Studying the signaling and metabolic factors involved in hemocyte progenitor maintenance and differentiation in *Drosophila melanogaster*

A Thesis

Submitted
By

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Dedicated to my Family and human curiosity

Declaration

The work presented in this thesis entitled "Studying the signaling and metabolic factors involved in hemocyte progenitor maintenance and differentiation in *Drosophila melanogaster*" has been carried out by me under the supervision of Dr. Lolitika Mandal at the Indian Institute of Science Education and Research (IISER) Mohali. This work has not been submitted in part or full for a degree, a diploma, or a fellowship to any 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 bona fide record of original work done by me and all sources listed within have been detailed in the references.

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

Date: Dr. Lolitika Mandal

Place: (Supervisor)

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Synopsis

Introduction:

One of the most intriguing questions in developmental and stem cell biology is how cells adopt their fate. A single cell zygote gives way to the whole metazoan body plan by orchestrating different cellular fates. Studies in past decades, both *in vivo* and *ex vivo* in multiple model systems have tried to unravel the factors implicated in cell fate specification. A battery of cell intrinsic and extrinsic factors has been implicated in regulating cell fate specification in normal development and in pathophysiological conditions.

Studies in multiple model systems have elucidated that the factors regulating a particular developmental process in normal metazoan development, say neurogenesis is generally conserved across taxa. However, the cohort or cocktail of factors regulating different processes of metazoan development might not be similar. For example, the factors controlling the fate specification of a neuron and a blood cell might be different or performing very different roles. Therefore, it is imperative to study the cell fate specification in different developmental processes and contexts.

Large timeline of the studies to unravel the factors controlling cell fate specification have been devoted to cellular signaling pathways, transcription factors and cofactors. Studies in only last couple of years have elucidated that apart from the aforesaid factors, cellular metabolism and metabolites can be one of the major factors controlling cellular fate. Therefore, metabolic state of a cell is not secondary consequence of its cellular fate but might be the governing factor regulating it. With past studies hinting on the collaboration of the cellular signaling pathways and metabolic state of the cell to govern the cellular fate, this study was conceived with an unbiased genome wide RNAi screen to unravel the factors dedicated in cell fate specification in normal development.

For our studies, we employed the genetically tractable organism *Drosophila melanogaster* as the model and chose *Drosophila* hematopoiesis as the developmental process to study cell fate specification. *In vivo* genetic analysis in *Drosophila* has divulged the stem cell properties in their resident microenvironment. *Drosophila* blood cell or hemocyte development in larval stages take place in an organ called lymph gland which starts forming in late embryogenesis. Lymph gland houses hemocyte progenitors, differentiated blood cells and the dedicated signaling cells or niche cells which provide the

microenvironment for hematopoiesis. Early instar lymph gland houses the self-renewing hemocyte progenitors and the niche cells. Third instar mature lymph gland houses the differentiated blood cells in periphery, in a region termed as cortical zone (CZ). The inner core of the late third instar lymph gland termed as medullary zone (MZ) houses hemocyte progenitors. The hemocyte progenitors found in MZ are akin to the vertebrate common myeloid progenitors (CMP). They are quiescent, have high levels of ROS, lack differentiation markers, are multipotent, and give rise to all *Drosophila* blood lineages. MZ cells are maintained by the signals from the niche cells also known as cells of Posterior Signaling Centre (PSC).

Objectives:

1. To carry out a genome wide RNAi based knock down study in hemocyte progenitors of *Drosophila melanogaster* larval stages with an aim to find out the candidate genes involved in progenitor maintenance.

2. Extensive genetic characterization of the few candidate genes whose role in hematopoiesis in light of hemocyte progenitor maintenance hasn't been well implicated.

Outcome of this study:

Genome wide RNAi screen to identify regulators of hemocyte progenitor maintenance

To find out the regulators of hemocyte progenitor maintenance, an unbiased genome wide RNAi screen was performed to pick out the candidate genes regulating this aspect of hematopoiesis. Around 120 UAS RNAi constructs were driven using progenitor based Domeless Gal4 having a temperature sensitive gal80^{ts} allele. This provided for a spatio temporal knockdown of 120 genes in hemocyte progenitors and also allowed to bypass any detrimental effect of the knockdown in early developmental stages. The status of the progenitors (MZ) was assessed by UAS GFP construct and the status of the differentiated zone was assessed by immunostaining for P1 nimrod which marks differentiated plasmatocytes. The relative increase or decrease in area of differentiated cells (CZ) compared to progenitor cells (MZ) in case of RNAi knockdown of a gene in progenitor established the outcome of the screen, based on which a gene can be categorized into one of three : (a) positive regulator ; (b) negative regulator ; (c) no changer of hemocyte progenitor

maintenance. The positive and negative regulators of progenitor maintenance were further grouped into major cell signaling, cell adhesion and metabolism subsets/cohorts. We picked up candidates of (A) metabolism cohort (B) cell adhesion cohort of integrin signaling which are negative regulators and (C) one major cell signaling Hippo signaling as positive regulator of hemocyte progenitor maintenance for further in depth analysis.

(A) Concomitant shift from glycolysis to Oxidative Phosphorylation and Fatty Acid Beta oxidation regulates hemocyte progenitor differentiation.

When we looked in the genetic screen, RNAi knockdown of three genes *Pdh-e1(Pyruvate dehydrogenase-e1 subunit)*, *Pdh-e2(Pyruvate dehydrogenase-e2 subunit)* and *Mpc1(Mitochondrial pyruvate carrier1)* involved in glycolysis to oxidative phosphorylation switch by pyruvate integration into mitochondrial TCA cycle, showed decrease in differentiation which means they are negative regulators of progenitor maintenance. To assess the metabolic state of the hemocyte progenitors, we looked at their mitochondrial architecture. Self renewing progenitors of second instar larval stage showed punctae and scant mitochondria while the quiescent progenitors of third late instar larval stage showed a reticulate and dense network of mitochondria, indicative of a glycolysis like state in early instar and a shift to oxidative phosphorylation in late third instar larval hemocyte progenitors. Expression analysis of genes involved in glycolysis and functional glucose uptake assay further validated high glycolysis/glucose metabolism in early self-renewing progenitors. This implies the conducive role of glycolysis to oxidative phosphorylation switch in regulation of progenitor differentiation.

When we looked at other metabolic genes which were part of the genetic screen, we came across two genes, *Whd* which is *Drosophila* CPT1 ortholog and *dHnf4*. Both of these genes are implicated in fatty acid beta oxidation. Hemocyte progenitor specific RNAi mediated knockdown of both the genes resulted in reduction of differentiation. Mutant analysis of both the genes further validated the loss of differentiation and higher maintenance of hemocyte progenitors. Mutant analysis of both the genes of beta-oxidation showed loss of quiescence in hemocyte progenitors as more of them were incorporating EdU. As self renewal of hemocyte progenitors is attributed to higher glycolysis rate, we wondered whether in fat oxidation mutants, glycolysis/glucose metabolism is upregulated. Glucose uptake assay revealed that indeed loss of fat oxidation has led the progenitors to switch onto higher glucose metabolism

and glucose uptake inhibition by glycolysis inhibitor 2-DG rescues the loss of quiescence seen in beta oxidation mutant progenitors. Concomitant increase in beta-oxidation by administering L-carnitine leads to advent of quiescence in self renewing early instar progenitors and precautious differentiation of progenitors implicating that beta oxidation of fatty acids is imperative to quiescence and differentiation of hemocyte progenitors.

(B) βPS/αPS1 integrin mediated cell-ECM adhesion regulates hemocyte progenitor differentiation.

In the genetic screen, RNAi mediated knockdown of *Beta-PS, Alpha-PS1*and *Talin* lead to diminished differentiation of hemocyte progenitors. Extensive expression analysis of integrins in lymph gland revealed their spatio temporal expression pattern. Early larval stage progenitors of second instar express *Beta*-ν/αPS2 pair of integrin heterodimer whereas; third instar hemocyte progenitors express *Beta*PS-*Alpha*PS1 integrins. Loss of differentiation in either RNAi mediated knock down of *Beta*PS or *Alpha*PS1 implicates cell-ECM adhesion being imperative for hemocyte progenitor differentiation. Apart from loss of differentiation, βPS/αPS1 integrin knockdown results in loss of quiescence of hemocyte progenitors. To implicate the mechanistic reason for loss of differentiation in integrin knockdown, we investigated the previously reported differentiation signal, Reactive Oxygen Species (ROS) in hemocyte progenitors. The level of ROS in hemocyte progenitors was very less compared to similar aged control lymph glands. Thus, ROS production is hindered in integrin knockdown in hemocyte progenitors leading to diminished differentiation. As we have seen that in quiescent hemocyte progenitors, intricate reticular mitochondrial architecture is there suggestive of oxidative phosphorylation dependent metabolic state which is known to generate cellular ROS. Integrin has been known to implicate mitochondria in generation of cellular ROS. Therefore, we looked at the mitochondrial architecture/morphology in βPS-αPS1 knockdown scenario in which mitochondrial network is less reticular and amount of mitochondrial load per cell is also less compared to control hemocyte progenitors. This might amount to loss of differentiation in integrin knockdown scenario in hemocyte progenitors.

In *Drosophila*, integrins have been implicated in cell-ECM adhesion. In integrin knockdown we looked at the cell-cell adhesion and ECM status. The expression of DE-Cadherin which mediates cell-cell adhesion is increased in integrin knockdown. We

looked at the expression of ECM component perlecan in integrin knockdown from hemocyte progenitor around which perlecan expression was increased. Thus, cell-ECM adhesion disruption by knockdown of integrins alters the fate of the progenitors by affecting the mitochondria, cell-cell adhesion and ECM components.

(C) Hippo signaling orchestrates the homeostasis of proliferation, survival and differentiation of blood progenitors to regulate hematopoiesis in *Drosophila*

When we analysed the positive regulators of hemocyte progenitor maintenance, we came across many players of Hippo signaling. Though, Hippo signaling has previously been implicated in *Drosophila* hematopoiesis, its hemocyte progenitor specific role hasn't been explored. Hippo signaling is characterized by a set of upstream kinases which phosphorylates and keep its transcriptional activator *Yorkie(yki)* from entering inside nucleus. *Yorkie*, once inside the nucleus controls plethora of cellular events. When we knocked down *yki* from hemocyte progenitors, their survival was compromised as we see induction of cell death and consequently, whole primary lobe of lymph gland is very small in size. Overexpression of *yki* achieved by RNAi mediated knock down of hippo signaling kinases *warts*, *expanded*, *merlin* and driving UAS *yki* construct leads to precautious differentiation of hemocyte progenitors. Expression analysis of transcriptional reporter of active hippo signaling *Diap1* GFP revealed that in self renewing progenitors *yki* mediated transcription is high whereas in quiescent progenitors *yki* mediated transcription is low, suggestive of hippo signaling kinases restricting yki in cytoplasm thereby mediating progenitor quiescence.

Drosophila adult fly has been shown to possess active sites of hematopoiesis termed as 'hematopoietic hubs' in their dorsal abdominal compartments. These hematopoietic hubs have been shown to be formed of *de novo* hematopoiesis as well as migration from circulation of fly. The signaling network that builds and maintains this hematopoietic hub is yet to be characterized. We looked at the status of hippo signaling in the hematopietic hub cells by looking at hippo signaling transcriptional reporter *Diap1* GFP expression in them. The expression of *Diap1* GFP suggested that *yki* activity is high in these cells. When we tried to disrupt hippo signaling by RNAi mediated knockdown of *warts* kinase in *hemolectin* positive bipotent precursor, we could see that the total size of hematopoietic hub is compromised and overall number of blood cells is very less. In *warts* RNAi knockdown we observe that actin cytoskeleton related components are upregulated and

PVR (PDGF-VEGF Receptor) downregulated. Both of these observation account for defects in migration and survival. When we looked at the migration pattern of the hemocyte in *warts* knockdown, we could see that hemocyte show abnormal migration pattern akin to what has been reported in *enabled* (*Enabled* is the sole *Drosophila* member of the Ena/VASP family) overexpression scenario, i.e., migration speed of hemocytes is higher to control hemocytes and hemocytes have also been shown to throw more fillopodia and less lamellopodia. These could amount to migration defects and hence less number of blood cells in hematopoietic hubs in warts knockdown scenario.

Conclusion

Biologists around the world are increasingly getting interested in the role of metabolism along with cellular signaling in shaping cell fate decisions in normal development and in pathophysiological conditions. Our findings not only find new players involved in blood progenitor maintenance but also uncover a new aspect of requirement of metabolism in progenitor quiescence and differentiation.

Not very long ago, it was the general belief of the field that cells derive their energy from glucose and only in times of starvation or fasting do we see the utilization of other energy sources such as fat. But, reports in last couple of years have challenged this belief with the increasing evidences pointing out that stem cells and progenitor cells preferably choose fatty acid metabolism to fuel themselves not only in terms of energy production but utilization of intermediary metabolites for various cellular processes.

To our knowledge, this is the first report which implicates the requirement of OXPHOS and beta oxidation of fatty acids in hematopoietic differentiation. In the knockdown, null mutants as well as pharmacological inhibition of beta oxidation, loss of differentiation and loss of quiescence seen points out at the preferential utilization of fat metabolism in late instar blood progenitors for setting up quiescence and differentiation. It is intriguing to note that early instar progenitors when self-renewing have high glucose utilization whereas, glucose utilization in late quiescent and differentiating progenitors is very less, at the same time when we knock down fat metabolism components, quiescence is compromised and differentiation is disrupted. This suggests at the possibility that there is a switch from glucose to fat metabolism when hemocyte progenitors move from self-renewal to quiescence and then to differentiation.

This study also corroborates cell adhesion complex mediated control of cellular fate via regulating metabolism. Integrins control the mitochondrial function and biogenesis thus contributes to differentiation of the hemocyte progenitors. Integrin mediates the production of ROS, which has been shown to induce differentiation in hemocyte progenitors by instigating JNK-Foxo signaling cascade. This study also points out the requirement of Hippo signaling effector yorkie in hemocyte progenitor survival, proliferation and differentiation and the concomitant requirement of hippo signaling in formation of adult hematopoietic hubs.

In total, this study not only focuses on characterization of few unexplored components of hemocyte progenitor maintenance, but also finds many new candidates in the genetic screen which might be imperative for future studies in hemocyte progenitors or hematopoiesis per se.

Chapter 1.

Materials and Methods

1.1 Fly culture

The flies were reared on standard corn meal food containing maize powder and sucrose as carbohydrates sources, yeast as protein source, propionic acid and Nepagin as preservatives and antifungal agent, respectively and agar for solidification. All fly stocks were grown at 25^{o}C (if not otherwise mentioned) in standard bottles/vials. For staging of larvae for timed dissections, flies were allowed to lay eggs on agar plates infused with fruit juice and yeast paste to promote egg laying for 2 hrs. Synchronised newly hatched first instar larvae from such a plate were transferred and cultured on food plate (25-30 larvae in each 60mmplate) and reared at standard temperatures till required stages.

1.2. Fly Stocks and Genotypes:

Most of the *Drosophila* stocks used in this study were procured from Bloomington Drosophila Stock Center, some UAS-RNAi and reporter lines from Vienna Drosophila Stock Center and some from Drosophila Genomics Resource Center, Kyoto. New genetic combinations were generated as per requirements by crossing or recombining with appropriate lines.

1)OreR: These flies are wild type *Drosophila*, with red eye. This stock is used as control in many cases.

2)w^{1118}: These flies carry recessive white mutation on first chromosome. As a result the homozygous mutants are white eyed. They were used as experimental control, in many cases.

1.2.1 GAL4 driver lines:

1) *Dome-Gal4, UAS-GFP/ FM7; Gal80^{ts}/+; +/+*

This is a transgenic line that expresses Gal4 under the control of DNA sequences in or near *Domeless (Dome)* promoter. This line shows expression in the blood progenitors of the larval lymph gland. The transgene insertion is not homozygous viable and therefore maintained by a FM7 balancer on chromosome 1. This stock was modified from the parent Dome-Gal4 stock, a kind gift from S. Noselli, CNRS, France.

2) *w^{1118}; P{w[+mC]=Hml-GAL4._ 2} P{w[+mC]=UAS-2xEGFP}AH2*

This is a transgenic line that expresses GAL4 under the control of DNA sequences near *Hemolectin (Hml)* promoter. This line shows expression in the differentiating hemocytes of the third instar larval lymph gland. The transgene insertion is homozygous viable on chromosome 2. This stock was obtained from Bloomington Stock Center (30140).

3)*y*w^{1118};P{w+mW.hs=GawB}NP7379/CyO,P{w-=UAS-lacZ.UW14}UW14*

This is a transgenic fly line that expresses Gal4 in a *Thioester containing protein 4 (Tep4)* specific manner. This fly line shows expression in the progenitors of the lymph gland. This stock is homozygous lethal and is maintained by a Cyo balancer on 2 chromosome. This fly stock was obtained by *Drosophila* Genomics Resource Center, Kyoto stock Center (105442).

4) *y[1] w[*]; P{w[+mC]=AyGAL4}25 P{w[+mC]=UAS-GFP.S65T}Myo31DF[T2]*

This is a transgenic fly line thatcauses ubiquitous expression of GAL4 under the control of the Act5C promoter following removal of a y[+] cassette by FLP recombinase. This line also expresses GFP.S65T under the control of UAS sequence. The transgenic insersion is on second chromosome and homozygously viable but may be segregating CyO, y[+].This stock was obtained from Bloomington Stock Center (4411).

5) *w^{1118}; P{y[+t7.7] w[+mC]=GMR50A12-GAL4}attP2/TM3, Sb[1]*

This is a transgenic line that expresses GAL4 under the control of DNA sequences in or near Hnf4. The insertion is on chromosome 3 and it is lethal when homozygous and thus balanced by TM3, Sb^1. This stock was obtained from Bloomington Stock Center (47618).

1.2.2. UAS Lines

1) w^{1118}; UAS-2xEGFP; +/+

This is a transgenic line that contains a UAS-driven green fluorescent protein. The transgene insertion is homozygous viable on chromosome 2. This stock was obtained from Bloomington Stock Center (6874).

2) w^{1118}; P{w[+mC]=UAS-mito-HA-GFP.AP}2/CyO

This transgenic fly line expresses GFP with a mitochrondrial import signal. The transgene insertion is not homozygous viable and therefore maintained by a Cyo balancer on chromosome 2.This stock was obtained from Bloomington Stock Center (8442)**.**

3) w[*]; P{y[+t7.7] w[+mC]=UAS-yki.V5.O}attP2

This transgenic fly line expresses V5-tagged yorkie protein under UAS control. The insertion is present on chromosome 2 and is homozygously viably. This stock was obtained from Bloomington Stock Center (28819)**.**

4) w^{1118}, P{UAS-bsk.DN}2 (Adachi-Yamada et al., 1999)

These transgenic flies have an insertion on first chromosome, which expresses a dominant negative form of *Drosophila* JNK, *basket* (*bsk*), under UAS control. This stock is homozygous viable. This transgenic insertion is on first chromosome. The stock was obtained from Bloomington Stock Centre (#6409).

5) UAS RNAi lines: The double stranded knockdown of genes was carried out using UAS RNAi lines obtained mainly from majorly Bloomington Stock Center. Some UAS RNAi lines from Vienna Stock Center were also obtained for this study. A comprehensive list of all the UAS-RNAi lines are attached in Table 2.1 in Chapter 2.

1.2.3. Mutant Lines:

1) ***whd*$^{[1]}$** (Levi and Henrich, 1991):This is a transgenic line having a spontaneous deletion on second chromosome in *withered, whd* coding region, a16bp deletion in exon2 that produces a frameshift which results in a stop codon in exon 3. The mutation is homozygously viable and procured from Bloomington Stock Center (#441).

2) ***Hnf4*$^{\Delta 17}$**(Palanker et al., 2009): This is a transgenic line with imprecise excision of *P{EP}Hnf4*^{EP2449} generating a 8193bp deletion, removing most of the *Hnf4* coding region along with three adjacent genes. The transgene is on chromosome 2 and is homozygous lethal and the balancer CyO carries an unknown GFP transgene marked with miniwhite. This line is obtained from Bloomington Stock Center (#44218).

3) ***Hnf4*$^{\Delta 33}$** (Palanker et al., 2009): This is a transgenic line with imprecise excision of *P{SUPor-P}Hnf4*^{KG08976} that generates a 1939bp deletion of *Hnf4* which removes sequence encoding most of the DBD and the entire LBD. The transgene is on chromosome 2 and is homozygous lethal and the balancer CyO carries an unknown GFP transgene marked with miniwhite. This line is procured from Bloomington Stock Center (#43634)

4) ***βv*^{2}** (Devenport and Brown, 2004): This is a transgenic line with imprecise excision of *P{EP}EP2030* has produced a 2494 bp deletion which removes first 8 codons of *βInt-v* and approximately 2kb of upstream regulatory sequence. The deletion is on chromosome 2 and is homozygous viable. This line was a kind gift from Adachi Yamada, Tokyo, Japan.

1.2.4. Protein Trap Lines:

1)*w*^{1118}*; P{w[+mC]=PTT-un1}ZCL1973:*This transgenic fly line is a protein trap line containing GFP sequence tagging the full-length vertebrate Perlecan homolog *Terribly reduced optic lobes (Trol)* expressed from its endogenous locus, allowing to observe its cellular and subcellular distribution. The transgene insertion is homozygous viable on chromosome 1. This fly line was generated as part of FlyTrap, a GFP Protein Trap project. This stock was a kind gift from U. Banerjee, UCLA,USA.

2) ***y[1] w[*]; Mi{PT-GFSTF.0}Glut1[MI02222-GFSTF.0]/TM6C, Sb[1] Tb[1]:***

This is a transgenic fly line containing Recombination Mediated Cassette Exchange of a Mi{MIC} insertion results in expression of *Glut1* tagged with EGFP-FlAsH-StrepII-TEV-3xFlag. Insertion is on chromosome 3 and homozygous lethal. This fly line has been procured from Bloomington Stock Center (# 59607).

3) *y[1] w[*]; Mi{PT-GFSTF.2}Tret1-1[MI06811-GFSTF.2] Roc2[MI0681-GFSTF.2-X]:* This transgenic fly line contain Recombination Mediated Cassette Exchange of a Mi{MIC} insertion results in expression of *Tret1-1* tagged with EGFP-FlAsH-StrepII-TEV-3xFlag. Insertion is on chromosome 2 and is homozygous viable. This fly line has been procured from Bloomington Stock Center (#66367).

1.3. Immunostaining and microscopic analysis

1.3.1. Lymph gland fixation and staining

Lymph glands from1 hour synchronized batches of larvae were dissected on ice cold1X PBS. The pull outs/tissues were fixed in 4% formaldehyde or paraformaldehyde prepared in 1X PBS (pH 7.2) for 40 min followed by two quick washings of1X PBS. Tissues were then permeablized by 0.3% PBT (0.3 triton-X in 1X PBS) for 30 min (3 washings, 10 min each). Blocking was done in 10% NGS (prepared in 0.3% PBT) for 45 min-1hr. Tissues were then incubated in primary antibody with appropriate dilution in 10% NGS (prepared in 0.3% PBT) for overnight at 4^{o}C. Post Primary antibody incubation, tissues were subjected to 3 washes of 15 min each with 0.3% PBT. Following which tissues were again blocked with 10% NGS (prepared in 0.3% PBT) for 30 min. Secondary antibody specific to primary antibody was added and kept at 4^{o}C overnight followed by washings with 0.3% PBT (15min X 3) followed by a wash of 1XPBS and incubated them in DAPI solution made in 1XPBS for 1 h at room temperature. DAPI was subsequently washed with 1X PBS and tissues were mounted in Vectashield (Vector Laboratories). For immunostaining of some membranous antibodies like anti-βv, instead of 0.3% PBT, 0.1% PBT was used for washings and antibody incubations were done in 1XPBS.

1.3.2. Adult fly dissection for Hematopoietic Hub isolation

For all the experiments, 8th day post emergence (unless mentioned) synchronised female flies were anesthetised and dissected in ice-cold 1X PBS (pH 7.2) using fine scissor and forceps. The detailed steps referred in (Ghosh et al., 2018) was followed.

1.3.3. Immunostaining of adult samples

The dissected samples were fixed in 4% formaldehyde or paraformaldehyde (Sigma) for 45 min in 1X PBS at room temperature (RT), washed thrice in 1X PBS for 10min followed by, 30 min incubation in 0.3%PBT (0.3% TritonX 100 in 1X PBS). This was then subjected to blocking in 10% bovine serum albumin (BSA) in 1X PBS for overnight at 4^0C. Next, the samples were incubated in primary antibody diluted in 1X PBS for 45hr at 4^0C (supplemented with 1µl of 0.02% Sodium azide. Sodium azide was used to control the chance of infection during this long incubation of antibody solution). After primary antibody incubation, samples were washed thrice in 1X PBS for 10min each and blocked in 10% BSA for 1hr in 1X PBS. Secondary antibodies were used for 45hr in 1X PBS at 4^0C and then washed 3 times in 1X PBS for 30 min. To mark the nucleus of cells , the tissues were incubated in DAPI solution prepared in 1X PBS at 4^0C for overnight and then washed 2 times 10 min in 1X PBS. Finally samples were mounted in mounting media Vectashield.

1.3.4. Mounting of adult fly samples

The mounting steps of the adult abdominal samples are critical for a successful observation of adult Hematopoietic Hub. For this the steps described in (Ghosh et al., 2018) was followed.

1.4. Primary antibodies for immunohistochemistry used in this study

1. Anti-Ci (2A1, Developmental Studies Hybridoma Bank, Iowa): This is a monoclonal antibody raised in rat that recognizes amino acids 700-850 at the C terminus of the Zn finger domain of Cubitus Interruptus protein in *Drosophila*. The working dilution for immunostaining is 1:3.

2. Anti-GFP (Sigma, G6539): This is a monoclonal antibody raised in mouse that recognises 49 kDa Green Fluorescent Protein (GFP) in GFP tagged fusion proteins. The working dilution is 1:50.

3. Anti-P1 (Nimrod): This is a monoclonal antibody raised in mouse that recognises a transmembrane protein P1 specific to macrophages: plasmatocytes in *Drosophila*. The working dilution is 1:50. This antibody was a kind gift from I. Ando, BRC, Hungary.

4. Anti-Phospho Histone 3 (PH3, Cell Signalling #9713): This is a polyclonal antibody raised in rabbit that recognizes amino terminus of histone H3 phosphorylated on Ser28. The working dilution is 1:500.

5. Anti-β-PS (CF.6G11, Developmental Studies Hybridoma Bank, Iowa):

This is a mouse monoclonal antibody that recognizes full protein of integrin subunit betaPS (myospheroid, Mys) of *Drosophila*. The working dilution is 1:5.

6. Anti-αPS1 (DK.1A4, Developmental Studies Hybridoma Bank, Iowa): This is a mouse monoclonal antibody that recognizes full protein of integrin subunit alphaPS1 (multiple edematous wing, mew) of *Drosophila*. The working dilution is 1:10.

7. Anti-αPS3: This is a polyclonal antibody raised in mouse that recognises alphaPS3 subunit (scb, scab) of integrin in *Drosophila*. The working dilution is 1:100. This antibody was a kind gift from Shigeo Hayashi, Kobe, Japan.

8. Anti-βv: This is a polyclonal antibody raised in rabbit that recognises integrin $\beta\upsilon$ subunit (itgbn, βv) in *Drosophila*. The working dilution is 1:200. This antibody was a kind gift from R.O. Hynes, MIT, USA.

9. Anti-Acetyl-Histone H3 (Lys9) (C5B11 (H3K9ac, Cell Signalling #9649): This is a monoclonal antibody raised in rabbit that recognizes amino terminus of histone H3 acetylated on Lys9. The working dilution is 1:200.

10. Anti-Hnt (1g9 anti-hindsight, Developmental Studies Hybridoma Bank, Iowa): This is a monoclonal mouse antibody that recognizes hindsight (hnt) protein in *Drosophila*. The working dilution is 1:3.

11. Anti-Ena (5G2 anti-enabled, Developmental Studies Hybridoma Bank,Iowa): This is a monoclonal mouse antibody that recognizes amino acids 105-370 of fusion Enabled (Ena) protein tagged with 6X-HIS. The working dilution is 1:20.

12. Anti β- galactosidase (Promega, Z3781): Monoclonal purified antibody raised in mouse against β-galactosidase protein of *E.coli*. The working dilution for immunostaining is 1:100.

1.5. Secondary antibodies for immunohistochemistry used in this study

1. **CyTM3-Conjugated affinipure donkey anti rabbit IgG (H+L) (Jacksons Immuno Research Laboratories, USA # 711-165-152)** This affinity purified secondary antibody conjugated with cyanine CyTM3 dye (absorption maxima/ emission maxima is 550 nm/ 570 nm) was used at a dilution of 1:600 to detect primary antibodies raised in rabbit.

2. (FITC)-Conjugated affinipure goat anti rabbit (Jacksons Immuno Research Laboratories, USA # 711-096-152): This affinity purified secondary antibody conjugated with Fluorescein FITC dye (absorption maxima/ emission maxima is 492 nm/ 520 nm) was used at a dilution of 1:300 to detect primary antibodies raised in rabbit.

3. CyTM3-Conjugated affinipure goat anti mouse (Jacksons Immuno Research Laboratories, USA # 115-166-062): This affinity purified secondary antibody conjugated with cyanine CyTM3 dye (absorption maxima/ emission maxima is 550 nm/ 570 nm) was used at a dilution of 1:600 to detect primary antibodies raised in mouse.

4. (FITC)-Conjugated affinipure goat anti mouse (Jacksons Immuno Research Laboratories, USA # 115-095-166): This affinity purified secondary antibody conjugated with Fluorescein FITC dye (absorption maxima/ emission maxima is 492 nm/ 520 nm) was used at a dilution of 1:300 to detect primary antibodies raised in mouse.

5. CyTM3-Conjugated affinipure donkey anti rat (Jacksons Immuno Research Laboratories, USA # 712-165-153): This affinity purified secondary antibody conjugated with cyanine CyTM3 dye (absorption maxima/ emission maxima is 550nm/ 570 nm) was used at a dilution of 1:600 to detect primary antibodies raised in rat.

6. CyTM5 649-Conjugated affinipure goat anti rat (Jacksons Immuno Research Laboratories, USA # 712-495-153): This affinity purified secondary antibody conjugated with cyanine 649 dye (absorption maxima/ emission maxima is 650 nm/ 670 nm) was used at a dilution of 1:300 to detect primary antibodies raised in rat.

7. Alexa FluorR 647 AffiniPure Goat Anti-Mouse IgG (H+L) (Jacksons Immuno Research Laboratories, USA # 115-605-003): This affinity purified secondary antibody conjugated with cyanine 649 dye (absorption maxima/ emission maxima is 650 nm/ 670 nm) was used at a dilution of 1:300 to detect primary antibodies raised in mouse.

8. Alexa FluorR 647 AffiniPure Donkey Anti-Rabbit IgG (H+L) (Jacksons Immuno Research Laboratories, USA # 711-605-152): This affinity purified secondary antibody conjugated with cyanine 649 dye (absorption maxima/ emission maxima is 650 nm/ 670 nm) was used at a dilution of 1:300 to detect primary antibodies raised in rabbit.

1.6. Stains used in this study

1. **Phalloidin- Texas Red (Molecular Probe, India # T7471):** It is a phallotoxin isolated from poisonous fungus Amanita phalloides, which specifically binds to polymeric filamentous actin and prevents its depolymerization. This F-actin probe is conjugated with red fluorescent Texas red dye (excitation maxima: 591nm, emission maxima: 608). The working dilution was 1:500. Tissues after secondary antibody treatment were washed with 1X PBS for three washes of 5 min each. Then, tissues were incubated in phalloidin Red (1:500) in 1X PBS for 1hr. at room temperature. The washing and counterstaining parts are similar as for immunostaining.

2. **DHE staining (Molecular Probes, Cat #D11347):** Dihydroethdium (DHE) is a superoxide indicator also called hydroethidine. It is oxidized by superoxide ions of the cell into 2-hydroxyethidium, which intercalates with the DNA and exhibits a bright red fluorescence. Third mid instar larvae reared at 29^{o}C were dissected in 1X Schneider's media (Invitrogen #217220024) on ice very quickly. This was followed by incubation in DHE (0.3 μ M in Schneider's media, Molecular Probes) for 5 minute at room temperature in dark. After washings with 1X PBS, brief fixation was done with 4% paraformaldehyde for 10 min. Tissues were mounted in vectashield mounting medium (#H-1200/H-1000)

3. **Nile red staining (Invitrogen #N-1142):** Nile red is a fluorescent lipid indicator, especially neutral lipid droplets within cells. Nile red has excitation/emission maxima at 552/636 nm in methanol. The working solution used was 10mM (1.6mg/500ulof DMSO). Mid third instar larvae were dissected in 1X PBS, fixed in 4% paraformaldehyde for brief period at room temperature. Tissues were rinsed in 0.3% PBT for half hour followed by one wash with 1X PBS. Tissues are incubated in Nile Red in1X PBS for half hour and washed with 0.3% PBT for half an hour. Tissues were mounted in DAPI vectashield (Vector labs, USA #H-1200) and imaged in confocal microscope LSM780 (Carl Zeiss).

4. **EdU staining (Click-It EdU, Invitrogen, #C10339):** Larval tissue was quickly pulled out in 1X PBS on ice (dissection for not more than 25 min and fat body and salivary gland cleared from the tissue). Then, incubation was done in EdU solution, Component A 1:1000 in 1X PBS on shaker at room temperature for 30-35min. EdU incubated tissues were fixed with 4% paraformaldehyde in 1XPBSand washed with 0.3% PBS-Triton four times at ten min interval followed by30-35 min of blocking in 10% NGS in 0.3% PBS-Triton. EdU staining solution as per manufacturer's instruction (for 50 μ l staining solution, 43 μl 1x EdU buffer, 2 μl CuSo4 solution, 5 μl 1x EdU buffer additive, 0.12 μl Alexa solution) was used to stain the sample for 30 min at room temperature. Two quick washes with 0.3%PBS-Triton was followed by a quick wash in 1X PBS. If no further antibody staining was required, nuclear staining by DAPI was done in 1X PBS and then mounted in vectashield. For further immunostaining, the protocol mentioned before was followed.

5. **DAPI (4',6-diamidino-2-phenylindole dihydrochloride, Invitrogen, # D1306):**This is blue fluorescent dye that binds to A-T rich region in dsDNA thereby use to stain nuclei in live as well as fixed tissues. Its absorption maxima/ emission maxima are 351nm/ 461nm. The working dilution was 1μ g/ml. Post staining, tissues were incubated in DAPI solution (1μ g/ml) in 1X PBS for 30-60 min at room temperature. After incubation, tissues were washed with 1X PBS and observed after mounting in vectashield.

6. **TO-PRO-3 iodide (Molecular Probe, # T3605):** This is a highly sensitive carbocyanine nucleic acid stain with far-red fluorescence. It is used as counter stain for nucleus and dead cell indicator. Its absorption maxima/ emission maxima are 642nm/ 661nm. The working dilution was 1:500. Post staining, tissues were incubated in TO-PRO-3 solution (1:500) in 1X PBS for 30-60 min at room temperature. After incubation, tissues were washed with 1X PBS and observed after mounting in vectashield.

7. **2-NBDG (2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) Amino)-2-Deoxyglucose, Molecular Probes #N13195)**

For *in vivo* assessment of glucose uptake, 2-NBDG uptake was measured. Dissected larvae were incubated in PBS with 0.50 mM 2-NBDG (Invitrogen) for 45 min at 25°C, washed twice in PBS for 5 min, fixed 20 min in PBS + 4% PFA, and washed again twice for 5 min in PBS. All washes and the fixation were done with pre-cooled 1X PBS (4°C). Lymph glands were rapidly dissected and mounted in Vectashield, and images were immediately collected

with a Zeiss LSM 780 confocal microscope. 2-NBDG fluorescence was excited at 488 nm and detected at 490–550 nm. (de la Cova et al., 2014)

8. **TMRM (Tetramethylrhodamine, Methyl Ester, Perchlorate, Molecular Probes #T668):** For having an assessment of the *in vivo* mitochondrial membrane potential, TMRM staining is performed on live samples followed by live imaging of the tissue/cell. The relative retention of the dye in mitochondria can be measured in terms of relative fluorescence intensity and quantified. This can be interpreted as a measure of mitochondrial membrane potential. Depolarised mitochondria are not able to sequester TMRM actively, which is reflected by low TMRM fluorescence intensity. Dissected larvae were incubated in PBS with 50 nM TMRM (Invitrogen) for 20 min at 25°C, washed thrice in PBS for 5 min. All washes and stain incubation were done at room temperature on shaker. Lymph glands were rapidly dissected and mounted in Vectashield, and images were immediately collected with a Zeiss LSM780 confocal microscope. TMRM fluorescence was excited at 561 nm and detected at 565–630 nm. (Frei et al., 2005)

1.7. Confocal microscopy: Immunofluorescence images from live samples or fixed samples were captured in Laser Scanning Confocal Microscopes (LSM 780 Carl Zeiss and Leica SP8). Confocal stacks of lymph glands were taken as optical section of 1µm. While imaging of cellular organelles, like mitochondria and lipid droplets, the optical sections were of 0.33µm.

1.8. Statistical analysis

All the experiments were repeated at least thrice to check for reproducibility. Data are expressed as mean ± standard deviation (SD) of values from three independent experiments. Statistical analysis was performed using two-tailed Student's t-test. Significance was accepted with *p-values* of <0.05; <0.001 and <0.0001, mentioned as *, **, *** respectively was considered as statistically significant.

1.9. Image analysis and Fluorescence quantification

Images were analysed by ImageJ software. For relative fluorescence intensity measurements, 12 bit confocal sections with optimally saturated signal were used to generate maximum intensity projections with ImageJ. Total fluorescence intensity per cell was measured as mean fluorescence intensity/ total cellular area. Based on this, relative fluorescent intensities per

cell to measure relative mitochondria/lipid or ROS were measured at different developmental time points and plotted. For each of these analyses, at least 3 biological replicates and 4 technical replicates of the experiment was conducted. To calculate fluorescence intensity of Ci^{155}, total fluorescent intensity was normalized by total area. Fluorescence intensity and the area of the selected expression domains were calculated by using measure function in ImageJ. For differentiation index, total area of P1 expressing cells was divided by the total area of the primary lobe in 3 middle stacks. For S-phase index of the progenitors, number of EdU incorporating cells were normalised by total number of MZ cells. For reproducibility of results, each experiment was repeated three times along with control and imaged at the similar parameter settings.

1.10. BrdU assay in third instar late lymph glands.

Third late instar larvae were dissected in 1X PBS and the pull out tissues were incubated in BrdU solution (1:1000 in 1X PBS) for 45 min for normal BrdU incubation at room temperature. The tissues were fixed in 4% paraformaldehyde for 45 min and then by using standard immunostaining protocol (as described above) staining with anti-GFP antibody was performed [Primary antibody: anti-GFP mouse (1:100) and secondary antibody: mouse FITC (1:400)]. Then the tissues were incubated in 2N HCL in 1X PBS for 25 min without shaking followed by a quick wash with 1X PBS and then fixing the tissues in 4% paraformaldehyde for 30 min. Following this, immunostaining with anti-BrdU antibody was performed [Primary antibody: anti-BrdU Rat (1:100) and secondary antibody: anti-Rat Cy3 (1:1000)]. Tissues were then washed thrice with 0.3% PBT for 10 min each followed by incubation in DAPI for 30 min. Finally, the tissues were washed twice with 1X PBS for 5 min each and then mounted in Vectashield.

1.11. ATP Assay:

ATP assay was performed from larvae of control oreR and whd^{1} homozygous. Around ten third instar late larvae were homogenized in ATP assay Lysis buffer. The samples were boiled at 95^{0}C for 5 min and diluted 1:1000 in dilution buffer provided in ATP luminescence kit HSII (Roche, 11699709001). Further assay was performed as instructed by kit's manual in Luminometer (Promega). Standard curve was generated and ATP concentrations were calculated. The ATP concentration was normalized with protein concentration.

1.12. Pharmacological drugs and Inhibitors used in this study:

Drug used	Source	Product Description and usage
Etomoxir (Singh et al., 2016)	Cayman Chemical. Catalogue number: Cay11969	(+)-Etomoxir (5 μM) is an irreversible inhibitor of carnitine palmitoyl transferase 1, a mitochondrial enzyme involved in fatty acid β-oxidation. It has been used at a concentration of 5 μM mixed in fly food and fed to larvae post second instar stages and analysis of lymph gland were done in late third instar stages. Control larvae had same vehicle control level mixed in fly food.
Mildronate (hydrate) (Singh et al., 2016)	Cayman Chemical. Catalogue number: Cay15997	Mildronate (100 μM) is a structural analog of γ-butyrobetaine (γBB), an intermediate in the biosynthesis of carnitine. It blocks carnitine synthesis by inhibiting γBB hydroxylase (IC_{50} = 62 μM) and, less potent, carnitine acetyl transferase (K_i = 1.6 mM). Through these actions, mildronate reduces the levels of free carnitine and long chain acyl carnitine. This leads to suppressed fatty acid metabolism and mitochondrial uncoupling during oxidative conditions. Used at a concentration of 100 μM and fed to larvae after second instar stages and vehicle control was fed to larvae which were controls.
L-Carnitine hydrochloride (Banerjee et al., 2013)	Sigma – Aldrich Catalog Number : C0283	L-Carnitine hydrochloride (100mM) has been used to allow the entry of palmitic acid

		into the mitochondria. L-Carnitine were used at 100mM concentrations in fly food and fed to larvae for 48 hrs in third instar analysis and for 24 hrs in second instar analysis. Same vehicle control fed larvae were used as controls.
2-DG (2-Deoxy-D-Glucose (2-Deoxyglucose)) (Singh et al., 2016)	Sigma – Aldrich Catalog Number : D8375	2-DG (100 mM) is a glucose analog that inhibits glycolysis via its actions on hexokinase, the rate limiting step of glycolysis. It is phosphorylated by hexokinase to 2-DG-P which cannot be further metabolized by phosphoglucose isomerase. This leads to the accumulation of 2-DG-P in the cell and the depletion in cellular ATP. 2-DG mixed in fly food at concentration of 100Mm and fed to third mid instar larvae for 24 hour period and analysis done in late third instar stage. Similar aged larvae fed on vehicle control served as controls.
Sodium Acetate	Sigma-Aldrich Catalog Number : 71196	Sodium Acetate Solution (50mM) is used as a supplement which can be converted into Acetyl CoA, elevating the Acetate levels *in vivo*. Mixed in fly food at concentrations of 50 mM and fed to larvae from second instar onwards and analysis done in late third instar stages. Similar aged larvae fed on vehicle controls served as controls.

1.13. RNA isolation from whole larvae

Appropriate number of (10 larvae for control) synchronously hatched larvae were collected and washed thoroughly with PBS to remove food particles. Larvae were then homogenized in TRiZOL (Invitrogen) and extracted with 200µl of chloroform (Sigma). Further purification was performed by using RNeasy Mini Kit (Qiagen - 74104) as instructed. To get rid of any residual DNA, 25 min incubation at 37^0c was given with RNase free 2U DNase (Qiagen-79254) in RDD buffer. RNA pellet was dissolved in nuclease free DEPC treated water (Sigma).

1.14. Total RNA isolation from Lymph gland

About 50 larval lymph glands were dissected on ice in 1X PBS and homogenized in 1 ml TRIzol reagent (Ambion # 15596018) by pipetting. After proper homogenization sample was kept at room temperature for 10 min for complete dissociation. 200µl of chloroform was added and incubated at room temperature for 10-15 min. The resulting mixture was then centrifuged at 4°C for 20 min at 13000 RPM. Three layers were separated out, uppermost aqueous layer containing RNA, middle layer containing proteins and lowermost organic layer containing DNA. The upper layer was carefully taken out in to a separate RNase-free tube so that middle layer remained unaffected. The sample was then incubated with 0.6 volumes of isopropanol for overnight at -80^0C. On the next day, sample was incubated for 10 min at room temperature to precipitate out the RNA and then centrifuge at 13,000 rpm for 15 min at 4 °C to pellet out the RNA. RNA pellet was washed with 70% ethanol and dissolved in DEPC water. Quantification of RNA was performed by using nanodrop spectrophotometer.

1.14. cDNA Synthesis

cDNA was synthesized using Verso c-DNA synthesis kit (Molecular Probe # AB1453A) following the manufacturer's recommended protocol. 500 ng of RNA was used for cDNA synthesis. RNA was incubated with cDNA reaction mix at 42˚C for 30 min and then kept on 95˚C for 2 min. c-DNA mix was prepared using 5X RT buffer, 500µM dNTP mix, Oligo dT and random Hexamer primer mix, RT Enhancer to remove contaminating DNA and Verso enzyme mix which acted as reverse transcriptase for conversion of cDNA, as shown in table 1.1. cDNA mix was added to the RNA and the reaction was mixed properly by pipetting and

short spin. This mixture was then incubated at 42˚C for first 30 min. Reaction was terminated by incubating at 95˚C for last 2 min cDNA samples were stored at -20˚C.

Table1.1: Preparation of Reaction Mixture for cDNA preparation.

Constituents	Final Concentration	Volume (µl) For 1 reaction
Water, nuclease-free		Volume make up to 20µl
5 X RT Buffer	1X	4
dNTPs Mix (500µM)	50 µM	2
RT Enhancer	1.5 mM	1
RNA Primer	1X	2
Verso Enzyme Mix	1 units	1
RNA (500ng/µl)	500ng	1-5µl
Total		20

1.15. RT PCR

Table1.2: Preparation of PCR Reaction Mixture

Constituents	Final Concentration	Volume (µl) For 1 reaction
PCR water		15.8
20 X Taq Buffer	1X	1.0
dNTPs Mix (10mM)	0.2mM	0.4
Forward Primer (10µM)	1µM	0.4
Reverse Primer (10µM)	1µM	0.4
Taq Polymerase (3 unit/ µl)	0.6 units	0.2
DNA (500ng/µl)	500ng	1
Total		20

PCR amplification reactions were carried out in a final volume of 20 µl. The reaction mixture was prepared by adding reagents in the following sequence: PCR water; 10X Taq Buffer; dNTPs mix; $MgCl_2$; Forward Primer; Reverse Primer; and Taq Polymerase.

The amplification reaction consisted of 40 cycles with initial denaturation at 94˚C for 5 min, followed by 39 cycles of denaturation at 95˚C for 30 sec, annealing at 59˚C (Variable for different set of primers) for 30 sec and extension at 72˚C for 30 sec. Reaction was terminated after final extension for 10 min at 72˚C. 10µl of PCR products were loaded on 1% agarose gel along with 50bp DNA ladder as the marker to check the amplification.

1.16. Real Time PCR

Table1.3: Preparation of PCR Reaction Mixture for qPCR

Constituents	Volume (µl) For 1 reaction
PCR water	7
SYBER Green Mix	10.0
Forward Primer (10µM)	1
Reverse Primer (10µM)	1
DNA (500ng/µl)	1
Total	20

Real time qRT PCR was performed using SYBR green mix Biorad on Biorad CFX96 instrument, following the instructions provided in the manual. Reaction mix was prepared as mentioned in table1.3, was set up at 95^0Cfor 15 sec and 59^0Cfor 15 sec and 72^0Cfor 15 sec for 40 reactions. Melting curve was performed from 95^0Cto 72^0Cfor 5 min, to analyse T_m of the amplicon. Expression analysis was performed on instrument using ΔΔCt method.

Expression level was normalized to Act level and then compared with respect to W^{1118}. Primers were designed from NCBI. We specifically designed primers from exon-exon junction to avoid any misinterpretation from DNA contamination. Specificity of primers were checked with primer blast.

Table 1.4 : List of Primers used

	Gene Name	Primer Sequence
1.	***srl***	AGAATCTGCTGATTTACAACGACT GCGATGGACCGATAGACCTG
2.	***TubB***	AGTGTGTGAAGCGGACAGTT AGATGATGTCCCAGATGGC
3.	***Act5C***	ACACATTTTGTAAGATTTGGTGTGT CCGTTTGAGTTGTGCTGT

1.17. Protein Isolation from larvae

Solutions used-

1. RIPA Buffer: 150 mM sodium chloride; 1.0% Triton X-100; 0.5% sodium deoxycholate; 0.1% SDS (sodium dodecyl sulfate); 50 mM TrisHCl (pH 7.4), pH 8.0; 5mM EDTA 2X Buffer was stored at -20^0C.

2. 2X Lamilli Buffer: 100 mM TrisHCl (pH6.8) ; 4% SDS; 0.2% Bromophenol Blue; 20% Glycerol. Buffer was stored at Room Temperature and 200mM β-mercaptoethanol was added freshly.

   For protein isolation from total larvae, they were homogenized in RIPA Buffer with 1% mammalian Protein Inhibitor Cocktail (Sigma, P8340) in RIPA on ice. The homogenate was centrifuged at 13000 rpm, for 15 min at 4^0C. The supernatant was mixed with equal amount of 2X Lamilli Buffer. The samples were boiled at 95^0C for 5 min and stored at -20^0C. The protein quantitation was done by Bradford Assay with a standard curve

generated freshly. For protein isolation from larval fat bodies, fat bodies were isolated from about 20 third mid larvae in directly in 1X Sample Buffer.

1.18. Histone Extraction from whole larvae: Histone extraction was carried out from whole *Drosophila* larvae by using Histone Extraction Kit from abcam #ab113476. Prescribed protocol provided by manufacturer was followed. Minimum 30 larvae for histone extraction were taken each for the experimental and control genotypes.

1. Around 30 third late instar *Drosophila* larvae were taken and kept in a pre-chilled homogenizer.

2. 200 µl of pre-lysis buffer supplemented with de-acetylase inhibitors and protease inhibitors were added to the homogenizer containing larvae.

3. Larvae were homogenized and the homogenized mixture was transferred to a 2 ml vial and centrifuged to 1000 RPM for 1 min to settle down the cells/tissues.

4. The pellet was re-suspended in three volumes of lysis buffer and incubated on ice for 30 minute.

5. The samples was centrifuged at 12,000 rpm for 5 min at 4°C and transferred the supernatant fraction (containing acid-soluble proteins) into a new vial.

6. Next, 0.3 volumes of the Balance-DTT Buffer was added to the supernatant immediately (e.g., 0.3 mL of Balance-DTT Buffer to 1 ml of supernatant).

7. The protein concentration was quantified by Bradford assay with BSA used as a standard.

8. Denaturing-PAGE of the isolated histones was carried out and the blots were probed for acetylated histones.

1.19. Poly Acrylamide Gel Separation of Protein Samples:

Solutions-

1. Solution A: 29gm Acrylamide, 1gm Bisacrylamide, Volume 100ml water (Autoclaved)
2. Solution B:1.5 M TrisHCl ;0.4% SDS; pH 8.8; Volume 200ml
3. Solution C:0.5 M TrisHCl ;0.4% SDS; pH 6.8; Volume 200ml
4. Running Buffer : 25 mM Tris Base; pH 8.3; 190 mM Glycine and 0.1% SDS
5. Transfer Buffer : 48 mM Tris Base ; 39mM Glycine; 0.04% SDS and 20% Methanol

Polyacrylamide resolving gel and stacking gel of different percentage were prepared as mentioned below in table 2.6. 40 ul protein sample was loaded and run at 70 – 120 mV.

Table1.5. : Reaction mixture for Resolving and Stacking Gels for PAGE.

Gel (%)	Resolving (9%)	Resolving (12%)	Resolving (15%)	Stacking (4.5%)
Solution A	2.7 ml	3.6 ml	4.5 ml	450ul
Solution B	2.25ml	2.25ml	2.25ml	-
Solution C	-	-	-	750ul
dH_2O	3.95ml	3.05ml	2.15ml	1.8ml
20% APS	100ul	100ul	100ul	20ul
TEMED	8ul	8ul	8ul	10ul

1.20. Semidry Transfer of protein samples:

The Gel was cut according to the areas of interest and transferred into Transfer Buffer for 15 min. Appropriate size of PVDF membrane was cut, soaked in methanol for 1-2 min. Extra thick filter paper cut according to the membrane size and the membrane were also kept in Transfer Buffer for 15 min. Sandwich was made in order, thick paper, blot, gel and paper again. Any trapped air in between these layers was rolled out. Transfer was done at 20V for 100 min.

1.21. Wet Transfer of protein samples:

For Wet transfer, sandwich was made in this order: foam, two thick papers, gel, blot and paper again, roll out gas trapped in between. The sandwich was kept in assembly and transfer was done at 90V for 120 min.

1.22. Immunoblotting:

Blot was washed three times in 0.1% Tween 20 in 1XTBS (0.1% TBST) for 15 min. Blot was then blocked in 5% skimmed milk in 0.1% TBST for 90 min at room temperature. This was followed by one wash with TBST and incubation in Primary antibody in 5% BSA for 12-18 hrs at 4^0C on shaker. Blot was next washed thrice in 0.1% PBST and incubated in HRP tagged secondary antibody in 0.1% PBST for 1 hour at room temperature on shaker. The imaging was done by pouring the HRP solution substrate (Roche) in LAS 2000. The images were analysed and band intensity was quantified in Fiji. The background intensity was subtracted from band intensity, ratios of test band from experimental to control was normalized for actin or tubulin as loading control in the same experiment. The antibodies used are mentioned as follows

1.23. Primary Antibody for western blot analysis

Anti-Actin (Mouse Anti-Actin (JLA20), Developmental Studies Hybridoma Bank, Iowa): This mouse anti-actin antibody recognizes the actin. This antibody is used as loading control for western blot was generated by Lin J.J. The working dilution of this antibody is 1:700.

Anti-Tubulin (Mouse monoclonal T6199 Anti αTubulin antibody sigma): This mouse anti-Tubulin antibody recognizes the Tubulin. This antibody is used as loading control for western blot. The working dilution of this antibody is 1:1000.

Anti-Histone H3 (Rabbit polyclonal to Histone H3 #ab1791): This Rabbit polyclonal antibody is specific to Histone H3. This antibody is used as a loading control for western blot. The working dilution of this antibody is 1:1000.

Anti-Histone H3K9 acetylation (Rabbit polyclonal to Acetyl-Histone H3 (Lys9) (C5B11) Rabbit mAb #9649, CST): This rabbit anti-H3K9acetylation antibody recognizes acetyl-H3 Histone at Lys9 residue. The working dilution of this antibody is 1:1000.

Anti-Histone H3K18acetylation (Rabbit polyclonal to Acetyl-Histone H3 (Lys18) (D8Z5H) Rabbit mAb #13998, CST): This rabbit anti-H3K18acetylation antibody recognizes acetyl-H3 Histone at Lys18 residue. The working dilution of this antibody is 1:1000.

Anti-Acetylated-Lysine (Rabbit polyclonal to pan-acetyl/L-Lysine Antibody #9441, CST): This rabbit anti-acetylated-Lysine antibody recognizes pan acetylations at L-Lysine residues. The working dilution of this antibody is 1:1000.

1.24. Secondary Antibody for western blot analysis

Anti Rabbit IgG HRP (Goat Anti-Rabbit IgG (H&L) [HRP] Polyclonal Antibody**,** A00098 GenScript**):** This is highly purified from goat antiserum by immunoaffinity chromatography and then conjugated to horseradish peroxidase. It reacts with rabbit IgG heavy and light chains.

Anti-mouse HRP (Goat Anti-mouse IgG (H&L) [HRP] Polyclonal Antibody, A00160 GenScript**):** This polyclonal antibody is highly purified from goat antiserum by immunoaffinity chromatography and then conjugated to horseradish peroxidase. It reacts with mouse IgG heavy and light chains.

Chapter 2

Genetic screen to identify the factor/s regulating hemocyte progenitor maintenance in *Drosophila melanogaster*.

2.1. Introduction

The advent of genetic screens, both forward as well as reverse approaches have enabled the de-convolution of gene function to a biological process. Forward genetic screen is based on the approach of identifying a gene or a set of genes responsible/regulating a particular phenotype. In this approach, the journey starts off with a phenotype and steers towards identification of the gene/s responsible for the phenotype. While reverse genetic screen commence with the disruption of a known gene followed by analyses of the effect of the disruption on the phenotype. Nonetheless, both forward and reverse genetic screens aim in determining gene function (St Johnston, 2002).

The most important aspect of a genetic screen is to have a visible and easy scoring phenotype that can be attributed to a gene function. In this regard, *Drosophila melanogaster* has emerged as the model organism of choice for carrying out an *in vivo* multicellular genetic screen (Nusslein-Volhard and Wieschaus, 1980; St Johnston, 2002; Wolf and Rockman, 2011). The set of genetic resources currently available in fruit fly is unmatched when compared to other model systems, thus endowing it an advantageous position for a successful genetic screen. From having short life cycle of ≈10 days to having access to different development stages i.e., fertilized egg, embryo, larvae, pupae to adult stages gives a unique opportunity to study many biological processes with easily scorable phenotypes. The unique advantage of fly genetics is the presence of balancer chromosomes that contain multiple inversions and suppress meiotic recombination. Hence, even recessive lethal mutations can be maintained in the presence of balancer chromosomes as stable stocks and followed in subsequent genetic crosses. *Drosophila* genetics has this unique advantage of having easily

observed physical traits associated with specific mutations, transgenes and balancers, e.g., the presence of a transgene is generally accompanied by a mini-white gene cassette, which results in a red eye color instead of white (Wolf and Rockman, 2011)). Apart from above advantages, absence of gene redundancy, smaller genome and presence of orthologue for most of genes which have been implicated in human diseases make *Drosophila* an attractive model organism for gene discovery by genetic screens (St Johnston, 2002).

Drosophila research employs different approaches for performing diverse genetic screens. Although, many visible mutations were isolated in *Drosophila* much before the advent of genetic screens, mostly they were spontaneous alleles from natural populations (St Johnston, 2002). The genetic screens only became possible once more sophisticated ways to generate mutations were developed. The first genetic screen to be carried on a large scale is the famous Heidelberg screen by Eric Wieschaus and Nusslein Volhard which led to the identification of patterning in *Drosophila* embryos which used Ethyl Methane Sulphonate (EMS) to induce mutagenesis (Nusslein-Volhard and Wieschaus, 1980). To identify the different components of a signaling pathway, the enhancer suppressor screen is performed which identifies modifiers of the mutant of the particular signaling component. Apart from these, another powerful yet efficient method of performing genetic screen is inducing mitotic clones and studying the function of a gene in the clones. However, the hurdle in these screening methods is to identify and map the disrupted gene. Not too long ago, genetic screen was also carried out by P-element or transposable element mutagenesis but is highly inefficient in the sense that many genes are cold spots for transposable element insertions.(St Johnston, 2002)

With the advent of twenty first century that saw the genomes of human and major model organisms sequenced, the major breakthrough in screening strategy was emergence of RNAi based targeted knockdown strategy. The RNAi based screening can be considered a forward genetic screen with reverse genetic approach in the sense that targeted knockdown of a particular gene is carried out (Ahringer, J., et al 2006). In *Drosophila* binary Gal4-UAS system is widely used for genetic studies (Brand and Perrimon, 1993). By combining Gal4-UAS and RNAi strategies, spatiotemporal knock down of a gene can be performed using its specific RNAi. Hence, by using RNAi based genetic screening, targeted knockdown of several genes can be performed to investigate their role in a particular biological process.

However, specificity, efficiency and off targets of the RNAi based knockdown must be kept in the mind while carrying out the screen.

Keeping in mind the aforesaid genetic screening strategies, for the current study a RNAi based knockdown strategy was chosen to embark upon the factors that are regulate larval hemocyte progenitor maintenance in *Drosophila melanogaster*.

In vivo genetic analysis in *Drosophila* has elucidated the stem cell and their precursor cell properties in their resident microenvironment. *Drosophila* blood cell development in larvae takes place in an organ called lymph gland, which starts forming in late embryogenesis. Lymph gland houses hemocyte progenitors, differentiating blood cells and the dedicated signaling cells or niche cells which provide the microenvironment for hematopoiesis and progenitor maintenance. MZ cells are maintained by the signals from the niche cells also known as cells of Posterior Signaling Centre (PSC). Early instar lymph gland have stem cells (Dey et al., 2016), self-renewing hemocyte progenitors, which lack any differentiation marker and the niche cells (Tate et al., 1990). Third instar mature lymph gland houses the differentiating blood cells in periphery, in a region termed as cortical zone (CZ). The inner core of the late third instar lymph gland termed as medullary zone (MZ) contains hemocyte progenitors which have now stopped self-renewal and are cell cycle arrested or quiescent (Tate et al., 1990). The hemocyte progenitors found in MZ are akin to the vertebrate common myeloid progenitors (CMP). They are quiescent, have high levels of ROS (Owusu-Ansah and Banerjee, 2009), lack differentiation markers and can give rise to all *Drosophila* blood cells and hence are multipotent (Jung et al., 2005). Therefore, hemocyte progenitors in larval lymph gland provides an excellent model system to carry out studies on progenitor maintenance, their self-renewal and quiescence in addition to unraveling interdependence of progenitor cell cycle state and maintenance. Thus, for this current study, the targeted knockdown via RNAi based screen to identify the factors regulating the maintenance and homeostasis of hemoycte progenitors was carried out.

Since, lymph gland in larval period is a developing organ and the differentiated cells at the onset of pupation leave the lymph gland and contribute to the circulation of pupae, disruption of genetic function in larval hemocyte progenitors necessarily doesn’t affect viability of larvae.

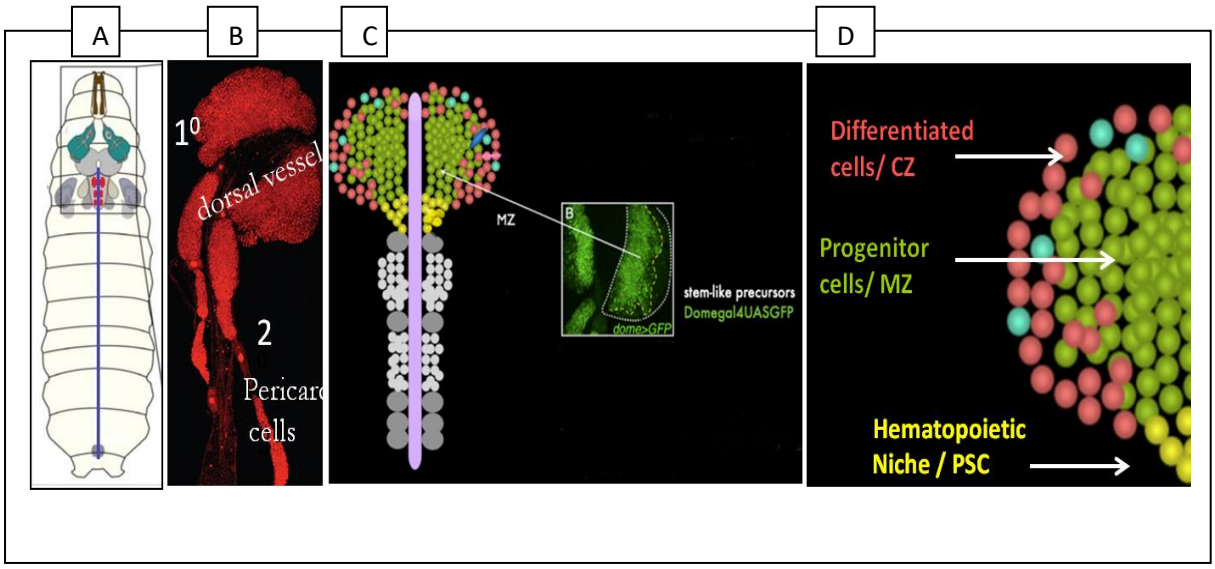

Fig 1 (A-D) Anatomical location and structure of *Drosophila* larval lymph gland. (**A)** Lymph gland stays closely associated with the dorsal aorta or the heart of the fly.**(B)** Confocal image showing third instar larval lymph gland with a pair of anterior lobes known as primary lobes and few slender posterior lobes known as secondary and tertiary lobes flanking the cardiac tube. Posterior lobes are separated by an array of cells which are nephroctyic in function known as pericardial cells. (**C-D)** The Primary lobe is divided into 3 different zones; The innermost zone known as medullary zone (MZ) houses stem like progenitors which upon differentiation moves toward the outer periphery forming the cortical zone (CZ). Posterior to these two zones there is Posterior Signalling Centre or the niche which maintains the homeostasis of both MZ and CZ. **(D)** *DomelessGal4UASGFP* positively marks the progenitors in MZ.

2.2. Strategy of the genetic screen

To carry out the RNAi based genetic screening, UAS-RNAi based transgenic fly lines were employed. There are three major stock centers that harbor UAS-RNAi based transgenic fly lines covering almost all the major genes in *Drosophila*. They are National Institute of Genetics (NIG-FLY) Japan, Transgenic RNAi Project (TRiP), Harvard Medical School and Vienna *Drosophila* RNAi Center, Austria. For the current study, majorly TRiP RNAi collection from Bloomington *Drosophila* Stock Centre (BDSC) was used. TRiP RNAi lines are known to be very efficient in the sense that mostly they don't have off target effects (refs). TRiP RNAi lines are made by site directed mutagenesis using very efficient set of vectors. The first generation of TRiP stocks that were made in the VALIUM1 and VALIUM10 vectors using long double-stranded hairpin approach. Long double-stranded hairpin approach sometimes requires UAS-Dicer2 for robust knockdown by RNAi. Second generation of TRiP stocks were constructed in the VALIUM20 vector using short hairpin microRNA technology which resulted in more robust knockdown in somatic cells negating the concomitant requirement of UAS-Dicer2 in the genetic background. Apart from the TRiP RNAi lines, RNAi fly stocks from VDRC for were used. These was for specific situations where genes whose TRiP RNAi line was not available or another independent RNAi line from different source was used to further validate the phenotype obtained by TRiP line.

The UAS-RNAi transgenic lines can be driven by Gal4 lines of choice depending upon the tissue where the gene function needs to be downregulated. To find out the regulators of hemocyte progenitor maintenance, an RNAi knockdown based genetic screen was performed to pick out the candidate genes involved in this process. Around 101 UAS RNAi constructs (Table 2.1) were driven using progenitor based *DomelessGal4* or *Tep4Gal4* having a temperature sensitive tubulin-gal80^{ts} allele in the genetic background. Gal80^{ts} is an inhibitor of Gal4 activity and at lower temperature around 18^{o} Celsius doesn't allow Gal4-UAS binding and inhibits the RNAi mediated knockdown. Whereas, at higher temperatures around 29^{o} Celsius, gal80^{ts} activity is minimum and hence Gal4 can bind to UAS sequence and RNAi mediated knockdown takes place. This provided a handle for a spatio-temporal knockdown of 101 RNAi lines in hemocyte progenitors and also allowed to bypass any detrimental effect of the knockdown in early developmental stages. The status of the progenitors (MZ) was assessed by fluorescent UAS-GFP/RFP expression tagged with the respective progenitor specific Gal4 while the status of the differentiated zone was evaluated by immunostaining for

P1 (Nimrod) which marks differentiated plasmatocytes. The relative increase or decrease in area of differentiated cells (CZ) compared to progenitor cells (MZ) in case of RNAi knockdown of a gene function established the outcome of the screen. Based on which, a gene was categorized into one of three: (a) no changer; (b) positive regulator; (c) negative regulator of hemocyte progenitor maintenance.

2.3. Results

2.3.1. No changers of hemocyte progenitor maintenance

In order to achieve RNAi mediated down regulation of a particular gene in hemocyte progenitors present in MZ of larval lymph gland, MZ based *DomelessGal4* or *Tep4Gal4* lines were crossed to the UAS-RNAi line. This spatial control of progenitor specific knockdown was further augmented with temporal control by using temperature sensitive $gal80^{ts}$ allele. From egg laying the early second instar F1 larvae were kept at 18^{o} Celsius to bypass RNAi knockdown in early development stages. From mid second instar onwards these larvae are shifted to 29^{o} Celsius to impart RNAi mediated knockdown of the particular gene. Out of the 101 UAS-RNAi lines that were screened, knockdown of 91 *Drosophila* genes was achieved. For ~10 genes, more than one RNAi lines were used. The RNAi list along with the gene it is knocking down is listed in Table 2.1. Out of the 101 fly RNAi lines, 39 lines didn't give us any phenotype regarding progenitor maintenance and hence, listed as no changers.

However, the result of the primary screening by RNAi mediated knockdown not showing any phenotype presents an outcome that these genes might not be regulating the hemocyte progenitor maintenance or homeostasis. Nonetheless, the above outcome should be tested by using other independent fly lines as well as classical bona-fide loss of function alleles before making a conclusive statement of their role in hematopoiesis as such.

2.3.2. Positive regulators of hemocyte progenitor maintenance

RNAi mediated knockdown of genes that resulted in an increment of differentiation are categorized as positive regulators of progenitor maintenance. Out of 101 RNAi lines that were screened, 33 RNAi lines corresponding to 33 fly genes were categorized as positive regulators of progenitor maintenance. The major class of genes which showed as positive regulators of progenitor maintenance belonged to mitochondrial dynamics genes,

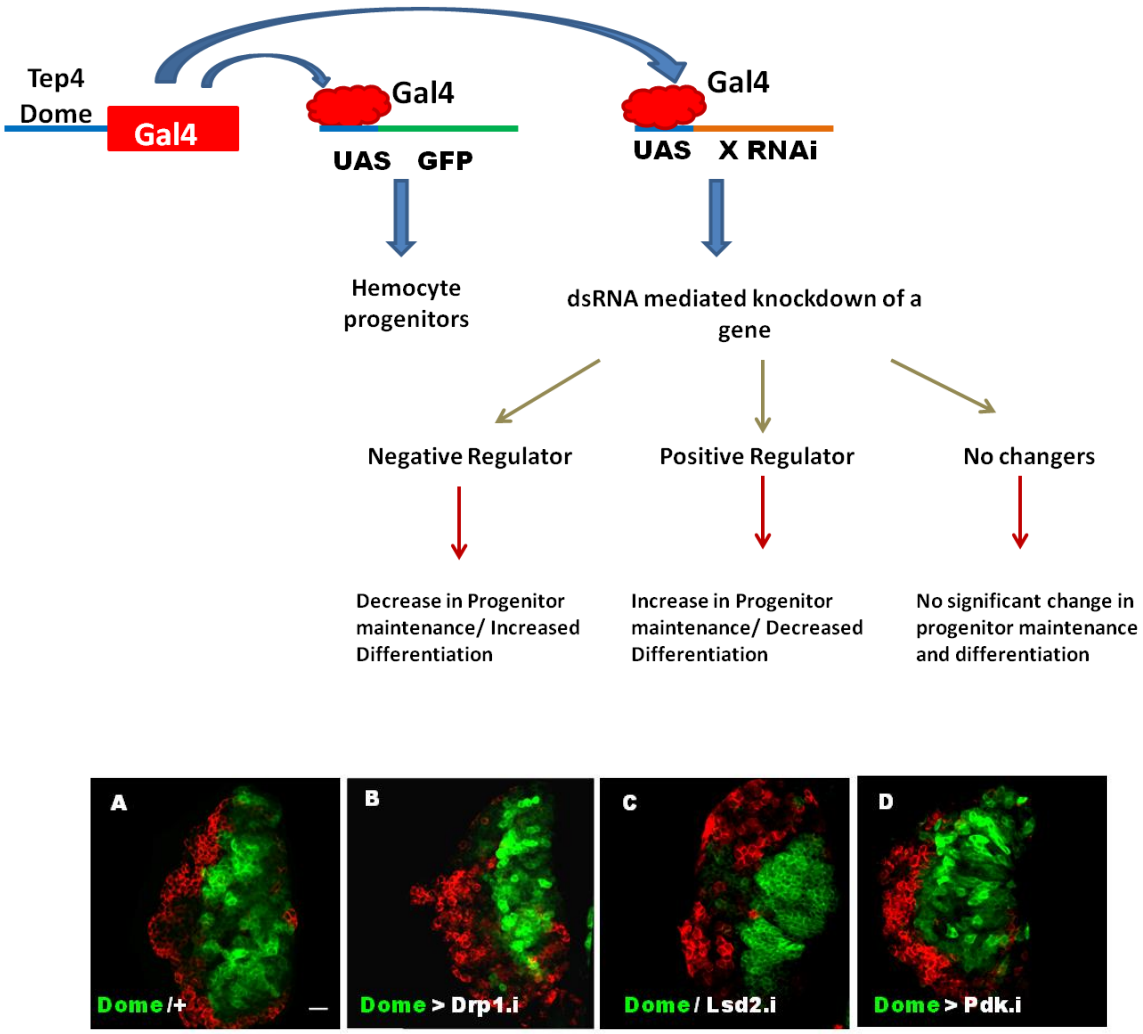

Fig1. Genetic strategy for progenitor specific knockdown of function of genes. Two independent progenitor specific GAL4 drivers *Domelessgal4* and *TepIVgal4* were used for the genetic screen where the progenitor population where positively labelled using UAS-GFP construct and UAS-X-RNAi was used to knockdown the function of gene of interest. Hits showing a decrease in the progenitor population compared to the wild type were categorised as **positive regulators**, increase in progenitor population was clubbed in **negative regulators**. **No changers** were those in which the progenitor population was comparable to that of wild type. **Few candidates representing the no changers category** dsRNA mediated knockdwn of genes including **(B)** *drp1*, **(C)** *Lsd2*, **(D)** *Pdk1* resulted in no significant change in the progenitor maintenance and differentiation compared to the **(A)** wildtype. Each experiment was performed atleast three times with n=10. Representative image depicts the average of the result. Scale bar: 20µm.

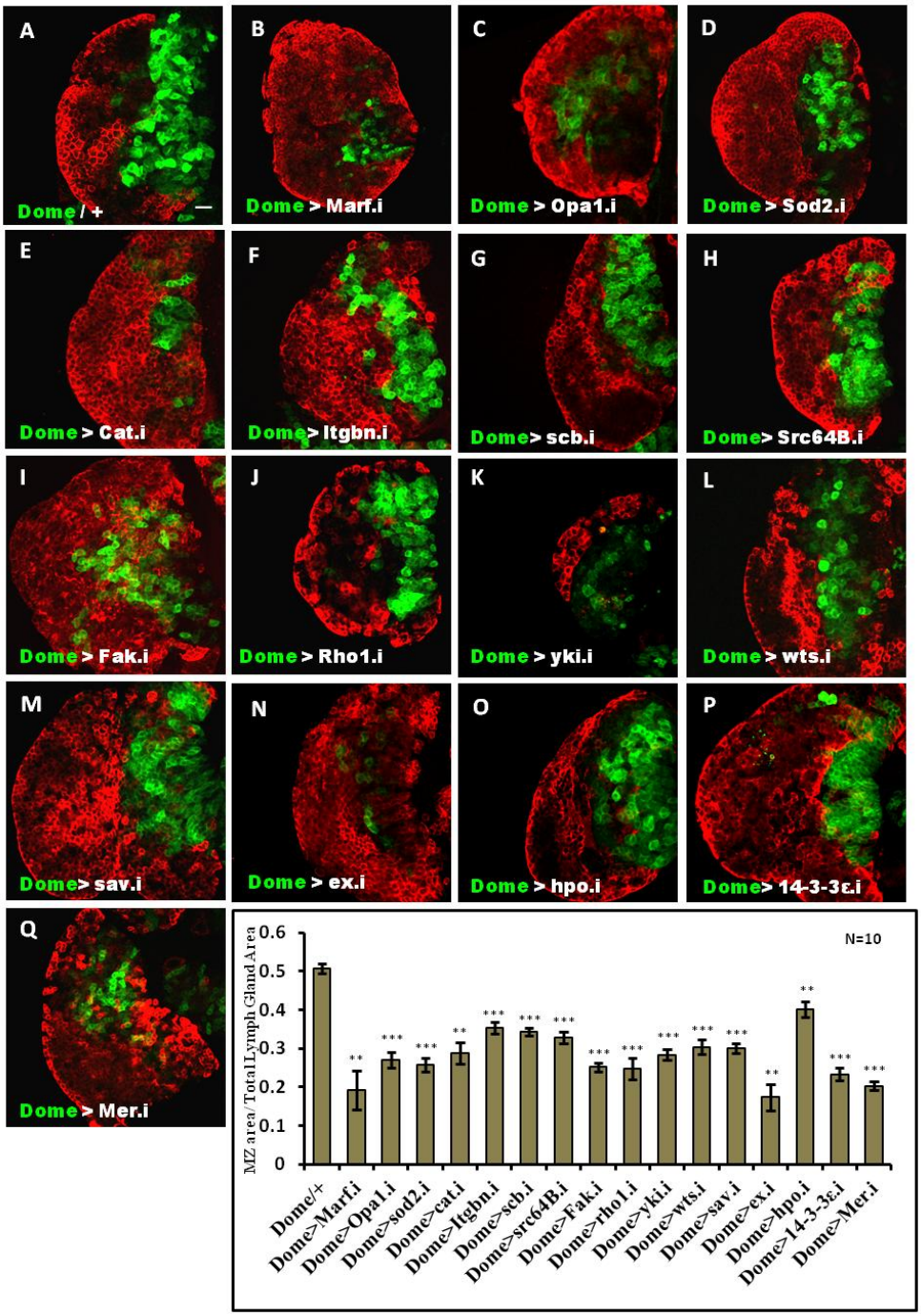

Fig 2. (A-R) Few positive regulators identified in the screen: dsRNA mediated knockdown of genes including *Marf* **(B)**, *Opa1* **(C),** *Sod2* **(D),** *Catalase* **(E)**, *Itgbn* **(F)**, *Src64B* **(H)**, *Fak* **(I)**, *Rho1* **(J)**, *Yorkie* **(K)**, *warts* **(L)**, *Salvador* **(M),** *Expanded* **(N)**, *hippo* (**O)**, *14-4-3 epsilon* **(P),** *merlin* **(Q)** resulted in decrease in progenitor cell population compared to that of wildtype **(A). (R)** Quantitative analysis showed statistically significant decrease in the pool of progenitor population compared to that of wild type. Red marks the differentiating cells and green marks the progenitor cell population. Each experiment was performed atleast three times with n=10. Representative image depicts the average of the result. Error Bar: S.D. NS, not significant, *p<0.05, **p<0.001, ***p<0.0001 (Student's *t*-test). Scale bar: 20µm.

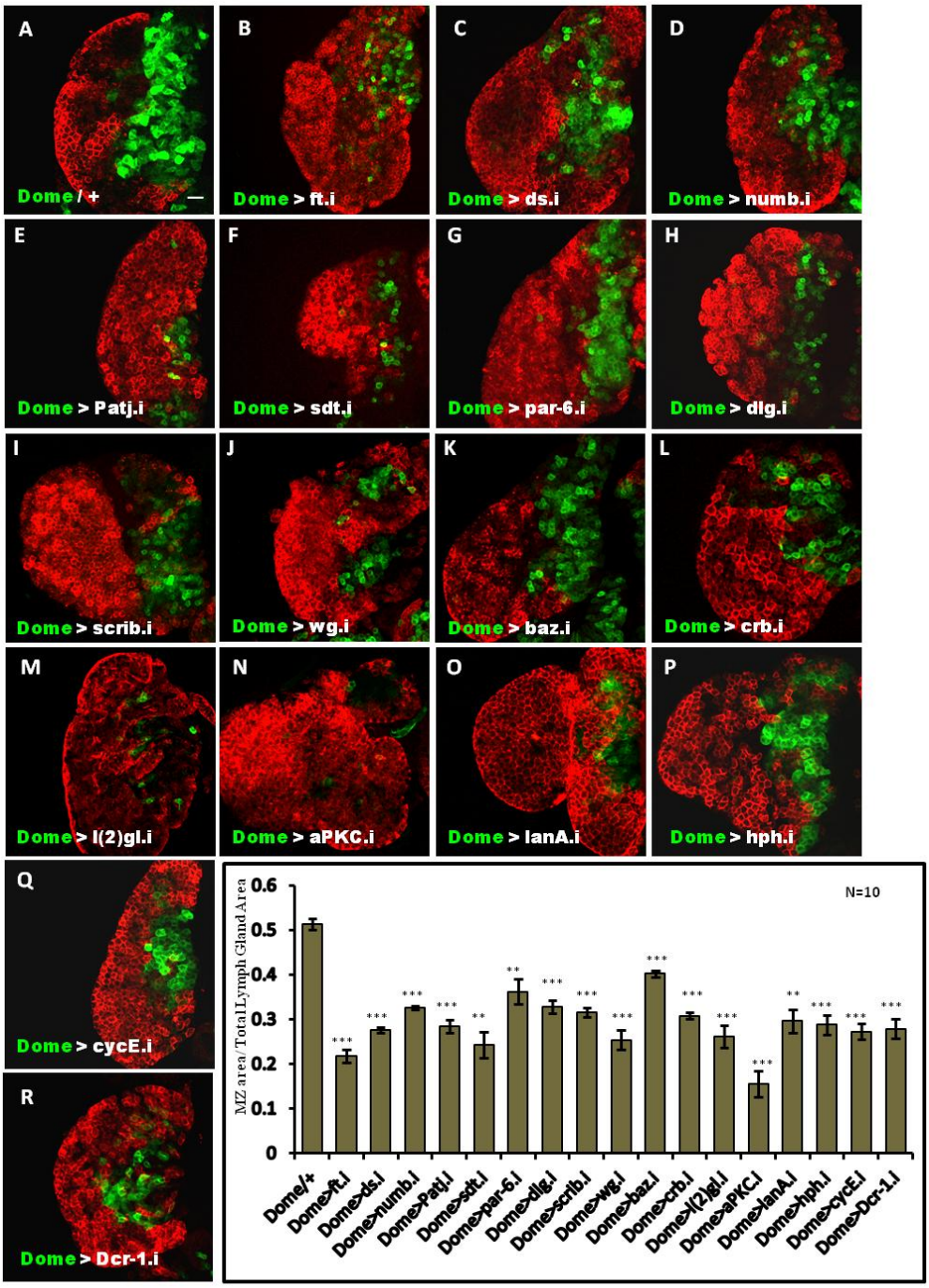

Fig 3. (A-S) Second set of positive regulators identified in the genetic screen : dsRNA mediated knockdown of the function of genes including *Fat* **(B)**, *Dachsous* **(C)**, *numb* **(D)**, Patj **(E)**, stardust **(F),** Par6 **(G),** *disc large* **(H),** *scribble* **(I),** *wingless* **(J),** *bazooka* **(K),** *crumbs* **(L),** *l(2)gl* **(M),** *aPKC* **(N),** *laminin A* **(O),** *hph* **(P),** *cyclin E* **(Q),** *Dicer1*(Valium 20) **(R)** resulted in decrease in progenitor cell population compared to that of wildtype (**A). (S)** Quantitative analysis showed statistically significant decrease in the pool of progenitor population compared to that of wild type. Red marks the differentiating cells and green marks the progenitor cell population. Each experiment was performed atleast three times with n=10. Representative image depicts the average of the result. Error Bar: S.D. NS, not significant, *p<0.05, **p<0.001, ***p<0.0001 (Student's *t*-test). Scale bar: 20µm.

mitochondrial redox regulators, Focal adhesion genes mediated by integrin signaling, Hippo pathway genes, cell polarity genes, ECM gene *LanA*, hypoxia gene *hph* and *Ecdysone Signaling Receptor*. Mitochondrial fusion genes *opa1* and *marf* positively regulate progenitor maintenance. RNAi mediated knockdown of genes regulating mitochondrial fusion leads to differentiation of blood progenitors along with the observation that Mitochondrial morphology in late instar progenitors are fused and reticular (Chapter 3. Fig.1. M-P) implicates that the mitochondrial dynamics play a significant role in regulating progenitor maintenance. Mitochondrial redox regulators *cat* and *sod2* serves as positive regulators of progenitor maintenance as previous reports have implicated ROS as positive regulator of differentiation; it sits well in the light of mitochondrial oxidation derived ROS imperative for progenitor maintenance/homeostasis.

Other major cohort that positively regulates hemocyte progenitor maintenance is cell polarity and hippo pathway genes. Interestingly, cell polarity genes have been shown to regulate cellular function by having control over hippo pathway output. Apart from apico-basal polarity, planar-cell-polarity genes also seem to be regulating progenitor maintenance. Cell polarity genes have been well studied in epithelia and single layer cells, but how cell polarity components are mediating progenitor maintenance in a mesodermal globular tissue is a challenge for future investigations.

2.3.3. Negative regulators of hemocyte progenitor maintenance

Around 30% of UAS-RNAi lines corresponding to 29 fly lines and 25 genes showed as negative regulators of progenitor maintenance. RNAi mediated knockdown of these genes stalled the differentiation of hemocyte progenitors. The negative regulators of progenitor maintenance are required for differentiation and hence in their knockdown, maintenance to differentiation switch is disrupted. Major class of genes acting as negative regulators of progenitor maintenance are: mitochondrial biogenesis, integrin signaling, lipid metabolism, pyruvate metabolism, JNK signaling, histone acetylation, ECM components and hypoxia regulation.

Current study reveals that as hemocyte progenitors move from self-renewal to quiescence, concomitant shift in mitochondrial amount per progenitor cells can be observed. Self-renewing progenitors have scanty mitochondria whereas mitochondrial load in quiescent progenitors primed for differentiation is pretty high. Therefore, knockdown of mitobiogenesis

genes showing disruption in differentiation imparts mitochondrial processes critical for progenitor differentiation.

Other major cohort is the presence of Integrin signaling and ECM components in negative regulators of progenitor maintenance. Interestingly, hemocyte progenitors are very tightly packed whereas the differentiated cells in primary lobe of lymph gland don't seem to be very densely packed relative to their MZ counterparts. The differential cellular and ECM architecture of progenitors and differentiated cells observed in the current study implied a possible role of integrin-ECM axis in progenitor homeostasis.

In *Drosophila*, integrin serves as cell-ECM adhesion complexes and knockdown of integrin as well as ECM components resulted in loss of progenitor homeostasis thereby implicating these factors in progenitor differentiation. This intriguing interplay of integrins and ECM in progenitor maintenance was selected for further in-depth characterization and analysis in Chapter 4.

It is the general belief of the biological field that cells derive their energy mainly from carbohydrates and fat moieties serve as reserve energy expenditure during starvation and other similar stress processes. Presence of several components of lipid metabolism as negative regulators of progenitor maintenance present an intriguing possibility that fat metabolism drives hemocyte progenitor homeostasis. A further in-depth characterization and analysis of this cohort was undertaken and described in chapter3.

Other important cohorts of negative regulators netted are *JNK* signaling, which has been implicated in previously linked with progenitor differentiation while Hypoxia regulator *sima* and redox regulator *Nos*, are yet to be implicated in hemocyte progenitor homeostasis.

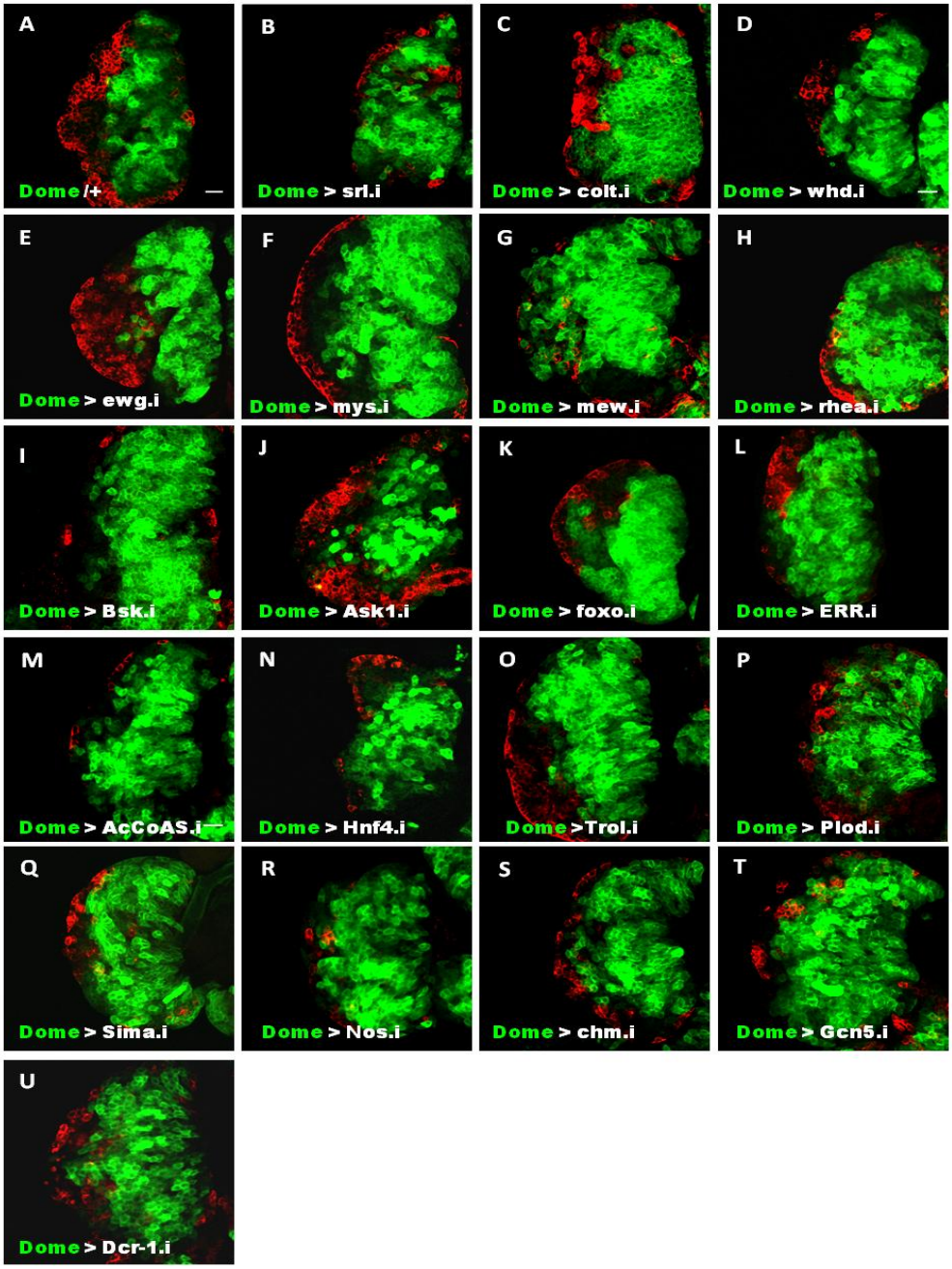

Fig 4 A-V Few negative regulators identified in the screen: dsRNA mediated knockdown of the function of genes including *spargel* **(B)**, *colt* **(C)**, *withered* **(D)**, *erect wing* **(E)**, *Myospheroid* **(F)**, *multiple edematous wing* **(G)**, *rhea* **(H)**, *basket* **(I)**, *Ask1* **(J)**, *foxo* **(K)**, *ERR* **(L)**, *AcCoAS* **(M)**, *Hnf4* **(N)**, *trol* **(O)**, *plod* **(P)**, *sima* **(Q)**, *nos* **(R)**, *chameau* **(S)**, *Gcn5* **(T)**, *Dicer1*(Valium 10) **(U)** resulted in decrease in progenitor cell population compared to that of wildtype **(A)**. **(V)** Quantitative analysis showed statistically significant increase in the pool of progenitor population compared to that of wild type. Red marks the differentiating cells and green marks the progenitor cell population. Each experiment was performed atleast three times with n=10. Representative image depicts the average of the result. Error Bar: S.D. NS, not significant, *p<0.05, **p<0.001, ***p<0.0001 (Student's *t*-test). Scale bar: 20µm.

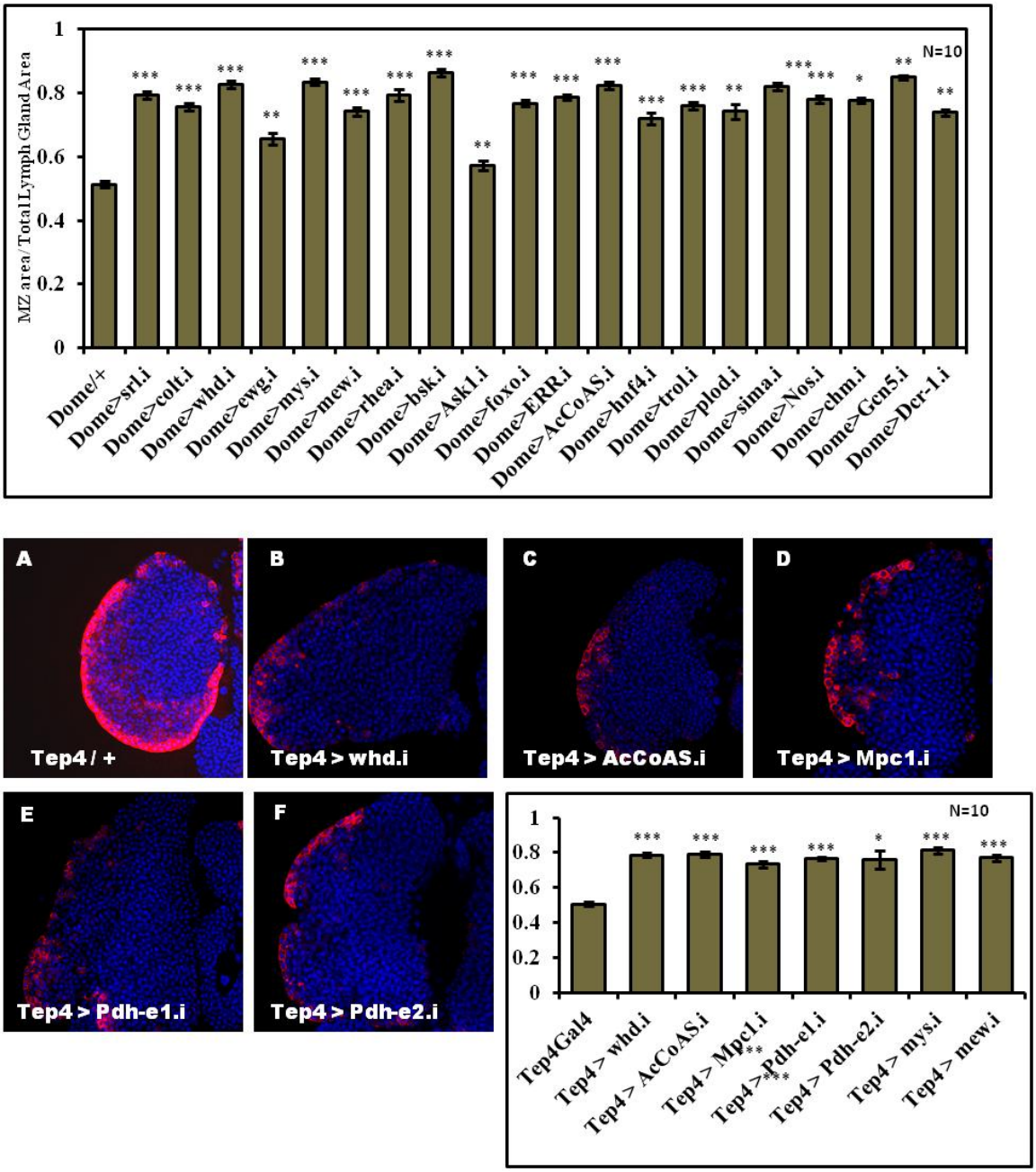

Fig 5A-G Few negative regulators identified in the screen using another independent driver *TepIV :* dsRNA mediated knockdwn of genes including *whd* **(B),** *AcCoAS* **(C),** *Mpc1* **(D)**, *Pdh-e1* **(E)**, *Pdh-e2* **(F)** resulted in a decrease in progenitor differentiation compared to that of control **(A).(G)** Quantitative analysis showed statistically significant decrease in the pool of differentiating cells compared to that of wild type. Red marks the differentiating cells and blue marks the nucleus. Each experiment was performed atleast three times with n=10. Representative image depicts the average of the result. Error Bar: S.D. NS, not significant, *p<0.05, **p<0.001, ***p<0.0001 (Student's *t*-test). Scale bar: 20µm.

2.4. Discussion and Conclusion

Since the beginning of developmental biology and embryology as a scientific field, the most important question people have tried to probe is how various cells of an embryo adopt a certain fate. The journey of life that begins from a single cell zygote takes its final form of trillions of cells in higher vertebrates all working in great synchrony to achieve a life form. The higher potency of embryonic cells doesn't fade away with the onset of adult life as almost all tissues are capable of repair and regeneration in different extent. Therefore, it is imperative to study the factors that regulate or govern the fate of a cell.

Study in multiple model systems catering to varied biological processes has divulged an array of signaling molecules and factors, which govern the fate of a cell. The factors regulating a particular developmental process in normal metazoan development, say neurogenesis is generally conserved across taxa. However, the cohort or cocktail of factors regulating different processes of metazoan development might not be identical. For example, the factors controlling the fate specification of a neuron and a blood cell might be different or might perform very different roles. Therefore, it is imperative to study the cell fate specification in different developmental processes and contexts.

In this context, current genetic screen was undertaken with an intention to identify the factors that regulate the hemocyte progenitor maintenance and homeostasis. The RNAi based primary screen enabled specific knockdown of different genes from larval hemocyte progenitors (in a defined spatiotemporal window) resulted in identification of various candidate genes crucial for progenitor maintenance. In addition to the genes whose RNAi lines when expressed in the progenitors didn't give a prominent phenotype (which are referred to as no changers), several genes were identified whose knockdown resulted in altered progenitor maintenance. They were binned into two groups: positive and negative regulators of progenitor maintenance. The positive regulators of progenitor maintenance are genes which when knocked down by RNAi resulted in loss of progenitor maintenance; On the other hand, the genes whose down regulation in the progenitors caused higher progenitor maintenance were referred as negative regulators. In simple analogy, positive regulators are the factors which are maintaining the progenitor status whereas, negative regulators move progenitor towards differentiation program.

Out of the various outcomes of the screen, one very intriguing revelation is the presence of nuclear encoded mitochondrial genes as both the positive and negative regulators of progenitor maintenance. There have been various studies in last couple of years, which have hinted at the collaboration of mitochondrial processes and fate of a cell regulating cell and tissue homeostasis (Bargiela et al., 2018), (Bertaux et al., 2018). But, the possibility that mitochondrial processes can impact the progenitor maintenance in both positive and negative way at the same time in a same cell type hasn't been explored in greater details. Mitochondrial redox regulators having an impact on progenitor maintenance has been reported in hemocyte progenitors (Owusu-Ansah and Banerjee, 2009), but components of mitochondrial dynamics required for hemocyte progenitor maintenance is to our knowledge an unexplored outcome of this screen.

Another very fascinating outcome of this study is the presence of metabolic factors regulating the homeostasis of progenitors. It has long been debated in the field that metabolic output is outcome of the fate and state of cell rather than the governing factor regulating cellular fate (Cliff and Dalton, 2017). Among the candidates netted in the screen, there are regulators of Pyruvate metabolism and Fatty acid metabolism whose down regulation affected progenitor maintenance. Post-primary screen, the involvement of the metabolic genes in hemocyte progenitor maintenance was further confirmed by employing bona-fide loss of function fly lines. The analyses affirmatively establish that both the pyruvate and fatty acid metabolism are required for differentiation as disruption of both these pathways lead to loss of differentiation and higher maintenance of hemocyte progenitors. We chose to further analyze the involvement of metabolic input in progenitor homeostasis with detailed characterization is described in Chapter3.

Besides the mitochondrial/metabolic cohort, another very interesting genetic cohort, which gave noticeable phenotype, is the progenitor specific loss of cell adhesion and ECM components. In *Drosophila*, integrins have been implicated in cell-ECM adhesion. Loss of differentiation seen in RNAi mediated knock down of *Beta*PS/*Alpha*PS1 implicates cell-ECM adhesion in progenitor homeostasis. This genetic cohort was also selected for further analysis. βPS/αPS1 integrin knockdown in progenitors resulted in loss of quiescence coupled with loss of differentiation. In integrin knockdown from progenitors, the cell-cell adhesion and ECM status was examined. The expression of DE-Cadherin, which mediates cell-cell adhesion, is increased in integrin knockdown scenario. On analyzing the expression of ECM component

Perlecan in integrin knockdown, surprisingly an elevated expression was seen around hemocyte progenitors compared to control. These present an intriguing collaboration of cell adhesion and ECM in determining the fate of hemocyte progenitors described in Chapter4.

When positive regulators of hemocyte progenitor maintenance were analysed, many components of hippo signaling were present. The hemocyte progenitor specific role of hippo signaling hasn't been explored in greater details. Down regulation of the function of transcription factor *yki* from hemocyte progenitors led to their compromised survival since induction of cell death can be seen. Moreover, as a consequence of cell death, entire primary lobe of lymph gland is reduced in size. Overexpression of *yki* achieved by RNAi mediated knock down of hippo signaling kinases *warts, expanded, merlin* as well as driving UAS *yki* construct leads to increased differentiation of hemocyte progenitors. The transcriptional reporter of active hippo signaling *Diap1* GFP expression is high in self renewing progenitors and low in quiescent progenitors, suggestive of hippo signaling kinases restricting yki in cytoplasm in late third instar thereby mediating progenitor quiescence. This genetic cohort was pursued for further analysis described in chapter5.

Apart from the aforesaid candidates, selected for detailed analyses, there are several candidate genes picked up from the primary screen that can be classified as positive or negative regulators of hemocyte progenitor maintenance, eg., *Nos1, scrib, CycE* etc. Interestingly, their involvement in hematopoiesis hasn't been reported previously. This screen thus sets a platform for further analysis and characterization of some of the candidate genes in hemocyte progenitor maintenance and hematopoiesis *per se* in future studies.

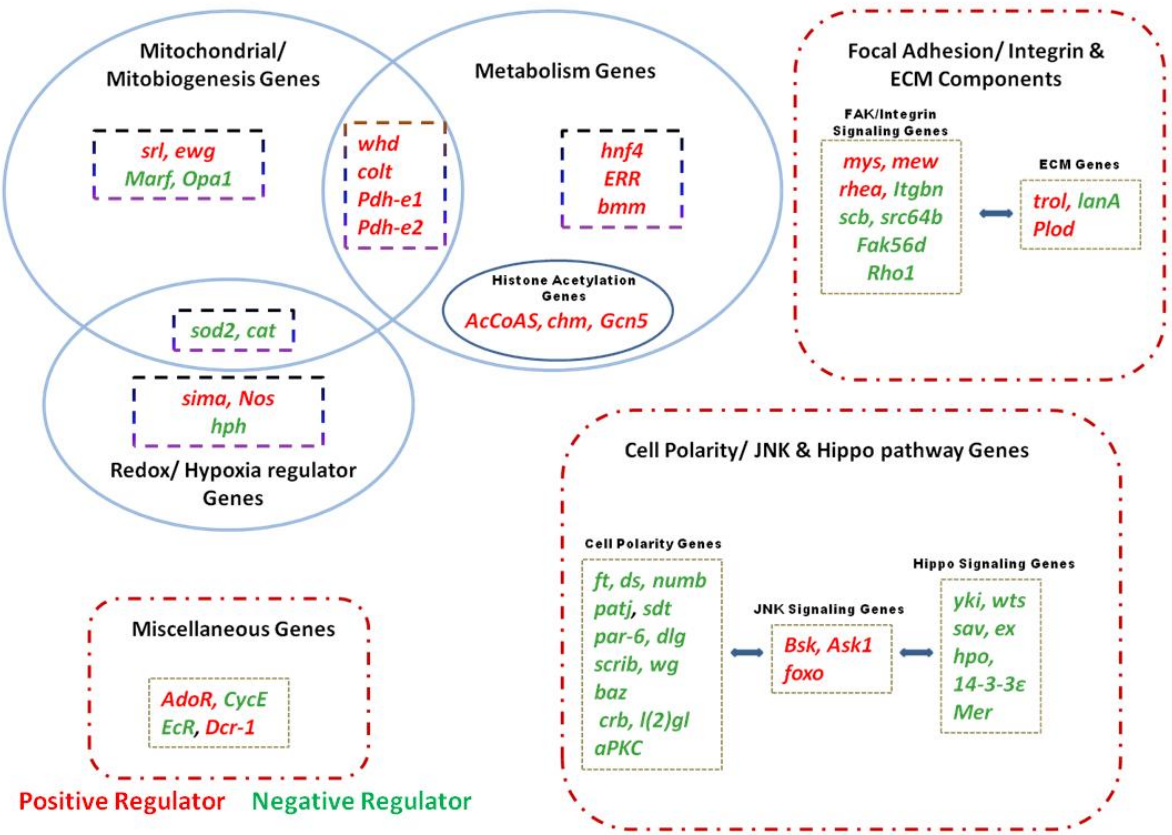

Fig 6. Comprehensive illustration of all candidate genes picked up in the genetic screen which are categorized as positive and negative regulators for progenitor cell maintenance.

Table 2.1. List of all the UAS RNAi lines used in the genetic screen

Modifier	Gene disrupted	Bloomington TRIP/VDRC RNAi stock number
Positive regulator	*srl*	33914
	colt	51798
	whd	34066
	ewg	31104
	mys	33642
	mew	27543
	rhea	32999
	bsk	32977
	Ask1	32464
	foxo	32427
	bmm	25926
	ERR	27085
	AcCoAS	41917
	hnf4	29375
	trol	29440
	Plod	34911
	sima	26207
	Nos	28792
	AdoR	27536
	Dcr-1	34826
	chm	27027
	Mpc1	KK103829

positive regulator	*gcn5*	33981
	Pdha	KK107209
	pdh-e2/muc	KK110735
	AcCoAS	KK100281
	mys	KK103704
	mew	KK109608
	whd	KK105400
Negative regulator	*marf*	31157
	opa1	32358
	sod2	32496
	cat	34020
	Itgbn	28601
	scb	27545
	src64b	30517
	Fak56d	29323
	Rho1	27727
	yki	31965
	wts	34064
	sav	32965
	ex	34968
	hpo	33614
	14-3-3ε	34884
	Mer	34958
	ft	34970
	ds	32964

Negative regulator	*numb*	35045
	patj	35747
	sdt	33909
	par-6	35000
	dlg	33620
	scrib	35748
	wg	32994
	baz	35002
	crb	34999
	l(2)gl	35773
	aPKC	34332
	lanA	28071
	hph	34717
	CycE	33654
	EcR	29374
No changers	*drp1*	31157
	Ilk	57308
	Rac1	34910
	RhoGEF2	34643
	kay	33379
	slpr	32948
	Tak1	33404
	Lsd-2	32846
	vkg	50895

No changers	*Col4a1*	44520
	Nox	32902
	tgo	26740
	RhoGAP19D	32361
	pfk	34336
	apc	34869
	PDZ-GEF	28928
	Epac	29317
	Pkc53E	34716
	Rap1	35047
	Rassf	27663
	shg	32904
	sparc	40885
	stan	35050
	zasp52	31561
	N	33611
	Ten-m	29390
	Iswi	32845
	par-1	32410
	sir2	32481
	tip60	28563
	sea	33976
	cbp	27724
	Rok	34324

No changers	*deltaCOP*	31764
	Nos	50675
	Lsd-2	34617
	srl	KK103355
	err	KK108349
	whd	33635

Chapter 3.

Fatty Acid Beta oxidation regulates hemocyte progenitor quiescence and differentiation.

3.1. Introduction

By driving cellular respiration and energy production, mitochondria sit at the centre of cellular metabolic processes. Apart from providing bioenergetics for cellular processes, mitochondria have been at the forefront of regulating cell fate decisions. Mitochondrial metabolic processes has been associated with regulating the cell fate decisions of various stem cells including, embryonic stem cells, hematopoietic stem cells, intestinal stem cells, muscle satellite stem cells and others (Anso et al., 2017), (Buck et al., 2016), (Anso et al., 2017), (Zhang et al., 2018). However, there is no unified mechanism by which mitochondria impacts stem cell fate decisions. Mitochondrial biogenesis, mitochondrial dynamics, increased mitochondrial metabolism, along with regulation of Calcium homeostasis and mitochondrial ROS production are the major aspects by which mitochondria has been shown to regulate stem cell differentiation (Chen et al., 2008), (Folmes et al., 2011), (Luchsinger et al., 2016), (Folmes et al., 2012).

Fatty Acid beta-Oxidation has been implicated to be feeding into the bioenergetics circuit of mitochondria (Eaton et al., 1996), (Bartlett and Eaton, 2004), (Kelley, 2005), however, its role in regulating cell differentiation remains to be elucidated (Wong et al., 2017), (Knobloch et al., 2017). Current study demonstrates that Fatty Acid beta-Oxidation is critical for differentiation of blood progenitors in *Drosophila melanogaster* which are akin to myeloid progenitors of vertebrates. Loss of function of genes associated with Lipid catabolism and Fatty Acid beta-Oxidation resulted in drastic reduction of differentiation of blood progenitors into differentiated blood cell types.

Drosophila blood cell or hemocyte development takes place in an organ called lymph gland which starts forming in late embryogenesis (Jung et al., 2005). Lymph gland houses

hemocyte progenitors, differentiated blood cells and the dedicated signaling cells or niche cells for providing the microenvironment for hematopoiesis (Crozatier et al., 2004; Jung et al., 2005), (Krzemien et al., 2007). Early instar lymph gland houses the self-renewing hemocyte progenitors and the niche cells. Third instar mature lymph gland houses the differentiated blood cells in periphery, in a region termed as cortical zone (CZ). The inner core of the late third instar lymph gland termed as medullary zone (MZ) houses hemocyte progenitors (Jung et al., 2005). The hemocyte progenitors found in MZ are akin to the vertebrate common myeloid progenitors (CMP). They are quiescent, have high levels of ROS, lack differentiation markers, are multipotent, and can give rise to all *Drosophila* blood lineages (Jung et al., 2005),(Crozatier et al., 2004), (Owusu-Ansah and Banerjee, 2009). MZ cells are maintained by the signals from the niche cells also known as cells of Posterior Signaling Centre (PSC).

The current genetic screen revealed that loss of function of genes belonging to lipid metabolism affected the differentiation of blood progenitors (Fig. 2C-D). Studies have implicated that lipid turnover is vital for cellular and tissue homeostasis (Liu and Huang, 2013), (Henne and Bundorf, 2008) but its role in regulating cell differentiation is unexplored. This chapter, therefore, intends to answer the role of lipid metabolism in blood cell differentiation.

Metabolism is the sum of two opposite processes: anabolism and catabolism. Anabolic processes involve use of energy to make larger macromolecules from smaller ones. Catabolic processes involve breakdown of larger macromolecules in smaller ones to release energy (Ito and Ito, 2016). Organismal survival and growth depends upon the continuous supply of ATP and metabolic intermediates generated by cellular respiration of different substrates, such as carbohydrates, amino and fatty acids in steady state. Excess calories in the body are stored as triacylglycerides (TAG) in specialized storage cells, called adipose cells (Schulz et al., 2015). During periods of prolonged starvation, TAG mobilization and catabolism becomes vital. Long chain Fatty acids (LCFA) are released from the glycerol backbone and activated by addition of a Coenzyme A (CoA) residue. LCFA-CoA undergoes beta-oxidation, either in mitochondria or in peroxisomes, but only mitochondrial beta-oxidation is coupled to ATP production (Van Veldhoven, 2010).

3.2. RESULTS

3.2.1. Progenitors switch from Glucose metabolism to fatty acid oxidation as they move on from self-renewal to quiescence and differentiation.

Studies in *Drosophila* have facilitated *in vivo* genetic analysis of stem and progenitor cells in their resident microenvironments (Van Veldhoven, 2010). Studies in *Drosophila* hematopoiesis has not only elucidated conserved nature of hematopoiesis across taxa (Mandal et al., 2004) but has also uncovered and implicated new and unique factors governing this process (Mondal et al., 2011), (Shim et al., 2013). *Drosophila* hemocyte progenitors are present in an organ called lymph gland which starts forming in late embryogenesis. The hemocyte progenitors are in a self-renewal state in early larval stages, as they actively incorporate EdU (Fig 1. A-B). The active state of cell cycle in early stage progenitors give way to cell cycle arrest or quiescence in late third instar hemocyte progenitors where EdU labeled cells are less in number. As blood progenitors move from an active state of cell cycle to quiet one, their fate also changes towards the differentiation program.

Although, the genetic circuitry controlling the transit of self-renewing blood progenitors to quiescence and eventually differentiation has been implicated (Mondal et al., 2011), the role of metabolism in this process is not known. Thus the first step was to analyse the metabolic signatures in the self-renewing and quiescent hemocyte progenitors. *In vivo* glucose uptake assay was performed by employing fluorescent derivative of glucose, 2-NBDG that revealed a stark difference in glucose uptake in self-renewing and quiescent progenitors. Self-renewing early stage progenitors showed noticeably elevated levels of glucose uptake (Fig 1. C) Compared to late stage quiescent progenitors (Fig1. D), indicating high glucose requirement/utilization in self-renewing state. The difference in glucose uptake in these progenitors is at the level of differential expression of glucose transporter is evident when a protein trap transgenic fly reporter Glut1-GFP was used. As shown in Figure Glut1 expression in self renewing progenitors is much higher compared to quiescent ones (Fig 1. E-F).

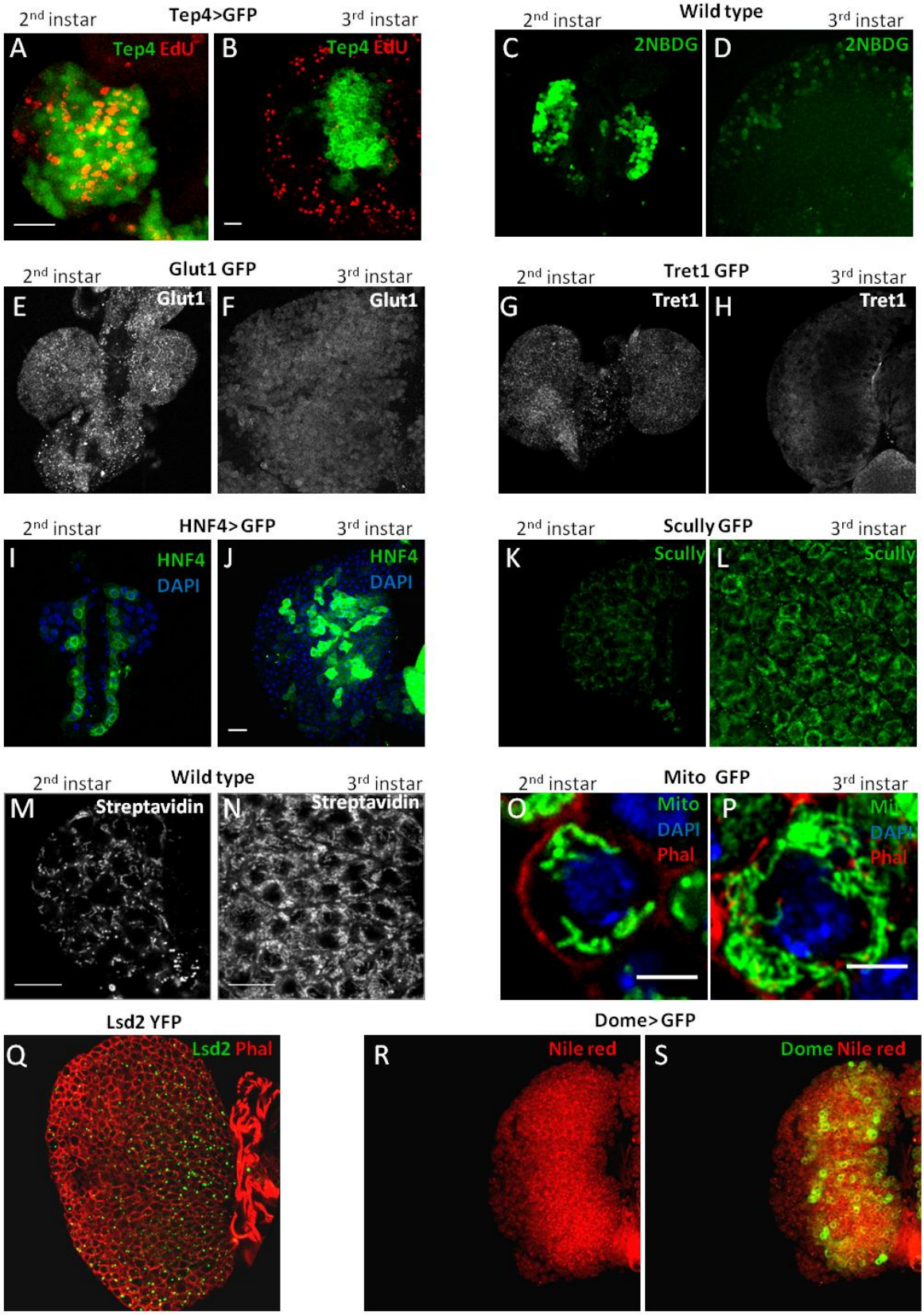

Figure1. **1A-B**: Levels of EdU incorporation in *Tep4* positive second instar progenitors (**1A**) compared to third instar late progenitors (**B**). **C-D**: Levels of 2-NBDG incorporation in second instar progenitors (**C**) compared to third instar late progenitors (**D**). **E-F**: Comparison of Glut1-GFP levels

in 2nd instar (**1E**) and 3rd instar late (**F**) larval lymph gland. **G-H**: Comparison of Tret1-GFP levels in 2nd instar (**1E**) and 3rd instar late (**F**) larval lymph gland. **I-J**: Levels of *Hepatocyte Nuclear Growth Factor4 (HNF4)* in HNF4>GFP lymph gland in 2nd instar (**I**) compared to and 3rd instar (**J**) larvae. **K-L**: Comparison of Scully>GFP in 2nd instar (**K**) and 3rd instar late (**L**) larval lymph gland. **M-N**: Comparison of Streptavidin fluorescence levels in 2nd instar (**M**) and 3rd instar (**M**) lymph glands. **O-P**: Zoomed in pattern of co-localization of *Mito-GFP* and Phalloidin in 2nd instar (**O**) larval lymph gland compared to late 3rd instar (**P**) larval lymph gland. **Q**: Phalloidin stain marking cell boundaries in *Lsd2-YFP* 3rd instar larval lymph gland. *Lsd2YFP* marks the lipid droplet. **R-S**: Nile Red staining marking neutral lipids in *Dome* positive progenitors in 3rd instar late larval lymph gland. Each experiment was performed atleast three times with n=10. Representative image depicts the average of the result. Scale bar: 20µm.

In *Drosophila*, the main energy supplying sugar is trehalose, a disaccharide formed by two α-glucose monomers linked in a 1,1-glycosidic bond (Slone et al., 2007). Trehalose is transported across plasma membrane by trehalose transporter (Slone et al., 2007). The reporter expression of one of the trehalose transporter Tret1-1 was next done. The protein trap transgenic fly construct tret1-1-GFP revealed higher expression in self renewing progenitors compared to quiescent ones (Fig 1. G-H). Hence, above results endorse the high glucose utilization/requirement in self renewing progenitors and as they become quiescent, the concomitant reliance on glucose uptake and/or utilization is drastically reduced.

As the development progresses, the self-renewing progenitors move on to quiescent state, which seems to be a prelude to the initiation of the differentiation programme. As there is less glucose/trehalose uptake/utilization in quiescent state, we reasoned that progenitors might be switching to some other energy source to meet the metabolic demands of differentiation.

Apart from sugar/carbohydrate metabolism, other major metabolic state active in stem and progenitor cells has been shown to be Fatty acid oxidation (FAO) (cartoon, (Knobloch et al., 2017),(Wang et al., 2018), (Ito et al., 2012) One of the key genes that have been implicated in larval FAO and lipid mobilization is *Hepatocyte Nuclear Factor 4 (HNF4)*. When *HNF4* expression was observed using binary GAL4/UAS system, it showed very high expression in quiescent progenitors compared to basal level expression in early self-renewing progenitors, indicating the shift to FAO in late quiescent progenitors (Fig 1. I-J). Also the expression of *Scully* (the hydroxy-acyl-CoA dehydrogenase implicated in FAO) was examined by using a protein-trap transgenic fly construct Scully-YFP. This also showed higher expression in quiescent progenitors (Fig 1. K-L). Expression of FAO genes exemplified the concomitant shift from glucose to fat metabolism, as the progenitors move from self-renewal to quiescence and differentiation.

Major facets of FAO take place in the mitochondria where fat moiety is broken down to generate acetyl CoA, NADH and $FADH_2$ (Bartlett and Eaton, 2004). Whereas, major part of glucose metabolism or glycolysis, take place in the cytosol (Lunt and Vander Heiden, 2011). To further ascertain the differential metabolic requirements of self-renewing and quiescent progenitors, the mitochondrial distribution in these two stages was next looked at. Early stage self-renewing progenitors had very scant and punctae mitochondria indicative of glycolytic state (Fig 1. M-N) whereas, late stage quiescent progenitors had reticulate network of

mitochondria (Fig 1. O-P). Mitochondrial distribution also supports the hypothesis of shift from sugar metabolism to fat metabolism as the progenitors quiesce and prepare to differentiate.

With the concomitant increase in mitobiogenesis, a significant level of neutral lipids/lipid droplets in blood progenitors (Fig1. Q-S) was observed. The presence of significant amount of lipid droplets in a non-lipid storage tissue raised the possibility that these lipids might have some developmental role per se. The expression of *HNF4* gene which has been implicated in lipid mobilization and in beta oxidation further strengthened the possibility of lipid turnover playing crucial role in hematopoiesis per se.

3.2.2. Fatty Acid beta-Oxidation is required for differentiation of blood progenitors

The significance of lipid homeostasis in starvation and oxidative stress has been well documented in light of providing the source of ATP and antioxidant role (Cahill, 2006), (Bailey et al., 2015), but its role in normal developmental context hasn't been explored in greater details. Fatty Acid beta-Oxidation is the process by which cellular lipids are broken down to produce energy. Cells store lipids in form of TAG (Tryacylglycerols) which are broken down to free Fatty Acids (FA) and glycerol. Fatty acyl-CoA synthase adds CoA group to fatty acids. CPT1 present on outer mitochondrial membrane converts long chain acyl-CoA to long chain acylcarnitine which is transported across the inner mitochondrial membrane by carnitine translocase. CPT2 present on inner mitochondrial membrane converts long chain acylcarnitine back to long chain acyl-CoA (Schulz et al., 2015). The long chain acyl-CoA can then enter beta-oxidation pathway resulting in formation of one acetyl-CoA from each cycle of beta-oxidation. The acetyl-CoA produced can enter the TCA cycle. The NADH and FADH2 produced by beta-oxidation along with TCA cycle can be utilized by electron transport chain to produce ATP (Schulz et al., 2015).

Drosophila homolog of CPT1, withered (whd) (Strub et al., 2008) is important for transport of fatty acid moiety inside the mitochondria for fatty acid beta-oxidation. Loss of function of *whd* would effectively block mitochondrial fatty-acid beta-oxidation (Strub et al., 2008). To investigate the role of FAO in hematopoiesis the TARGET system (McGuire et al., 2004) was employed to down regulate *whd* expression in progenitors using RNAi construct of *whd*. Two different Gal4 based progenitor drivers, *Dome-Gal4* (Jung et al., 2005) and *TepIV-Gal4* (Shim et al., 2013) and two different RNAi constructs (from two sources, KK lines from

VDRC and TRIP from Bloomington: BDSC). Progenitor specific down regulation of *whd* expression results in a halt in differentiation as evidenced by the decline in number of differentiated plasmatocytes (P1 (red): Fig 2. B-F) along with the strong reduction in differentiated cell domain, the Cortical Zone (CZ) area. Similarly, knockdown of another key player in lipid metabolism *HNF4* by RNAi results in reduction in differentiation of progenitors, suggesting the role of FAO in hematopoietic differentiation of hemocyte progenitors. Blood progenitor specific knockdown of lipid metabolism genes strengthen the cell-autonomous role of Fatty Acid Beta-Oxidation in differentiation of blood progenitors and implicates a unique role of lipid metabolism in hematopoiesis.

To further validate the role of FAO in progenitor differentiation a null allele of *withered*, whd^{1} was employed. In order to examine the differentiation status in the lymph gland, P1 immunostaining was used to positively mark the differentiated plasmatocyte and the area of differentiated cell type, CZ. Remarkably, whd^{1} homozygous primary lobes have drastically reduced number of differentiated hemocytes when compared to similar aged controls. The complete loss of function of *dHNF4* was analyzed employing transheterozygous combination of two alleles, *dHNF4Δ33*/*dHNF4Δ17* (Palanker et al., 2009). Mutant *dHNF4* lymph glands had similar reduction in differentiated cell, CZ area as evidenced by P1 immunostaining, further illustrated that FAO disruption leads to compromise in differentiation of blood progenitors (Fig 2. G-I). The state of progenitors in the loss of function null background was observed by Dome>GFP expression which revealed that all the non-differentiated cells are in hemocyte progenitor state (Fig 3. A-B).

Apart from genetic knockdown and loss of function analysis pharmacological inhibition of FAO in *Drosophila* larvae was performed. Two bonafide FAO inhibitors, etomoxir (Pike et al., 2011) and mildronate (Singh et al., 2016) were used to knock down FAO. In both cases, a drastic drop in differentiation as seen in genetic analysis was evident (Fig 3. C-D).

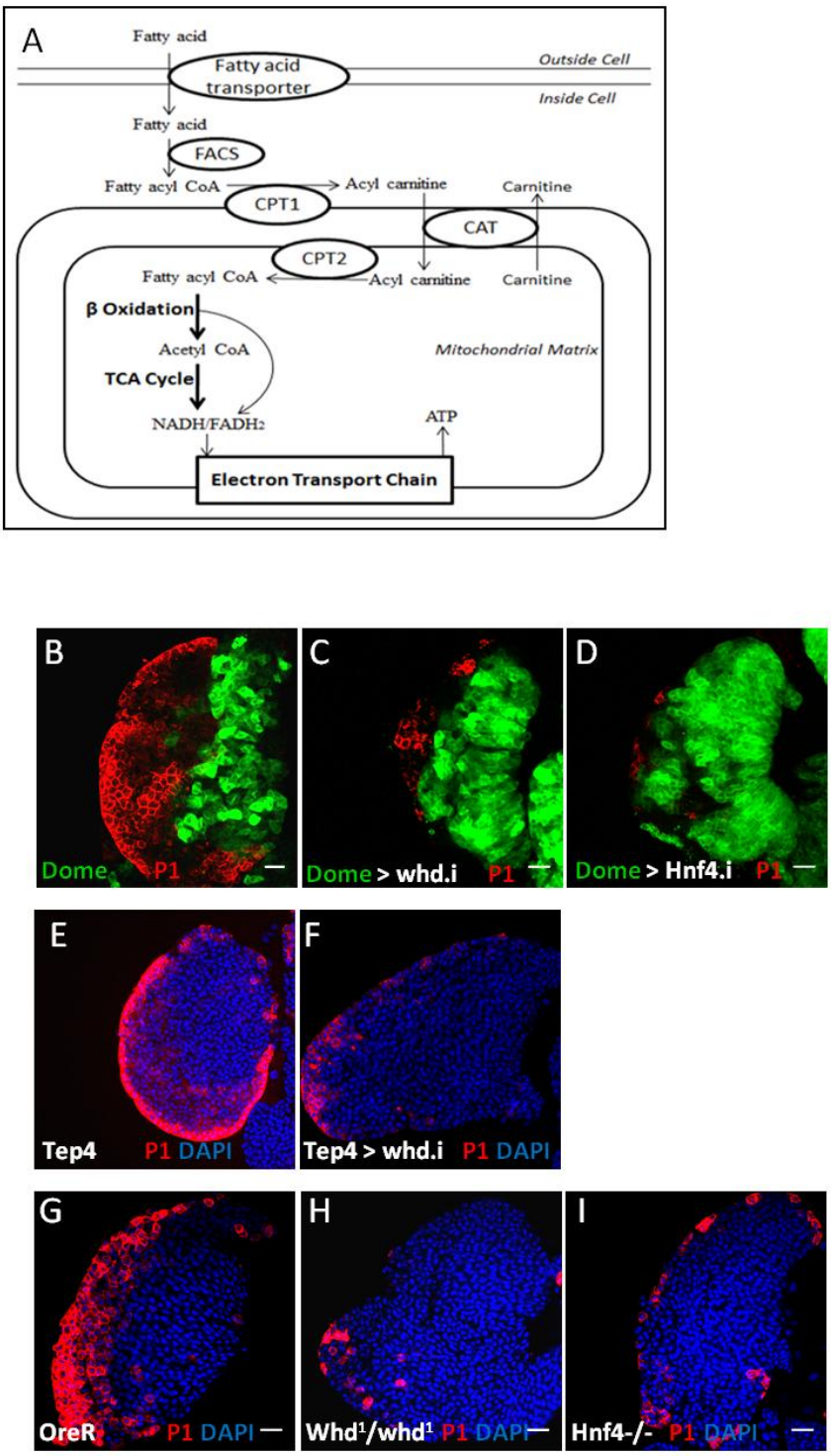

Fig.2.A: Schematic representation of Fatty Acid β oxidation in cell. **B-D:** Status of P1 antigen in lymph gland of *Dome* driving *whd* RNAi (**C**) and *Hnf4* RNAi (**D**) compared to control (**B**). Figure **E-F**: Status of P1 antigen in lymph gland of *Tep4* driving *whd* RNAi (**F**) compared to control (**E**). **G-I**: Status of P1 antigen in *whd*^{1} mutant (**H**) and *Hnf4* mutant (**I**) compared to wildtype (**G**). Each experiment was performed atleast three times with n=10. Representative image depicts the average of the result. Scale bar: 20µm.

Since perturbation of differentiation of hemocyte progenitors was noted, the next agenda was to examine the status of differentiation and maintenance factors per se in these progenitors where FAO is compromised. Hedgehog (hh) signaling has been implicated in the maintenance of the progenitors (Mandal et al., 2007). Hematopoetic niche also known as Posterior Signaling Centre (PSC) relays hh which are received by the progenitors and the active hh signal can be assayed in the progenitors by looking at the expression of hh signal transducer Cubitus interruptus (Ci), its active form Ci^{155}. Interestingly, upon immunostaining elevated levels of Ci^{155} was observed in *whd*^{1} mutant lymph gland correlating with high maintenance and less differentiation (Fig 3. E-H).

The major signal that has been implicated in differentiation of the hemocyte progenitors is Reactive Oxygen Species (ROS). High levels of developmentally generated ROS in progenitors trigger Jun Kinase (JNK) signaling which sets these progenitors towards differentiation programme. Since, in FAO loss of function *whd*^{1} mutant lymph glands, differentiation is compromised an attempt was made to examine the status of differentiation signal, ROS in *whd*^{1} mutant lymph glands by dihydroxy ethedium (DHE) staining. Surprisingly, when compared to the control, there was no compromise in levels of ROS in *whd*^{1} mutant progenitors, suggesting that the progenitors are not undergoing differentiation despite having normal levels of differentiation signal, ROS (Fig 3. I-L). Therefore, the stall in progenitor status in FAO loss of function is not due to the imbalance of maintenance vs differentiation signals. Rather these blood progenitors and have the differentiation signal ROS levels comparable to control.

To further characterize the lymph gland progenitors in FAO loss of function mutants, cell cycle status was investigated employing EdU labeling. Mutant *whd* and *dHNF4* lymph glands had significantly higher number of EdU labeled progenitor cells compared to similar aged controls, suggesting that progenitors in FAO loss of function are in active state of self-renewal instead of cell cycle arrest/ quiescence found in control third instar late progenitors.

Together these results assert that FAO disruption in progenitors doesn't allow them to quiesce and subsequently differentiate. Despite the presence of differentiation signal progenitors are not able to move into differentiation. It suggests an intriguing possibility of FAO acting downstream of differentiation signal ROS to commemorate differentiation programme.

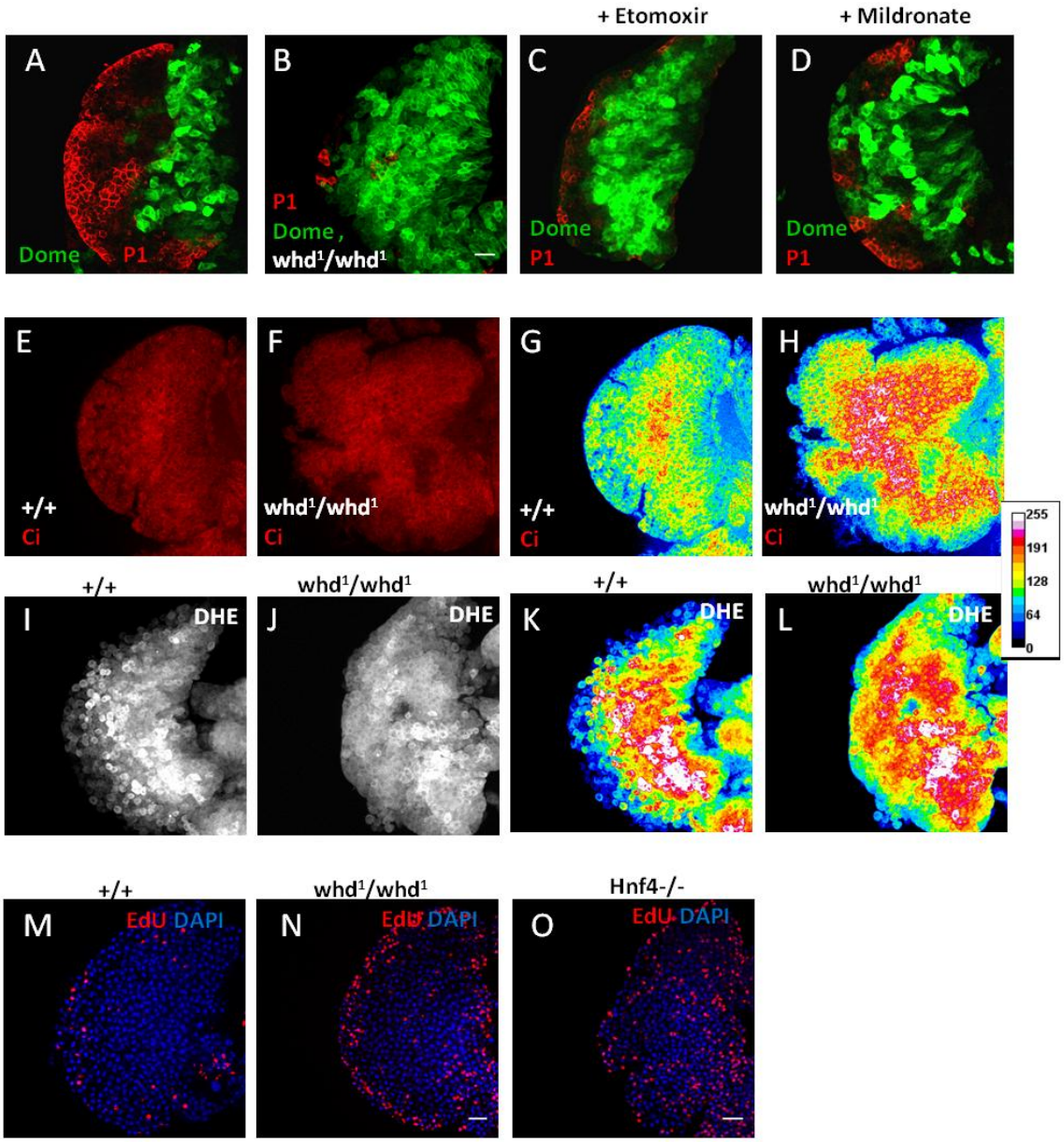

Fig 3. A-D: Comparison of P1 levels in the lymph gland of 3rd instar control larvae (**A**), *Dome>GFP*; whd1 homozygous mutants (**B**) with Etomoxir fed (**C**) and mildronate fed (**D**) larvae. **E-F**: Status of Ci levels in lymph gland of *whd*^{1} mutants (**F**) compared to wildtype (**E**). **G-H**: Heat map representing the Intensity of Ci in the lymph gland of *whd*^{1} mutants (**G**) compared to wildtype (3H). **I-J**: Comparison of DHE levels in the lymph gland of *whd*^{1} mutants (**J**) and wildtype (**I**). **K-L**: Heat map representing the Intensity of ROS by DHE staining in the lymph gland of *whd*^{1} mutants (**L**) compared to wildtype (**K**). **M-O**: EdU incorporation in the lymph gland of 3rd late instar wildtype larvae (**M**) compared to *whd*^{1} mutants (**N**) and *Hnf4* mutants (**O**). Each experiment was performed atleast three times with n=10. Representative image depicts the average of the result. Scale bar: 20µm.

3.2.3. L-carnitine supplementation leads to increased and precocious differentiation and onset of quiescence in blood progenitors.

The transport of Long chain fatty acid (LCFA) moiety in mitochondria requires the carnitine shuttle present on the mitochondrial membrane. Carnitine aided by other enzymes sits at the centre of the carnitine shuttle. LCFA moiety once transported inside the mitochondria can undergo beta-oxidation (Van Veldhoven, 2010). Carnitine is a small soluble compound which can be synthesized by the body as well as acquired by food products. Individuals suffering from defects in carnitine biosynthesis primarily rely on carnitine uptake from food products where carnitine becomes an essential nutrient. Due to the central role of carnitine in fat metabolism, it is also used as a supplement for enhancing fat metabolism/burning (Marcovina et al., 2013). It has been shown that carnitine supplementation shifts the cellular metabolism from carbohydrate dependent energy generation to fat dependent energy generation (Li et al., 2017). Therefore, administering L-carnitine effectively upregulates FAO (Li et al., 2017).

Drosophila larvae were fed L-carnitine supplemented fly food and the third instar late mature lymph glands 96 AEH were analyzed. Strikingly, hemocyte progenitors upon administering L-carnitine exhibit enhanced or increased differentiation. The progenitor zone area marked by progenitor marker *Dome>GFP* is drastically reduced and the differentiated cell area marked by P1 immunostaining is increased compared to control lymph glands (Fig 4. A-B). Apart from the upregulated differentiation in the primary lobe of lymph glands in larvae fed with L-carnitine, the progenitors of secondary and tertiary lobes which are undifferentiated at this time point (Jung et al., 2005) seems to have initiated differentiation program precociously as evidenced by presence of P1 positive cells and decline in number of *Dome* expressing progenitor cells (Fig 4. A-B). Apart from the increased differentiation of hemocyte progenitors in L-carnitine fed condition, a decrease in size of the lymph glands prompted an investigation of the cell cycle status of these progenitors. Increase beta oxidation due to L-carnitine feeding resulted in drastic reduction of EdU incorporation in hemocyte progenitors.

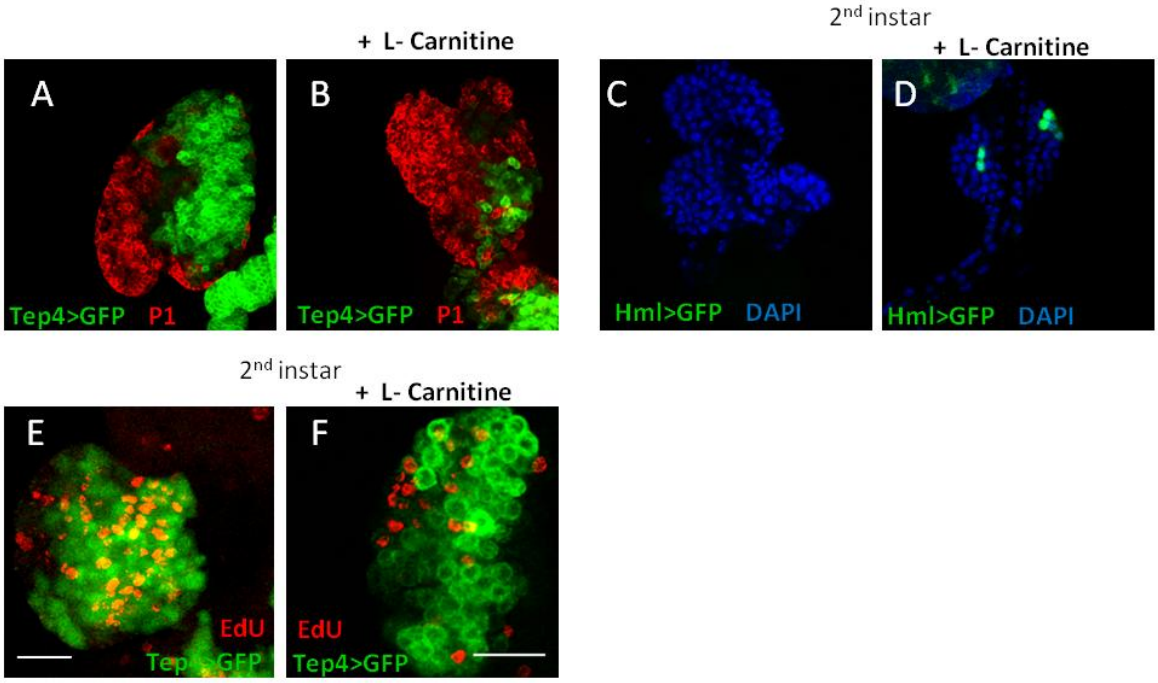

Fig.4.A-B: Comparison of P1 levels in *Tep4>GFP* lymph gland of control (**A**) and L-Carnitine administered (**B**) larvae. **C-D**: Induction of differentiating *Hml>GFP* 2nd instar lymph gland of control (**A**) and L-Carnitine administered (**B**) larvae. **E-F**: EdU incorporation levels in *Tep4>GFP* 2nd instar lymph gland of control (**E**) and L-Carnitine administered (**F**) larvae. Each experiment was performed atleast three times with n=10. Representative image depicts the average of the result. Scale bar: 20µm.

However, during normal scenario, late third instar progenitors seldom incorporate EdU suggesting their quiescent status at this time point. Therefore, to investigate the cell cycle status in L-carnitine fed condition, actively self-renewing second instar hemocyte progenitors were analyzed. Interestingly, EdU incorporation in L-carnitine fed third early and mid instar hemocyte progenitors drastically affected compared to similar aged controls, suggesting cell cycle arrest/quiescence brought about in L-carnitine supplementation (Fig 4E-F).

Whether increase in differentiation is coupled with advent of quiescence in hemocyte progenitors upon L-carnitine administration needed to be tested. To investigate such possibility the stage 36 hrs AEH was chosen. This is because at this stage i.e., mid second instar lymph gland consists of primarily self-renewing progenitors with differentiation yet to be initiated. First instar larvae were reared in fly food supplemented with L-carnitine for 24 hrs and assayed. The differentiation status was examined by looking at the expression of *Hml>GFP*, a differentiating cell marker in fed vs. non-fed controls. In similar aged control lymph glands no *Hml>GFP* positive cell are seen whereas in L-carnitine lymph glands *Hml>GFP* positive cells are present (Fig 4. C-D), suggesting that L-carnitine supplementation leads to precocious differentiation of hemocyte progenitors. On investigating the cell cycle status by EdU assay at this time point it was evident that L-carnitine fed condition the EdU incorporation is drastically reduced compared to similarly aged larvae (Fig 4. E-F).

Collectively, all these results suggest that upregulating fat oxidation by administering L-carnitine leads to precocious differentiation and quiescence of hemocyte progenitors.

3.2.4. FAO loss of function leads to increased glucose uptake in hemocyte progenitors

As hemocyte progenitors move from self-renewal to quiescence/cell-cycle arrest, a concomitant drop in glucose uptake was observed (Fig 1. C-D). The loss of quiescence seen in FAO loss of function mutant *whd*^{1} led to the hypothesis that in absence of fat utilization, progenitors have reverted back to high glucose utilization/ metabolism. To gain an insight into the sugar metabolism status in hemocyte progenitors, glucose uptake assay was performed by employing fluorescent derivative of glucose, 2-NBDG which revealed an upregulated glucose uptake in FAO mutant late instar progenitors compared to similar aged controls (Fig 5. A-C).

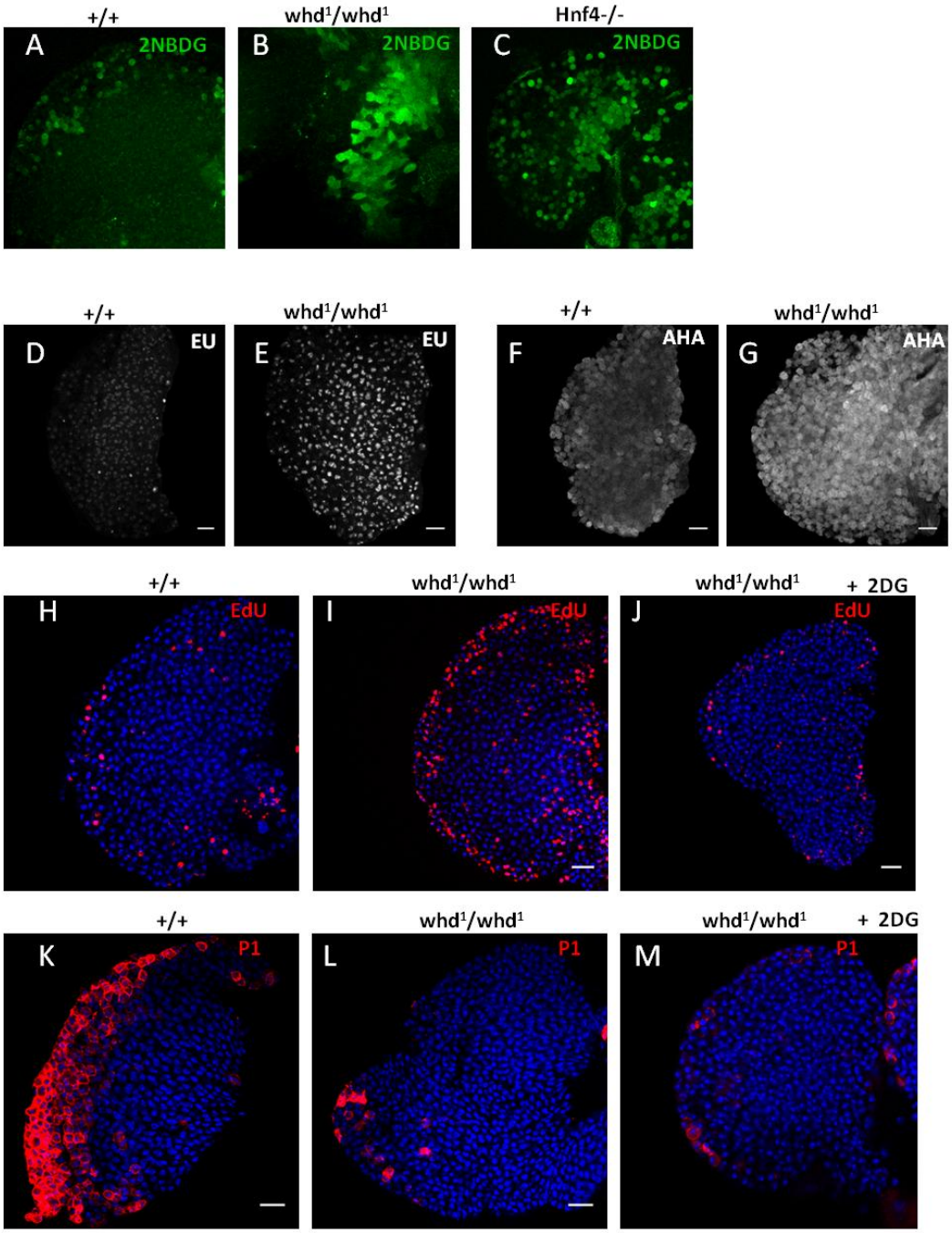

Fig.5. A-C: Levels of 2NBDG in 3rd instar larval lymph gland in *whd*^{1} mutant (**B**) and *Hnf4* mutant (**C**) compared to wildtype (**A**). Figure D-5E: Comparison of levels of EU incorporation in the lymph gland of wildtype (**D**) and *whd*^{1} mutant larvae (**E**). **F-G**: Levels of AHA in the larval lymph gland in *whd*^{1} mutant (**G**) compared to wildtype (**F**). **H-J**: Rescue of EdU incorporation by administration of 2-DG in *whd*^{1} mutants (**J**) compared to wildtype (**H**) and *whd*^{1} mutants (**I**). **K-M**: Comparison of levels of P1 in wildtype (**K**), *whd*^{1} mutants (**L**) and 2-DG administered *whd*^{1} mutants (**M**). Each experiment was performed atleast three times with n=10. Representative image depicts the average of the result. Scale bar: 20µm.

To concur whether loss of differentiation and quiescence observed in FAO mutant progenitors can be ascertained to the surge in glucose uptake/metabolism, glycolytic inhibitor 2-Deoxy-D-glucose (2-DG) (Woodward and Hudson, 1954) was employed. Upon feeding third instar mutant *whd*^{1} larvae with fly food supplemented with 2-DG, it was observed that the active self-renewing progenitors now have quietened down with 2-DG supplementation, as a consequence EdU incorporation reverted back to wild type like state (Fig 5. H-J). However, the abrogated differentiation seen in mutant *whd*^{1} didn't revert back to wild type like state upon glycolytic block by feeding 2-DG. Hence, the loss of quiescence seen in FAO loss of function can be attributed to the surge in glucose uptake/metabolism but this surge isn't responsible for the higher maintenance or disrupted differentiation of hemocyte progenitors. Thus, these set of results further implicates the FAO in hemocyte progenitor differentiation.

3.2.5. Histone acetylation is altered in FAO loss of function

Beta oxidation of fatty acids results in formation of one acetyl-CoA from each cycle of reaction. The acetyl-CoA produced can be employed to produce energy by entering TCA cycle or can be used for acetylation of various proteins, majorly known of such proteins are mitochondrial proteins and histones (Drazic et al., 2016). Hence, disrupted differentiation of hemocyte progenitors in FAO mutants may be attributed to either altered ATP levels or/ and difference in acetylation levels. To ascertain whether loss of differentiation seen in FAO mutant progenitors is due to compromised ATP levels, ATP assay was performed which showed similar or non-significant changes in ATP levels in *whd*^{1} mutants compared to similar aged controls (Fig 6. A). Apart from the active cell cycle state of *whd*^{1} mutant progenitors, an analysis of their transcription and translation profiling by EU (Fig 5. D-E) and AHA (Fig 4. F-G) labeling respectively, revealed a concomitant increase in transcription as well as translation along with earlier observed increased proliferation. The upregulation of ATP demanding processes like cell division, transcription and translation further affirms that in *whd*^{1} mutants, ATP levels are not compromised.

Thus, the next step was to ascertain whether disrupted FAO in *whd*^{1} mutants leads to altered histone acetylations. Incidentally, the genetic screen done in this study had netted few acetylation genes whose downregulation from the hemocyte progenitors affected their differentiation. Specifically RNAi mediated knockdown of Histone Acetyl Transferase

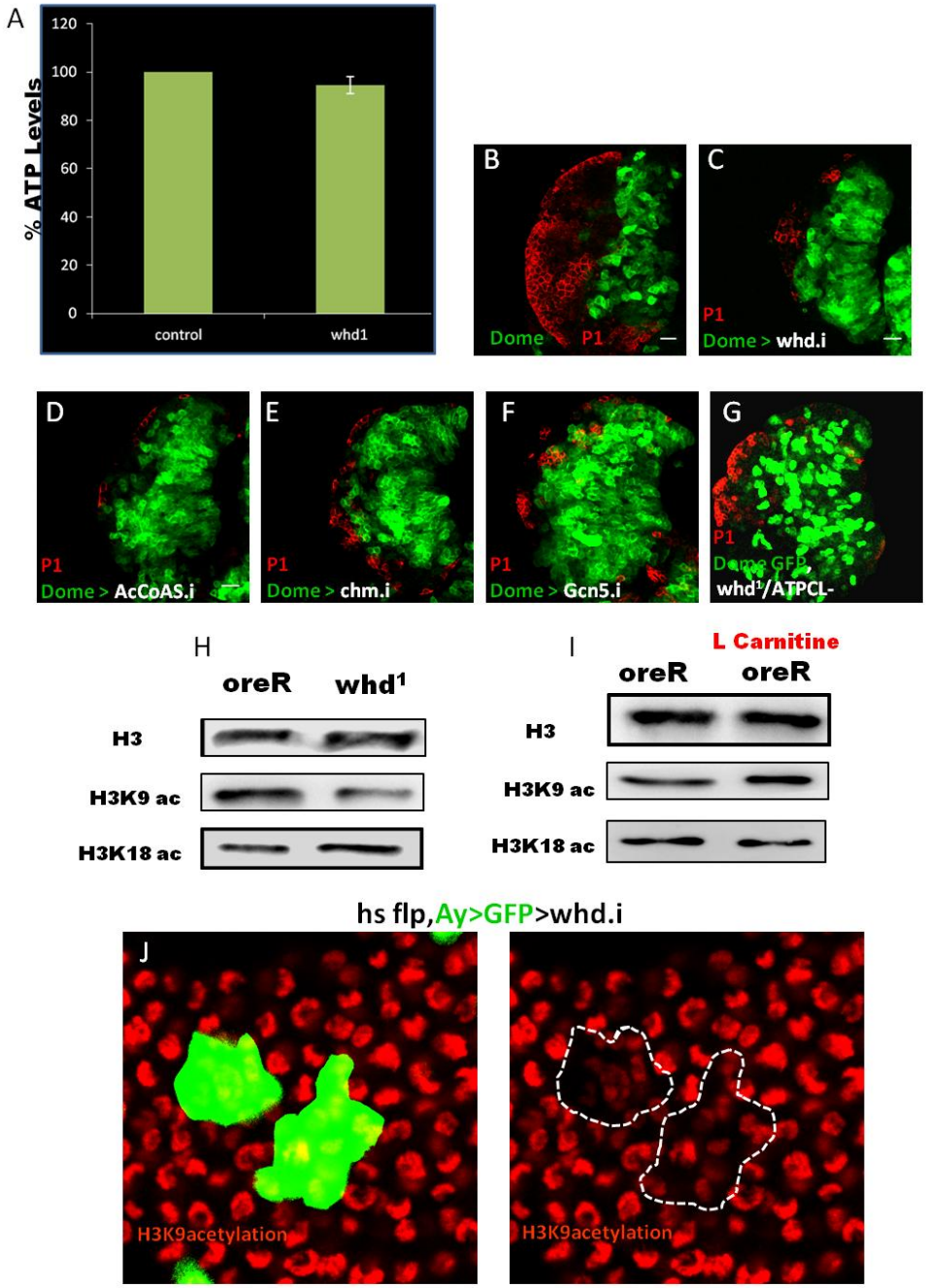

Fig.6.A. Graph showing the percentage of ATP levels in wildtype compared to *whd*^{1} mutant larvae. **B-G**: Level of P1 antigen in control (**B**) compared to *Dome>GFP* driving (**C**) *whd-RNAi*, (**D**) *AcCoAS-RNAi*, (**E**) *chm-RNAi*, (**F**) *Gcn5-RNAi* and (**G**) *DomeGFP; whd*^{1}*/Atpcl*$^{-}$ heterozygous one copy mutant of *whd* and *ATPCL*. **H**: Western blot showing the comparison of Acetylated H3K9 and Acetylated H3K18 in *whd*^{1} mutants compared to wildtype. H3 was used as loading control. **I**: Western blot showing the comparison of Acetylated H3K9 and Acetylated H3K18 in L-carnitine administered larvae compared to wildtype. H3 was used as loading control. **J-K**: *hsflp; AyGal4* based *whd-RNAi* mosaic clones with H3K9 acetylation immunostaining in background. Each experiment was performed atleast three times with n=10. Representative image depicts the average of the result. Error Bar: S.D. NS, not significant, *p<0.05, **p<0.001, ***p<0.0001 (Student's *t*-test). Scale bar: 20µm.

(HAT) genes, *Gcn5* and *chm*, *Chameau* phenocopies FAO loss of function in hemocyte progenitors both in terms of differentiation as well as quiescence defects (Fig 6. E-F). Apart from HAT genes, RNAi mediated knockdown of another metabolic gene which has been widely implicated in histone acetylation, *Acetyl Coenzyme A Synthase/ AcCoAS* the *Drosophila* orthologue of *ACSS2*, phenocopied the differentiation defects seen in FAO loss from hemocyte progenitors (Fig 6. D). This prompted the epistatic interaction analysis of whd^{1} allele with classical loss of function of major acetylation gene, *ACLY Drosophila* orthologue, *ATPCL*. Trans-heterozygous one copy loss of function of whd^{1} and $ATPCL^{01466}$ phenocopied FAO loss of function hemocyte progenitor in whd^{1} homozygous mutant larvae (Fig 6. G). Above set of genetic correlation analysis seen endorsed the histone acetylation alteration in FAO loss of function.

Histone acetylation status in whd^{1} homozygous mutant larvae were next analysed compared to similar aged wild type control larvae. Histones were extracted and probed by western blotting for Histone 3 acetylation status primarily by anti-H3K9 acetylation and anti-H3K18 acetylation antibodies. Anti-Histone3 served as the loading control. Compared to wild type, H3K9 acetylation was compromised in whd^{1} mutants whereas, H3K18 acetylation was upregulated (Fig 6. H). Downregulation of specific H3K9 acetylation led to the hypothesis that the differentiation defects in whd^{1} mutants are outcome of this acetylation alteration. To check this hypothesis, whd^{1} mutant larvae were fed sodium acetate and their differentiation status was analysed. Intriguingly, sodium acetate feeding rescues the differentiation defects seen in FAO loss of function whd^{1} mutant hemocyte progenitors (Fig 7. A-D). Moreover, when H3K9 acetylation status was analysed in whd^{1} mutants fed with sodium acetate, H3k9 acetylation levels were rescued compared to non fed whd^{1} mutants. To ascertain whether the histone acetylation status seen in whd^{1} mutant larvae replicated in the cells of interest, hemocyte progenitors, mitotic clonal analysis (Lee et al., 2005) was undertaken. Mosaic clones using *hsFLP/ay-GAL4* mediating RNAi knockdown of *Drosophila CPT1* orthologue *whd* were generated and immunostaining was carried out using anti-H3K9 acetylation. The clonal patches positively marked with GFP showed a remarkable drop in H3K9 acetylation levels (Fig 6. J-K) validating the H3K9 acetylation defects seen in FAO loss of function hemocyte progenitors. To further abrogate the H3K9 acetylation alteration seen in FAO loss of function, in L-carnitine fed scenario where the upregulation of beta-oxidation leads to upregulated differentiation, H3K9 acetylation profile was analysed by western blotting. Wild

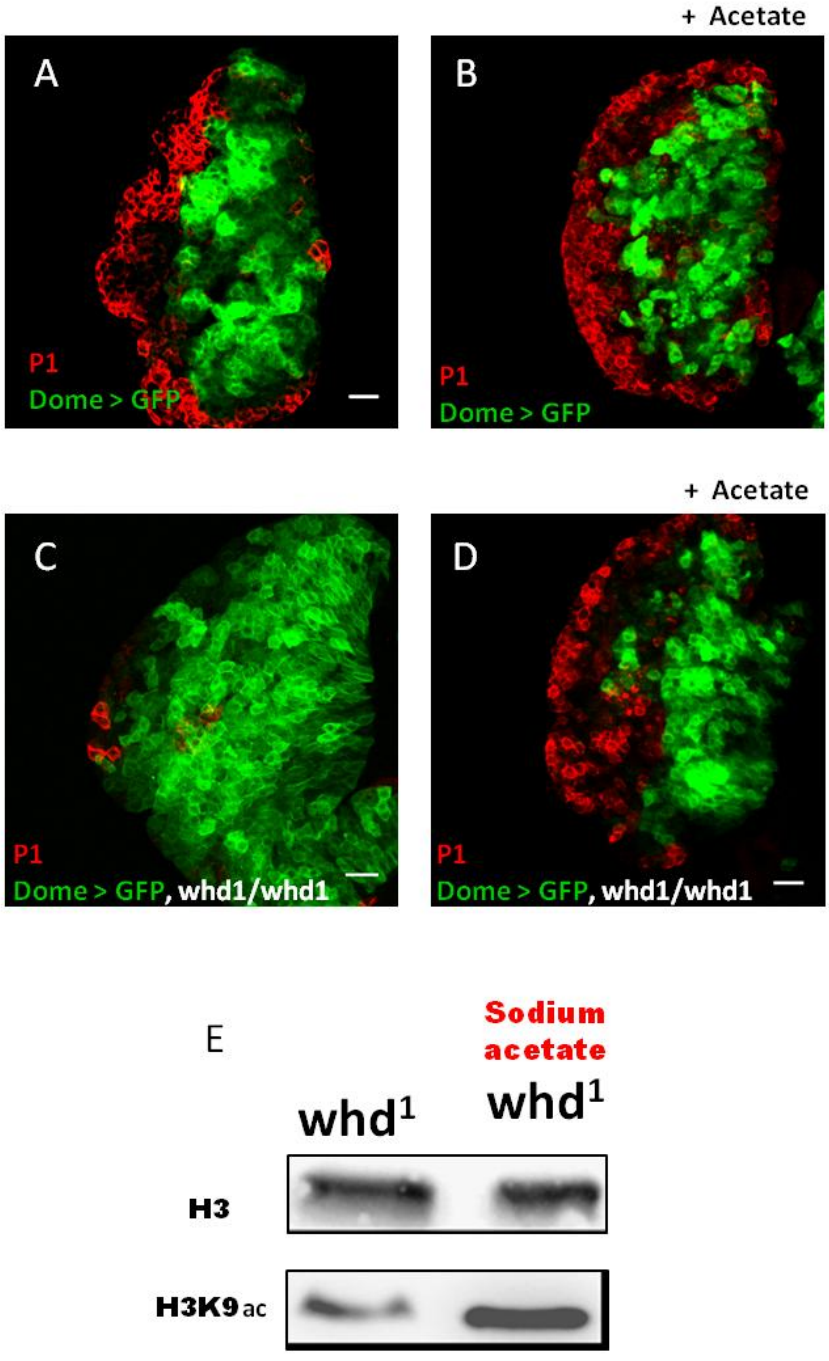

Fig.7. **A-B**: Status of P1 antigen in *Dome>GFP* larval lymph gland in control (**A**) compared to Sodium Acetate administered larvae. **C-D**: Rescue of P1 in *Dome>GFP; whd*^{1}*/whd*^{1} mutant larval lymph gland (**D**) by administering Sodium Acetate. **E**: Western blots showing increment in H3K9 acetylation levels in Sodium Acetate administered *whd*^{1} mutant larvae compared to control. Each experiment was performed atleast three times with n=10. Representative image depicts the average of the result. For the western blot experiments three biological replicates were done with n=3. Error Bar: S.D. NS, not significant, *p<0.05, **p<0.001, ***p<0.0001 (Student's *t*-test). Scale bar: 20µm.

type larvae when fed with L-carnitine had upregulated H3K9 acetylation levels compared to similar aged non-fed controls, further implicating FAO mediated H3K9 acetylation being central to hemocyte progenitor differentiation (Fig 6. I).

3.3. Discussion

Researchers around the world are increasingly getting interested in the role of metabolism along with cellular signaling in shaping cell fate decisions in normal development as well as in pathophysiological conditions. Current findings uncover a new aspect of requirement of metabolism in progenitor quiescence and differentiation.

The conventional belief of the biological field has been that cells derive their energy from glucose and only in times of starvation or fasting do we see the utilization of other energy sources such as fat. But, reports in last couple of years have challenged this belief with the increasing evidences pointing out that stem cells and progenitor cells may actually preferentially choose fatty acid metabolism not only for energy production but utilization of intermediary metabolites for various cellular processes.

To our knowledge, this is the first report which implicates the requirement of Oxidative Phosphorylation and beta oxidation of fatty acids in progenitor hemocyte differentiation. In the knockdown, null mutants as well as pharmacological inhibition of beta oxidation, loss of differentiation and loss of quiescence seen points out at the preferential utilization of fat metabolism in late instar blood progenitors for setting up quiescence and differentiation. It is intriguing to note that early instar progenitors when self-renewing have high glucose utilization whereas, glucose utilization in late quiescent and differentiating progenitors is very less, at the same time when we knock down fat metabolism components, their quiescence and differentiation are compromised. This suggests at the intriguing possibility that there is a switch from glucose to fat metabolism when hemocyte progenitors move from self-renewal to quiescence and then to differentiation in normal development.

FAO upregulation by L-carnitine supplementation further endorse the role of beta oxidation in hemocyte progenitor differentiation program as the differentiation index increase upon L-carnitine feeding. L-carnitine supplementation led to differentiation of reserve hemocyte progenitors of secondary and tertiary lobes of lymph gland which in wild type conditions

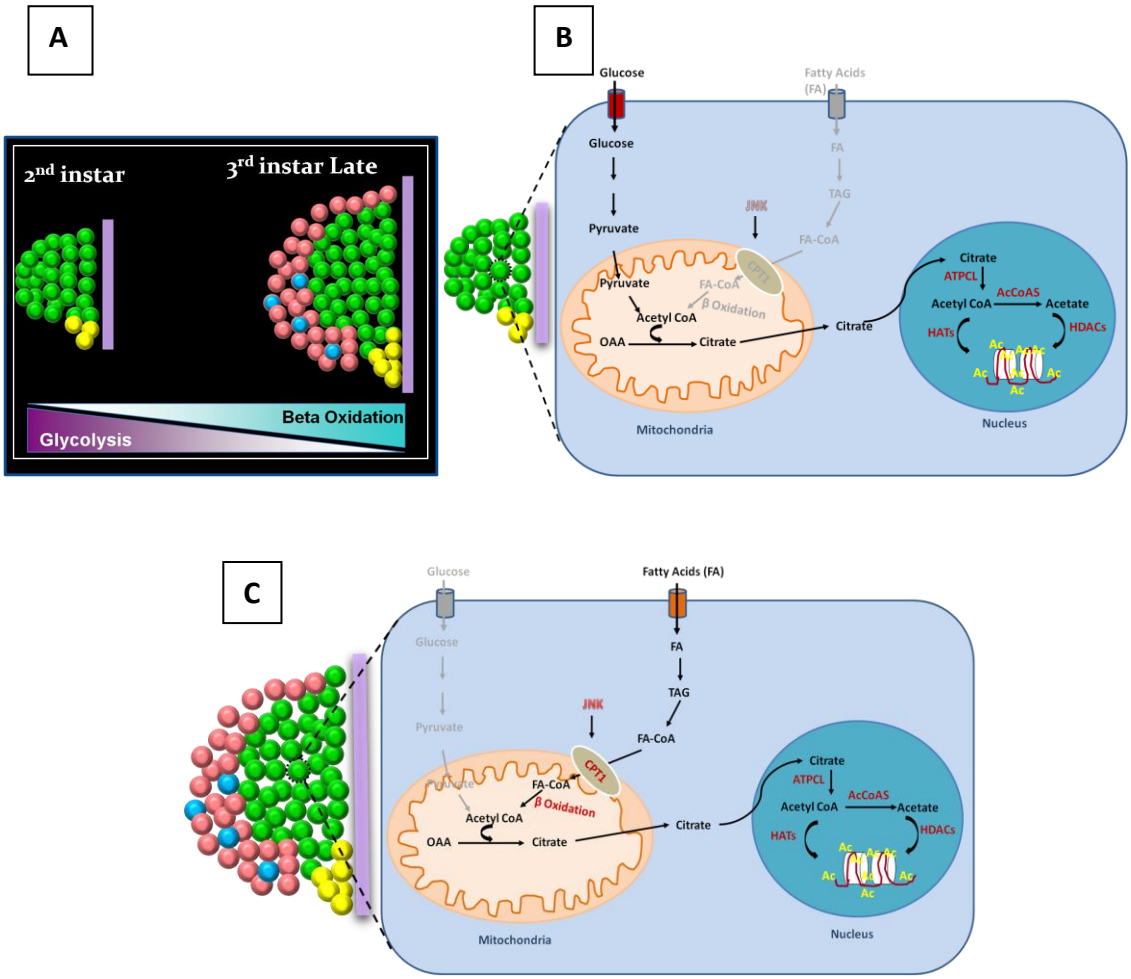

Fig.8. A-C: Schematic representation of the metabolic state of self-renewing second instar progenitora and quiescent third instar late progenitors.

don’t show any differentiation at this time point. Moreover, apart from the precocious differentiation of hemocyte progenitors, their self-renewal was also compromised in both the progenitors of primary as well as reserve population of secondary lobe of lymph gland. Thus, implicating that FAO upregulation via L-carnitine supplement brings about precocious quiescence and differentiation in hemocyte progenitors. L-carnitine is a widely used supplement around the world for enhancing fat burning, however, this study points out at the hematopoietic effects of L-carnitine supplement. Though, further studies in multiple model systems should be carried out to endorse the effects of L-carnitine on different physiological aspects.

Another very interesting outcome of this study is the rescue of hematopoietic anomalies associated with FAO loss of function by feeding sodium acetate. It not only rescues and endorses that differential acetylation profile might exist in FAO mutants in normal physiological scenarios, but also paves the way for clinical investigations on the use of Sodium acetate as a probable therapeutic agent in hypo-acetylation conditions and specifically different acetylation specific defects seen in inborn errors of metabolism conditions.

Increased glucose uptake in FAO mutants leading to loss of quiescence seen in hemocyte progenitors being rescued by glycolytic inhibition correlated the loss of quiescence to the surge in sugar metabolism but, glycolytic inhibition by 2-DG having no appreciable effect on the hemocyte progenitor differentiation defects further implicated that hemocyte progenitor differentiation is FAO driven rather than the outcome of sugar metabolism surge seen in FAO mutants. FAO related metabolic state preferentially moving hemocyte progenitors to differentiation state further gains strength with the profiling of specific Histone H3 acetylation. Whereas, some H3 acetylations like H3K18 acetylation show upregulation in FAO mutants, H3K9 acetylation specifically shows decreased levels in FAO mutants. Differential response: This differential acetylation in FAO mutants evoke an intriguing probability that fat moiety might be doing specific acetylations to modulate cell fate.

Sodium acetate feeding not only rescues hematopoietic defects but also rescues H3K9 acetylation thereby implicating FAO mediated H3K9 acetylation regulating hemocyte progenitor differentiation. The result of H3K9 acetylation defects in FAO mutants seen in western blots of histone extracts from whole larvae further endorsed by generation of RNAi

mediated knockdown of *whd* in heat shock Ay-Gal4 based mosaic clones in hemocyte progenitors. Immunostaining of these clones with an antibody against anti-H3K9 acetylation reveals the compromised H3k9 acetylation levels in clones compared to neighbouring wild type progenitors. Thus, this study not only tries to characterize the hematopoietic defects seen in FAO loss of function but also attempts to find the mechanistic basis for the same.

Chapter 4

Integrin mediated cell-ECM adhesion regulates hematopoiesis by maintaining larval hemocyte progenitor homeostasis in *Drosophila melanogaster*.

4.1. Introduction

Integrin mediated cell adhesion by formation of focal adhesion complex is implicated in cell attachment to the ECM (Hynes, 2002). Integrins are heterodimeric cell membranous receptors consisting of one α subunit and one β subunit. These focal adhesion complex acts as a docking site which links the ECM components to the intracellular cytoskeleton (Hynes, 2002). In this manner it serves as a bridge between cell and its ECM. By acting at the cellular membranes, integrins can coordinate morphological cellular processes by regulating the cytoskeleton. In addition to this, because of its attachment to ECM components it is postulated that integrin signaling is coordinated with ECM components which acts as the ligands for integrin receptors (De Arcangelis and Georges-Labouesse, 2000). Integrin complex can also interact with growth factor receptors and other cellular signaling mediators to regulate milieu of cellular processes like cell survival, proliferation, migration and differentiation (Hynes, 2002). In *Drosophila*, till now there are five characterized α integrin subunits: αPS1 (encoded by the gene *multiple edematous wings*, *mew*), αPS2 (encoded by *inflated*, *if*), αPS3 (encoded by *scab*, *scb*), αPS4 and αPS5 as well as two β integrin subunits: βPS (encoded by the gene *myospheroid*, *mys*) and βν (Bokel and Brown, 2002). The two integrin β subunits can individually form heterodimer pair with each five α integrin subunit and thus can make ten different integrin combinations in *Drosophila* (Humphries et al., 2006). Though, not all ten theoretically possible integrin heterodimer combinations have been reported in *Drosophila* tissues. The most prominent integrin heterodimer combinations which have been implicated in *Drosophila* development and tissue homeostasis are- βPS/αPS1, βPS/αPS2, βPS/αPS3 and βν/αPS3 (Nonaka et al., 2013), (Fraichard et al., 2010).

In *Drosophila* Integrin signaling has specifically been implicated in stem cells and their microenvironment interactions, especially in *Drosophila* GSCs (Germline Stem Cell),

(O'Reilly et al., 2008) and ISCs (Intestinal Stem Cells, (Lin et al., 2013)). In the *Drosophila* ovary, integrin mediated cell adhesion has been implicated in follicle stem cell maintenance in the germarium. Specifically, βPS/αPS1and βPS/αPS2 integrin heterodimer pairs are important for both positioning and proliferation of follicle stem cells (O'Reilly et al., 2008). In the *Drosophila* testis, β integrin mediated adhesion along with a mediator of integrin adhesion by *Lasp*, are required for anchoring the GSC niche (or hub) to the anterior tip of the ovary. Integrin loss of function leads to hub detachment and GSC delocalization (Lee et al., 2008), (Tanentzapf et al., 2007). Integrin mediated adhesion and signaling is important for ISC maintenance and proliferation in *Drosophila*. Out of the various α and β subunits βPS, αPS1, αPS3 and βν have specifically been shown to regulate ISC fate and its self-renewal (Lin et al., 2013). Integrins have also been implicated to regulate the asymmetric division of adult ISC in conjunction with Par complex of *Drosophila* (Goulas et al., 2012). The expression pattern analysis and loss of function mutant analysis suggest that βPS is the primary β integrin expressed in *Drosophila* tissues through developmental and adult stages and it is indispensible for organismal survival as its homozygous loss of function mutations are lethal (Bunch et al., 1992; Lin et al., 2013). However, βν homozygous loss of function null alleles survive to adult stages owing to its restricted expression in fewer tissues compared to widespread expression of βPS integrin (Hashimoto et al., 2004; Lin et al., 2013). Other major α integrins like αPS1, αPS2, and αPS3 are also indispensible for organismal survival as their homozygous loss of function null alleles are lethal (Brower et al., 1995; Lin et al., 2013).

Integrins in *Drosophila* have also been widely implicated in blood cells or hemocyte migration and phagocytosis during different developmental stages (Moreira et al., 2013). In immune challenged conditions, a special blood cell type known as lamellocye is produced which highly expresses βPS and αPS4 integrins but the hematopoiesis specific role of integrins hasn't been investigated in *Drosophila* with greater details.

4.2. Results

4.2.1 Integrin expression analysis in larval lymph gland

The expression pattern of integrins was analysed by immunostaining for two β integrin antibody specific for βPS and βν and three α integrin antibodies specific for αPS1, αPS2 and αPS3. Spatiotemporal expression pattern of integrins in the larval hematopoietic organ lymph

gland was analyzed by immuno-staining with these five specific antibodies for integrin in different developmental stages. The chosen developmental stages corresponded to second larval instar early (26+/-2 AEH), second instar late (45+/-2 AEH), third instar early (60+/-AEH) and third instar late (96+/-AEH). The extensive expression analyses of integrins reveal that out of the five integrin, the expression of four can be detected in the lymph gland. The spatiotemporal co-localization of these integrins reveals that in lymph gland during normal development, there are two sets of integrin hetero-dimer expressed in different cell types at different time points. The integrin βν and αPS3 showed robust expression in both second early and second late instar hemocyte progenitors (Fig 2. A-B, E-F, I-J, M-N) whereas; their expression from hemocyte progenitors of MZ couldn't be detected in third early as well as third late instars (Fig 2. C-D, G-H, K-L, O-P). On the other hand, integrin βν and αPS3 expression is evident in differentiating hemocytes of Cortical Zone (CZ) in late third instar (96+/- AEH) (Fig 2. G-H, O-P). The spatiotemporal co-expression of integrin βν and αPS3 implicate that both are acting as a heterodimeric integrin pair.

The expression of other integrin pair βPS and αPS1exclusively in hemocyte progenitors of MZ from second instar stage to late third instar stage (Fig 1. B-I) suggested that both of these are acting as integrin heterodimer pair. However, the expression of βPS and αPS1 was more robust in third instar hemocyte progenitors compared to early second instar progenitors.

Therefore, the expression pattern of two heterodimeric integrin pair βPS/αPS1 and βν/αPS3 observed in this study suggest that βν/αPS3 integrin might have a role in second instar progenitors whereas post second instar, the blood progenitors switches integrin pair and now expresses βPS/αPS1.

4.2.2. βPS/αPS1 integrin mediated cell-ECM adhesion regulates hemocyte progenitor quiescence and differentiation.

The genetic screen carried out in progenitors revealed that RNAi mediated knockdown of integrin *βPS, αPS1* and its downstream focal adhesion component *talin* lead to diminished differentiation of hemocyte progenitors. The detailed expression analyses of integrins in lymph gland revealed their spatio temporal expression pattern. Early larval stage progenitors of second instar express *β*ν/αPS3 pair of integrin hetero-dimer (Fig 2. A-B, I-J) whereas; third instar hemocyte progenitors expresses *β*PS/*α*PS1 integrins (Fig 1. D-E, H-I).

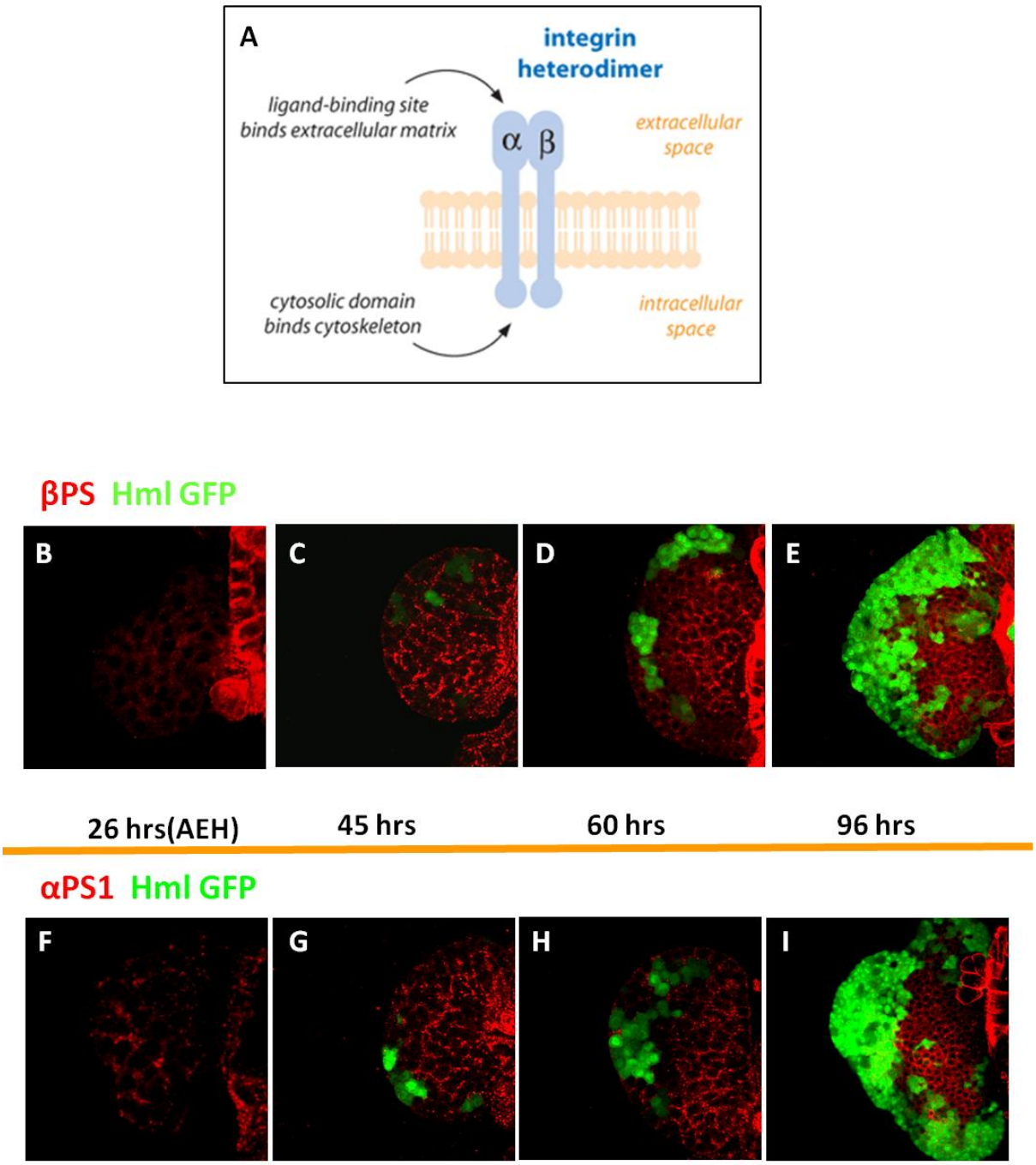

Figure 1. A. Integrin heterodimer showing two subunits α and β. Extracellular ligand binding domain of integrin binds to the Extracellular Matrix and cytosolic domain binds to the cytoskeleton of cell. **(B-E)** Temporal expression pattern of βPS in hemocyte progenitors marked by *Hml>GFP* from 26 hrs AEH to 96 hrs AEH of *Drosophila* larvae. **(F-I)** Temporal expression pattern of αPS1 in in hemocyte progenitors marked by *Hml>GFP* from 26 hrs AEH to 96 hrs AEH of *Drosophila* larvae. Each experiment was performed atleast three times with n=10. Representative image depicts the average of the result.

Loss of differentiation in either RNAi mediated knock down of βPS or αPS1 implicates cell-ECM adhesion mediated by βPS/*α*PS1 integrin is critical for hemocyte progenitor differentiation. Apart from loss of differentiation, βPS/αPS1 integrin knockdown results in loss of quiescence of hemocyte progenitors. RNAi mediated knockdown of βPS resulted in elevated BrdU labeled hemocyte progenitors compared to similar aged controls ((Fig 3. E-F). The loss of differentiation observed in hemocyte progenitors in integrin knockdown prompted us to investigate the previously reported differentiation signal, Reactive Oxygen Species (ROS) in hemocyte progenitors. The level of ROS was measured by DHE (Dihydroxy ethedium) staining in lymph gland. The control lymph glands of late third instar stage had high ROS levels in hemocyte progenitors which has been implicated to serve as the differentiation factor by instigating JNK-Foxo dependent signaling cascade. Hemocyte progenitors in βPS/αPS1 integrin knockdown had compromised levels of ROS compared to similar aged controls (Fig 1. G-I). Thus, ROS production is hindered in integrin knockdown in hemocyte progenitors leading to diminished differentiation.

As evident from the current study (Chapter3 and Fig1. M-P), the quiescent hemocyte progenitors of late third instar stage have intricate reticular mitochondrial architecture and is therefore suggestive of oxidative phosphorylation dependent metabolic state which is known to generate cellular ROS as a byproduct in ETC (Electron Transport Chain). Integrin has been known to implicate mitochondria in generation of cellular ROS (Werner and Werb, 2002). Therefore, the mitochondrial architecture/morphology in βPS/αPS1 knockdown scenario was next analyzed using Streptavidin staining. The mitochondrial network was observed to be less reticular and amount of mitochondrial load per cell was also less compared to control hemocyte progenitors (Fig 3. J-K). The observation of mitochondria in βPS/αPS1 knockdown was further validated using mito-GFP expression which also revealed similar drop in mitochondrial amount and change in their architecture (Fig 4. A-D). The loss of differentiation in integrin knockdown scenario in hemocyte progenitors may thus be a consequence of deficit in ROS levels due to diminished pool of mitochondria. The decrease in mitochondrial levels observed prompted an investigation whether there is drop in expression of mitobiogenesis genes. The primary mitobiogenesis gene in *Drosophila* is *PGC-1* or *spargel (srl*,(Baltzer et al., 2009*)*. The Real Time-PCR analysis of *srl* transcripts revealed ~40% drop in *srl* transcription in βPS knockdown from

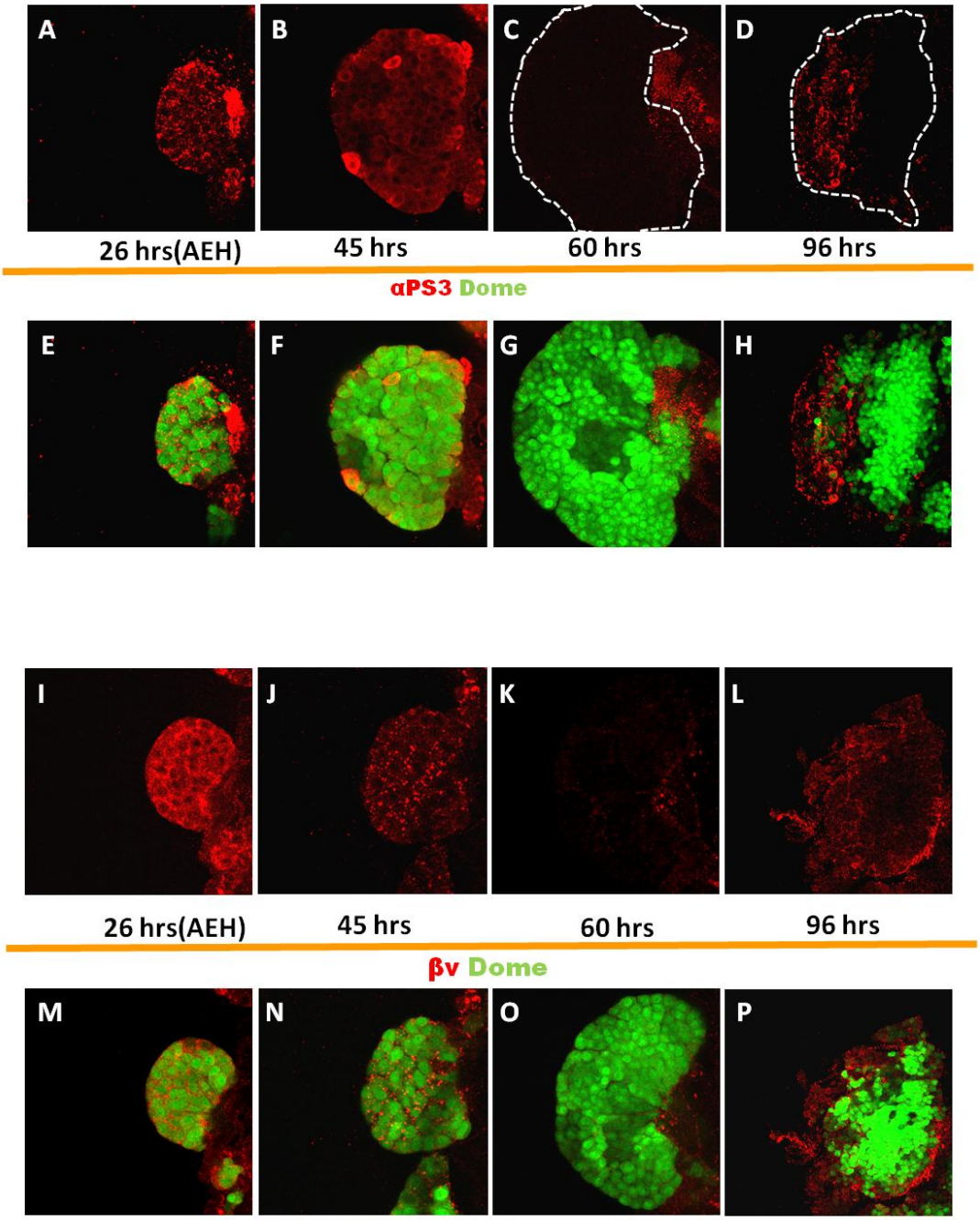

Figure 2. (A-H) Temporal expression of αPS3 in the lymph gland of *Drosophila* larvae from 26 hrs AEH to 96 hrs AEH. (E-H) shows the co-localization pattern of αPS3 and *Dome* positive cells. **(I-P)** Temporal expression of βv in the lymph gland of *Drosophila* larvae from 26 hrs AEH to 96 hrs AEH. **(E-H)** shows the co-localization pattern of βv and *Dome* positive cells. D, H, L, P captured at lower zoom to accommodate whole primary lobe of late third instar lymph gland. Each experiment was performed atleast three times with n=10. Representative image depicts the average of the result.

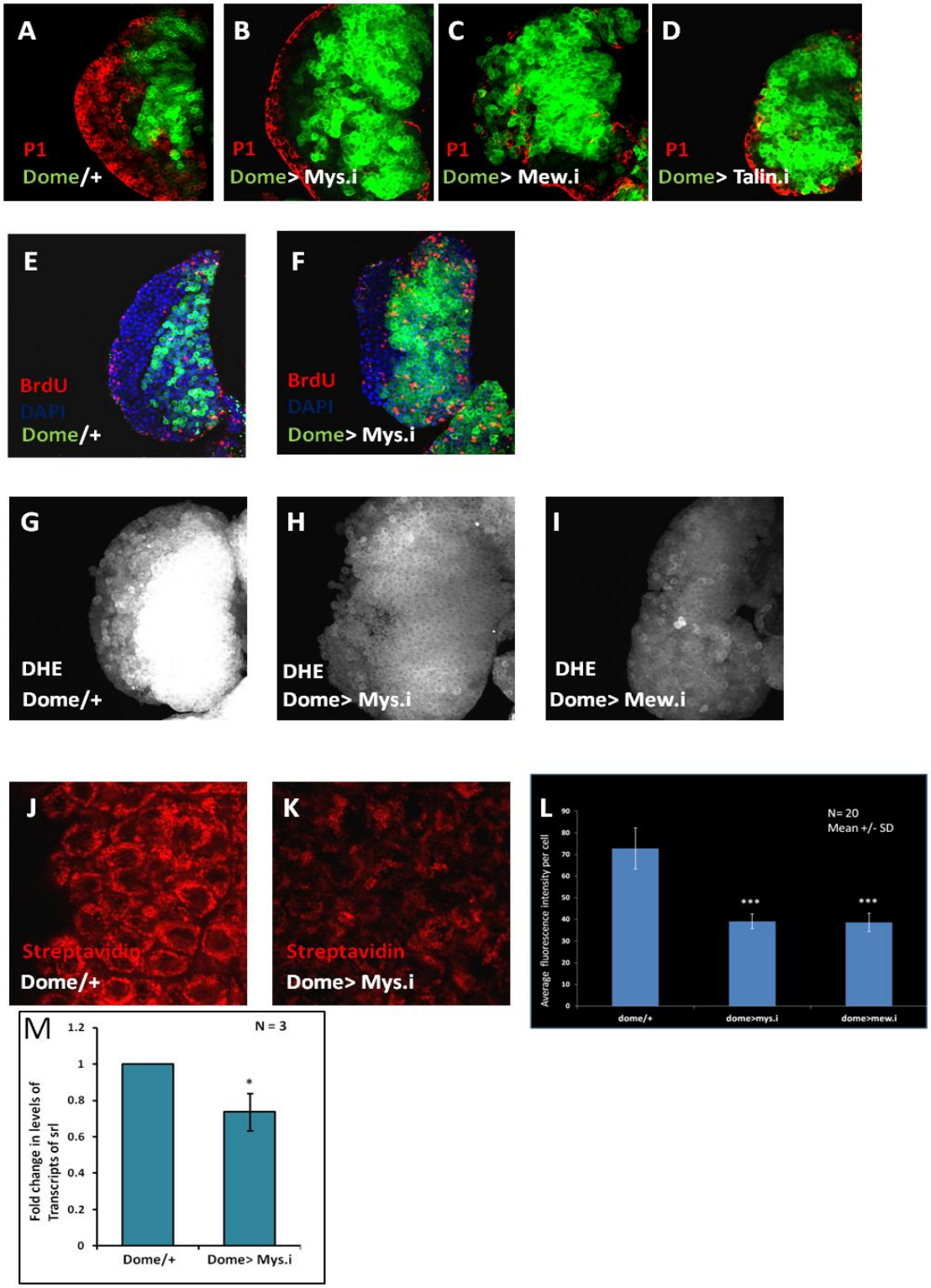

Figure 3 A Control 3rd Instar lymph gland showing status of P1 antigen along with Dome positive progenitor cell population. (B) P1 staining in *Mys-RNAi* from *Dome* positive progenitor cells. **(C)** P1 staining in *Mew-RNAi* from *Dome* positive progenitor cells. **(D)** P1 staining in *Talin-RNAi* from *Dome* positive progenitor cells. **(E-F)** Levels of BrdU incorporation in *Dome* positive cells **(E)** and *Dome>Mys RNAi* **(F)**. **(G-I)** DHE staining showing the levels of ROS in *Dome* positive control lymph gland **(G)** compared to

DomeGal4 driving *Mys-RNAi* **(G)** and *DomeGal4* driving *Mew-RNAi* **(I). (J, K)** Zoomed in images of 3rd Instar lymph gland showing Streptavidin staining in control **(J)** and *DomeGal4* driving *Mys RNAi* **(K)**. **(L)** Graph showing the Quantification of Streptavidin. Fluorescence Intensity in *Domegal4* driving *Mys-RNAi* and *Mew-RNAi* compared to control. Each experiment was performed atleast three times with n=10. Representative image depicts the average of the result. Each experiment was performed atleast three times with n=10. Representative image depicts the average of the result. **(M)** Graph showing the fold change in transcript levels of *srl*. Error Bar: S.D. NS, not significant, *p<0.05, **p<0.001, ***p<0.0001 (Student's *t*-test). Scale bar: 20μm.

hemocyte progenitors correlating with the same amount of mitochondrial drop as revealed by fluorescence intensity analysis (Fig 3. L-M).

Mitochondria serve as the hub of various metabolic processes undergoing in a cell. Apart from the different facets of oxidative phosphorylation including TCA (Tri Carboxylic Acid) cycle and ETC (Electron Transport Chain), Fatty Acid Oxidation (FAO) also takes place in mitochondria. Integrin knockdown scenario had compromised levels of mitochondria in hemocyte progenitors. Hence, to investigate whether compromised mitochondria had effect on fat metabolism, nile-red staining marking the neutral lipids was done in lymph glands. βPS knockdown from hemocyte progenitors resulted in elevated lipid levels in hemocyte progenitors compared to similar aged control progenitors (Fig 4. E-M), as elucidated by nile-red stain. The increased lipid levels observed upon integrin downregulation from the blood progenitors might be either a consequence of a drop in mitochondrial FAO because of the diminished mitochondrial function or can be an outcome of an anabolic increase in lipid synthesis. By and large it can be concluded that lipid metabolism is altered in integrin knockdown from hemocyte progenitors.

In *Drosophila*, integrins have been implicated in cell-ECM adhesion (Hynes, 2002). Hemocyte progenitors in lymph gland show high cell-cell adhesion mediated by E-cadherin (Fig 5. A-B). They are also densely packed by ECM component perlecan orthologue Trol, which show an intricate ECM packaging network around hemocyte progenitors (Fig 5. E-F). Robust expression of βPS/αPS1 integrin (observed in this study) further adds to this adhesion network which serves as a bridge between ECM and cellular cytoskeleton. In βPS integrin knockdown, the cell-cell adhesion and ECM status of the hemocyte progenitors in lymph gland were next analysed. The expression of DE-Cadherin which mediates cell-cell adhesion is increased in βPS integrin knockdown from hemocyte progenitors compared to control (Fig 5. C-D). Hence, cell-ECM adhesion disruption mediated by integrin knockdown led to ECM adhesion mediator integrin knockdown in hemocyte progenitors alters their fate along with disrupted mitochondrial function, altered cell-cell adhesion and changed ECM milieu.

4.2.3. βv/αPS3 integrin mediated cell-ECM adhesion regulates fate of hemocyte progenitors of MZ and bipotent progenitors of CZ

The current study revealed the expression pattern of βv/αPS3 in self-renewing and undifferentiated early second instar progenitors. To investigate whether the self-renewal of

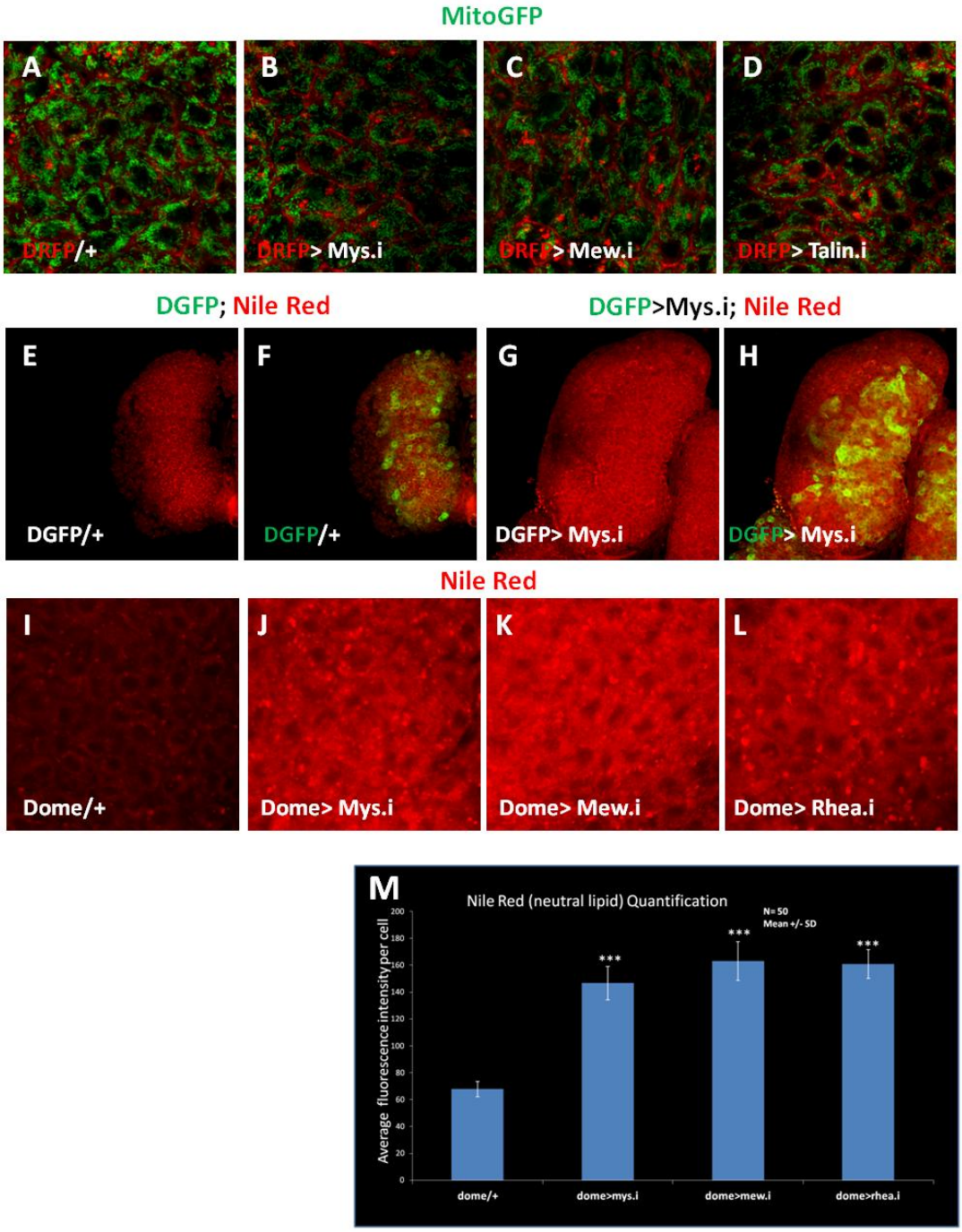

Figure 4 A-D Expression of Mito-GFP in *Dome>RFP* driving *Mys-RNAi* (B), *Mew-RNAi* (C) and *Talin-RNAi* (D) compared to control (A). 3rd Instar lymph gland. **(E-H)** Expression of Nile Red in the lymph gland of 3rd Instar *Dome>GFP* control **(E-F)** and *Dome>GFP* driving *Mys-RNAi* **(G-H)**. **(I-L)** Expression of Nile Red in 3rd Instar lymph gland of Control **(I)** compared to *Dome* driving *Mys-RNAi* **(J)**, *Mew-RNAi* **(K)** and *Rhea-RNAi* **(L)**. **(M)** Graph showing the Quantification of fluorescence intensity of Nile Red in *Dome* driving *Mys-RNAi*, *Mew-RNAi* and *Rhea-RNAi* compared to control. Each experiment was performed atleast three times with n=10. Representative image depicts the

average of the result. Error Bar: S.D. NS, not significant, *p<0.05, **p<0.001, ***p<0.0001 (Student's *t*-test). Scale bar: 20µm.

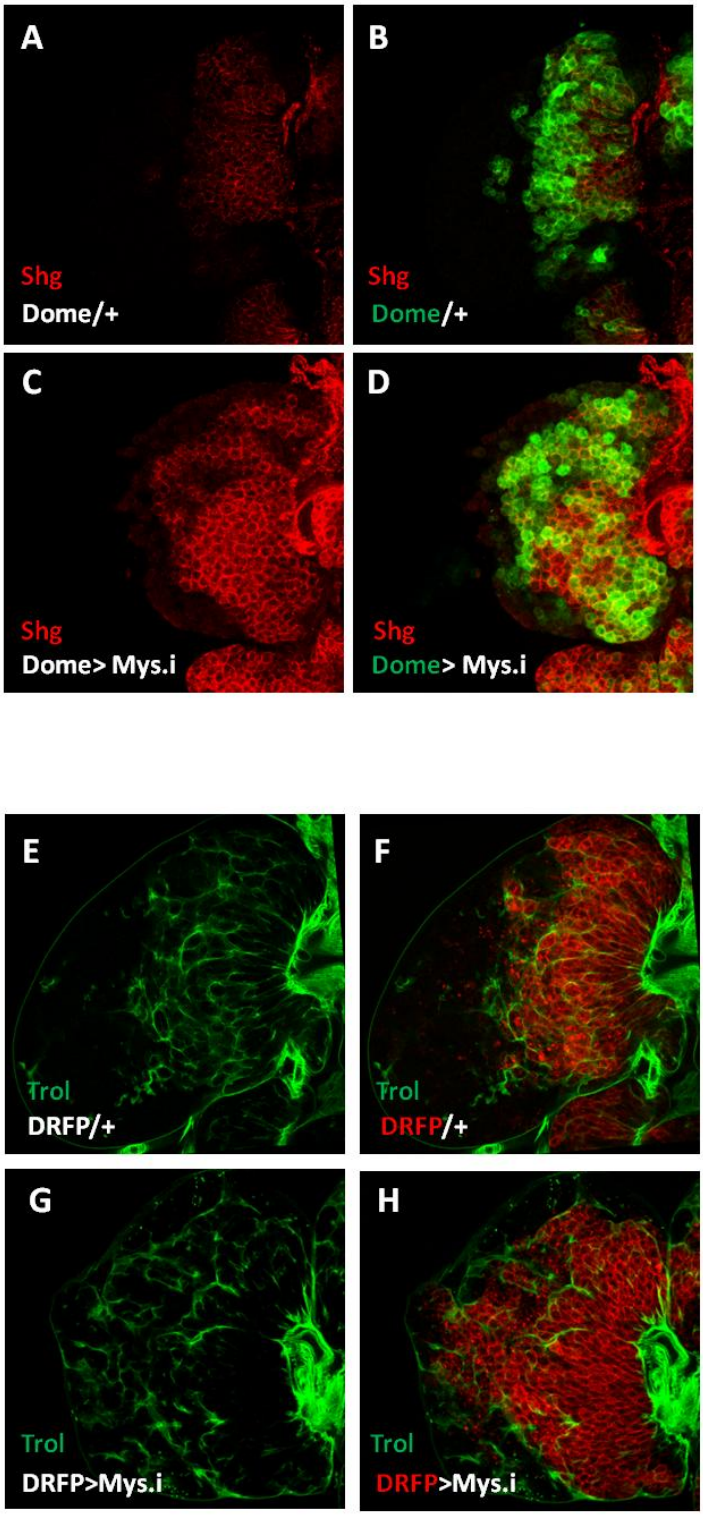

Figure 5 (A-D) Expression of *Drosophila* E-Cadherin (*Shotgun/Shg*) in *Dome>GFP* driving *Mys-RNAi* (C-D) compared to control **(A-B). (E-H)** Expression of *Trol-GFP* in *Dome>RFP* driving *Mys-RNAi* **(G-H)** compared to control **(E-F)**. Each experiment was performed atleast three times with n=10. Representative image depicts the average of the result.

hemocyte progenitors in second instar is βν/αPS3 integrin dependent, a total loss of function homozygous null allele of *βν, βν²* was subjected to detail analysis. The number of S-phase cycling cells marked by EdU label indicated no difference between loss of function of βν integrin and similar aged control progenitors of second instar larval stage (Fig 6. I-J), suggesting that self-renewal of progenitors in second instar is not regulated by integrin signaling.

Early hemocyte progenitors of second instar are not differentiated. Moreover, differentiated cell markers characteristic of plasmatocyte, nimrod P1 (Kurucz et al., 2007) and of crystal cell, ProPo (Muller et al., 1999) only start appearing in lymph gland post second larval instar stages when βν/αPS3 integrin expression is no more detectable (Fig 2. C-D, G-H, K-L, O-P). Based on this mutually exclusive expression pattern, it was hypothesized that βν/αPS3 integrin might be involved in early hemocyte progenitors maintenance. To ascertain this, the expression of βν and αPS3 was downregulated from the early progenitors and differentiation index was evaluated. This down regulation of the integrin function resulted in increase in hemocyte progenitor differentiation (Fig 6. A-C). The RNAi result was further validated with the classical homozygous loss of function null allele of *βν, βν²*, which further showed more differentiation index (Fig 6. G-H) compared to control.

Interestingly, RNAi mediated knockdown of other members of βν/αPS3 integrin signaling cascade including ECM ligand *LanA*, downstream effectors *Fak* and *Src64B* kinases lead to similar compromise in progenitor maintenance and increase in differentiation (Fig 6. D-F), thus, launching a possibility that loss of βν/αPS3 elicits differentiation of hemocyte progenitors precociously.

The expression pattern revealed that apart from being expressed in early hemocyte progenitors of second instar larval stage, βν/αPS3 expression is also seen in differentiating cell population of Cortical Zone (Fig 2. D, H, L, P). Hemocytes of Cortical Zone bears two types of differentiated blood cell types, plasmatocytes (marked by nimrod P1) and crystal cells (marked by Hindsight, hnt). The third differentiated hemocyte lamellocyte is not seen in normal development and only appears in stress scenarios such as immune challenged conditions. The two differentiated blood cell type lamellocyte and crystal cell has a common bipotent precursor marked by *Hemolectin*, *hml* expression (Sinenko et al., 2009). To explore

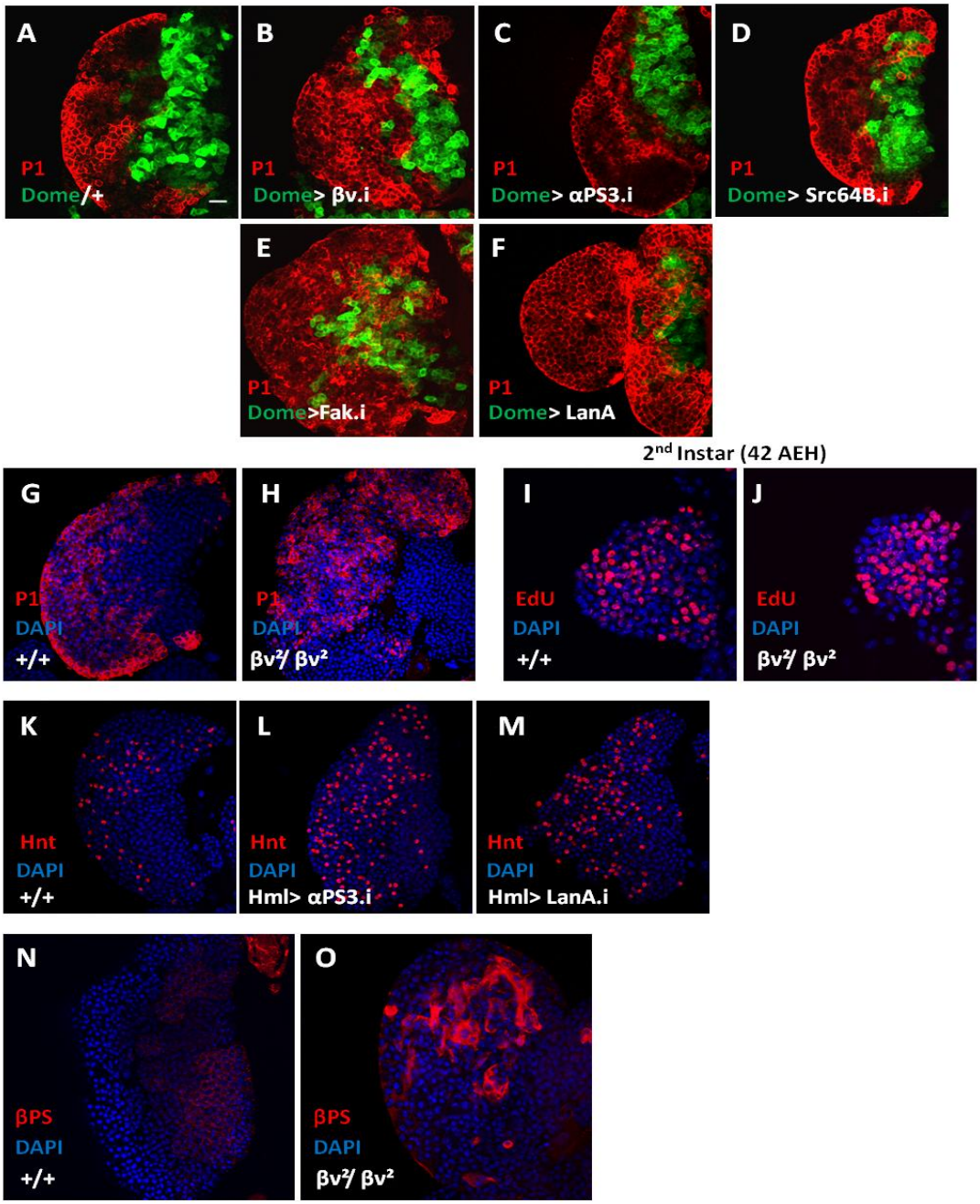

Figure 6 (A-F) Status of P1 antigen in *Dome>GFP* driving *βv-RNAi* (B), *αPS3-RNAi* (C), *Src64B -RNAi* (D), *Fak-RNAi* (E) and *LanA-RNAi* (F) compared to control (A). (G-H) Status of P1 antigen in *βv²/ βv²* 3rd Instar lymph gland compared to wildtype. **(I-J)** Comparison of EdU incorporation *βv²/ βv²* 2nd Instar lymph gland (42 hrs AEH) and wildtype. **(K-M)** Levels of Hindsight (Hnt) in *Hml* driving *αPS3-RNAi* **(L)** and *LanA-RNAi* **(M)** compared to control **(K). (N-O)** Lamellocyte marked by beta-PS antibody in *βv²/ βv²* 3rd Instar lymph gland and wildtype beta-PS antibody expression. Each experiment was performed atleast three times with n=10. Representative image depicts the average of the result. Scale bar: 20µm.

the possibility of βν/αPS3 mediated integrin signaling playing any role in this hematopoietic decision of bipotent precursor, RNAi mediated knockdown of βν/αPS3 integrin, its ligand *LanA* or downstream effectors, *Fak* or *src64B* kinases was performed. Upregulation (upto two fold) in number of crystal cells was noted in each case, thereby, implicating the βν/αPS3 integrin signaling in bi-potent precursor cell fate decision and crystal cell formation. Employing the homozygous loss of function null allele of βv, βv^2, it was observed that apart from the altered differentiation profile of crystal cell increase, lamellocyte were also formed in certain cases (Fig 6. K-O).

4.3. Discussion

This study demonstrates a requirement for integrin in hematopoiesis in *Drosophila*. Several integrin subunits and integrin signaling components are expressed at high levels in hemocyte progenitors and bipotent precursors of larval lymph gland. The spatiotemporal expression analysis of integrin subunits reveals their dynamic expression pattern in lymph gland hemocytes. Especially, expression analysis of integrin in hemocyte progenitors reveal that the integrin heterodimers switch their expression when self-renewing second instar progenitors move to quiescence and differentiation in third instar larval stages. The robust expression of βν/αPS3 in second instar hemocyte progenitors give way to higher expression of another heterodimer integrin pair βPS/αPS1 from late second instar onwards.

The disruption of differentiation seen in βPS/αPS1 integrin knockdown indicates its role in hemocyte progenitor differentiation program. The loss of quiescence observed in βPS/αPS1 knockdown scenario points out at the concomitant requirement of this integrin pair in progenitor homeostasis by mediating their quiescence as well as their differentiation. The differentiation defects can be attributed to the failure of ROS build up in the βPS/αPS1 knockdown from hemocyte progenitors. High levels of ROS has been implicated to propel hemocyte progenitors into differentiation and it has been proposed that without a certain threshold of ROS build up (Owusu-Ansah and Banerjee, 2009), these progenitors don't move into the differentiation programme. Mitochondrial respiration is the major source of ROS generation in a cell where free radicals are produced as a byproduct of electron transport via ETC in mitochondrial membrane. Interestingly, the expression of βPS/αPS1 integrin in hemocyte progenitors picks up at the end of second larval instar and simultaneously, mitochondrial mass and its network also start to build up in these progenitors. Therefore, it

can be postulated that integrin mediated signaling play a role in mitobiogenesis and mitochondrial dynamics, thereby building up the ROS with increase in mitochondrial respiration in third instar hemocyte progenitors. The Real Time-PCR analysis revealed a drop in transcript levels of major mitobiogenesis gene *PGC1/srl* thereby implicating βPS/αPS1 mediated integrin signaling in mitochondrial metabolism and subsequent differentiation of hemocyte progenitors.

Integrin mediated focal adhesion is the centre of cell-ECM adhesion and interaction. Therefore, the impact of integrin knockdown on cell-cell adhesion and ECM was analysed by looking at the expression of cell-cell adhesion mediator E-cadherin and ECM component which is generally enriched in MZ hemocyte progenitors, *Drosophila Perlecan* orthologue *Trol*. Surprisingly, the disruption of cell-ECM adhesion mediated by integrin knockdown resulted in upregulation of cell-cell adhesion as well as ECM component Trol. This observation is suggestive of the possibility that the hemocyte progenitor adhesion to its ECM is crucial in maintaining their homeostasis. Although the mechanism remains to be elucidated, it can be hypothesized that achieving the right balance of cellular tension mediated by other components like cytoskeleton and cell-cell adhesion complexes is necessary for this process.

The expression of βν/αPS3 in second instar hemocyte progenitors of MZ and late bi-potent precursors of CZ in conjunction with the loss of function analysis revealed the role βν/αPS3 in maintaining the homeostasis of both the progenitor cell populations in the developing lymph gland. The other signaling components of βν/αPS3 including the ligand and downstream effector kinases further endorsed the concomitant role of βν/αPS3 integrin signaling in maintaining the hemocyte progenitors as well as bi-potent precursor. The formation of lamellocytes in $\beta\nu^2$ homozygous larvae suggest a stress being build up in the animal in absence of the integrin $\beta\nu^2$. However, the formation of lamellocyte either by RNAi mediated knockdown of βν from bipotent precursor of CZ or from hemocyte progenitor of MZ wasn't achieved, suggesting that there is some external input for lamellocyte formation other than these two cell types or knockdown of βν wasn't upto the levels to induce lamellocyte formation.

Chapter 5

Hippo signaling orchestrates the homeostasis of proliferation, survival and differentiation of blood progenitors to regulate hematopoiesis in *Drosophila.*

5.1. Introduction

Hippo signaling pathway has been regarded as the major regulator of growth and organ size in a diverse range of animals, from *Drosophila* to mammals (Harvey and Tapon, 2007; Johnson and Halder, 2014; Pan, 2010). In *Drosophila*, this pathway comprises of a core kinase cascade involving four kinases linking two kinase complexes—Hippo (Hpo)-Salvador (Sav) and Warts (Wts)-Mob as tumor suppressor (Mats). Studies have illustrated that Hippo can also be regulated by upstream protein complexes of tight junction, polarity determining protein complexes and actomyosin components (Badouel and McNeill, 2011; Yu et al., 2015; Zhao et al., 2011). These upstream kinases phosphorylates and keep its transcriptional activator *Yorkie (yki)* from entering inside nucleus (Li et al., 2010; Staley and Irvine, 2010; Tumaneng et al., 2012; Yu and Guan, 2013). *Yorkie*, once inside the nucleus controls plethora of cellular events by functioning as a transcriptional co-activator for the expression of target genes involved in cell growth, proliferation, and survival (Staley and Irvine, 2010; Yu and Guan, 2013). Studies in diverse model systems integrating various biological processes implicate that the Hippo signaling integrates multiple biological inputs coming from cell polarity, cell adhesion, mechanical tensile force and signal input from other signaling pathways (Boggiano and Fehon, 2012); (Piccolo et al., 2014); (Yu and Guan, 2013). In *Drosophila*, tight junction complex of Fat and Dachsous proteins activates Merlin (Mer) and Expanded (Ex) complex which turns on the Hippo signaling cascade (Su et al., 2017). Sav serves as a scaffolding protein which aids Hippo to phosphorylate Wts, which further phosphorylates and inactivates Yorkie (Yki), the fly homologue of YAP (Yu et al., 2015). In absence of Wts mediated phosphorylation and inactivation, active Yki enters nucleus and

forms complex with Scalloped (Sd) and regulates expression of many genes mainly *CycE* and *DIAP1* to regulate cell cycle and cell survival respectively(Peng et al., 2009; Zhao et al., 2011). Cell growth is mediated by expression of *myc* and micro-RNA *bantam* (Neto-Silva et al., 2010; Tumaneng et al., 2012; Yu et al., 2015; Zhao et al., 2008). Hippo pathway controls non-autonomous cellular growth by transcription of secretary cytokines such as UPDs (Ohsawa et al., 2012; Shaw et al., 2010; Staley and Irvine, 2010).

5.2. Results

5.2.1 The expression analysis of Hippo pathway genes

The expression of Hippo in lymph gland hemocytes was analyzed using anti-Hpo immunostaining. The expression of Hpo protein was detected in all cells of the lymph gland during various developmental stages, though the expression in MZ hemocyte progenitors was correspondingly higher than other cell types (Fig 1B and 1C). Apart from the core kinases of hippo cascade, the expression of Polarity Protein Complex component Discs Large (Dlg) by immunostaining lymph gland with anti-Dlg revealed expression in all hemocytes of lymph gland with robust expression in hemocyte progenitors (Fig 1D). The expression of Hpo and Dlg confirmed the presence of Hippo signaling components in hemocytes of lymph gland. However, to analyze the functional status of Hippo signaling cascade, the transcriptional readout of yki activity: DIAP1-GFP was employed. High Diap1-GFP corresponds to inactive hippo and other upstream kinases whereas low or no Diap1-GFP expression reflects active hippo kinases mediated phosphorylation and inactivating of yorkie. The Diap1-GFP expression pattern is suggestive of a very dynamic Hippo signaling regulation in hemocyte progenitors. The self-renewing progenitors of second instar had high Diap1-GFP expression (Fig 1E to 1M) indicative of high yorkie activity in nucleus. Interestingly, from early third instar onwards, the expression of Diap1-GFP in hemocyte progenitors begin to dwindle (Fig 1N to 1P). Third instar progenitors have very low levels of Diap1-GFP expression (Fig 1Q to 1S) corresponding to low yorkie mediated transcriptional activity and high restriction of yki activity by Hippo signaling kinases. Although the lowering down of expression of Diap1-GFP in hemocyte progenitors is evident as they move from self renewal to quiescence, cells of CZ, have robust Diap1-GFP expression (Fig 1Q to 1S) indicative of high yorkie activity in differentiation.

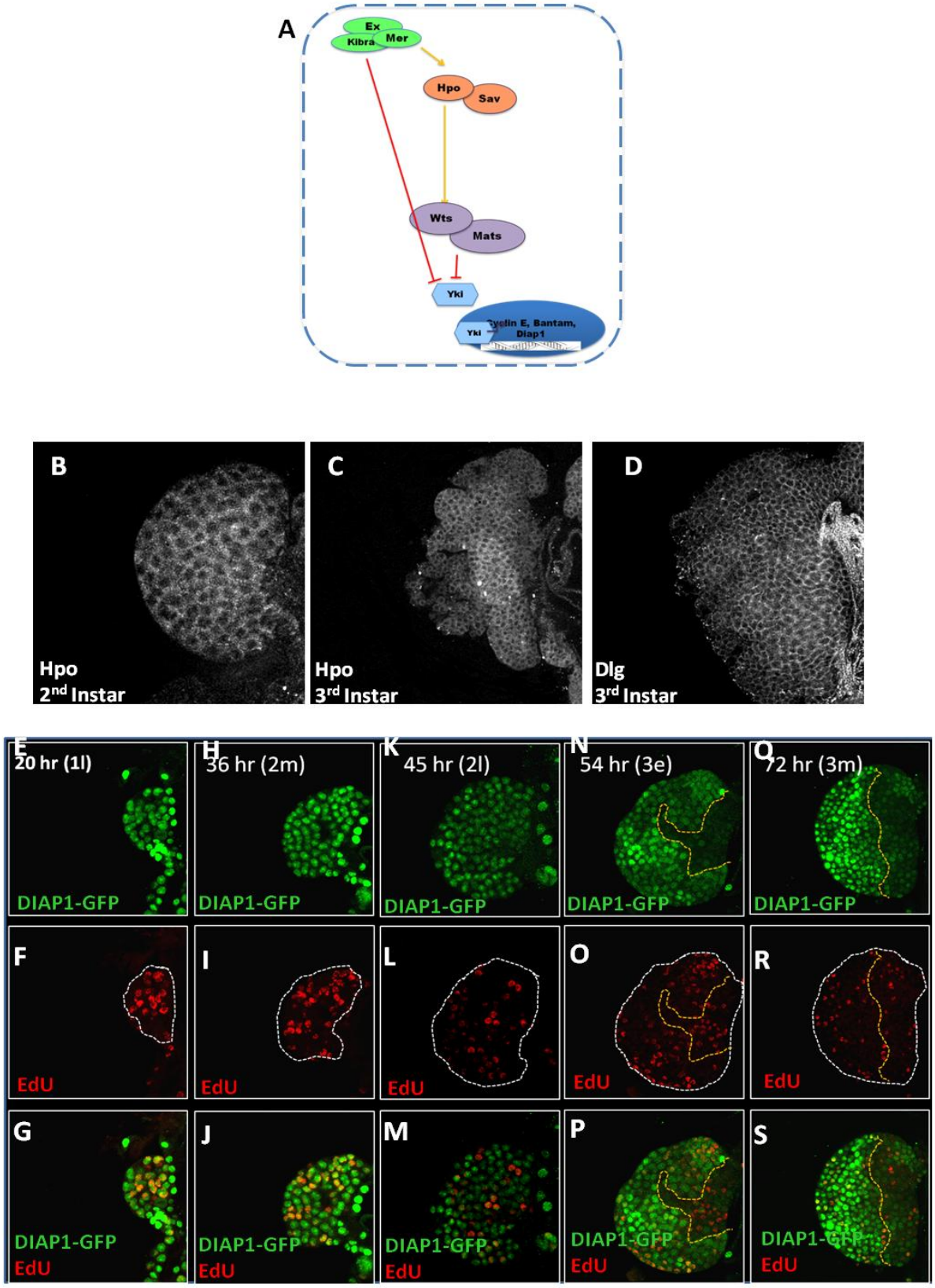
A
Ex
Kibra
Mer
Hpo
Sav
Wts
Mats
Yki
Cyclin E, Bantam, Diap1
Yki
B
Hpo
2nd Instar
C
Hpo
3rd Instar
D
Dlg
3rd Instar
E
20 hr (1l)
DIAP1-GFP
H
36 hr (2m)
DIAP1-GFP
K
45 hr (2l)
DIAP1-GFP
N
54 hr (3e)
DIAP1-GFP
Q
72 hr (3m)
DIAP1-GFP
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Figure 1 (A) Schematic depiction of the core Hippo pathway. (B, C) Anti-hpo expression in 2nd & 3rd instar lymph gland. **(D)** Anti-Dlg expression in 3rd instar lymph gland. **(E-S)** Diap1-GFP expression co-localization with EdU Labeling in 1st instar, 2nd instar mid and late, 3rd instar early and mid stages lymph gland. Each experiment was performed atleast three times with n=10. Representative image depicts the average of the result.

5.2.2 The knockdown of Hippo pathway genes leads to precocious differentiation of hemocyte progenitors.

In addition to negative regulators, there were several positive regulators of hemocyte progenitor maintenancepicked up in the genetic screen. This positive regulators consist of many direct components of Hippo signaling and/or protein complexes which acted upstream of hippo kinases, eg., Polarity Protein Complex. Current study attempted to characterize a positive regulator in addition to the negative regulators of progenitor maintenance (Chapters3 and 4). Since, all the major players of Hippo signaling including its upstream polarity protein complexes all show robust phenotype by positively regulating hemocyte progenitor maintenance, Hippo pathway was chosen for further detailed characterization and analysis. Though, Hippo signaling has previously been implicated in *Drosophila* hematopoiesis (Ferguson and Martinez-Agosto, 2014; Milton et al., 2014), its hemocyte progenitor specific role hasn't been explored in greater details.

The RNAi mediated knockdown of core kinases of Hippo pathway genes *wts* and *hpo* resulted in higher differentiation index of hemocyte progenitors (Fig 2B and 2C). Knockdown of other components of Hippo pathway including Sav, 14-3-3ε as well as upstream activator of hippo signaling cascade Ex and Mer resulted in severely compromised hemocyte progenitors and elevated differentiation index (Fig. 2D to 2G). Knockdown of the Hippo pathway genes *wts, hpo, sav*, *14-3-3ε*, *ex* and *mer* essentially leads to high yorkie activity in late hemocyte progenitors where the yorkie activity is normally low as revealed by low Diap1-GFP expression levels in control hemocyte progenitors. To directly upregulate yorkie in hemocyte progenitors, *UAS-yki* was driven in third instar larval stages and the differentiation index analysis indicated the elevated differentiation levels (Fig. 2H).

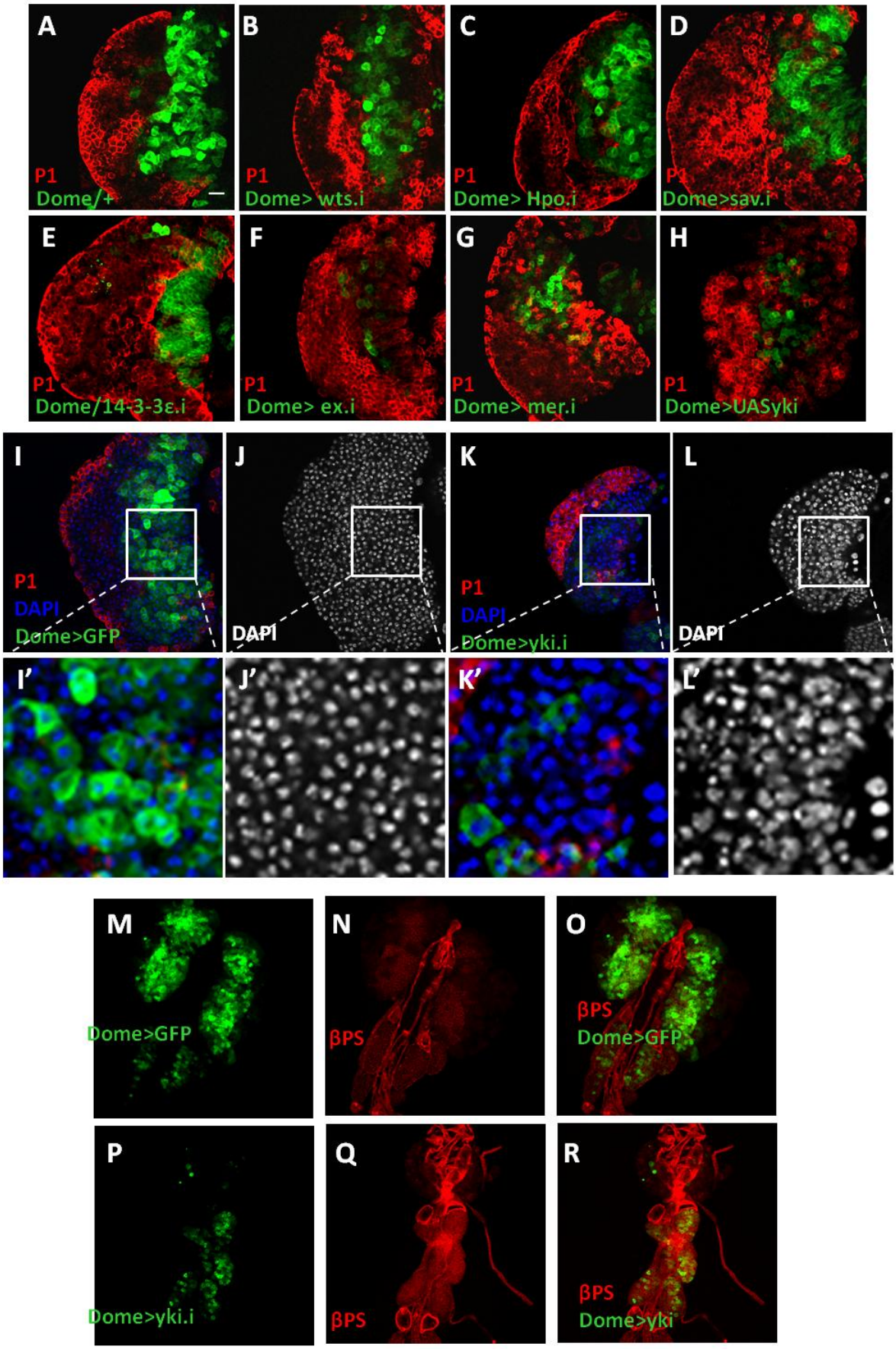
Next, to embark upon the role played by yorkie in hemocyte progenitors, RNAi mediated knockdown of yki expression was carried out. The differentiation index analysis of late third instar lymph glands revealed a drastic drop in size of primary lobes of the lymph gland (Fig. 2K and 2L). In the corresponding primary lobes, the expression of progenitor marker *Dome>GFP* was diminished (Fig. 2K) but these cells were not positive for differentiated cell marker too (Fig. 2K). A closer examination of these primary lobes indicated that the cell membranes of the hemocyte progenitors are ruptured upon down regulation of yki expression.

Cell membrane rupture/ blebbing were easily observed because of the GFP construct used to mark progenitors is membranous mCD8GFP (Fig. 2K). A further analysis of yki knockdown hemocyte progenitor nuclei by DAPI staining revealed the presence of disintegrated broken nuclei of various shapes and sizes (Fig. 2L). Membrane blebbing and nuclear disintegration suggested the requirement of yki mediated cell survival of hemocyte progenitors. Thus, Hippo signaling plays a unique role in integrating cellular proliferation, maintenance, differentiation and cell survival to bring about homeostasis of hemocyte progenitors.

5.2.3 The role of Hippo signaling in formation and maintenance of adult hematopoietic hubs in *Drosophila*.

Drosophila adult fly has been shown to possess active sites of hematopoiesis termed as 'hematopoietic hubs' in their dorsal abdominal compartments. These hematopoietic hubs enriched with ECM milieu are formed of *de novo* hematopoiesis as well as migration from circulation of fly (Ghosh et al., 2015). The cellular signaling network and pathways that builds and maintains this hematopoietic hub is yet to be characterized. Hippo signaling sits at the crossroads of cellular signaling by integrating various inputs coming from cell adhesion, cell polarity, tensile forces and other signaling inputs to mediate cell survival, proliferation, differentiation, migration and other cellular events (Boggiano and Fehon, 2012);(Piccolo et al., 2014); (Yu and Guan, 2013)). Therefore, the Hippo pathway involvement in hematopoietic hub formation and maintenance was analyzed. The status of hippo signaling in hematopoietic hub cells was examined by Diap1-GFP expression (a transcriptional reporter of hippo signaling). It was intriguing to note that the hub resident hemocytes expresses *Diap1*-GFP (Fig. 3A), which suggest that yki activity is high in these cells. Upon analyzing the expression pattern of Diap1-GFP in hub cells at different time points post eclosion, it was evident that the expression of Diap1-GFP decreases with ageing (3A to 3F).

Since Diap1 was observed in the CZ, attempt was made to disrupt Hippo signaling by RNAi mediated downregulation of *warts* kinase from *hemolectin* positive bipotent precursor *(HmlGal4-UASGFP>wts-RNAi)*. It was observed that *wts* knockdown lead to compromised size of hematopoietic hub and overall number of blood cells declined (Fig. 3G to 3J). Moreover, upon *warts* down regulation, actin cytoskeleton related components were


A
P1
Dome/+
B
P1
Dome> wts.i
C
P1
Dome> Hpo.i
D
P1
Dome>sav.i
E
P1
Dome/14-3-3ε.i
F
P1
Dome> ex.i
G
P1
Dome> mer.i
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Dome>UASyki
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Figure 2 A. *Dome>GFP* lymph gland with P1 marking CZ. **(B-G)** ds-RNA mediated knockdown of hippo pathway genes from *Dome>GFP* progenitors **(H)** Overexpression of Yki in *Dome>GFP* progenitors. **(I, J)** Control, *Dome>GFP* lymph gland with P1 marking CZ. DAPI marking nuclei. **(J)** DAPI stained nuclei marked in grey. **(K-L')** dsRNA mediated knockdown of yki from *Dome>GFP* progenitors. **(L)** DAPI stained nuclei marked in grey. **(M, N, O)** *Dome>GFP* marked control progenitors of 1^{o} and 2^{o} lobe of lymph gland. Red is BetaPS integrin expression. **(P,Q, R)** dsRNA mediated knockdown of yki from Dome>GFP progenitors of 1^{o}&2^{o} lobes. Red is BetaPS integrin expression. Each experiment was performed atleast three times with n=10. Representative image depicts the average of the result. Scale bar: 20µm.

upregulated and PVR (PDGF-VEGF Receptor) expression was downregulated. Both of these observations accounted for defects in hemocyte migration and survival. When the migration pattern of the hemocyte in *warts* knockdown was observed, hemocyte showed abnormal migration pattern akin to what has been reported in *enabled* (*Enabled*: an actin remodeler, is the sole *Drosophila* member of the Ena/VASP family) overexpression scenario, i.e., migration speed of hemocytes is higher to control hemocytes and hemocytes have also been shown to throw more fillopodia and less lamellopodia (Fig. 4A to 4D).

The migration pattern of the hemocyte in *warts* knockdown was observed, hemocyte showed abnormal migration pattern akin to what has been reported in *enabled* (*Enabled*: an actin remodeler, is the sole *Drosophila* member of the Ena/VASP family) over-expression scenario, i.e., migration speed of hemocytes is higher to control hemocytes and hemocytes have also been shown to throw more fillopodia and less lamellopodia (4A to 4D). These could be due to migration defects and hence less number of blood cells in hematopoietic hubs in warts knockdown scenario. PVR signaling has also been implicated in survival of circulating hemocytes in *Drosophila* (Bruckner et al., 2004). Since upon *wts* down regulation Pvr expression is compromised, it might also be possible that hemocyte survival is also affected upon down regulation of *wts*. This can also contribute towards the decline in number of hemocytes in the hub of *wts* loss of function.

5.3 Discussion

This study not only finds a new role of Hippo signaling in hemocyte progenitor survival but also implicates Hippo signaling in formation and maintenance of adult hematopoietic hubs. Previous reports implicating Hippo signaling in hematopoiesis have focused on yorkie and scalloped dependent transcription of *Lozenge*, which determines the fate of crystal cells (Milton et al., 2014), (Ferguson and Martinez-Agosto, 2014). Moreover, total mutants of warts kinase used in those studies showed precocious differentiation and hyper-proliferation of whole of lymph gland. But, Hippo signaling has been shown to non-autonomously control cell growth and hemocyte progenitors can also be regulated cell autonomously in the vicinity by cells of hematopoietic niche (PSC) as well as differentiating cells of CZ in the same tissue. Therefore, instead of the holistic effect seen in classical loss of function mutants of Hippo signaling on hematopoiesis as such, current study intended to reveal the non-cell autonomous role of hippo signaling only in hemocyte progenitor population by targeted knockdown. To

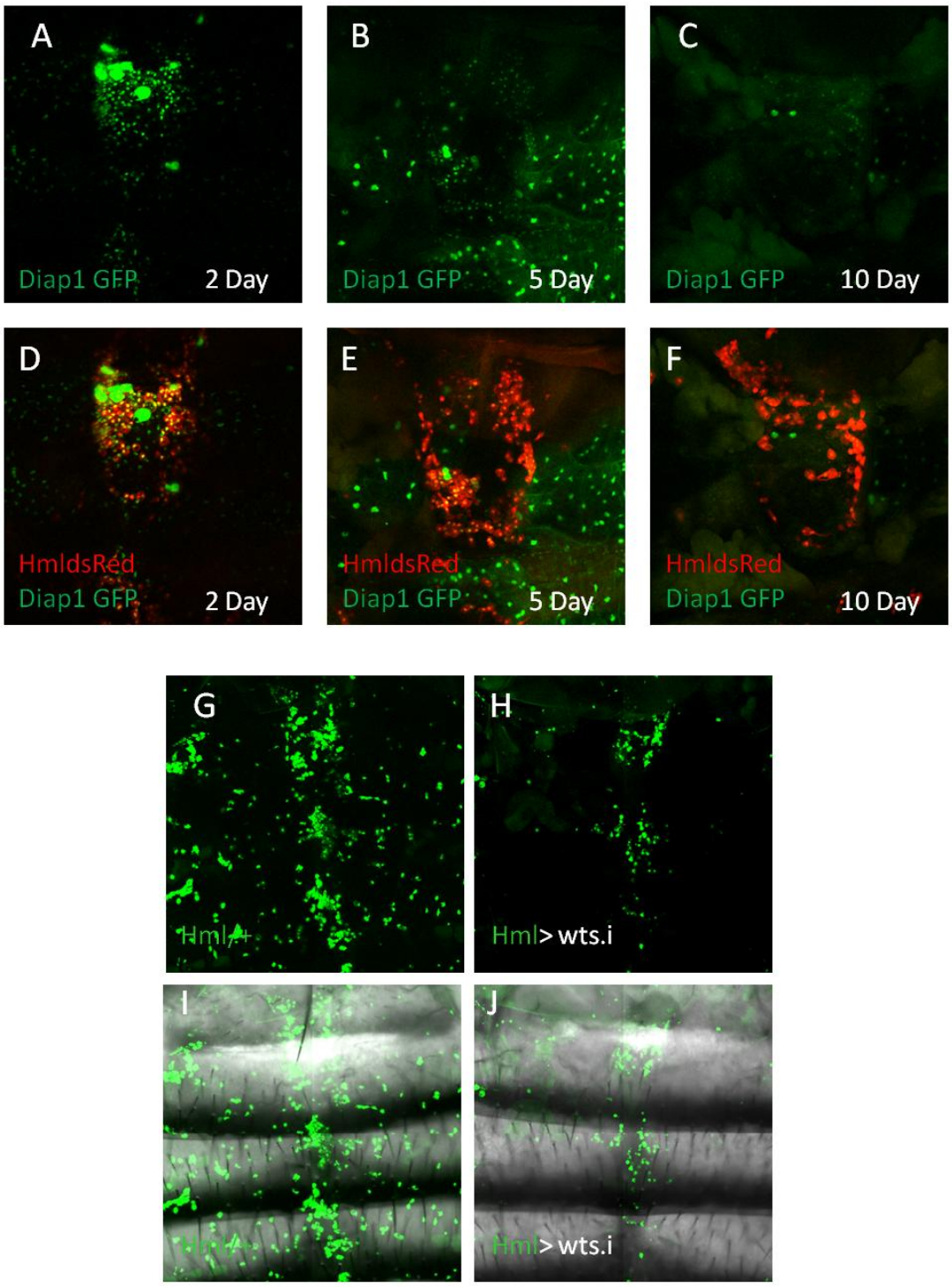

Figure 3 (A, B, C) ***Diap1GFP*** **expression post eclosion 2, 5 and 8 day in first cluster of hematopoietic hub**. **(D, E, F)** *Diap1GFP* expression in **(A, B, C)** colocolized with *HmldsRed* expression of first cluster of hematopoietic hub. **(G)** Control *Hml>GFP* marked hemocytes of dorsal hematopoietic hub. **(H)** DsRNA knockdown of *wts* from *Hml>GFP* hemocytes and hematopoietic hub in 8 day post eclosion. **(I, J)** Hematopoietic clusters of G and H merged with their respective dorsal epidermis. Each experiment was performed atleast three times with n=10. Representative image depicts the average of the result.

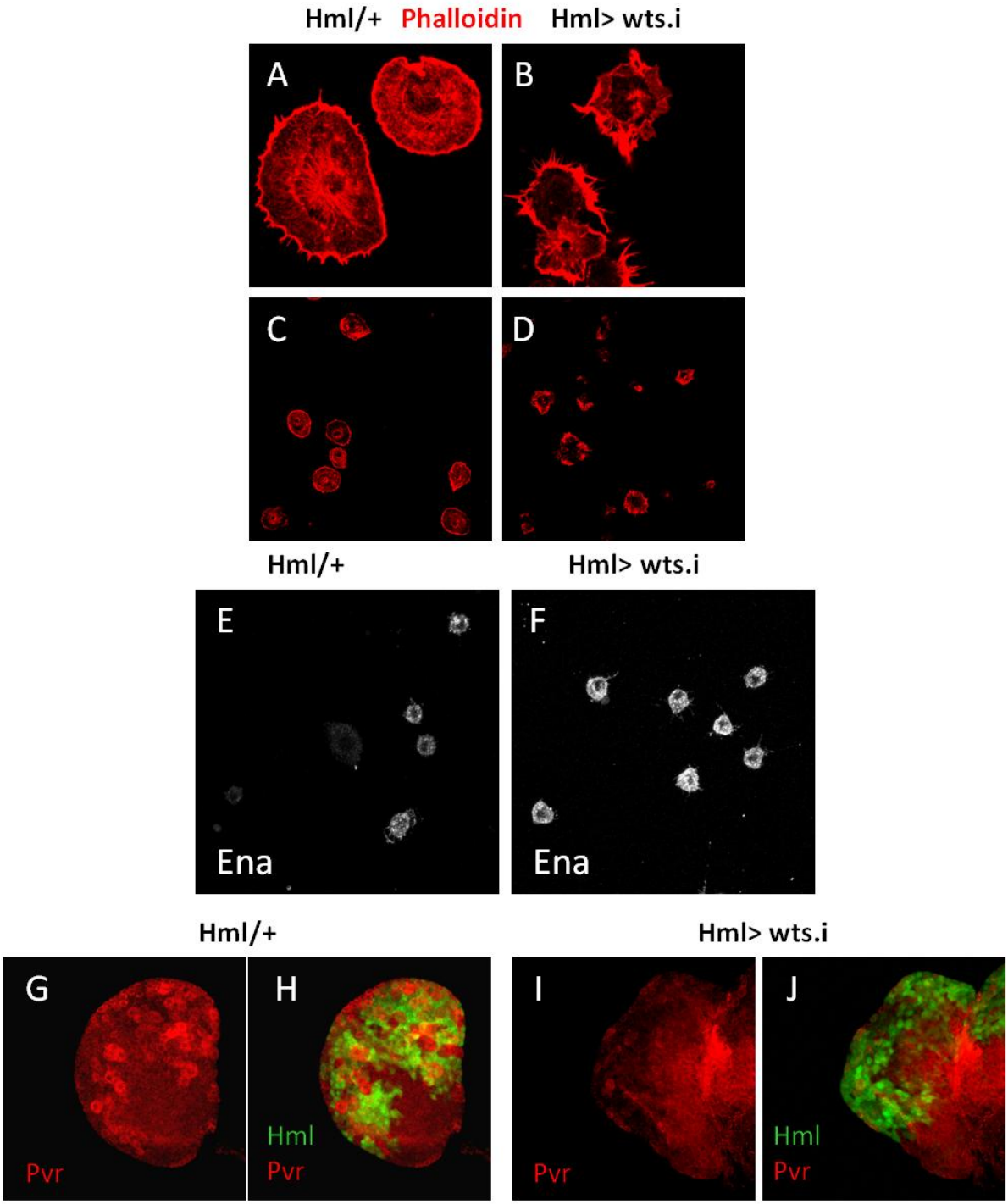

Figure 4. A. Zoomed in image of Pupal circulating control hemocyte 5APF of *Hml/+* stained with phalloidin. **4B**. Zoomed in image of Pupal circulating hemocyte 5APF of *Hml>wts RNAi* stained with phalloidin. **4C.** Zoomed out image of Pupal circulating control hemocyte 5APF of *Hml/+* stained with phalloidin. **4B**.Zoomed out image of Pupal circulating hemocyte 5APF of *Hml>wts* RNAi stained with phalloidin. **4E**. Larval bleed stained with anti-Ena in control *Hml/+* larvae. **4F**. Larval bleed stained with anti-Ena in Hml>wts.RNAi larvae. **4G, H**. Larval lymph gland immunostained with anti-Pvr in control *Hml/+* larvae. **4I, J**. Larval lymph gland immunostained with anti-Pvr in control *Hml>wts-RNAi* larvae. Each experiment was performed atleast three times with n=10. Representative image depicts the average of the result.

this end, it became evident that apart from hippo signaling regulation in hemocyte progenitors by other cell types, the cell intrinsic over activity of yorkie achieved by knockdown of *ex*, *mer*, *hpo*, *sav*, *wts*, *14-3-3ε* or by expressing *UAS-yki* construct in progenitor specific manner resulted in compromised progenitor maintenance and precocious differentiation.

Interestingly, RNAi mediated knockdown of *yki* from second instar onwards compromised hemocyte progenitor survival as observed by cell membrane blebbing and disintegrating nuclei. Hence, to this end it became imperative that yorkie activity subsides in hemocyte progenitors post second instar larval stages with the onset of quiescence but this diminishing level of yorkie expression seems to be important for the cell survival of hemocyte progenitors.

The concomitant requirement of hippo signaling in adult hematopoietic hub formation and maintenance by possibly regulating cell migration events implicates hippo in mediating cell migration of hemocytes. As seen in *wts* knockdown, expression of *ena* increases in circulating hemocytes coupled with more filopodia and lesser lamellopodia formation. This in turn might affect migration of the hemocytes and eventually the formation of hematopoietic hubs. Together, these results reveal new insights in hippo signaling in context of hematopoiesis both in larval stages and adult stages of *Drosophila*.

Chapter 6

Conclusion & Future Perspective

Stem cells and progenitors cells in normal development and tissue homeostasis choose differential metabolic states. Recent studies have implicated the role of metabolic state of a cell in conjunction with cellular signaling pathways in regulating cell fate decision of stem and progenitor cells both in normal development and in pathophysiological conditions. The current study reveals the importance of cellular metabolism and the corroborating cellular signaling pathways in determining hemocyte progenitor cell fate decision. This study not only finds new players involved in blood progenitor maintenance but also uncover a new aspect of requirement of metabolism in progenitor quiescence and differentiation.

The conventional belief in the field of metabolism and animal physiology is that cellular energy is carbohydrate derived and fat moieties serve as storage molecules to be relied on in times of cellular/ organismal stress, primarily in starvation. But, reports in last couple of years have challenged this belief with the increasing evidences pointing out that stem cells and progenitor cells preferably choose fatty acid metabolism to fuel themselves not only in terms of energy production but utilization of intermediary metabolites for various cellular processes and depriving them of this metabolic state is deleterious to their state and fate.

To our knowledge, this is the first report which implicates the requirement of oxidative phosphorylation and β–oxidation of fatty acids in hematopoietic differentiation. In the knockdown, null mutants as well as pharmacological inhibition of β-oxidation, quiescence is lost and differentiation does not take place. This points out at the preferential utilization of fat metabolism in late instar blood progenitors for setting up quiescence and differentiation. It is intriguing to note that early instar progenitors when self-renewing have high glucose utilization whereas, glucose utilization in late quiescent and differentiating progenitors is very less, at the same time when we knock down fat metabolism components, quiescence is compromised and differentiation is disrupted. This suggests at the possibility that there is a switch from glucose to fat metabolism when hemocyte progenitors move from self-renewal to quiescence and subsequently to differentiation. It will be very interesting to know what are the molecular factors governing this switch from sugar metabolism to fat metabolism in the

hemocyte progenitors. Likewise, the bioenergetic compensatory mechanism regulating hemocyte progenitor homeostasis becomes an important outcome of this study and future studies might unravel new players regulating this aspect. The change in mitochondrial dynamics might be imperative for this change in metabolic state and energy homeostasis as major facets both fat oxidation and oxidative phosphorylation takes place in the mitochondria.

The subsequent drop in H3K9 acetylation levels and recovery by acetate supplementation along with rescue of differentiation defects in hemocyte progenitors further embodies the fat dependent acetylation crucial for hematopoiesis. A drop in only H3K9 acetylations but not say H3K18 points out a differential histone acetylation profile regulated by fat moiety and can be a subject of future detailed analysis. Recovery of acetylation defects by acetate feeding further endorses acetate supplementations in inborn errors of metabolism and several other pathophysiological scenarios governed by hypoacetylation defects.

The current study apart from finding out the metabolic requirements of hemocyte progenitor quiescence and differentiation also aimed to find the factors which work in collaboration with the metabolic circuitry of progenitor. This study also corroborated cell adhesion complex mediated control of cellular fate via regulating mitochondrial metabolism. Integrins control the mitochondrial function and biogenesis as both of them are affected in integrin knockdown from hemocyte progenitors. Integrin mediates the production of ROS by employing mitochondrial function. Optimal ROS production has been shown to induce differentiation in hemocyte progenitors by instigating JNK-Foxo signaling cascade. The knockdown of integrin which mediates cell-ECM adhesion, seem to affect cell-cell as well as ECM components. It will be an interesting aspect to investigate whether these changes in cellular and ECM adhesion are due to integrin mediated changes in metabolic circuitry or they themselves govern this metabolic and cell fate output. Likewise, how integrins corroborate the mitochondrial dynamics/ biogenesis looks to be very intriguing aspect to investigate.

Additionally this study also points out the requirement of Hippo signaling effector yorkie in hemocyte progenitor survival, proliferation and differentiation and the concomitant requirement of hippo signaling in formation of adult hematopoietic hubs. It is interesting to note that previously in other studies, hippo signaling has been implicated in regulating various facets of metabolism in different cell types and by controlling the various cellular

aspects like proliferation, cell survival, migration etc, might be coupling the metabolic state to other cellular processes. In hematopoiesis, the different roles of Hippo we see may be attributed to the ability of Hippo signaling to influence the metabolic state of the hemocyte progenitors. A detailed analysis in this light might open up new avenues of Hippo signaling role in hematopoiesis.

Apart from the three major candidate gene cohorts; a) metabolism b) integrin and c) hippo signaling which were picked up in the genetic screen and subsequently characterized in details in this study, there are several genetic candidates which show a positive or negative regulation of hemocyte progenitor maintenance. Some of the genes haven't been implicated in progenitor maintenance. For eg., hypoxia and redox regulator genes *sima* and *nos1* knockdown show that they are negative regulator of progenitor maintenance. It will be interesting to probe whether these genes feed into the metabolic regulatory network of hemocyte progenitors, as redox and hypoxia states are related to metabolic network.

Overall this study unravels the crucial role of metabolism in hemocyte progenitor homeostasis. Previous works have demonstrated combinatorial signaling essential for progenitor homeostasis. It will be intriguing to map them with the metabolic circuitry identified in this current study.

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