relish. We analyzed the hematopoietic niche at 4 different time points, +28hours, +48hours, +72hours and +96hours post egg hatching. We found no GFP expression in the Antennapedia positive cells at +28hours and +48hours.By +72hours post hatching Relish starts expressing in few cells in the niche (Figure 11).By +96hours we could see robust expression of Relish in the niche. Through this experiment we were able toshow for the first time the presence of an IMD pathway component in hematopoietic niche of *Drosophila melanogaster*.

III.IV Validation of RNAi phenotypes

Classical loss of function mutant's analysis

As a part of validating our RNAi lines, we tried to check the status of niche in classical loss of function mutants of IMD pathway components.We raised the mutants at 25^{o}C, dissected them at late third instar stage and labeledthe lymph gland using niche specific marker Antennapedia.Compared to the control there was an increase in the niche cell number in loss of function mutants IMD pathway components. (Figures 12.1 and 12.2)The niche cell number was quantified and the increase was found to be statistically significant. Although the increment in niche cell number was not as profound as that of RNAi. We believe that the total loss of function was affecting several processes and tissues where yet to be identified developmental role of relish need to be explored. However it does validate that within the hematopoietic niche it was operating to control the niche cells number and thus its functionality.

Presence of Relish protein using Relish antibody

Wefurther went on validating our RNAi phenotype by looking at the expression of Relish protein in the niche. Prof. Neal Silverman gifted the relish antibody and we could standardize the antibody in our tissue of interest. In order to confirm that we had indeed removed the Relish protein from the niche in *relish*RNAi background we did Relish antibody labeling. Compared to the control, no Relish expression at protein level could be detected in case of relish RNAi in the niche (Figure 14).This reduction in the Relish protein expression in the niche clearly suggests that our *relish*RNAi was working and the phenotype that we got was indeed due to the loss of Relish proteinfrom the niche.

In summary, we were able to show for the first time the presence of IMD pathway component Relish in the hematopoietic niche of *Drosophila melanogaster*. Removing IMD pathway components from the niche not only kicked in proliferation in the niche which otherwise remains quiescent but also affected the functionality of the niche. By using Relish anybody and with the help of classical loss of function mutants we were able to validate our RNAi phenotype.

III.IV Discussion

Since the time it was first identified (mid 1990), people were more fascinated in finding out how IMD pathway is fine-tuning the humoral immune responses in *Drosophila melanogaster*.Eventhough the developmental role of Toll pathway, which is another independent humoral immune pathway in Drosophila,has been established since decades, the role of IMD pathway in developmental scenario remained unexplored. Here we were able to show for the first time the role played by IMD pathway in hematopoietic niche maintenance in *Drosophila melanogaster*. We believe that this study will be extremely helpful for understanding involvement of NF-kB signaling in hematopoiesis. Very recently a group of researchers were able to show that deletion of NF-kB subunit from the bone marrow severely impairs Hematopoietic Stem Cell function. They have also shown that when NF-kB subunit is deleted, there was an accumulation of phenotypic HSC's, decrease in progenitor number, extra medullary hematopoiesis, and differentiation defects (21), something similar to that of the developmental defects that we observed when we removed IMD pathway components from the hematopoietic niche. So if we could further nail down the key players involved in this process we can better understand the aberrant, non functional niches found in many diseased conditions and through this knowledge we can further explore and find out therapeutic methods through which we can tackle such a problem.

Chapter IV

CONCLUSION AND FUTURE PROSPECTS

IV CONCLUSION AND FUTURE PROSPECTS

Through this study we were able to not only nail down one such pathway which is involved in hematopoietic niche maintenance, but also were able to show for the first time the role of IMD pathway in developmental context. Since loss of IMD pathway components form the niche is kicking in proliferation in the niche, as future endeavors, we would like to explore the involvement pathways involved in cell proliferation, cell cycle regulation etc. in the same. One such potential candidate would be Hedgehog which has been already shown to be synthesized by the niche cells and transported to the medullary zone, where it plays a major role in maintaining the progenitor pool (15). Several evidences are there which talks about the role ofHedgehog in cell proliferation for example recently a study has shown that HH signaling is essential for the proliferation of the ovarian somatic stem cells (22)

We also would like to do rescue experiments before wrapping up the project, in which we want to show that if we somehow manage to ectopically provide the downstream components of IMD pathway to the niche from where the upstream components are removed, we could rescue the non functional big niche phenotype and get back wild type functional niche. In recent study researchers has reported about a negative regulator of IMD pathway- *pirk*, which is getting highly expressed upon Gram-negative bacterial infection in *Drosophila* in vitro and in vivo. *pirk* encodes a cytoplasmic protein and they have shown that it co-immunoprecipitates with Imd and the cytoplasmic tail of peptidoglycan recognition protein LC (PGRP-LC). RNA interference-mediated down-regulation of Pirk also caused Imd pathway hyperactivation upon infection with Gram-negative bacteria, while overexpression of *pirk* reduced the Imd pathway response both invitro and in vivo (23). These data clearly indicates that Pirk is a negative regulator of IMD pathway. So if we could bring Pirk mutant in which there will be IMD pathway overexpression in the background of our RNAi's,theoretically we should see a recue through which we can further confirm that the phenotype that we are seeing is exclusively mediated by the RNAi mediated knockdown of IMD pathway components from the niche.

Through our study we were able to unravel the novel role played by IMD pathway in *Drosophila* hematopoiesis,and we believe that this study will be helpful to further explore and understand the

molecular basis behind various hematopoietic malignancies where we have aberrant non-functional niches.

Chapter V

Bibliography

V Bibliography

1 *Drosophila melanogaster*: a fly through its history and current use: R Stephenson,NH Metcalfe,J R Coll Physicians Edinb 2013; 43:70–5 2013 RCPE

2 *Drosophila*, the golden bug, emerges as a tool for human genetics: Ethan Bier,*Nature Reviews Genetics* 6, 9-23 (January 2005) doi:10.1038/nrg1503

3 Steroid regulated programmed cell death during *Drosophila* metamorphosis.Jiang C, Baehrecke EH, Thummel CS

4 *Drosophila*, the golden bug, emerges as a tool for human genetics
Ethan Bier(*Nature Reviews Genetics* 6, 9-23 (January 2005))

5 Yeast Gal4: a transcriptional paradigm revisited-Ana Traven,[1] Branka Jelicic, and Mary SoptaEMBO Rep. 2006 May; 7(5): 496–499.doi: 10.1038/sj.embor.7400679

6 Refining GAL4-driven transgene expression in *Drosophila* with a GAL80 enhancer-trap: Suster ML, Seugnet L, Bate M, Sokolowski MB.

7 Potent and specific genetic interference by double-stranded RNA in*Caenorhabditis elegans:* Andrew Fire, SiQun Xu, Mary K. Montgomery, Steven A. Kostas, Samuel E. Driver & Craig C. Mello*Nature* 391, 806-811 (19 February 1998)

8 RNA Interference: Biology, Mechanism, and Applications : Neema Agrawal, P. V. N. Dasaradhi, Asif Mohmmed, Pawan Malhotra, Raj K. Bhatnagar, and Sunil K. MukherjeeMicrobiol Mol Biol Rev. 2003 Dec; 67(4): 657–685.

9 *In Vivo* RNAi-Based Screens: Studies in Model Organisms: Miki Yamamoto-Hino and Satoshi GotoGenes (Basel). 2013 Dec; 4(4): 646–665

10 Ontogeny of the Hematopoietic System: Annual Review of Immunology
Vol. 25: 745-785

11 Genetic Dissection of Hematopoiesis Using*Drosophila* as a Model System: Cory J. Evans, Sergey A. Sinenko, Lolitika Mandal , Julian A. Martinez-Agosto,Volker Hartenstein,andUtpal Banerjee

12 The GATA family (vertebrates and invertebrates) Patient, Roger K., and James D. McGhee.Current opinion in genetics & development 12.4 (2002): 416-422

13 Evidence for a fruitfly hemangioblast and similarities between lymph-gland hematopoiesis in fruit fly and mammal aorta-gonadal-mesonephros mesoderm. Mandal, Lolitika, Utpal Banerjee, and Volker Hartenstein Nature genetics 36.9 (2004): 1019-1023

14 The *Drosophila* lymph gland as a developmental model of hematopoiesis. Jung, Seung-Hye, et al. Development 132.11 (2005): 2521-2533

15 A Hedgehog- and Antennapedia-dependent niche maintains Drosophilahaematopoietic precursors: Lolitika Mandal Julian A. Martinez-Agosto Cory J. Evans Volker Hartenstein and UtpalBanerjee

16 Interaction between differentiating cell-and niche derived signals in hematopoietic progenitor maintenance: Mondal, Bama Charan, et al. Cell 147.7 (2011): 1589-1600

17 *Drosophila* cellular immunity: a story of migration and adhesion: Marie-Odile Fauvarque and Michael J. Williams

18 Toll and IMD Pathways Synergistically Activate an Innate Immune Response in *Drosophila melanogaster*-Takahiro Tanji, Xiaodi Hu, Alexander N. R. Weber, and Y.Tony Ip Mol Cell Biol. 2007 Jun; 27(12): 4578–4588.

19 Bacterial recognition and signalling by the *Drosophila* IMD pathway: Takashi Kaneko and Neal Silverman,Cellular Microbiology (2005)

20 Nimrod,a putative phagocytosis receptor with egf repeats in *Drosophila* plasmatocytes-Eva Kurucz,Robert Markus,Istvan Ando

21 Deletion of the NF-κB subunit p65/RelA in the hematopoietic compartment leads to defects in hematopoietic stem cell function: Sarah J. Stein and Albert S. BaldwinBlood. 2013 Jun 20; 121(25): 5015–5024.

22 Hedgehog acts as a somatic stem cell factor in the *Drosophila* ovary.

Zhang Y, Kalderon D.Nature. 2001 Mar 29;410(6828):599-604

23 Pirk Is a Negative Regulator of the*Drosophila* Imd Pathway **:** Anni Kleino, Henna Myllymäki, Jenni Kallio, Leena-Maija Vanha-aho,Kaisa Oksanen, Johanna Ulvila, Dan Hultmark, Susanna Valanne andMika Rämet The Journal of ImmunologyApril 15, 2008vol. 180 no. 8 5413-5422

FIGURES

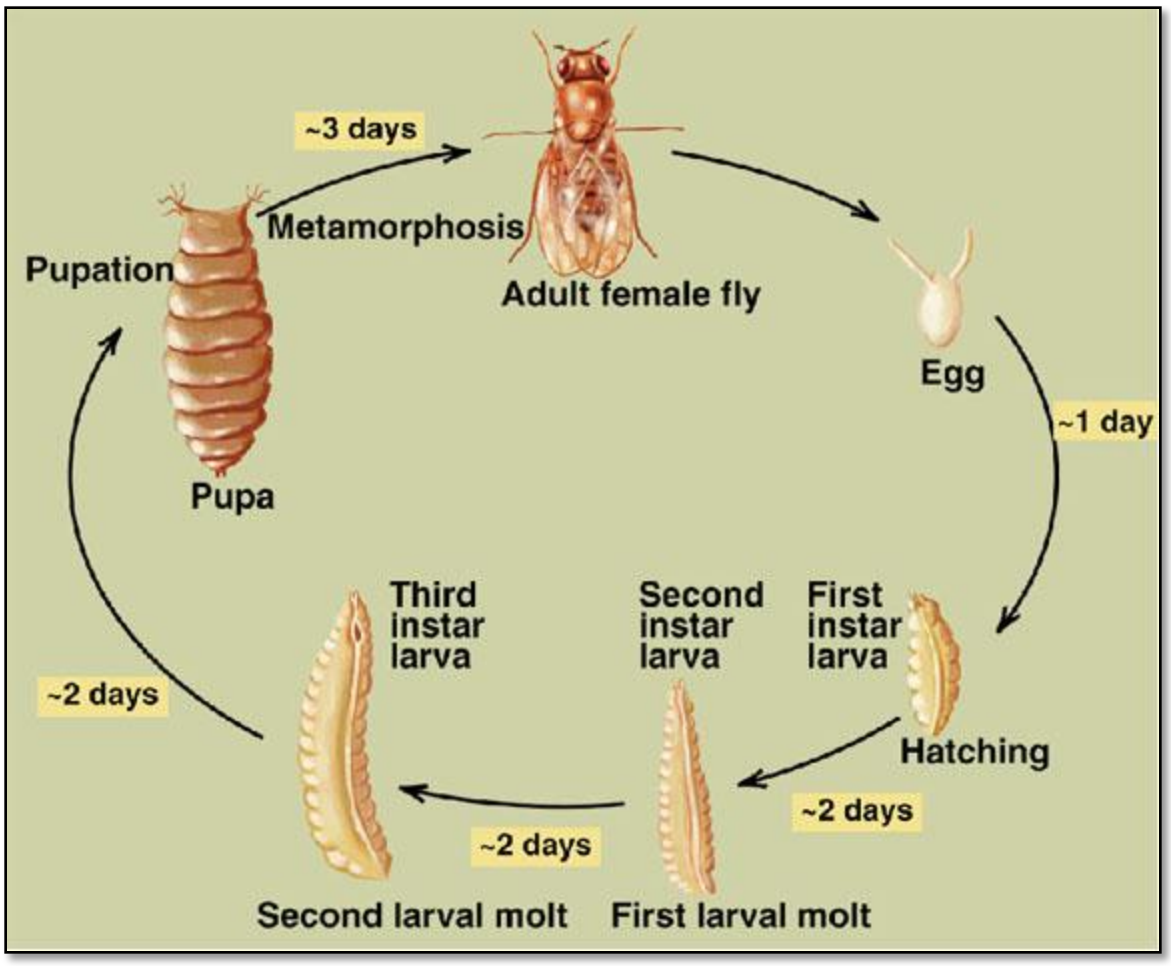

Figure 1: Life cycle of *Drosophila melanogaster* (http://www.zoology.ubc.ca/)

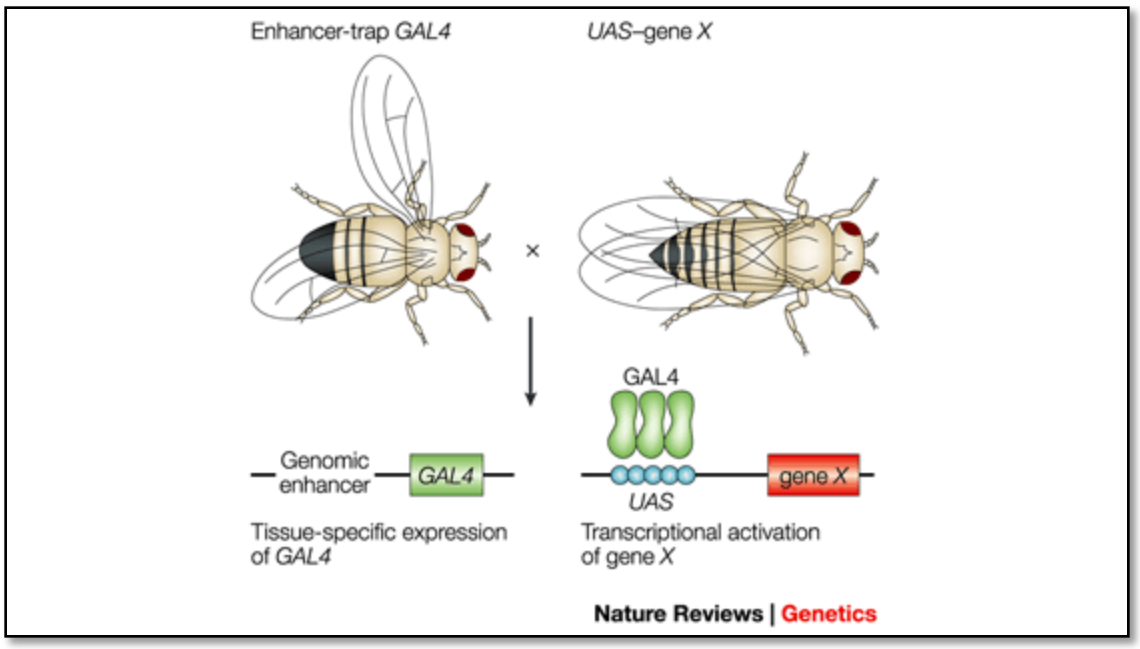

Figure 2: UAS-GAL4 system: Through this genetic tool we can express our gene of interest in a tissue specific manner(Nature Reviews Genetics 3, 176-188 (March 2002))

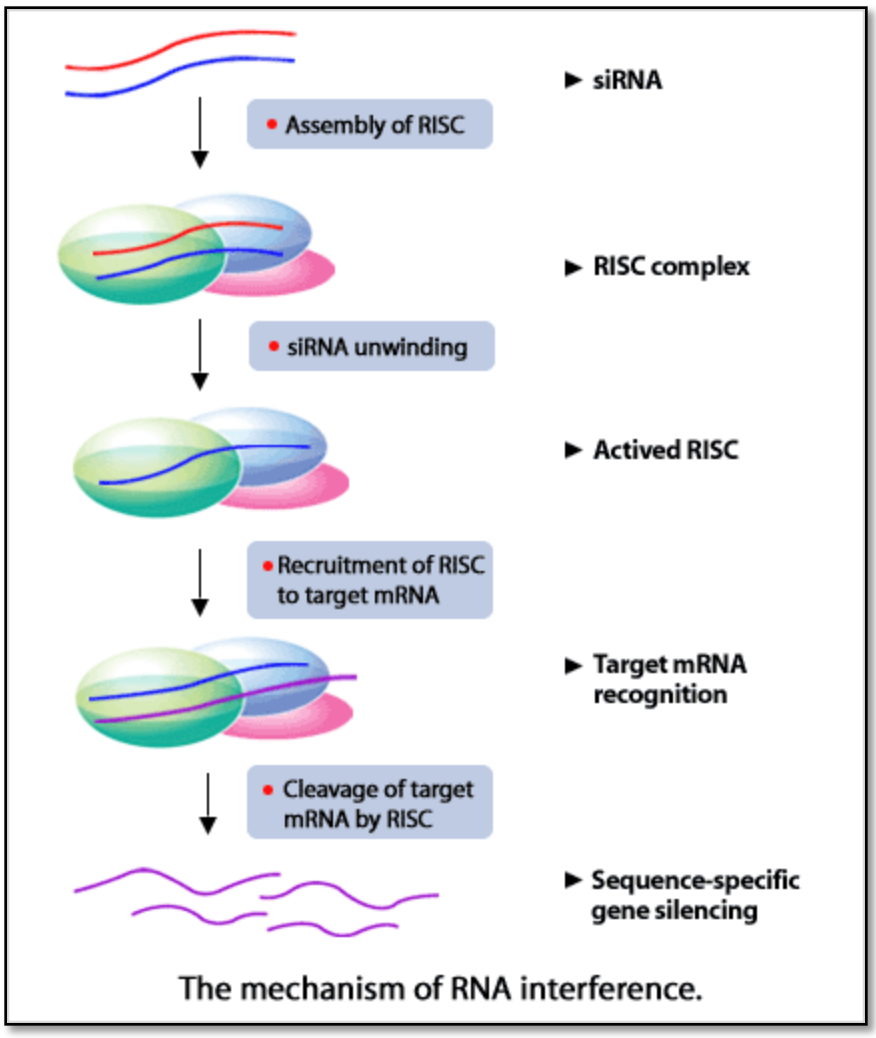

Figure 3: Schematic representation showing RNA interference mechanism

(http://www.bioneer.co.kr/)

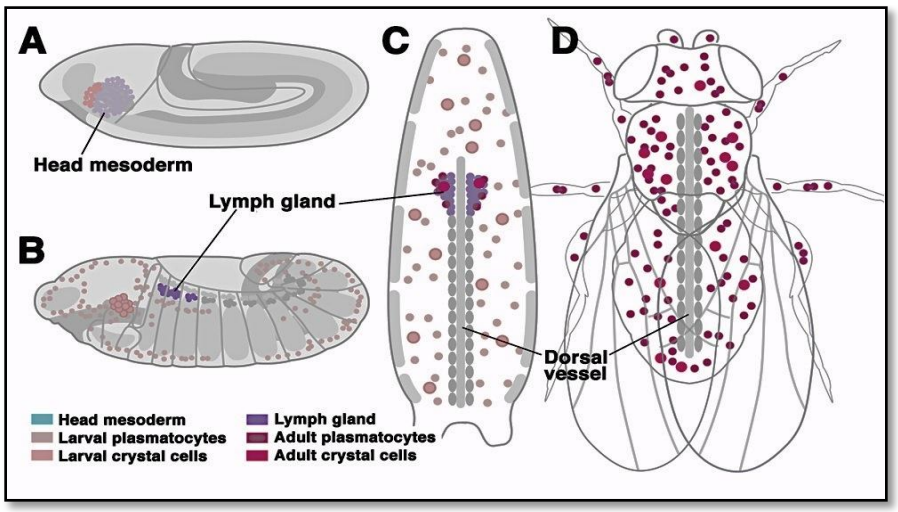

Figure 4: Two waves of *Drosophila* hematopoiesis

- **A.** Emryonic head mesoderm,site of primitive hematopoiesis
- **B.** Genesis of larval hematopoietic organ in the dorsal region of embryonic cardiogenic mesoderm.
- **C.** Lymph gland
- **D.** Hemocytes populating the hemolymph of adult fly.

(Cory J 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

Table 1: Transcription factors involved in hematopoiesis are highly conserved between *Drosophila* and mammals.

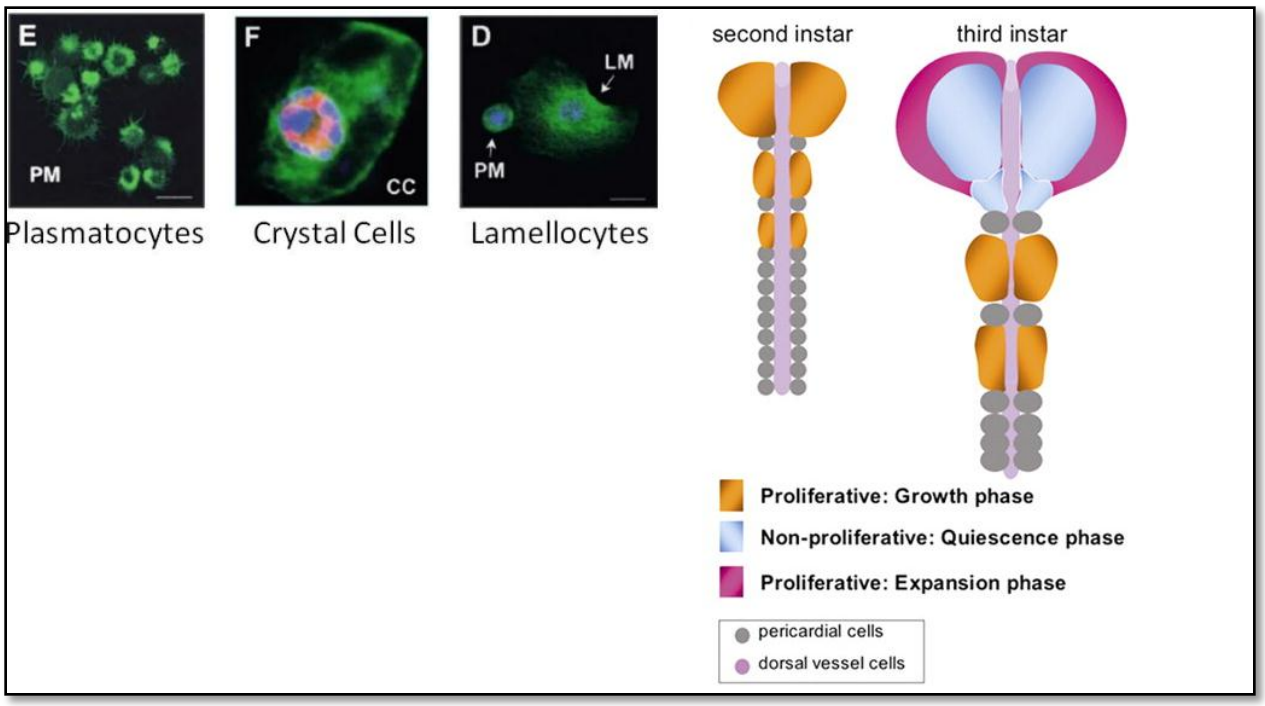

Figure 5: The larval hematopoietic organ,Lymph gland.There are 3 terminally differentiated blood cell types in Drosophila,Plasmatocytes,crystal cells and Lamellocytes(SeungHye Jung et al, Development, 2005, Cory J Evans et al.,Developmental cell, 2003)

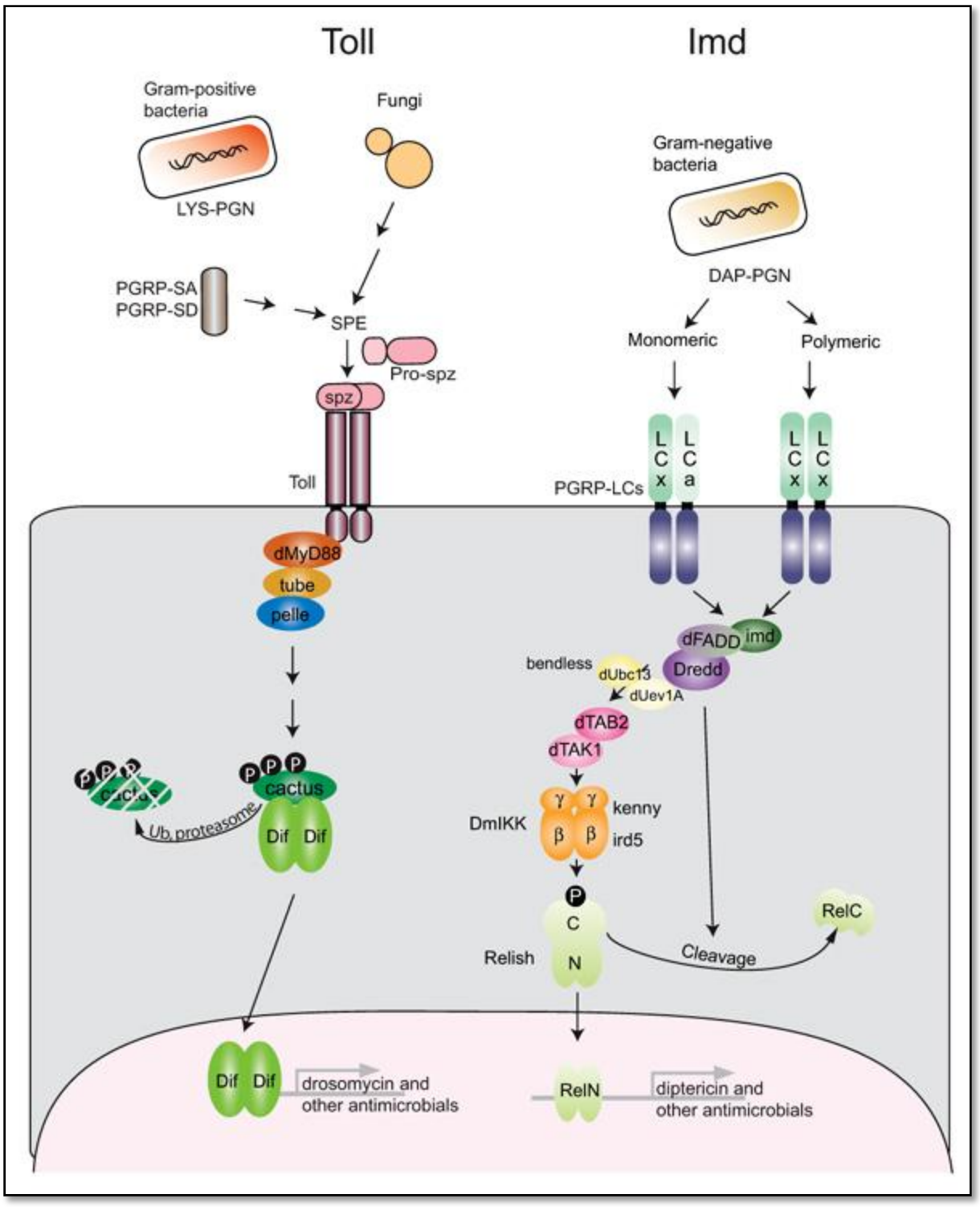

Figure 6: Schematic representation showing two humoral immune pathways in Drosophial;Toll pathway and Immune deficiency pathway(Nature Immunology - 7, 911 - 917 (2006))

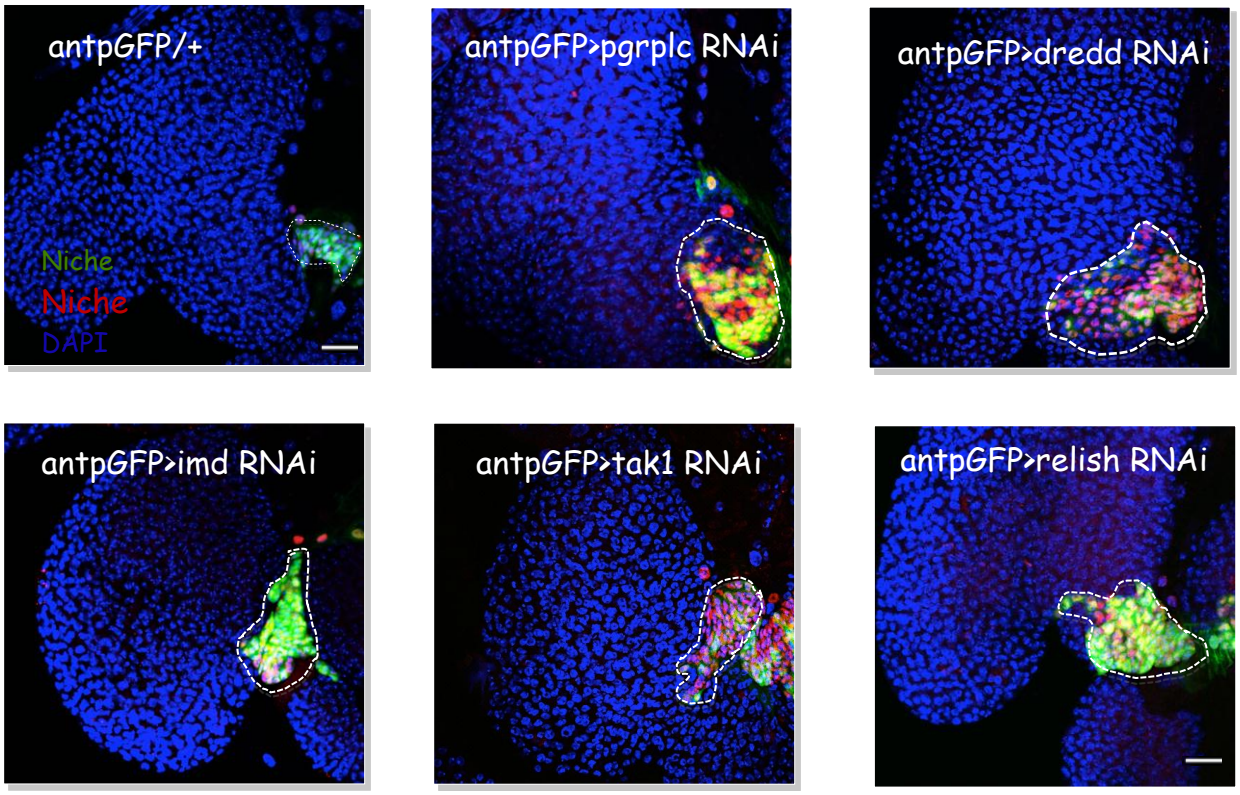

Figure 7: Knockdown of IMD pathway components caused drastic increment in niche cell numbers.

The niche cells which are expressing the GFP reporter is stained with antibody against Antennapedia (red) which is expressed in the niche.DAPI(Blue) is marking the nucleus.(scale bar-20 micron)

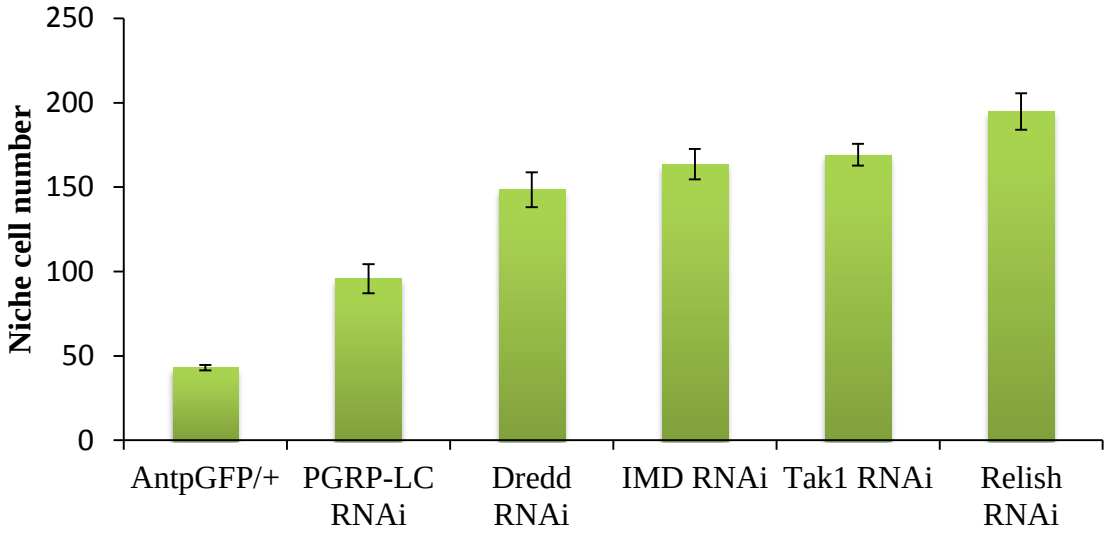

Figure 8: Statistical tests showed that the increment in the niche cell number is highly significant. Maximum increase was found when Relish is removed from the niche.

NichePH3

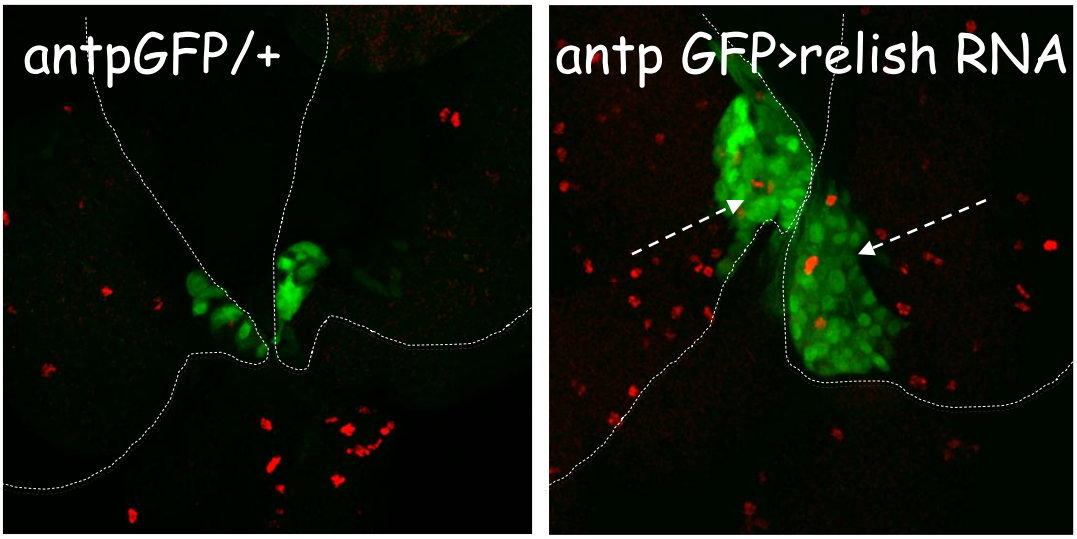

Figure 9: Relish knockdown in quiescentthird instar hematopoietic niche (green) forces cells to re-enter into proliferation mode (Arrow indicates the niche cellswhich are PH3 (red)positive).

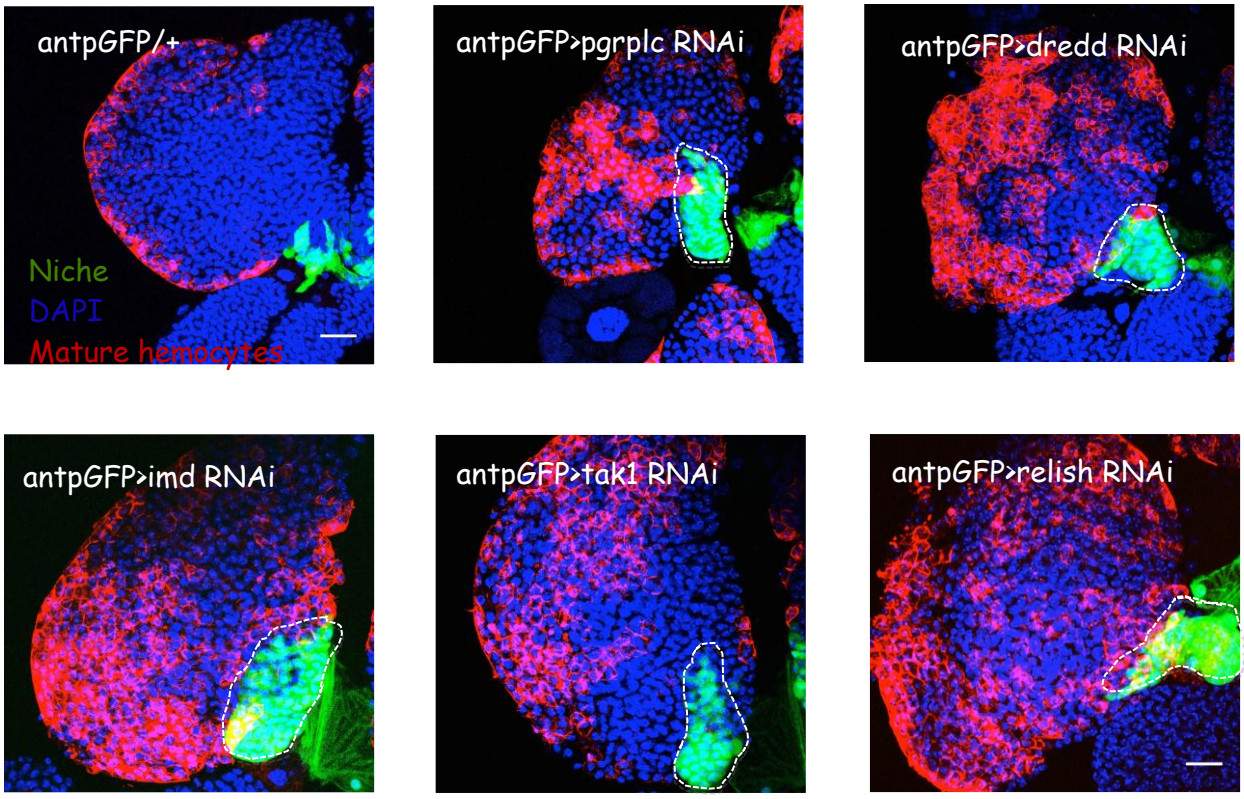

Figure 10:The number of plasmatocytes(red) increased tremendously when IMD pathway components are removed from the niche(green) compared to the control.This data clearly indicates that the progenitors are undergoing ectopic differentiation.(Scale bar-20 micron)

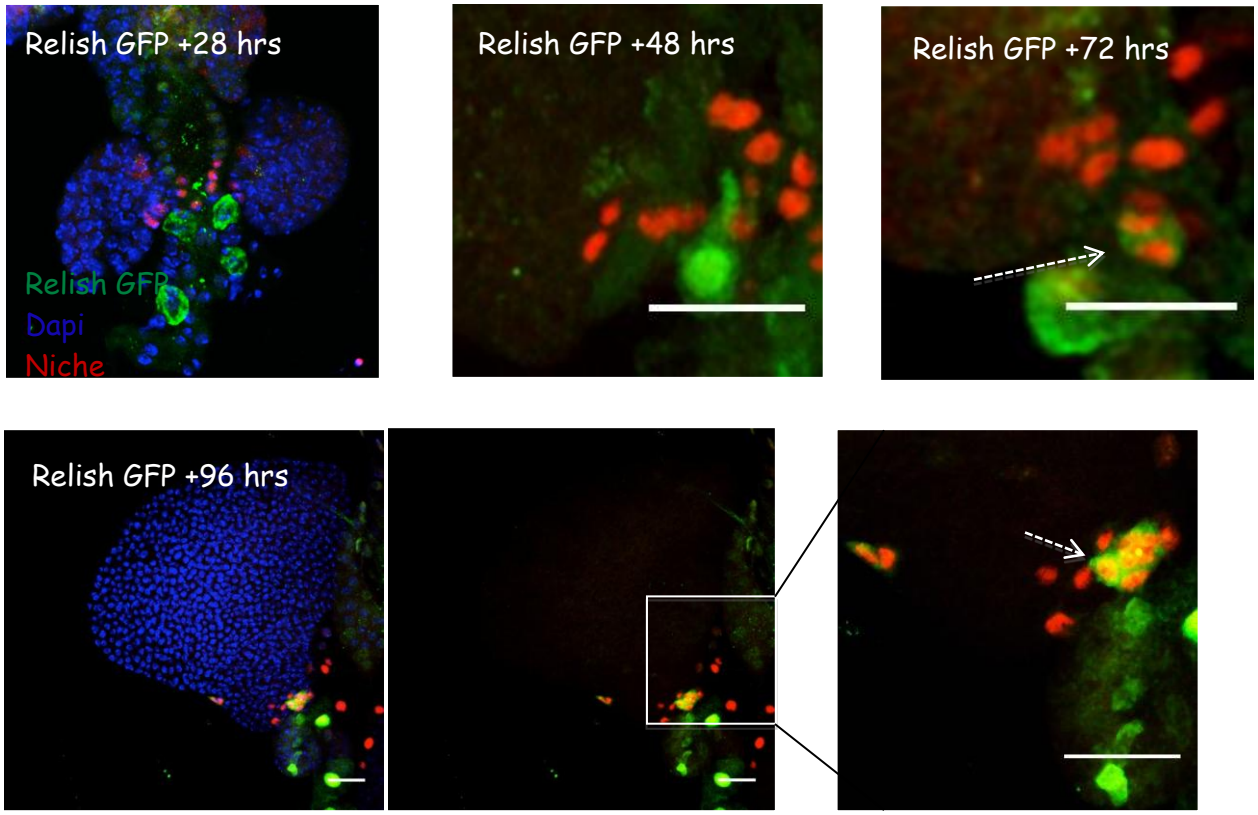

Figure 11: Relish GFP expression was not detected in the Antennapedia(red) positive cells at +28hours and +48hours.By +72hours relish(green) started expressing in few cells in the niche. By +96hours we could see robust expression of Relish in the niche. (Arrowhead)(Scale bar = 20 micron).

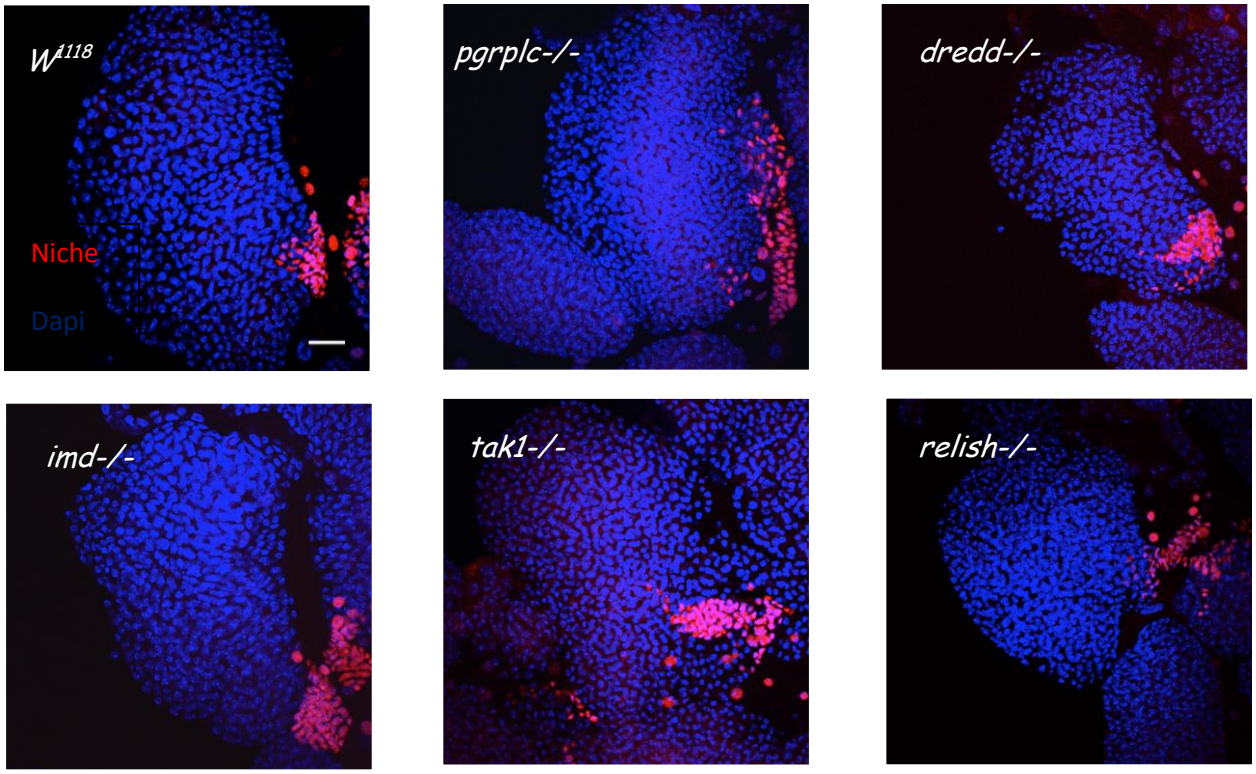

Figure 12.1: Compared to the control there was an increase in the niche cell number in loss of function mutants IMD pathway components even though the increment was not as profound as that of RNAi.(Scale bar = 20 micron)

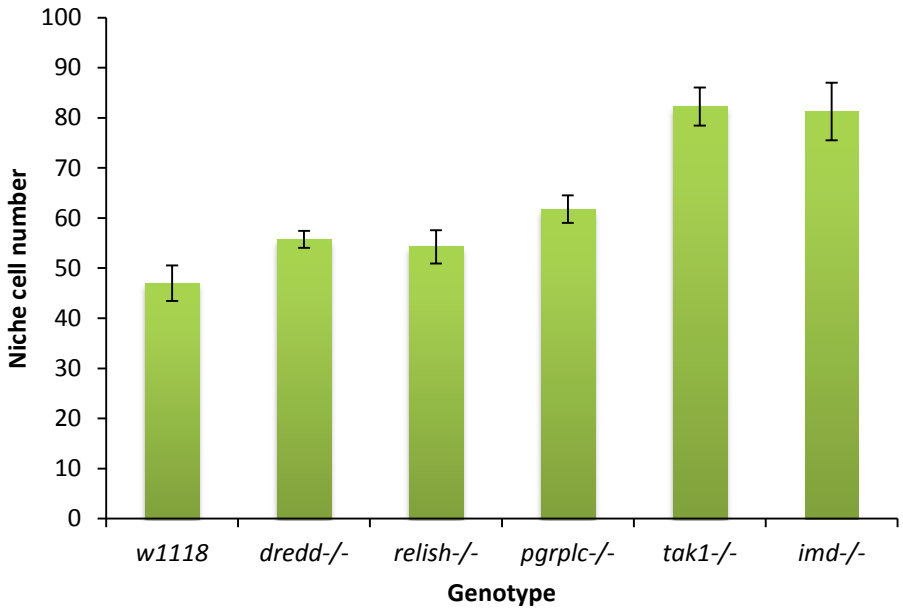

Figure 12.2: There was no huge increment niche cell number in case of loss of function mutants but compared to the control the increment was significant in case of *tak1-/-*,*imd-/-* and *pgrplc-/-*

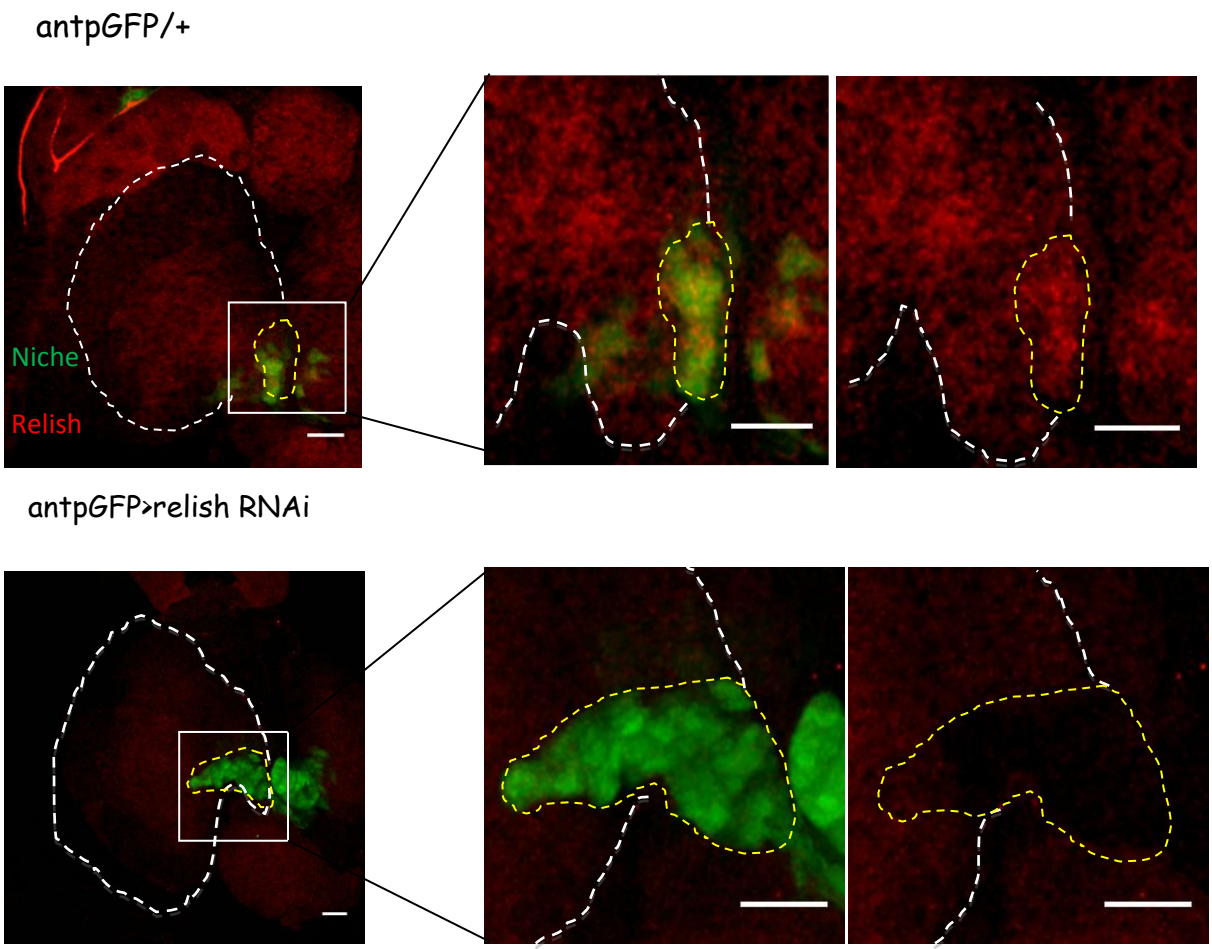

Figure 13: Compared to the control, in case of relish RNAi there is no or reduced relish expression(red) at protein level in the niche(green) This clearly suggests that our RNAi was able to inhibit relish mRNA translation and hence there is reduction in the relish protein expression in the niche(Scale bar = 20 micron).