Metabolic regulation of the hematopoietic niche in *Drosophila melanogaster*

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Certificate of Examination

This is to certify that the dissertation titled "metabolic regulation of the hematopoietic niche in drosophila" submitted by Mr. Jugal Pant (Reg. No. MS09065) for the partial fulfilment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Sudip Mandal **Prof. Somdatta Sinha** **Dr. Lolitika Mandal (Supervisor)**

Dated:

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. Lolitika Mandal at the Indian Institute of Science Education and Research Mohali. This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Jugal Pant
(Candidate)
Dated: April 25, 2014

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

Dr. Lolitika Mandal
(Supervisor)

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Abstract

Stem cells are identified as the source of virtually all differentiated cells that are replenished during the lifetime of an individual. Stem cells serve as a resource to take care, to maintain a critical balance between stem and differentiated cell population which is crucial for the long term maintenance of functional tissue types. They do so by choosing one of several alternate fates: self-renewal, commitment to differentiate and senescence or cell death.

Microenvironment in which the stem cells reside and maintain their stemness is commonly known as the stem cell niche. A specific signaling pathway or a cell adhesion molecule allows the niche cells to maintain contact with stem cells and in the absence of this signal, the stem cells leave their niche and/or either divide, differentiate or apoptose. Question of how stem cells are maintained by niche has been actively investigated. But how the stem cell niche is maintained has not been investigated in detail. For investing into the question of stem cell niche maintenance we are probing *Drosophila* larval hematopoietic system, the lymph gland.

Lymph gland, with its three very well characterized zones has been exploited to understand the Signaling network between niche, progenitor and differentiated cells. The three distinct zones namely, the outer peripheral zone, Cortical Zone or CZ houses the differentiating hemocytes, the Medullary Zone or MZ in which the stem like cells reside, and the third zone is the Posterior Signaling Centre or PSC that serves as the niche for the MZ cells. The small group of 40-45 PSC/niche cells control the fate of thousands of blood cell progenitors of the medullary zone intrigued us to hypothesize on the active metabolic nature of these Signaling cells and led us to probe into the role of mitochondrial genes in niche maintenance and functionality.

We have undertaken a loss of function RNAi based screening for nuclear encoded mitochondrial genes in the niche. We got few candidate genes in which the niche

morphology was perturbed i.e., either the niche cell number increased or decreased compared to the control niche number.

In this scenario in which the niche was perturbed, we looked at the functional status of the aberrant niche by looking at the status of progenitor cells and the differentiating cells. We also tried to investigate the mechanism leading to the perturbation of niche cell number and functionality, by looking at the stress signals like the Reactive Oxygen Species or ROS generation in this mitochondrial loss of function background in the niche.

It will be interesting to address whether this loss of function of the nuclear encoded mitochondrial genes in the hematopoietic niche has led to switching on an alternate metabolic pathway like aerobic glycolysis. Or as reported in some malignant and developmental scenarios, the niche cell is still able to keep the OXPHOS metabolic pathway on.

1 INTRODUCTION

1.1 *Drosophila melanogaster*:

Drosophila commonly known as the fruit fly, which belongs to the order Diptera (Greek di = two, and ptera = wings) in the family *Drosophilidae*. It is one of the most widely studied organism for studying cellular and developmental processes in genetics and developmental biology.

1.2 Physical appearance:

Drosophila is covered in a chitinous exoskeleton having three main body segments and three pairs of segmented legs**.** The common wild type fruit fly is normally a yellow brown color with brick red eyes and transverse black rings across the abdomen. It has a rounded head with large, red, compound eyes; three smaller simple eyes, and short antennae. They exhibit sexual dimorphism i.e., females are slightly larger than male flies. The dorsal surface of the abdomen has black stripes which can be used to determine the sex of the fly. Males have a greater amount of black pigmentation concentrated at the posterior end of the abdomen. Males have a row of dark bristles known as sex comb on the first pair of their legs whereas females don't have this structure.

1.3 Life cycle of *Drosophila melanogaster*

One of the biggest advantages of *Drosophila melanogaster* is the rapid growth and development. The whole life cycle of *Drosophila melanogaster* under standard laboratory conditions (25 °C) is about 10 days. Both the life span and rate of its development varies with temperature as it is an ectothermic species. Life span of *Drosophila melanogaster* at 25 °C is of 45 days whereas at 29°C the life span is of 30 days. At 25°C, its developmental time is of 10 days and at 16 °C, it becomes 24 days. Mutant fly lifespan varies according to the corresponding mutation. There are four phases of the life cycle egg, larvae, pupa and adult.

The formation of embryo, embryogenesis takes place within the egg and larvae hatch after 22 hours of egg laying. The larval growth process continues for about 6 days and the larvae molts twice during this time to develop into 1st instar, 2nd instar and 3rd instar. During this time, the larva increases approximately 200-fold in weight. The larvae in the first instar generally crawl over the food but as it molts and reaches the second instar it moves inside the food having its head upside down. Towards the end of the third instar, the larva stops feeding and leaves the food in search of a dry place for pupariation. The larvae are transparent, and one can see the inside organs. Metamorphosis takes place in the pupal case. The emerging adult flies are about 3 mm in length with females being slightly larger than males.

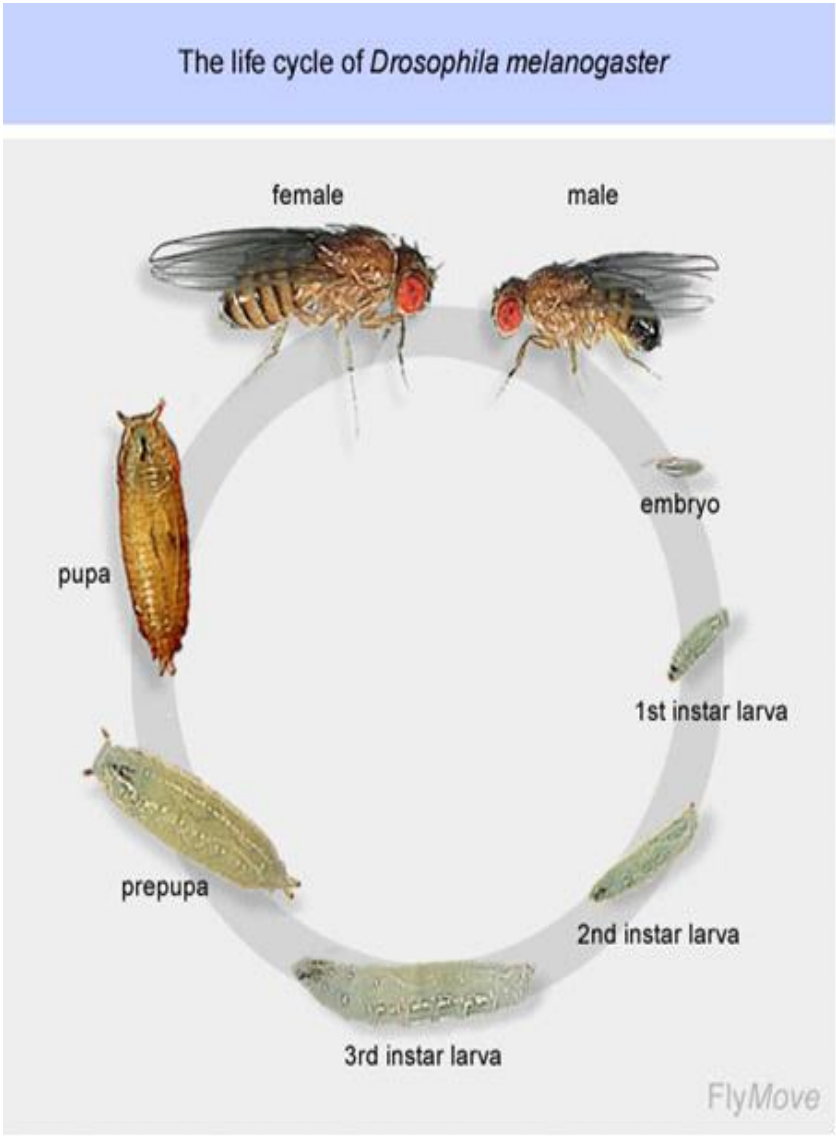


Figure 1: Life Cycle of *Drosophila melanogaster*.

1.4 *Drosophila* as a model system

The fruit fly *Drosophila melanogaster* has been extensively studied for over a century as a model organism for genetic investigations. It has several characteristics which makes it an ideal organism for the study of animal development and behavior, neurobiology, and human genetic diseases. Some of the features of *Drosophila* that make it a good model organism are:

- *Drosophila* has a short life cycle which makes it convenient to handle and study the specifically induced mutations.
- The genome of *Drosophila* has been fully sequenced which makes it possible to manipulate almost any gene.
- Approximately 60% of a group of readily identified genes that are mutated, amplified or deleted in a diverse set of human diseases have a counterpart in *Drosophila*.
- It does not spread any disease or contamination.
- With *Drosophila* there is an array of genetic tools like the GAL4-UAS system which makes it easier to perform genome wide manipulations and upregulation or downregulation of the transgenes.

1.5 Genetic tools in *Drosophila*

GAL4-UAS system is a powerful tool to study gene expression and since its development, it has been widely used as a genetic toolbox for various model organisms, particularly the fruit fly, *Drosophila melanogaster.*

It consists of two parts: GAL4 which is the yeast transcription factor and the UAS (Upstream Activation Sequence) which is an enhancer to which GAL4 binds to activate gene transcription. The transcription of specific genes is regulated by GAL4 as it binds to UAS enhancer and expresses those specific genes.

In a work by Fischer et al. (1988), it was shown that GAL4 is capable of stimulating the transcription of reporter gene under UAS control in *Drosophila*. Thus the expression of the gene of interest which is next to UAS is reported by the reporter gene.

Controlling the gene expression spatially has been one of the major achievements for addressing gene function. The gene of interest, the responder is controlled by UAS element, which is inactive due to the absence of GAL4. In order to attain the gene expression by activating the transcription of responder gene, the flies are mated with the flies having GAL4 in them. The selected progenies would then have the responder gene expressed which would be visible due to the expression of reporter gene such as GFP.

GAL4-UAS expression systems are used to artificially express genes in organisms carrying both the constructs. This system is widely used to identify the genes involved in the process of interest, to analyze the loss of function phenotypes, to identify specific genes which hamper the process of interest. Also by using RNAi of a specific gene under the control of UAS, this system can be used for down regulating a specific gene. The GAL4 system is sensitive to the temperature. Its activity is minimized at 16°C and is maximized at 29°C without compromising fertility and viability. Thus a wide range of expression level is achieved by manipulating the system under various temperatures.

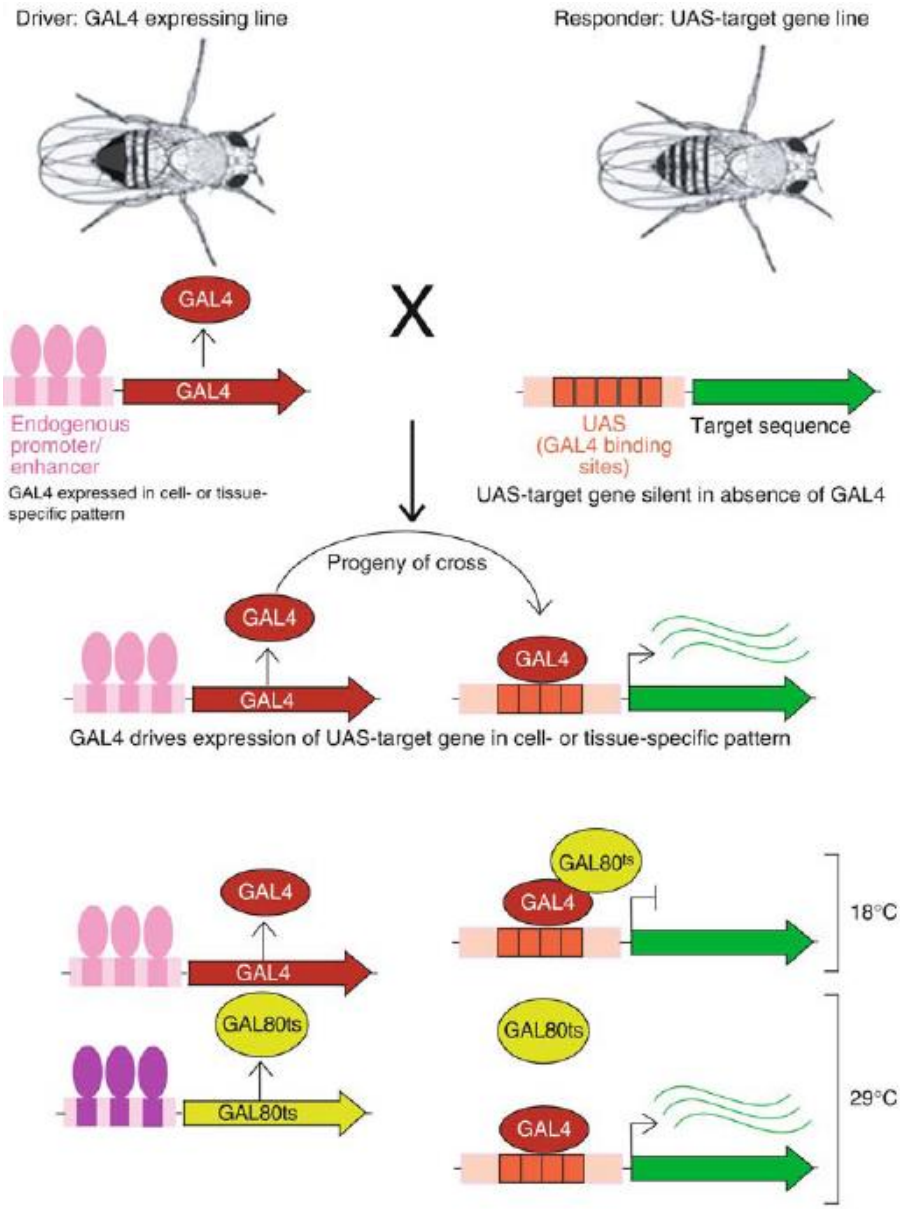


Figure2: Image showing how the GAL4UAS system works. David A Elliott and Andrea H Brand:The GAL4UAS system 2008.

1.6 Enhancer Trap

Enhancer Trap is a mobile DNA element with a reporter gene fused to a weak or a basal promoter. This engineered transposable element thus on its own cannot drive detectable expression but can respond to the transcriptional enhancers that lie in the neighborhood of its integration site. Therefore the expression of the reporter is dictated / regulated by the genomic enhancer which now is said to be trapped by the transposable element. This leads to the identification of the genomic enhancer.

Enhancer traps allows us to follow cells throughout development, even though morphogenetic movements or cell shape changes may make them otherwise hard to track. This system also endows scientist a chance to follow cell types and trace out their fate in different mutant background. But the main drawback with this system is, since the insertion of reporter gene is random, it could disrupt endogenous gene which may affect the phenotype and expression and the insertion could be lethal to the cell.

1.7 Hematopoiesis

The process by which immature precursor cells develop into mature blood cells is known as Hematopoiesis. Monophyletic theory which explains the functioning of this process is the theory that is accepted currently and it describes that a single type of stem cell give rise to all mature blood cells in the body. This stem cell is called the pluripotential (pluripotent) stem cell.

1.7.1 Hematopoiesis in vertebrates

Hematopoiesis in mammals is biphasic as there are two phases known as the primitive phase and the definitive phase. The origin of primitive hematopoiesis is from the embryonic yolk sac, whereas the definitive hematopoiesis originates from the AGM (aorta gonad mesonephros) region. Although several hematopoietic activities are found in fetal, spleen, liver, and placenta, the hematopoietic site for adult stage is the bone marrow where adult hematopoiesis takes place. The mammalian hematopoietic stem cell give rise

to two types of progenitor cells lymphoid and myeloid, which give rise to the T lymphocyte, B lymphocyte, dendritic cells and all types of phagotic macrophages.

1.7.2 Hematopoiesis in *Drosophila*

In *Drosophila*, hematopoiesis is biphasic- Primitive Hematopoiesis and Definitive Hematopoiesis. There is spatial and temporal difference in these two phases during the development of *Drosophila* (Lebestky et al., 2000; Evans et al., 2003; Hartenstein, 2006). The hematopoiesis begins with the onset of specific hemogenic molecular signature in mesoderm population of cells.

1.7.3 Primitive hematopoiesis

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

Another important factor in specifying blood cell lineage is Tinman (Tin) which is *Drosophila* homolog of vertebrate Nkx2.5 (Tin; Azpiazu and Frasch, 1993; Bodmer, 1993). Initially Tin is expressed throughout the entire mesoderm of head and trunk. Then expression of buttonhead in the cylindrical domain of embryonic head activates Srp and represses Tin (Yin et al., 1997)

Plasmatocytes are specified by the expression of Glial-cells-missing proteins (Gcm-1 and 2) which are the conserved transcription factors that function in *Drosophila* as determinant of glial cells and plasmatocytes cell fates (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996; Bernardoni et al., 1997; Lebestky et al., 2000; Kammerer and Giangrande, 2001; Alfonso and Jones, 2002). Initially the expression of Gcm co-localizes with Srp expression (Bataille et al., 2005). Then anterior subpopulation of Gcm positive cells then expresses Lz. Majority of these Lz positive cells down regulate Lz and become the plasmatocytes and rest others become crystal cell precursors (Lebestky et al., 2000; Bataille et al., 2005).

1.7.4 Definitive Hematopoiesis

Definitive Hematopoiesis, which is the second phase of hematopoietic development in *Drosophila,* occurs in a specialized organ called lymph gland which originates during the late embryonic stage and is present throughout the larval stages of fly carrying on the process of hematopoiesis. Lymph gland arises from cardiogenic mesoderm. Cardiogenic mesoderm then gives rise to cardioblasts, pericardial cells and cells of lymph gland lineage. Like mammals, where the blood vascular system is supposed to be derived from a common precursor cells called hemangioblast (Murray, 1932; Choi et al., 1998), there is evidence for the existence of hemangioblast like cells in case of *Drosophila* also ((Mandal et al., 2004).

The cardiogenic mesoderm is specified by the activity of Tin and a GATA factor Pannier (pnr). The Tin expression in this case is maintained by Decapentaplagic (dpp) and Heartless (htl) signaling pathways. Then activity of Wingless (Wg) signaling in the cardiogenic mesoderm is required to promote all the cell type fates. The Notch signaling plays role both during the specification of cardiogenic mesoderm as well as cardioblasts (Mandal *et al*., 2004). During the 12th stage of embryo, the Tin and Pnr expression in the

cardiogenic mesoderm starts restricting to cardioblasts. Then a zinc finger protein Odd-Skipped is expressed in the cell clusters of T1 to A6 segment cardioblasts. The T1 to T3 segment become lymph gland progenitors whereas abdominal clusters form pericardial cells (Mandal *et al*., 2004). Then these lymph gland progenitor cells start expressing Srp to convert it to the true component of hematopoietic system. The lymph gland is restricted to the thoracic segments only because of expression of ultrabithorax (Ubx) in the abdominal segments (Mandal *et al*., 2004).

The lymph gland is divided into three distinct zones which include the cortical zone, medullary zone and posterior signaling centre. The cortical zone is the zone of differentiated blood cells and the medullary zone is the zone of progenitor cells. The posterior signaling center is the region which acts as niche for the maintenance of these two populations.

Investigations have revealed that each zone of mature lymph gland is marked by distinct molecular markers. The cortical zone in the periphery of the lymph gland is populated by mature blood cells which is evident by markers like Hemolectin (Hml, Goto et al., 2003), Peroxidasin (Pxn, Nelson et al., 1994), and Lz (Lebestky et al., 2000). The inner core, medullary zone, expresses distinct cellular markers like *Drosophila* E Cadherin (DE-cadherin), domeless Gal4 (Bourbon et al., 2002) and unpaired3 Gal4 (Agaisse et al., 2003) and ZCL 2897. These cells, like stem cells are quiescent, pluripotent, label retaining and niche dependent. The PSC region expresses the Notch ligand Serrate (Ser, Lebestky et al., 2000) Antennapedia (Mandal et al., 2007), Collier (Crozatier et al., 2004) and can also be visualized by Dorothy-Gal4 (Dot), and Upd3-Gal4 (Jung et al., 2005).

We are basically working on definitive hematopoiesis which takes place in a specialized organ known as the lymph gland.

1.8 The lymph gland

Pluripotent blood cell progenitors proliferate and mature into differentiated hemocytes which comprises of 3 domains.

- Cortical Zone where the differentiated cells reside.
- Medullary Zone harbors the stem like cells. The cells in this zone are slow cycling, label retaining and multipotent.
- Posterior Signaling Centre (PSC) where the stem reside in a microenvironment known as the stem cell niche.
- The PSC not only regulate the fate of stem like cell of the medullary zone but also can regulate a signal generated by distant differentiating cells of the cortical zone. Interestingly, this signal impinges on the self-renewing progenitor to initiate quiescence.

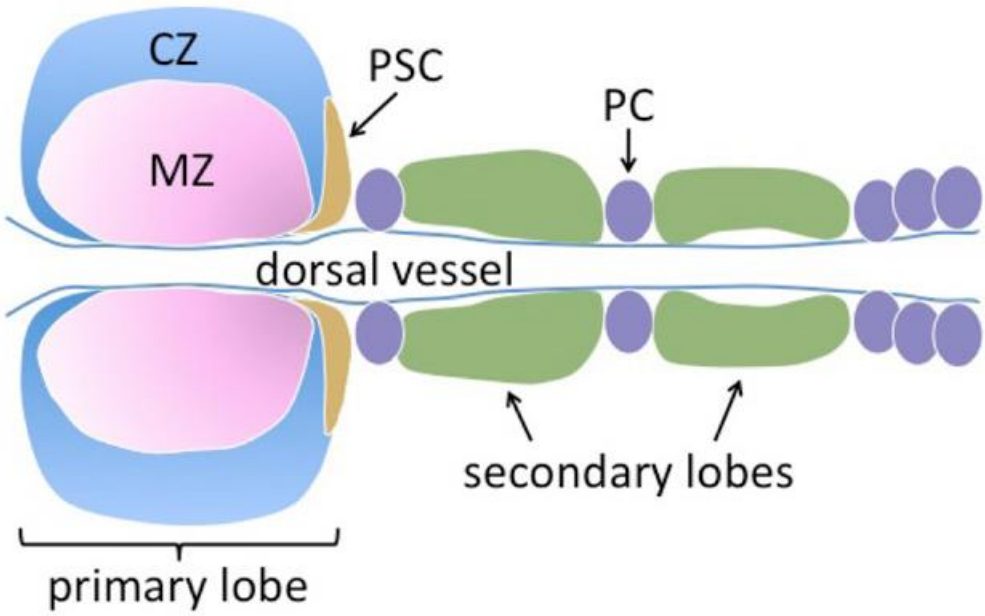


Figure3: Image showing the hematopoietic organ known as the lymph gland in Drosophila larvae. Definitive hematopoiesis takes place in this specialized organ. (Seung-Hye Jung et al, 2005, Development)

1.9 *Drosophila* blood cell types

The blood cells or hemocytes of *Drosophila* are of three types:

- Plasmatocytes
- Crystal cells
- Lamellocytes

These 3 blood cell types can be seen into circulation with plasmatocytes representing more than 90% of the total hemocyte population (Rizki, 1956, 1978). Most of the remaining hemocytes are crystal cells, with only a few or no lamellocytes present under normal growth condition. However, specific stimuli, such as immune challenge by parasitic wasps, induce the differentiation of large numbers of these cells (Rizki and Rizki, 1992; Lanot et al., 2001; Sorrentino et al., 2002).

The main function of plasmatocytes is phagocytosis during various developmental stages including apoptotic clearance of cell debris in metamorphosis and as a key mediator of innate immunity.

In contrast to the phagocytic role of plasmatocytes, the main function of crystal cells appears to be in the process of melanization. This 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 class of enzymes called phenol oxidase (PO).

Lamellocytes are large cells which are generally not seen in normal healthy lymph glands but are induced in special immune challenged conditions like parasitic wasp infestation to encapsulate the large wasp eggs.

2 MATERIAL AND METHODS

2.1 *Drosophila* strains

Different RNAi fly stocks that were used in this study were obtained from Bloomington Stock Centre. The following stocks were used in the study:

Tubgal80^{ts20};antpgal4uasgfp/tm2, UAS Pink1 RNAi, UAS Blw RNAi, UAS ATP synthase gamma RNAi, UAS CG12262 RNAi, UAS Snf4 gamma, UAS Nox1 RNAi.

2.2 Protocol for Immunostaining in larvae (antennapedia and P1 antibodies)

- Larvae were dissected in 1×PBS and fixed in 4% formaldehyde for 30 minutes.
- Washed 3 times in 0.3% PBT for 45 minutes.
- Then the sample was blocked in 10%NGS for 30 minutes and incubated in primary antibody at 4^0C overnight.
- Next day samples are first washed with 0.3%PBT for 45 minutes and again blocked with 10%NGS and then secondary antibody is made in 0.3%PBT and kept overnight at 4^0C.
- Next day the samples are washed with 0.3%PBT then samples are incubated in DAPI solution for 30 minutes then 2 washes of PBS and then mounting is done with vectashield from Vector Laboratories.

2.3 Protocol for DHE staining

- Dissect larvae in Schneider's medium and don't keep fat body, salivary gland, proventriculus in sample and dip the sample properly.
- Incubate in DHE in Schneider's medium, cover it (1µl in 1ml) for 8 minutes.
- Discard the stain and now wash 2 times in Schneider's medium.
- Quick wash in 1×PBS.
- Fix in 4% paraformaldehyde for 12 minutes.
- Wash 3 times in 1×PBS.
- Mount in Vecta shield with Dapi.

2.4 Antibodies used in the study:

Primary antibodies that were used are: **P1 (1:50)** a kind gift from Prof. I Ando, **Antp (1:5, DSHB)**, **pJNK (1:100, Cell Signalling)**

Secondary antibodies that were used are: Anti Mouse Alexa 568, 594 from Molecular Probes and Cy3 from Jackson Laboratories.

2.5 Mounting medium

Vectashield and vectashield with Dapi from Vector laboratories.

Name of antibody	Reagents	Marker	Cell type marked
P1	Nimrod	Cortical zone	Terminally Differentiated plasmatocytes
Integrin	**β**-ps	Medullary zone	lamellocytes
Antp	Hox gene	Hematopoietic Niche	Niche cell

Table 1: Table showing different antibodies and their functions.

I have also used DHE (dye) which is known to mark the superoxides thereby indicating the ROS level.

2.6 Screening strategy

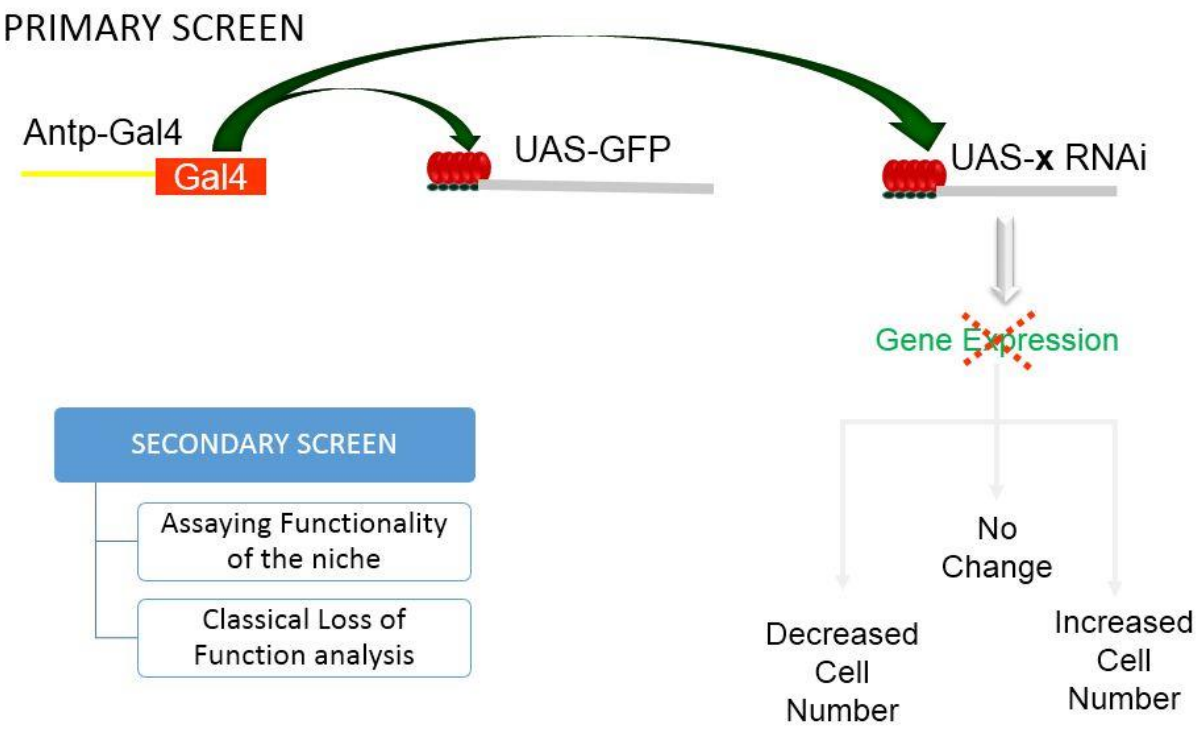


Figure 4: Schematic representation of the strategy followed in the current study.

The experiments were done with the above screening strategy. When the niche specific Antp-Gal4UASGFP virgin fly is crossed to a male fly having UAS X RNAi as the genotype, in the F1 progeny the expression of the gene X is silenced in the niche and in such a genetic background we explored the alteration in the PSC cell number. The phenotypes either decrease in niche cell number and increase in cell number helped us to categorised genes as positive regulators or negative regulators of niche cell number.

2.7 Cross scheme

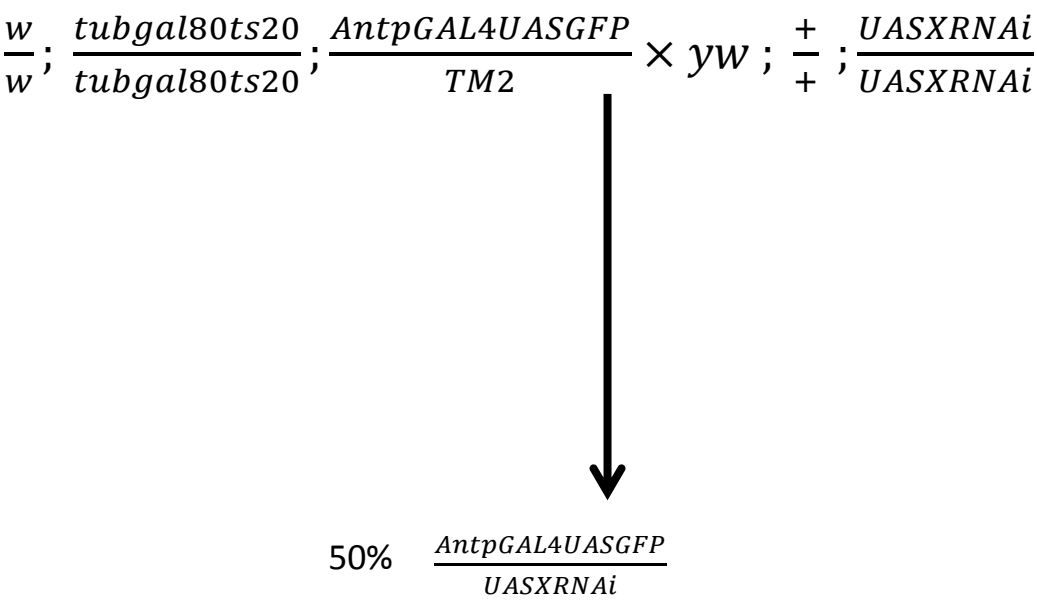


Figure 5: Shown is the cross scheme followed to attenuate the mitochondria by specific RNAi lines with a temporal regulation of a temperature sensitive allele of gal80.

The basic cross scheme that was being followed for the screening of all the 25 lines is depicted above. With this cross scheme the selection was much easier as the larvae with GFP is selected as in the same larvae the RNAi for the specific gene has silenced the expression of that gene of interest. To avoid lethality caused by using a driver like antennapedia gal4, a temporal regulation was employed using $gal80^{ts}$.

3. Results

3.1 RNAi based screening of nuclear encoded mitochondrial genes in PSC

In mature 3rd instar larvae, the niche cell number in the lymph gland is about 40-45 cells. In order to investigate the metabolic regulation of the hematopoietic niche, different nuclear encoded mitochondrial genes were attenuated by RNAi and the effects on the niche or the posterior signalling centre (PSC) were studied. In all cases AntpGal4UASGFP served as a control. The responder line by its self were also analysed and in both cases the niche number tallied.

Figure 6 shows a list of the genes whose RNAi lines were screened for this study and the ones that showed a phenotype.

3.2 Screen harvest

TRIP LINE	Result
TRIP CG8993 RNAI	NO CHANGE
TRIP PDK RNAI	NO CHANGE
TRIP WAL RNAI	NO CHANGE
TRIP PPl RNAI	NO CHANGE
TRIP DNK RNAI	NO CHANGE
TRIP CYPE RNAI	NO CHANGE
TRIP CG12262 RNAI	INCREASED NICHE
TRIP ND42 RNAI	NO CHANGE
TRIP DEBCl RNAI	NO CHANGE
TRIP CPTI RNAI	NO CHANGE
TRIP BLW RNAI	INCREASED NICHE
TRIP CG4389 RNAI	NO CHANGE
TRIP CG8728	NO CHANGE
TRIP Nd42 RNAI	NO CHANGE

TRIP LINE	Result
TRIP ATP SYN GAMMA RNAI	INCREASED NICHE
TRIP SOD2 RNAI	NO CHANGE
TRIP SOD2 RNAI	NO CHANGE
TRIP ND23 RNAI	NO CHANGE
TRIP ND42 RNAI	NO CHANGE
TRIP PDSW RNAI	NO CHANGE
TRIP COVA RNAI	NO CHANGE
TRIP ND75 RNAI	NO CHANGE
TRIP ND75 RNAI(3)	NO CHANGE
TRIP Pink1 RNAI	INCREASED NICHE
TRIP Park RNAI	NO CHANGE

Figure 6: List of screened RNAi lines. The ones marked in red resulted in increased niche.

Out of these 25 RNAi lines, the 4 lines marked in red led to increase in niche cell number upon driving it with niche driver AntpGal4. The candidate genes that resulted in increased niche cell number were selected for further analysis. Therefore, the following genotypes were analysed in the current study: Antp>Blw RNAi, Antp>Pink1 RNAi, Antp>ATP synthase gamma RNAi and Antp>CG12262 RNAi.

The statistical analysis of the niche cell number increase is demonstrated in Figure 7.

As mentioned in the previous section, 4 out of 25 lines in the nuclear encoded mitochondrial screen resulted in an observable phenotype. Interestingly, all of these turned out be negative regulator of niche cell number, i.e., when the function of these genes are attenuated, the niche cell number increases tremendously. Further analysis of the niche cell number in all these cases was carried out and it was found that there was approximately 1.73-2.3 fold increase as compared to the normal niche cell count (~45 cells).

3.3 Statistical analysis of the niche cell number increase

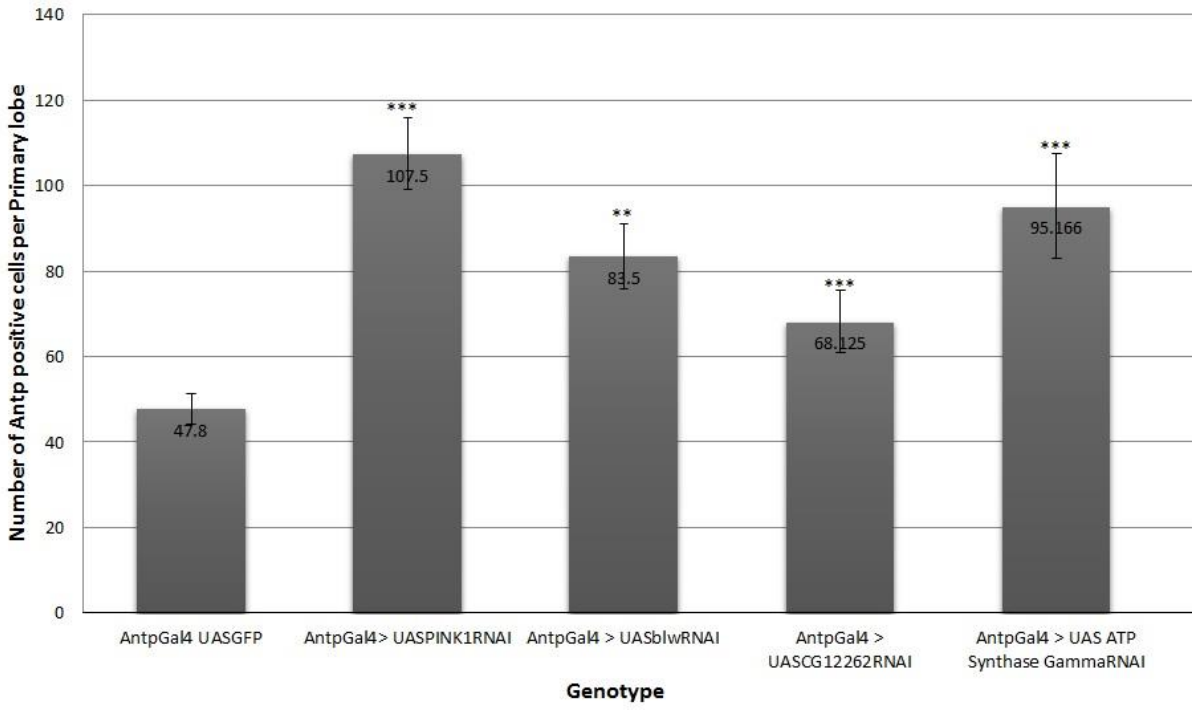


Figure7: Increase in the number of cells in the niche by attenuating the respective nuclear encoded mitochondrial genes.

Genotype	P-value
Pink1 RNAi	0.0015
Blw RNAi	0.0595
CG12262 RNAi	0.0076
ATP Synthase Gamma RNAi	0.0086

Table 2: Table showing p-value for the statistical significance of the data.

3.4 Functional Analysis of the increased niche

The hematopoietic niche or PSC maintains the stem like population/precursors housed in the medullary zone. In normal developmental scenario, the number of niche cells is 40-45 which regulates the cells of the medullary zone and maintains the balance between differentiation and progenitor maintenance. In cases of increased niche cell number by mitochondrial gene knockdown we were interested to know whether this increase in niche size has affected its functionality. To check how the hematopoietic system as a whole is altered in such a case, the next assay was to check whether the bigger niche is able to maintain the progenitor population. Simultaneously, for assaying the differentiation in the lymph gland, P_1 immunostaining marking the differentiated plasmatocyte population was carried out onto all of these three genotypes i.e., Antp>ATP synthase gamma RNAi, Antp>Blw RNAi, Antp>Pink1 RNAi with respect to Antp>GFP (control) shown in figure10.

Interestingly, increase in differentiated plasmatocytes in all these genotypes implies that even though the niche is huge it is unable to function properly. To probe whether due to this manipulation the progenitors are compromised or not, we then used a blood cell progenitor marker Integrin. This marker (unpublished) was identified by doctoral student Satish Tiwari in the laboratory (figure 9). Integrin antibody was earlier reported as a marker for Lamellocytes which is rarely seen in the lymph gland. In control lymph gland of Antp>GFP Integrin is thus seen marking the cell membranes of the medullary zone cells and there is no lamellocyte induction as expected in a healthy individual.

Interestingly, in genotypes where ATP synthase gamma and Bellwether (Blw) is knocked off from the niche, there is a drastic induction of lamellocytes. Contrary to the above cases, in Pink1 loss from the niche does not evoke similar response. We hypothesize that in case of antp>Blw RNAi and antp>ATP syn gamma RNAi the lymph gland is mounting a response similar to an immune challenged condition.

3.5 Induction of ROS levels in the niche

Previous reports have shown that a minimum threshold of Reactive Oxygen Species (ROS) is necessary for the proper functioning of the progenitors in the lymph gland (Owusu-Ansah & Utpal Banerjee 2009). The ROS is never detected in a healthy hematopoietic niche. We wondered whether by attenuating the mitochondria what effect it has on the levels of ROS in the niche.

Upon DHE staining which marks the superoxide radicals, we detected that in all genotypes i.e., Antp>ATP synthase gamma RNAi, Antp>Blw RNAi, Antp>Pink1 RNAi with respect to Antp>GFP (control) the ROS levels were very high in the niche. Although the increase in ROS levels in Antp>Pink1 RNAi was not very much high compared to the other genotypes, but it was high with respect to the control. As shown in figure 11.

3.6 Discussion and Future Aspects

The intriguing possibility of metabolic regulation of niche cell number and functionality thereby regulating the cell fate of blood cell progenitor motivated us to undertake the RNAi based screening of nuclear encoded mitochondrial genes in the PSC.

Out of the 25 genes that were part of this screening, four of the candidate genes led to increase in niche cell number, namely Pink1RNAi, Bellwether RNAi, CG12262 RNAi and ATP Synthase Gamma RNAi.

Pink1 is a member of the complex1 of the electron transport chain. Pink1 acts as a scout to probe for damaged mitochondria in conjugation with parkin. Without Pink1, parkin cannot efficiently localize to damaged mitochondria, while an over-expression of Pink1 causes parkin to localize to even healthy mitochondria. It will be interesting to probe in

how the accumulation of damaged mitochondria in Pink1 knockdown in niche is leading to an increase in niche cell number.

Bellwether: it is a protein coding gene that helps in ATP binding and proton transporting ATP synthase activity. It is a member of the complex5 of the electron transport chain

ATP synthase gamma: Gamma subunit of ATP synthase f1 complex forms the central shaft that connects the f0 rotary motor to the f1 catalytic core. It is a member of the complex 5 of the electron transport chain. Both Blw and ATP-Syn gamma forms the part of ATP Synthase Complex and attenuating their expression in PSC might be affecting the ATP levels of PSC cells which is leading to an deregulation in niche cell number, and affecting the niche functionality.

Very high levels of ROS are deleterious for the cells but a minimum threshold of ROS is required for the progenitor cells to start differentiation. Previous works have shown (Owusu-Ansah & Utpal Banerjee 2009) that, by the third instar, progenitor population in wild type lymph gland medullary zone have high levels of ROS compared to their neighbouring differentiated progeny which is necessary for the differentiation of blood cell progenitors. To measure the ROS levels in the lymph gland, we did DHE staining which showed high levels of ROS in the niche in mitochondrial loss of function which might be affecting the niche cell functionality.

How this metabolic insult translates into a signalling pathway is a matter of great interest and we wish to investigate in the future. In a brief duration of my master thesis, an attempt was made to understand the downstream immediate effects after insulting the metabolic aspect of the niche by attenuating mitochondrial genes.

There are various reports which suggest that the ROS generation evokes downstream stress signals such as JNK, P38MAPK and/or NFκB (Owusu-Ansah & Utpal Banerjee 2009). The reporter activity of these stress sensors is to be probed in the candidate genes which are giving us an altered niche. If ROS is evoking one or more of these stress sensor pathways that is affecting the niche cell number as well as functionality, then down regulation of the increased ROS levels should rescue the ROS driven phenotypes. To address this I am bringing following genetic combinations together to genetically alter the ROS levels in the niche.

3.7 Reactive oxygen species generation and disposal in the mitochondria.

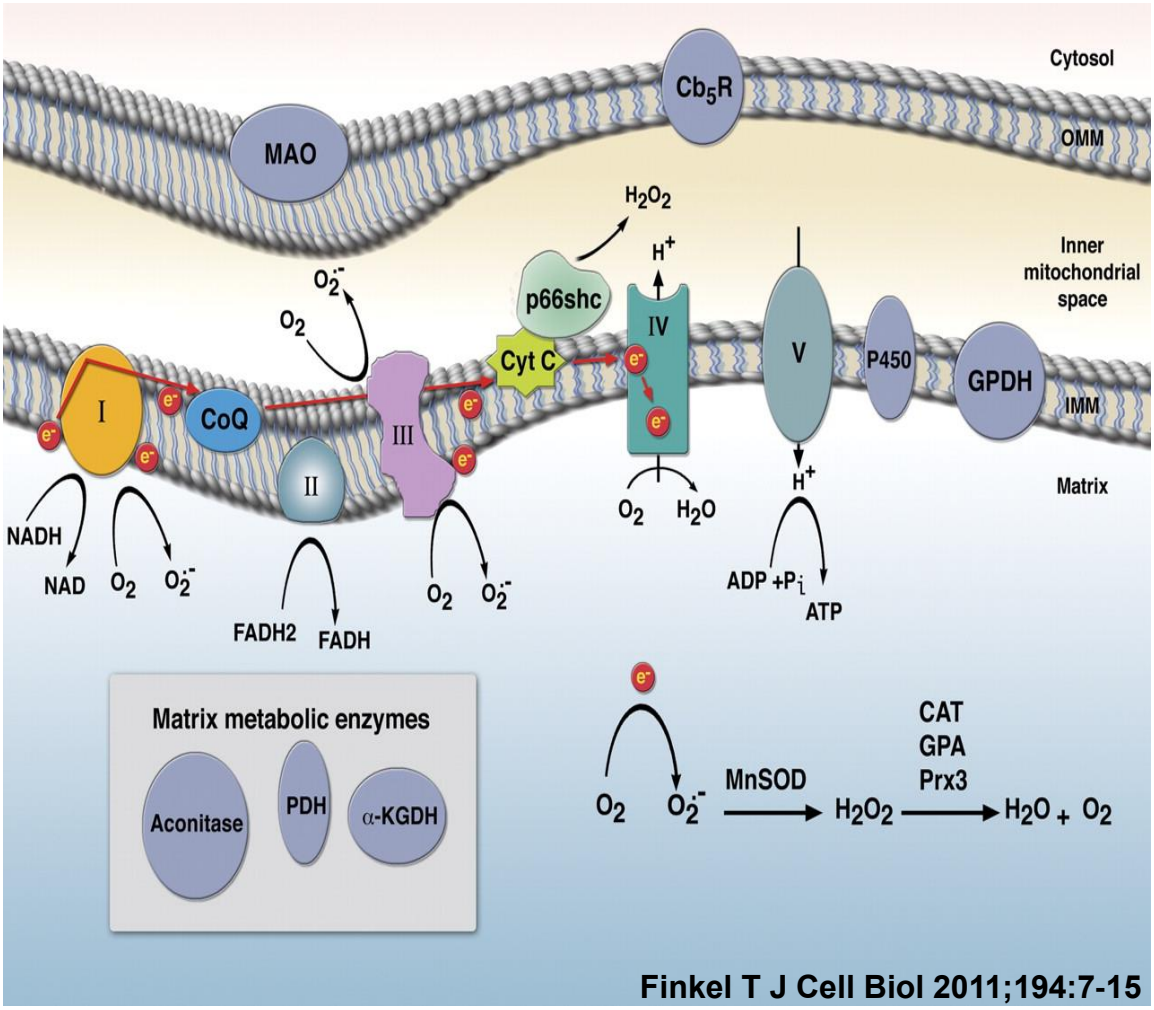


Figure 8: Figure above shows ROS generation and disposal in mitochondria.

Reactive Oxygen Species (ROS) are side products of electron transport in the mitochondrial respiratory chain, the principal component of energy transformation in mitochondria. ROS generation starts with the formation of a superoxide radical (O^-_2) as a result of interaction between molecular oxygen and free radicals.

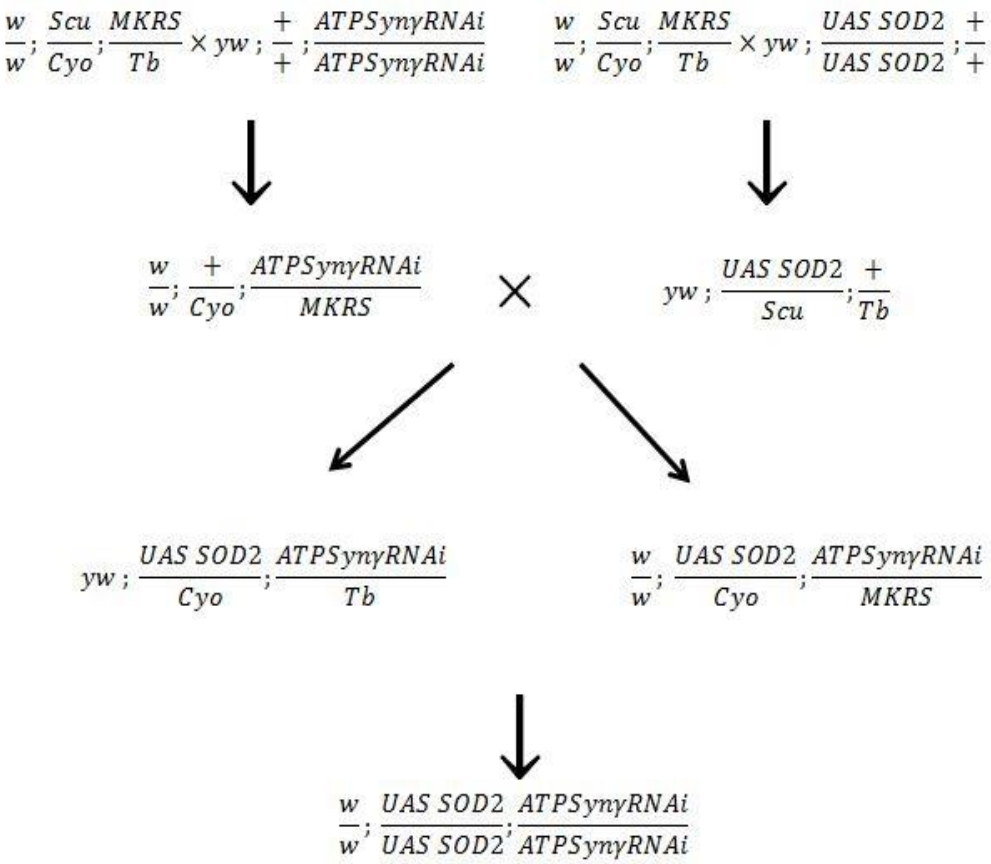
$$O_2+Q^-\rightarrow O_2^-+Q \quad Q\text{: semiquinone}$$

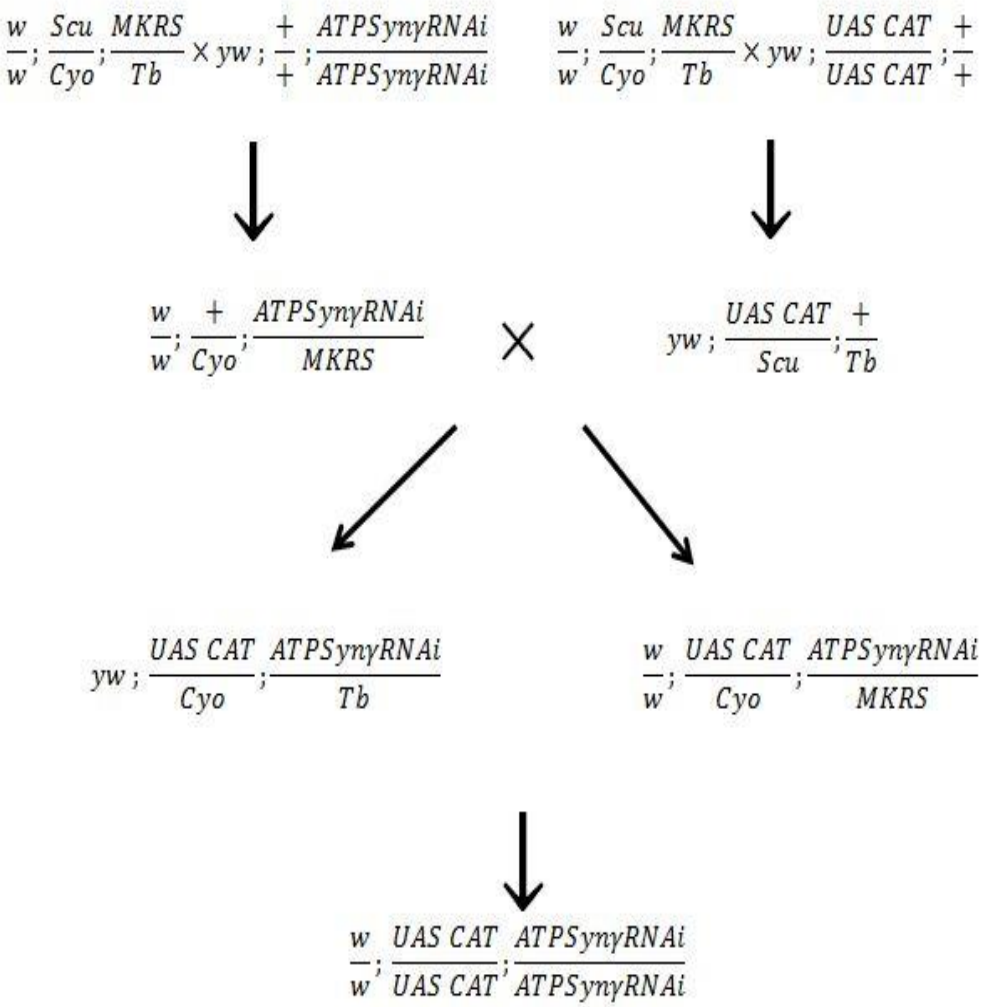
This extremely active compound can be deactivated in cells, mainly through superoxide dismutase. H_2O_2 formed in this process can interact with various intracellular compounds to produce ROS. ROS production serves as a metabolic signal. When released in excess in stress conditions such as hypoxia, ROS can directly damage cells.

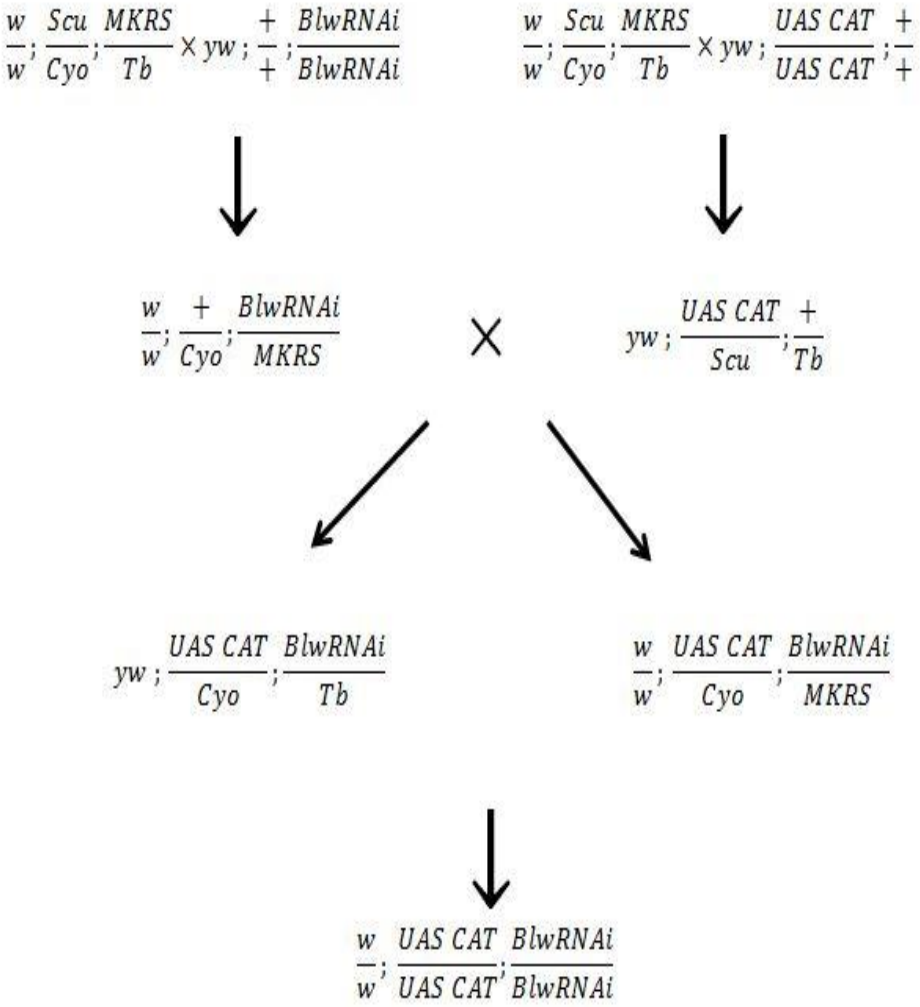
3.8 Cross scheme used to make new lines for rescuing ROS levels in the hematopoietic niche:

As mentioned in Fig 8 the ROS generated can be scavenged by either SOD2 (Super oxidase dismutase2) or by Cat (catalase). Therefore we decided to genetically bring the construct in background of these above mutation. This will enable us to scavenge ROS generated in the niche due to the perturbation of mitochondria.

If ROS is responsible for such phenotype than it should rescue the phenotype on such manipulation.


$\frac{w}{w}; \frac{Scu}{Cyo}; \frac{MKRS}{Tb} \times yw; \frac{+}{+}; \frac{ATPSynγRNAi}{ATPSynγRNAi}$
$\frac{w}{w}; \frac{Scu}{Cyo}; \frac{MKRS}{Tb} \times yw; \frac{UAS\ SOD2}{UAS\ SOD2}; \frac{+}{+}$
$\frac{w}{w}; \frac{+}{Cyo}; \frac{ATPSynγRNAi}{MKRS}$
×
$yw; \frac{UAS\ SOD2}{Scu}; \frac{+}{Tb}$
$yw; \frac{UAS\ SOD2}{Cyo}; \frac{ATPSynγRNAi}{Tb}$
$\frac{w}{w}; \frac{UAS\ SOD2}{Cyo}; \frac{ATPSynγRNAi}{MKRS}$
$\frac{w}{w}; \frac{UAS\ SOD2}{UAS\ SOD2}; \frac{ATPSynγRNAi}{ATPSynγRNAi}$


w/w; Scu/Cyo; MKRS/Tb × yw; +/+; ATPSynγRNAi/ATPSynγRNAi
w/w; Scu/Cyo; MKRS/Tb × yw; UAS CAT/UAS CAT; +/+
w/w; +/Cyo; ATPSynγRNAi/MKRS
×
yw; UAS CAT/Scu; +/Tb
yw; UAS CAT/Cyo; ATPSynγRNAi/Tb
w/w; UAS CAT/Cyo; ATPSynγRNAi/MKRS
w/w; UAS CAT/UAS CAT; ATPSynγRNAi/ATPSynγRNAi


w/w; Scu/Cyo; MKRS/Tb × yw; +/+; BlwRNAi/BlwRNAi
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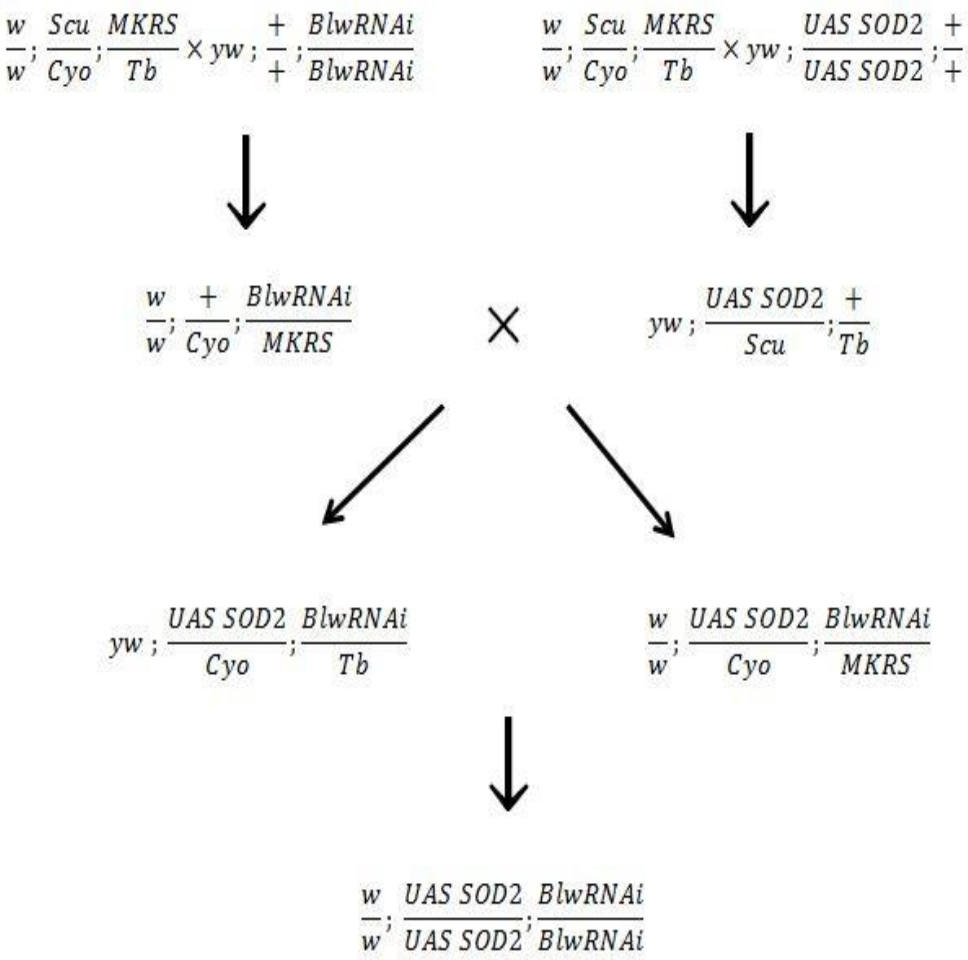
w/w; +/Cyo; BlwRNAi/MKRS
×
yw; UAS CAT/Scu; +/Tb
yw; UAS CAT/Cyo; BlwRNAi/Tb
w/w; UAS CAT/Cyo; BlwRNAi/MKRS
w/w; UAS CAT/UAS CAT; BlwRNAi/BlwRNAi


$\frac{w}{w}; \frac{Scu}{Cyo}; \frac{MKRS}{Tb} \times yw; \frac{+}{+}; \frac{BlwRNAi}{BlwRNAi}$
$\frac{w}{w}; \frac{Scu}{Cyo}; \frac{MKRS}{Tb} \times yw; \frac{UAS\ SOD2}{UAS\ SOD2}; \frac{+}{+}$
$\frac{w}{w}; \frac{+}{Cyo}; \frac{BlwRNAi}{MKRS}$
$\times$
$yw; \frac{UAS\ SOD2}{Scu}; \frac{+}{Tb}$
$yw; \frac{UAS\ SOD2}{Cyo}; \frac{BlwRNAi}{Tb}$
$\frac{w}{w}; \frac{UAS\ SOD2}{Cyo}; \frac{BlwRNAi}{MKRS}$
$\frac{w}{w}; \frac{UAS\ SOD2}{UAS\ SOD2}; \frac{BlwRNAi}{BlwRNAi}$

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3.10 Figures

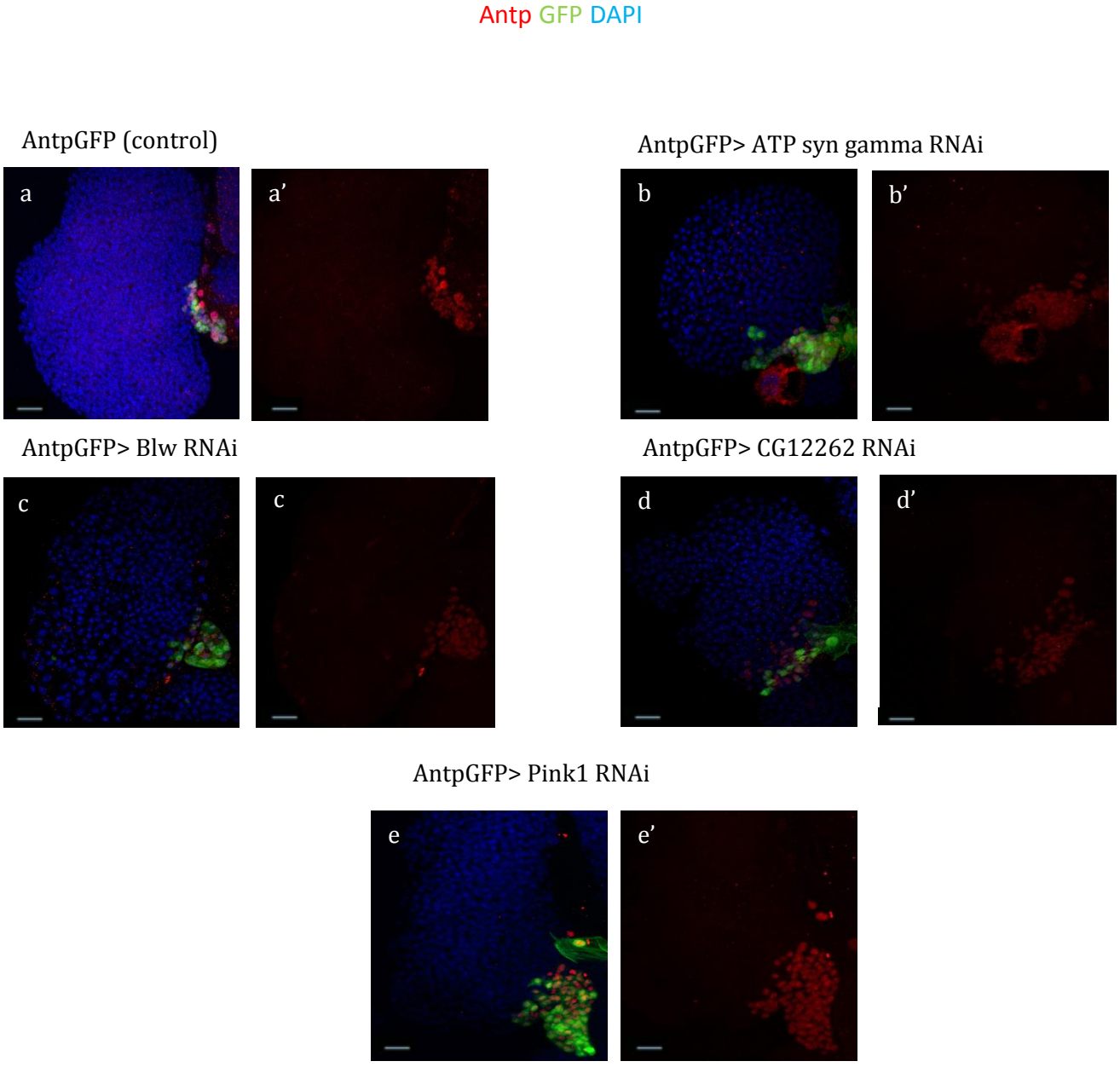

Fig 9: Antp immunostaining to positively mark the PSC cells(shown in red). Generally there are 40-45 cells in the PSC in control(a) antp>GFP when driving specific RNAi in (b) antp>ATP syn gamma RNAi, (c) antp>Blw RNAi, (d) antp>CG12262 RNAi and (e) antp>Pink1 RNAi with respect to control the increase in PSC cell number is 1.75 to 2.3 folds. Green marks the AntpGFP population and the DAPI in blue marks the nucleus.

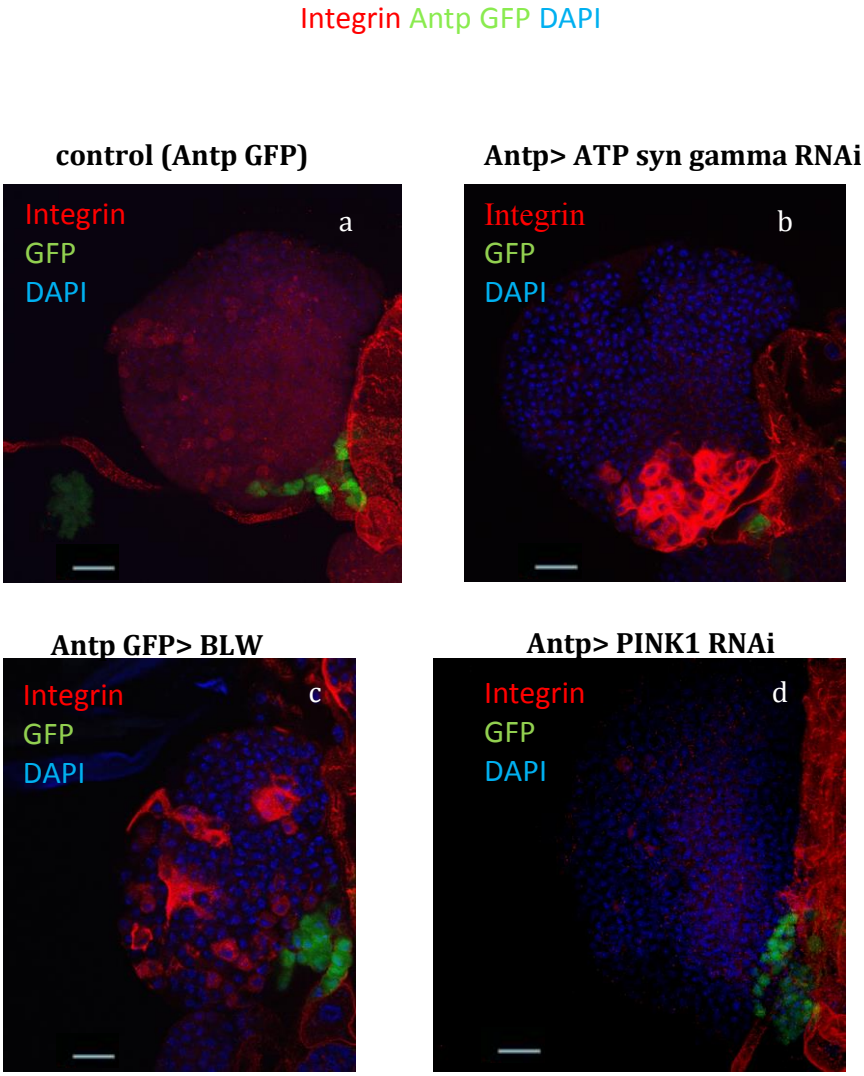

Fig 10: In control lymph gland (a) antp>gfp there is no lamellocyte induction. In (b) antp>ATP syn gamma RNAi and (c) antp>BLW RNAi there is lamellocyte induction in the lymph gland and in (d) whereas antp>pink1RNAi there is no lamellocyte induction.

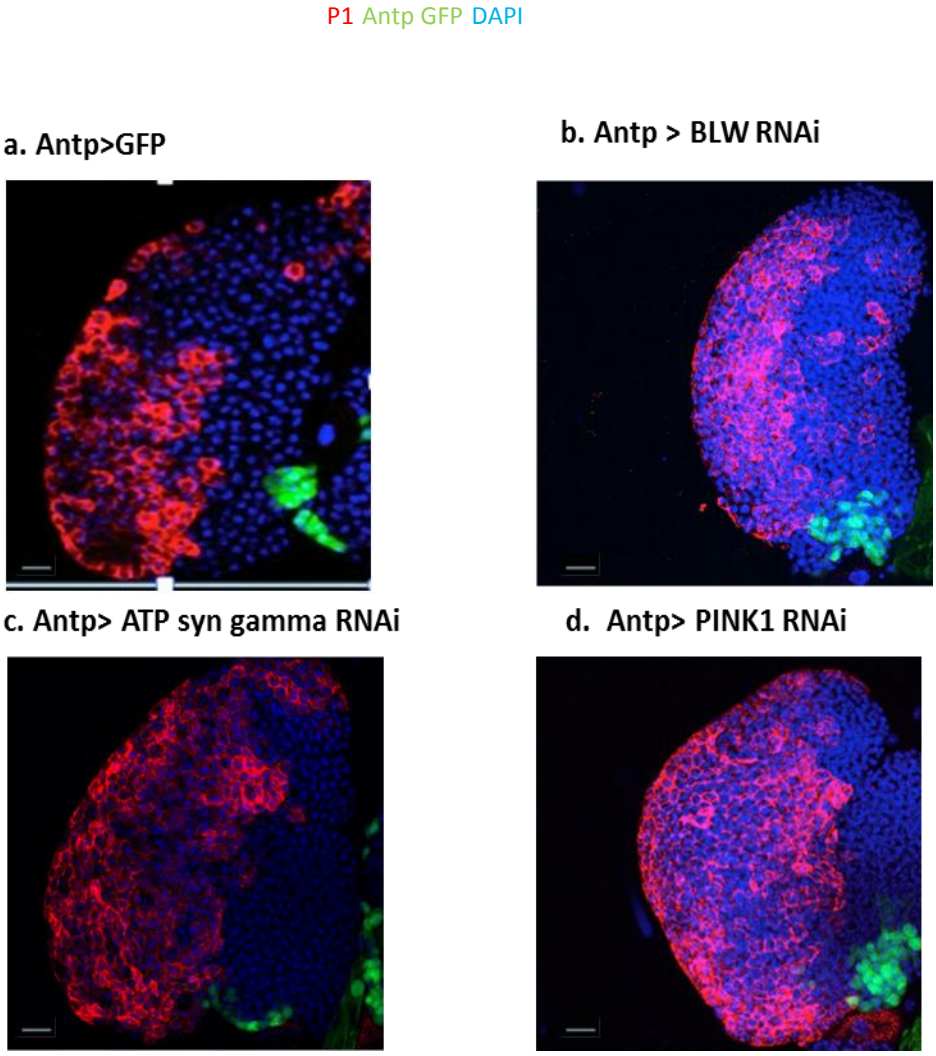

Fig 11:The loss of function of nuclear encoded mitochondrial genes from the hematopoietic niche affects the cell number and also the functionality of the niche. The progenitor cells are unable to maintain themselves and there is increment of mature hemocytes in the lymph gland. As shown in red by P1 immunostaining, in control image (a)antp>GFP differentiated plasmatocytes are mainly lying at the periphery whereas in other genotypes the differentiation is more with respect to the control in images (b)antp>blw RNAi,(c)antp>ATP syn gamma RNAi and (d)antp>pink1 RNAi.

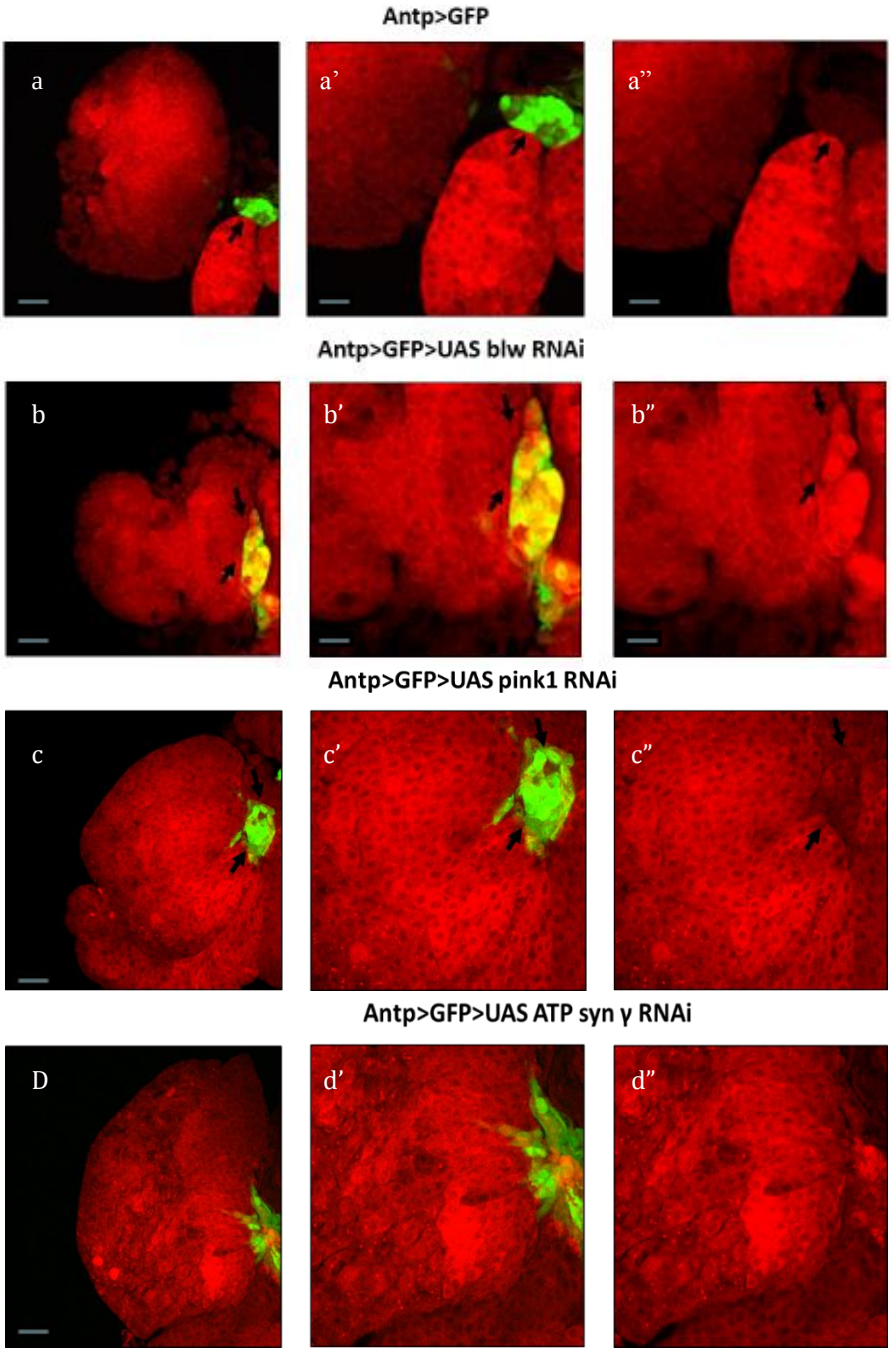

Fig 12:Normally ROS levels are barely detectable in control (a) antp>GFP lymph gland niche but upon driving specific RNAiin figure(b) antp>uas blw RNAi,(c) antp>uas ATP syn gamma RNAi and(d) antp>uas pink1 RNAi the ROS levels are highly up regulated. In (d) pink1 RNAi the increase in ROS level is not that high but the level of ROS in this genotype was also greater than the control. a', a'', b', b'', c', c'', d', d'' all are zoomed in view of the niche. Green color marks antp>GFP and marking the niche.

3.11 Conclusion

Disruption of nuclear encoded mitochondrial genes within the niche by RNAi resulted cell proliferation even with the compromised ATP level. Though the niche size increased but the functionality was hugely affected. This kind of insult has increased the ROS level in the niche. Whether this deregulated ROS level impinged onto combinatorial signaling or not would be a next question.