Role of extrinsic and intrinsic factors in regulation of the hematopoietic niche

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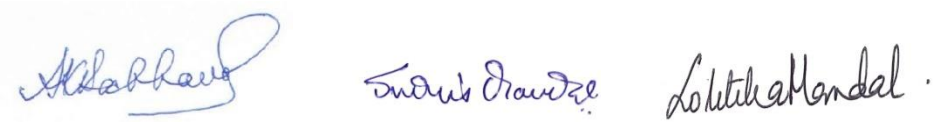

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Dated: May 07, 2021

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. Lolitika Mandal at the Indian Institute of Science Education and Research Mohali.

This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Harshath Amal

(Candidate)

Dated: May 07, 2021

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

Dr. Lolitika Mandal

(Supervisor)

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Abstract

Hematopoiesis is the developmental process of the formation of blood cells. In both vertebrates and the well studied model organism *Drosophila melanogaster*, hematopoiesis takes place in two waves; primitive and definitive hematopoiesis. Definitive hematopoiesis in *Drosophila* takes place in a specialized organ known as the lymph gland, and shares several similarities with vertebrate definitive hematopoiesis in the aorta-gonad-mesonephros region, making *Drosophila* an excellent model to study hematopoiesis and related morbidities. The lymph gland houses mainly three types of cells, each occupying a distinct domain of the lymph gland, dividing the lymph gland into distinct zones. The differentiating blood cells form the outermost layer and constitute the cortical zone, the progenitor cells are found inner to the cortical zone and constitute the medullary zone and the niche cells located in the innermost region constitute the posterior signaling centre. The various zones of the lymph gland are known to cross talk with each other for the maintenance of the progenitor population. But, a signal from the differentiating cells to the niche has not been found. Here, we show that Upd2, a ligand of the JAK-STAT signaling pathway is produced by the differentiating cells and is crucial for the maintenance of the niche cell population. Upd2 activates the canonical JAK-STAT pathway, leading to the activation of STAT92E in the medullary and intermediate zones. Loss of Upd2 results in an increased proliferation of the niche, along with precocious differentiation in the medullary zone. We also show that this deregulation of niche cell homeostasis is at least in part due to an upregulated insulin signaling coupled with a downregulation in Dpp signaling. This is the first report of a cytokine molecule regulating insulin signaling in the lymph gland. Upon loss of Upd2 expression, the niche cells downregulate the expression of Hedgehog, a molecule known to be involved in progenitor maintenance. We conclude that downregulated Hedgehog expression contributes to the precocious differentiation of the progenitor population. We also show that overexpressing STAT92E in the differentiating cells, although leads to a decrease in progenitor index and overall lymph gland size, does not affect the niche cell population. Our study throws light on the role of JAK-STAT signaling in maintaining the hematopoietic niche, and consequently overall hematopoiesis in the lymph gland. The high degree of conservation in hematopoietic processes between *Drosophila* and humans means that building on our results

could give us a better understanding of the role of JAK-STAT signaling in hematopoietic malignancies in humans that arise due to misregulation of JAK-STAT signaling.

CHAPTER 1

INTRODUCTION

1.1. THE LIFE CYCLE OF *Drosophila melanogaster*

Drosophila melanogaster, commonly known as the fruit fly, is a holometabolous insect. It passes through four stages during its life cycle; embryo, larva, pupa, and adult (Fernández-Moreno *et al.* 2007) (Figure 1). The females can mate up to six times in nature and lay about 80 eggs per day (Aranha *et al.* 2018). Eggs can be laid shortly after penetration by the sperm or retained in the uterus during the early stages of embryonic development (Parvathi *et al.* 2009). The *Drosophila* life cycle is sensitive to external temperature. At 25 ^{o} C, it lasts about 10 days, while it takes 15 days for completion at 20 ^{o} C (Demerec & Kaufman, 1996, pp. 4-8). It is fastest at about 30 ^{o} C. Development below 12 ^{o} C and above 32.5 ^{o} C is not viable (Economos *et al.* 1986).

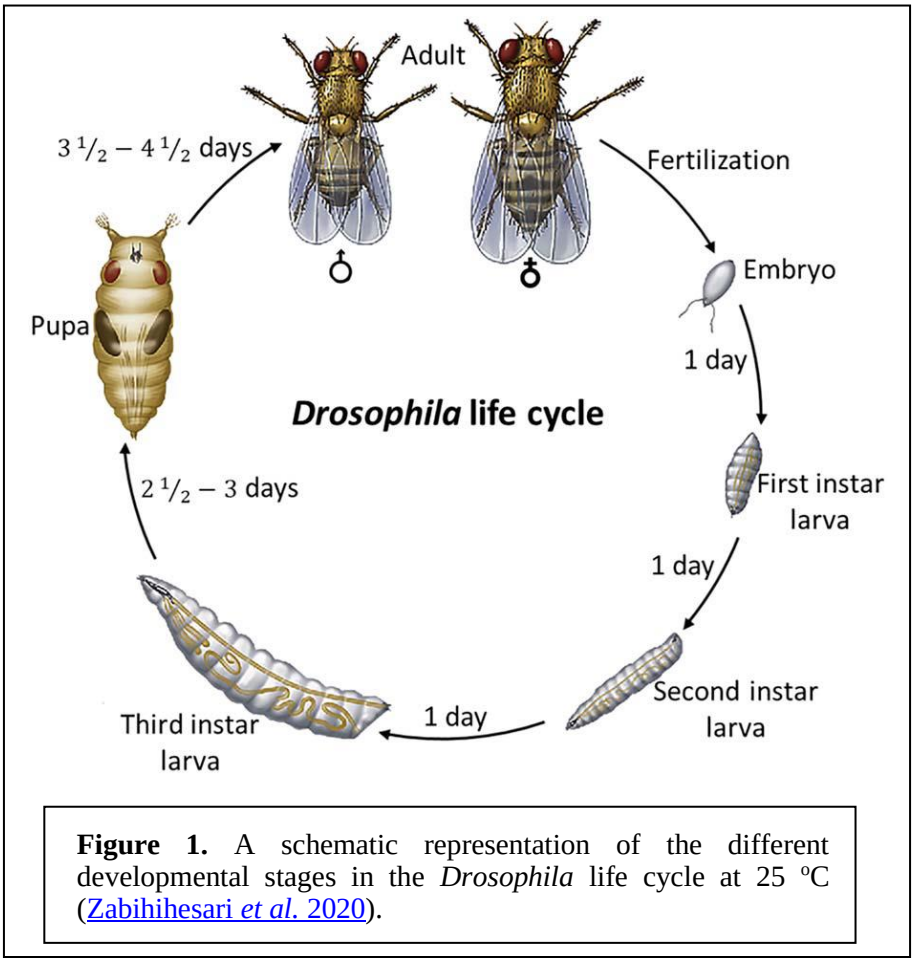


Figure 1. A schematic representation of the different developmental stages in the *Drosophila* life cycle at 25 ^{o}C (Zabihihesari *et al.* 2020).

Embryonic development lasts for 24 hours at 25 ^{o} C. An external membrane, the chorion, covers the egg. The sperm enters the egg through an opening in the chorion called the micropyle. The

egg also has a pair of dorsal appendages that function in gas exchange and anchorage onto the surface (Osterfield *et al.* 2017; Demerec & Kaufman, 1996, pp. 4-8). The eggs hatch, giving rise to the first instar larvae, which feed on the surface and undergo a molt after about 24 hours of egg hatching to develop into the second instar larvae. The second instar larvae burrow into the food and undergo another molt 24 hours post the first one, giving rise to the third instar larvae. Once they are preparing to pupate, they crawl up the walls to find a dry region to pupate, after spending 2-3 days in the third instar stage (Fernández-Moreno *et al.* 2007; Hales *et al.* 2015). Upon pupariation, the larva becomes covered by a dark covering of chitin. Metamorphosis ensues soon after, resulting in a loss of the larval structures and the formation of those seen in adults (Fernández-Moreno *et al.* 2007). The pupal stage lasts for 3-4 days, before the emergence of the adult fly.

1.2 *Drosophila* AS A MODEL SYSTEM

The fruit fly, *Drosophila melanogaste*r, is an ideal model organism and has been used in research for well over a century. There are several reasons for the popularity of the use of *Drosophila* as a model system. The short life cycle of *Drosophila* makes it possible to carry out experiments and get results faster compared to vertebrate models such as mice. The females lay a huge number of eggs allowing for large numbers of biological replicates for experiments if required (Jennings *et al.* 2011). It is easy and relatively cheap to maintain stocks and culture the flies. *Drosophila melanogaster* exhibits sexual dimorphism and the male and female flies are easily distinguishable. They also only have four sets of chromosomes, of which one has very few genes (called the dot chromosome), making it easy to study genetics in the fly model. Although it has fewer genes compared to humans, the *Drosophila* genome is 60 % homologous with humans, and about 75% of human disease-causing genes have homologs in the fly genome (Mirzoyan *et al.* 2019). A wide range of genetic tools such as the GAL4-UAS system, FLP-FRT system, Mosaic analysis with a repressible cell marker (MARCM), and gal4 technique for real-time and clonal expression(G-TRACE) make the Drosophila model highly susceptible to genetic manipulation (Allocca *et al. 2018;* Evans *et al.* 2009). Combined with the fact that mutants often

show a visible phenotype, *Drosophila melanogaster* has been established as a powerful model system to study development, genetics, neurobiology, and evolution.

1.3 THE GAL4-UAS SYSTEM

Gal4 is a protein of 881 amino acids identified in the yeast *Saccharomyces cerevisiae* as a galactose dependent gene regulator that binds to a stretch of DNA similar to eukaryotic enhancers to activate gene transcription (Duffy *et al.* 2002). The region of DNA that the GAL4 protein binds to is called Upstream Activating Sequence (UAS). GAL4 can activate transcription of a gene placed downstream of UAS, in several species, including other model systems, mammals, plants, and *Drosophila* (Halpern *et al*. 2008; Kakidani *et al.* 1988; Radoeva *et al.* 2016; Fischer *et al.* 1988).

The GAL4-UAS system is bipartite and employs a driver and responder line. The transgenic driver line has GAL4 placed under a tissue-specific enhancer, whose activation, in turn, activates GAL4 transcription (Brand *et al.* 1993). The reporter line has the genetic construct of interest placed downstream of UAS. This bipartite system ensures that the gene of interest is not activated in the responder line unless it is crossed with the driver line (Duffy *et al.* 2002). Upon crossing the two lines, the GAL4 and UAS constructs are brought together in the same fly. Subsequently, in the tissues expressing the GAL4 driver, GAL4 protein binds to UAS, activating the transcription of the downstream construct (Figure 2). Since GAL4 and consequently the gene construct of interest is only transcribed in tissues that express the specific enhancer, the GAL4-UAS system allows tissue-specific expression of a gene of interest (Duffy *et al.* 2002).

Several improvements to the GAL4-UAS system have made it possible to use it to temporally and spatially control the expression of a responder gene placed downstream of the UAS. A temperature sensitive Gal80 gene, a Gal4 inhibitor in yeast, can be used to control Gal4 expression. $Gal80^{ts}$ activity is highest at 18 ^{o} C (Zeidler *et al*. 2004). So in stocks kept at 18 ^{o} C, Gal4 activity and consequently that of the responder gene, will be downregulated. At a temperature of 29 ^{o} C, which is non-permissive for $GAL80^{ts}$, the GAL4 activity would be highest due to the lack of $Gal80^{ts}$ expression. This allows for tissue specific gene expression or

knockdown over time. In addition to Gal80^{ts}, other methods such as the ligand dependent GeneSwitch is also available for temporal regulation of Gal4 activity (Duffy *et al.* 2002). This targeted approach allows the study of gene expression patterns using a UAS-GFP line, gene function studies through knockdown or overexpression experiments using a UAS-geneX-RNAi or UAS-gene-X lines, or even induce cell death in tissues of interest by expressing pro-apoptotic proteins like reaper and hid.

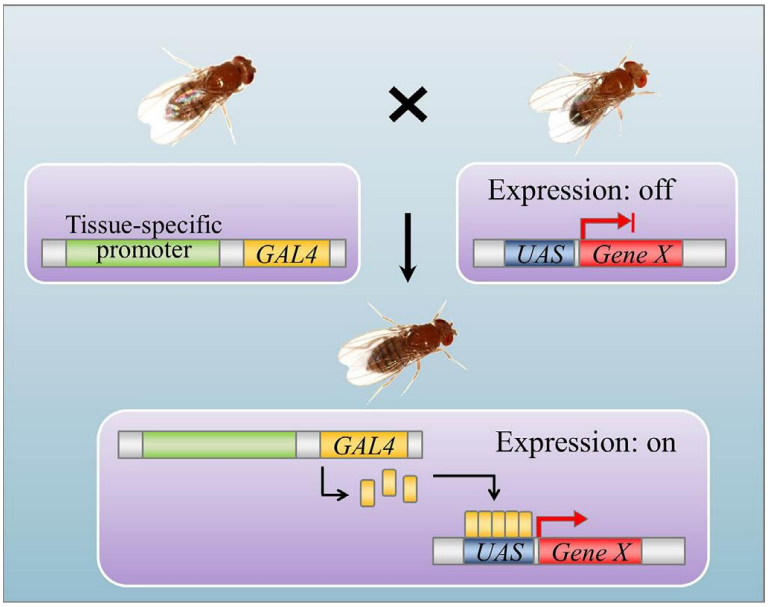


Figure 2. The working of the bipartite Gal4-UAS system in *Drosophila*. The 'gene X' can be a marker like GFP, a pro-apoptotic gene, an RNAi construct, or an overexpression construct (Cho, Bang & Toh, 2014, pp327-336).

1.4 *Drosophila* AS A MODEL SYSTEM FOR THE STUDY OF HEMATOPOIESIS

1.4.1 SIMILARITIES BETWEEN *Drosophila* AND VERTEBRATE HEMATOPOIESIS

Hematopoiesis is the developmental process of the formation of blood cells from a group of mesodermal precursors, often to compensate for their limited lifetime or in response to increased demand as a consequence of injury and infection (Banerjee *et al.* 2019). Hematopoiesis in *Drosophila* gives rise to three blood cell types that are functionally similar to the myeloid cells of

vertebrates (Evans *et al*. 2003). Hematopoiesis in the vertebrate system takes place in two waves, termed "primitive" and definitive" hematopoiesis. Primitive hematopoiesis in vertebrates is initiated in the embryonic yolk sac and gives rise to erythrocytes and macrophages (Tremblay et al. 2018), while definitive hematopoiesis begins in the aorta-gonad-mesonephros(AGM) region and gives rise to hematopoietic stem cells(HSC), that migrate and colonize the fetal liver, thymus, spleen, and eventually the bone marrow. The placenta has also been implicated in hematopoiesis during the AGM to fetal liver period (de Bruijn *et al*. 2000; Orkin *et al*. 2008). Similarly, *Drosophila* hematopoiesis also consists of two waves. The first wave, primitive hematopoiesis takes place in the embryonic procephalic mesoderm, and gives rise to plasmatocyte and crystal cells. Definitive hematopoiesis is initiated during the larval stage, in a specialized organ known as the lymph gland, giving rise to all three blood cell types (Holz *et al.* 2003).

Hematopoiesis in the procephalic region of the *Drosophila* embryo shares several similarities with primitive hematopoiesis of vertebrates at the molecular level. The GATA proteins GATA 1, -2, and -3 play a major role in hematopoiesis. While GATA-2 promotes the survival and proliferation of early progenitors, GATA 1and -3 are involved in the differentiation of various blood cell lineages (Fossett *et al.* 2001). The *Drosophila* GATA homolog Serpent (Srp) is the master regulator of hematopoiesis (Banerjee *et al*. 2019). Similar to GATA-2 mutant embryos, loss of Srp results in a loss of all mature hemocytes (Fossett *et al.* 2001). Srp contributes to the differentiation of all three blood cell lineages in *Drosophila*. Srp activates Glial cells missing (Gcm) and Gcm-2 to promote plasmatocyte fate while regulating Lozenge (LZ) and U-shaped expression to specify crystal cells (Bataillé *et al.* 2005; Fossett *et al.* 2003). Srp also regulates lamellocyte differentiation through the regulation of Ush (Banerjee *et al*. 2019).

Friend of GATA proteins (FOG) contributes to the repression of various blood cell lineages in vertebrates. This function is conserved in *Drosophila*, where the FOG homolog U-shaped (Ush) is responsible for suppressing crystal cell fate. The expression of murine FOG-1 and -2 can suppress crystal cell formation in Drosophila (Fossett *et al.* 2001). The RUNX-1 protein, also called Acute myeloid leukemia-1 (AML-1), plays a similar role as its homolog in *Drosophila*,

Lozenge. Both are involved in cell fate determination and regulation of differentiation (Fossett *et al.* 2001).

In addition to conservation in the role of several proteins, there is also conservation in the role of various signaling pathways. BMP signaling in the bone marrow contributes to the maintenance of the HSC population through the regulation of niche size. Loss of BMP signaling in the mouse bone marrow resulted in an increase in the number of osteoblasts and HSCs (Zhang *et al.* 2003). This is mirrored in *Drosophila* lymph glands, where Dpp signaling maintains the homeostasis of the progenitor population through negative regulation of niche cell number and the maintenance of the pre-progenitor population seen in the first instar lymph gland (Pennetier *et al.* 2012; Dey *et al.* 2016). Dpp and FGF signals are required in definitive hematopoiesis for the dorsal mesoderm specification, similar to its role in vertebrates where BMP and FGF signals are required for the specification of the AGM region from the lateral plate mesoderm (Banerjee et al. 2019). Also, Notch signaling in vertebrates acts to determine the arterial program, the loss of which results in a lack of AGM hematopoiesis. It is also involved in regulating cell fate (Bigas *et al.* 2012). Notch recapitulates these roles during *Drosophila* development, playing crucial roles in cardiogenic mesoderm specification and the determination of crystal cell fate and that of lamellocytes upon wasp parasitization (Duvic *et al.* 2002). It is also worth noting that both systems have hemangioblasts which act as the common precursor for hemocytes and the heart and aorta (Mandal *et al.* 2004).

1.4.2 THE LYMPH GLAND

The lymph gland is a multi lobed hematopoietic organ that is seen flanking the dorsal vessel in the *Drosophila* larvae (Figure 3). It is first seen during the mid embryonic stages and continues to develop throughout the larval stage before rupturing during pupation, releasing the blood cells into circulation. The anterior-most lobes of the lymph gland are called the primary lobes. The secondary, tertiary, and quaternary lobes are located posterior to the primary lobe and are collectively called posterior lobes (Banerjee *et al*. 2019).

The lymph gland develops from the dorsal mesoderm during the embryonic stages. The dorsal mesoderm also gives rise to the pericardial nephrocytes and the dorsal vessel, the latter of which serves as the *Drosophila* heart aorta later on. It is the cardiogenic mesoderm, which arises from the dorsal mesoderm, that gives rise to both the lymph gland as well the dorsal vessel (Banerjee *et al.* 2019; Mandal *et al.* 2004). The specification and maintenance of the cardiogenic mesoderm require several factors, including Dpp, Heartless(Htl), Wnt, Tinman(Tin), Pannier(Pnr), and Notch. Cells of cardiogenic mesodermal origin that express Odd-skipped gives rise to the lymph gland in the T1-T3 segments, while such cells give rise to pericardial nephrocyte cells in the abdominal segments (Mandal et al. 2004). At the end of embryogenesis, the lymph gland is composed of niche cells and hematopoietic progenitor cells (Krzemien *et al.* 2010).

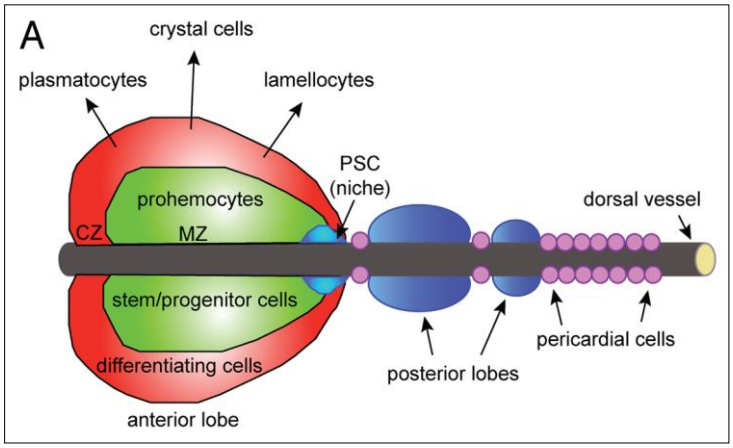


Figure 3. A schematic of the third instar lymph gland with all the different lobes (Amoyel *et al.* 2012).

The first differentiated blood cells begin to appear in the primary lobe of the lymph gland during the mid to late second instar (Lan *et al.* 2020). By the third instar stage, the primary lobe is divided into distinct zones made up of cells expressing different sets of markers (Figure 4). The outermost zone is called the Cortical zone (CZ). The CZ houses a population of maturing hemocytes that will eventually contribute to the circulating and sessile pools of the adult fly. Three types of mature blood cells can be seen in the adult fly, all of which are also seen in the lymph gland; plasmatocytes, crystal cells, and, although rarely, lamellocytes (Evans *et al.*2003).

The plasmatocytes constitute 90-95 % of the total blood cells (Ramond *et al.* 2020). Plasmatocytes are macrophages and are phagocytic in nature. The differentiation of plasmatocytes in the lymph gland depends on the expression of Pannier (Pnr). The plasmatocyte fate is inhibited by the matrix protein tiggrin, regulated by Wee1, a cell cycle regulator (Yu *et al.* 2018). The circulating plasmatocytes express markers such as Draper and Croquemort that aid in identifying phosphatidylserine on cells to be engulfed (Tung *et al.* 2013; Franc *et al.* 1999). Plasmatocytes contribute to tissue remodeling by phagocytizing cells undergoing apoptosis, deposition of extracellular matrix, and associated molecules like Collagen IV and laminin, intestinal stem cell activation in response to injury, regulation of glucose metabolism, wound healing, wasp parasitization response, and response to infection (Gold *et al.* 2015*;* Letourneau *et al.* 2016). They express the transmembrane proteins Nimrod C1 Eater that help in the identification of bacterial cells. Plasmatocytes are also capable of secreting anti-microbial peptides and molecules like Unpaired (Upd) to mount an immune response (Shin *et al.* 2020; Charroux *et al.* 2009).

The platelet-like crystal cells comprise 2- 5 % of the *Drosophila* blood cell population. (Vlisidou *et al.* 2015; Lan *et al.* 2020). Specification of the crystal cell lineage depends on Notch and Hippo signaling pathways (Yu *et al.* 2018). These cells contain crystalline inclusions and are involved in a melanization cascade in response to injury and innate immune responses. Melanization at wound sites and around invading pathogens helps defend the organism from further infection (Chen *et al.* 2012). The crystalline inclusions contain the Phenoloxidase enzyme responsible for melanin biosynthesis at the target site (Vlisidou *et al.* 2015). The Phenoloxidase enzyme in crystal cells is synthesized in and released in an inactive form and is activated through proteolytic cleavage to generate Phenoloxidase. Phenoloxidase oxidizes phenol into quinones, which subsequently polymerizes to form melanin (Binggeli *et al.* 2014). *Drosophila* has three PPO genes, two of which, the PPO1 AND PPO2, are expressed in crystal cells, while the PPO3 gene is expressed in the lamellocytes and does not require activation by proteolytic cleavage. (Chen *et al.* 2012). Melanization has been implicated in the survival of the organism upon microbial infections (Binggeli *et al.* 2014).

Lamellocytes are large, flat, disc-shaped cells rarely seen in the lymph gland. They possess a higher number of lysosomes and phagocytic vacuoles compared to plasmatocytes. The cortical cytoplasm of lamellocytes is devoid of organelles (Shrestha *et al.* 1982; Williams *et al.* 2007). The production of lamellocytes is seen to increase upon injury or wasp parasitization (Dudzic *et al.* 2015). Lamellocytes contribute to the encapsulation of pathogens or wasp eggs that are too large to be phagocytized (Williams *et al.* 2007).

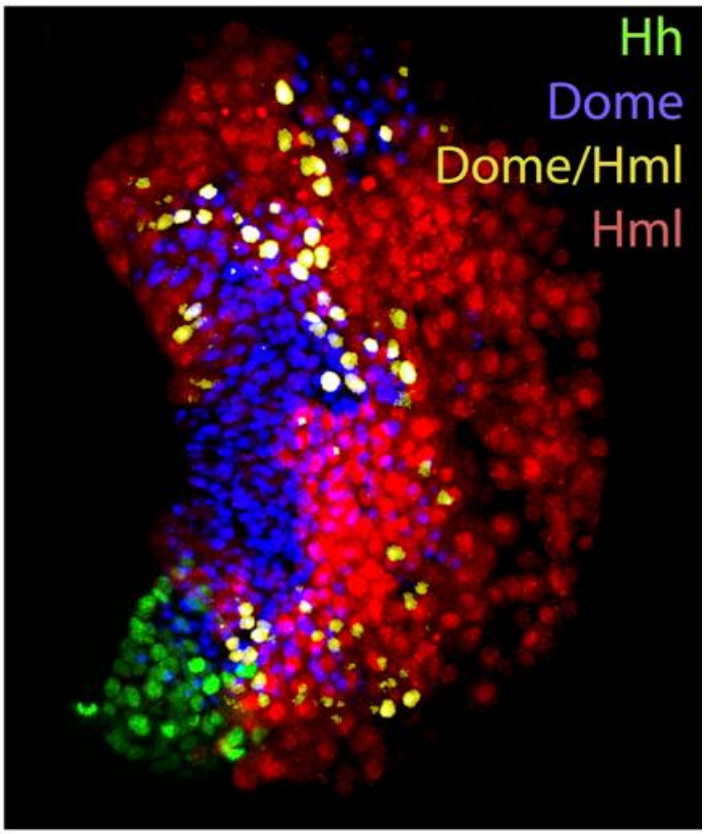


Figure 4. A confocal image of the lymph gland showing all the different zones. (1)Hml represents the CZ (2)Dome/Hml represents the IZ (3)Dome represents the MZ (4) Hh represents the PSC (Banerjee *et al.* 2019).

Inner to the cortical zone is the medullary zone (MZ), which houses a population of progenitor cells that can differentiate into any of the mature blood cells. The progenitors dominate during the first and early second instar lymph glands (Jung *et al.* 2005). The progenitors of the MZ exhibit extensive proliferation during the first instar and initial stages of the second instar until

the appearance of the mature blood cells, beyond which point proliferation is drastically lowered (Banerjee et al. 2019). The prohemocytes of the posterior lobes do not exhibit quiescence and continue to proliferate during the third instar stage. The progenitor cells of the MZ are tightly packed and show high expression of ECM markers such as Drosophila E-Cadherin (DE-Cadherin) (Jung *et al.* 2005).

The maintenance of the progenitor population depends on various signals that originate from different regions of the lymph gland (Banerjee *et al.* 2019). The posterior signaling center secretes Hedgehog, which binds to the Patched receptor present in the medullary zone to maintain activated Ci, and thus maintain multipotency (Mandal *et al.* 2007). Signals emanating from the cortical zone also contribute to progenitor maintenance by regulating the continued expression of activated Ci. Pvf1 ligands released from the PSC bind to the Pvr receptors of the CZ, leading to the downstream non-canonical activation of STAT92E. STAT92E activates the transcription of Adenosine deaminase growth factor-A, which converts adenosine to inosine, thus inhibiting the adenosine-dependent AdoR signaling in the MZ. This results in a downregulation of Protein kinase-A (PKA) activity and consequently maintenance of activated Ci (Mondal *et al.* 2011). Upd3 activated STAT92E directly activates U-shaped (Ush) transcription in the MZ. Loss of Ush results in increased levels of differentiated blood cells in the lymph gland (Gao *et al.* 2009). Ush achieves this through the maintenance of DE-Cadherin expression in the MZ, the loss of which results in a similar phenotype (Gao *et al.* 2013). Wingless signaling in the MZ inhibits the loss of progenitor cells by inhibiting the differentiation of a population of progenitor cells undergoing a fate transition. Loss of wingless results in an increase in the number of cells expressing both MZ and CZ markers (Sinenko *et al.* 2009). The angiotensin converting enzyme (Ance) contributes to progenitor maintenance through the regulation of DE-Cadherin, resulting in the maintenance of the full length activated Cubitus interruptus (Ci) in a PKA independent manner (Sharma *et al.* 2019). Other factors like Collier (Col), which is expressed in low levels in the MZ, also contribute to maintaining the multipotency of the progenitor population (Benmimoun[a] *et al.* 2015). The balance between maintenance and differentiation is also maintained by factors such as Jumu, which inhibits Col to induce proper differentiation, ROS, which primes the prohemocytes for differentiation and

subsequently lowers in level after it has happened, and the FGF signaling pathway (Banerjee *et al.* 2019).

Some quiescent progenitor cells of the third instar MZ, located furthest away from the posterior signaling center, express early differentiation markers such as Haemolectin (Hml) and constitute the intermediate zone (IZ). These cells are still multipotent and will eventually give rise to differentiated blood cells of the cortical zone. After committing to a fate, they start to proliferate (Banerjee *et al*. 2019).

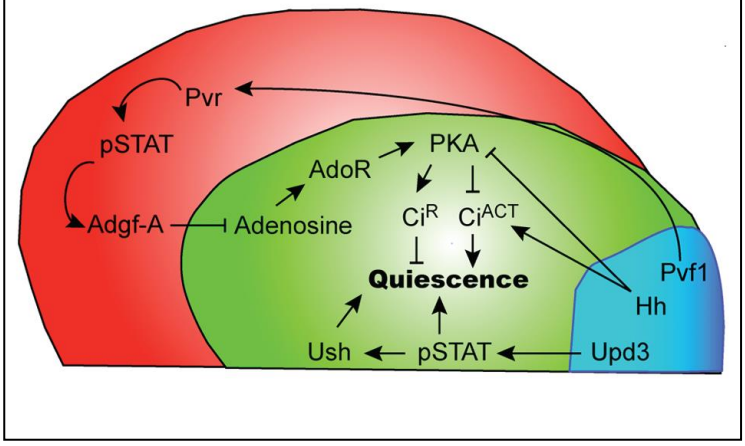


Figure 5. A schematic indicating the various pathways and molecules involved in the maintenance of the progenitor population (Amoyel *et al.* 2012).

The posterior signaling center (PSC), commonly referred to as the hematopoietic niche, constitutes the innermost zone of the lymph gland. The PSC develops from a group of 5-6 Odd expressing cells of the T3 segment of the stage 16 embryo that also express Antennapedia (Antp) (Mandal *et al.* 2007). Collier is expressed in the PSC from embryonic stage 16 and is required to maintain the PSC. In the absence of Col, the PSC forms but is lost by the third instar (Mandal *et al.* 2007). The proliferation of the niche cells is positively regulated by intrinsic factors such as Wg, while Dpp inhibits niche cell proliferation (Sinenko *et al.* 2009*;* Pennetier *et al.* 2012). The non-cell-autonomous factor Slit, a ligand secreted from the dorsal vessel, binds to its receptor

Robo, expressed in the PSC to regulate PSC size negatively, while dILPs from the brain, neurosecretory cells, and fat body regulate niche size positively (Morin-Poulard *et al.* 2016; Benmimoun *et al.* 2012*;* Nässel *et al.* 2015).

The niche cells secrete Hh, which maintains U-shaped expression to maintain the progenitor population (Baldeosingh *et al.* 2018). It has been recently claimed that the ablation of PSC does not lead to a loss of the progenitor population, and suggested the PSC might not be an essential element in progenitor maintenance (Benmimoun[b] *et al.* 2015). The loss of Col, on the other hand, can cause massive differentiation in the lymph gland. Col is expressed in a subset of the progenitor population and is suggested to act cell-autonomously to maintain the multipotency of these cells (Oyallon *et al.* 2016; Benmimoun[b] *et al.* 2015). The Col expressing population is maintained intrinsically and might be resistant to external differentiation signals, while the Col negative progenitor population is maintained by the Hh signal from the niche, and responds to differentiation signals (Baldeosingh *et al.* 2018).

The niche plays a crucial role in maintaining the pre-progenitor population seen in the first instar stage. The pre-progenitors express reporters of Notch signaling, Homothorax (Htx) and STAT92E but do not express Dome, a marker of all progenitor cells. The pre-progenitor cells are multipotent and can give rise to both progenitor cells as well as differentiated blood cells. The loss of this population significantly reduces the size of the third instar lymph gland. Dpp released from the PSC activates pMad in the pre-progenitor population, resulting in its self-renewal and maintenance (Dey *et al.* 2016).

1.5 JAK-STAT SIGNALING IN *Drosophila melanogaster*

JAK-STAT signaling plays several key roles in *Drosophila* and shows high degree of conservation with vertebrates (Zeidler *et al.* 2000). As is the case in vertebrates, JAK-STAT signaling in *Drosophila* involves the binding of the ligand to a cytoplasmic tyrosine kinase-associated transmembrane receptor. Ligand binding is followed by receptor dimerization and trans-phosphorylation of the JAK kinases, leading to their activation and consequent

phosphorylation of the receptors. STAT proteins bind to the phosphorylated tyrosine residues of the receptor and subsequently get phosphorylated themselves. The phosphorylated STAT then separates from the receptor and forms a dimer translocating to the nucleus to activate transcription (Hombría *et al.* 2002) (Figure 6). The STAT molecules form dimers via the interaction between the SH2 domain of one and the phospho-tyrosine residue of the other (Arbouzova *et al.* 2006). Studies have shown that STAT dimers can exist even before phosphorylation in the HeLa cell line through interaction via their N-terminal domains, although it is suggested that these cannot activate gene transcription (Braunstein *et al.* 2003; Arbouzova *et al.* 2006).

Mammals possess 4 JAK kinases and 7 STAT proteins. The ligands for this pathway include erythropoietin, interferons, interleukins, and growth factors (Rawlings *et al.* 2004). More than 50 different ligands falling into these categories have been identified in mammals (Morris *et al.* 2018). JAK-STAT activation can happen through a wide range of receptors, including cytokine receptors, GPCRs, RTKs, and homodimeric hormone receptors, of which the most common are the cytokine receptors (Bousoik *et al.* 2018). By contrast, there are only three JAK-STAT ligands in *Drosophila*; unpaired 1, unpaired 2, and unpaired 3. Domeless (Dome), which shows similarities with the IL-6 receptor family, is the only JAK-STAT receptor in *Drosophila* (Rawlings *et al.* 2004; Brown *et al.* 2001). Domeless associates with the *Drosophila* JAK kinase, Hopscotch (Hop). STAT92E is the sole STAT protein in *Drosophila*. The Hop kinase shares highest similarity with the mammalian JAK2 kinase, while STAT3 and STAT5 are the closest in similarity to STAT92E (Amoyel *et al.* 2012).The JAK-STAT pathway regulation is done through Suppressors Of Cytokine Signaling (SOCS), Protein Inhibitors of Activated STAT (PIAS) and phosphatases like PTP61F and the transcriptional repressor 'Ken and Barbie' (KEN), which suppress JAK-STAT activity and Signal Transducing Adaptor Molecules (STAM), which positively regulates JAK-STAT activity (Arbouzova *et al.* 2006; Zeidler *et al.* 2000). This simplicity, coupled with the high degree of conservation and the high degree of amenability of the system, makes Drosophila an excellent model for understanding the JAK-STAT pathway.

JAK-STAT pathway in *Drosophila* plays a critical role in hematopoiesis, immune responses, wound healing, stem cell maintenance, regeneration of the gut and wing disc, development of the

eye, wing and leg, sex determination, and cell migration during oogenesis (Morin-Poulard *et al.* 2013; Lee *et al.* 2017; Herrera *et al.* 2019; Luo *et al.* 2001; Avila *et al.* 2007; Ghiglione *et al.* 2002). In the lymph gland, JAK-STAT signaling is essential for progenitor maintenance. Upd3 in the medullary zone binds to Dome, activating the JAK-STAT signaling cascade resulting in the maintenance of DE-Cadherin (Morin-Poulard *et al.* 2013; Gao *et al.* 2013). Pvf1 ligands from the PSC binds Pvr receptors of the CZ to activate non-canonical JAK-STAT signaling, which also plays a role in progenitor maintenance through the downregulation of AdoR signaling in the MZ (Mondal *et al.* 2011). JAK-STAT signaling in the MZ also inhibits lamellocyte differentiation under normal conditions. JAK-STAT signaling is turned off upon wasp parasitization through the expression of the dominant-negative Dome-like receptor, latran, to allow lamellocyte differentiation (Makki *et al.* 2010).

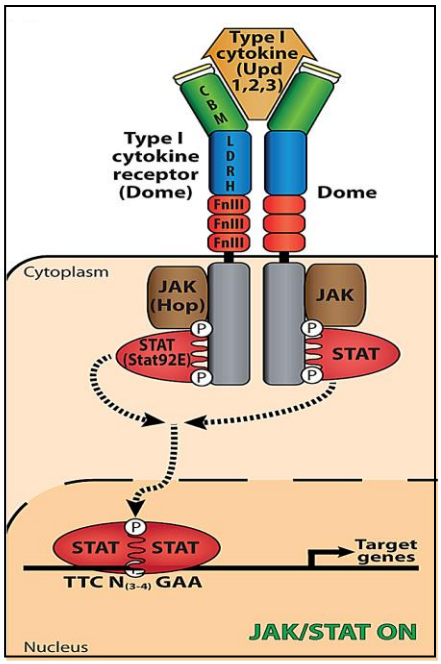


Figure 6. A schematic of the *Drosophila* JAK-STAT pathway activation (Morin-Poulard *et al.* 2013).

1.5.1 CONSERVATION OF JAK-STAT SIGNALING BETWEEN DROSOPHILA AND VERTEBRATES

JAK-STAT signaling in *Drosophila* shares several similarities with that of vertebrates (Zeidler *et al.* 2000) (Figure 7). There is a high level of similarity between *Drosophila* and vertebrates in terms of the components of the cascade such as the ligands, receptors, the kinases, and the signal transducers. The Upd molecules are analogs of mammalian leptins, and expression of leptins have been shown to rescue Upd loss of function phenotypes (Beshel *et al.* 2017). Dome, Hop, and STAT92E all have known mammalian counterparts (Amoyel *et al.* 2012). Homologs of the *Drosophila* JAK-STAT regulators SOCS, PIAS, and STAM have been identified in humans (Zeidler *et al.* 2000). In addition to conservation in the overall signaling cascade, there is also conservation in the function. JAK-STAT signaling regulates proliferation in both systems, as evidenced by the induction of several cancers. In humans, constitutive STAT has been seen in leukemias, melanomas, renal carcinomas, breast cancers, and brain tumors, among others (Calò *et al.* 2003). Similarly, the activating mutations in the *Drosophila* JAK kinases have been shown to be responsible for the induction of melanotic masses. The $Hop^{tum\text{-}1}$ and Hop^{T42} mutations, both of which possess a missense mutation resulting in a hyperactive kinase, leads to increased DNA binding of STAT and subsequent overproliferation and aggregation of plasmatocytes, forming melanotic masses. Furthermore, a mutation similar to Hop^{T42} has also been shown to hyperactivate JAK2 in mice (Luo *et al.* 1997).

JAK-STAT also plays a role in immune response in the two systems. It is essential for Type 1 T helper cell differentiation, IFN-γ production from T- and NK cells, and the differentiation, development, and expansion of T-cells (Watford *et al.* 2003). In *Drosophila*, JAK-STAT plays a vital role in response to septic injury, wasp parasitization, and antiviral responses, and mutations can lead to increased susceptibility to immune challenges (Banerjee *et al.* 2019; Shelly *et al.* 2009).

Stem cell maintenance in both *Drosophila* and vertebrates requires JAK-STAT signaling. It was shown that murine embryonic stem cells require the activation of STAT3 by LIF and IL-6 family cytokines (Niwa *et al.* 1998). However, STAT3 alone is not sufficient to maintain the

pluripotency of human embryonic stem (ES) cells. Other STAT molecules could be contributing to the stem cell maintenance of human ES cells (Arbouzova *et al* 2006). In *Drosophila*, JAK-STAT signaling contributes to the maintenance of the male germline stem cells. The apically located stem cell niche of the testis termed hub cells secretes Upd ligands that promote self-renewal of the GSCs during asymmetric division. The cell further away from the hub turns off JAK-STAT signaling and undergoes differentiation (de Cuevas *et al.* 2011). JAK-STAT signaling in the *Drosophila* gut contributes to the maintenance and self renewal of the intestinal stem cells (ISCs) (Lin *et al.* 2010). Loss of JAK-STAT signaling in the ISCs results in a loss of ISC population and ISC quiescence (Lin *et al.* 2010).

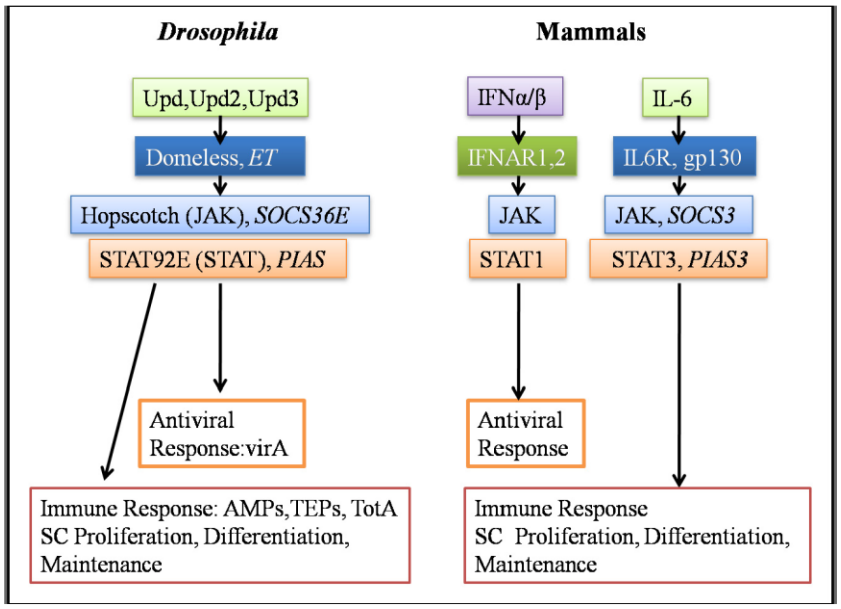


Figure 7. Conservation in the overall cascade and functions of the JAK-STAT signaling pathway in Drosophila and mammals (Panayidou *et al.* 2013).

1.6 BACKGROUND

The third instar lymph gland can be differentiated into distinct zones based on the expression of specific markers. While the different zones of the lymph gland are known to crosstalk with each other, mainly for progenitor maintenance, a signal from the CZ to the PSC is yet to be revealed.

In a loss of function screen done at the laboratory, Upd2 was identified as a potential regulator of lymph gland hematopoiesis. Work has been done focusing on the effect of Upd2 loss from the differentiating cells on the maintenance of the progenitor population, and it had also shown a disruption in niche cell homeostasis. Previous work from the laboratory had established '84 hours post egg laying' as the phenocritical time point for the phenotype to become full-blown. My thesis focuses on identifying the signal transduction involved in the cascade and emphasizes the effect of Upd2 loss on the niche.

Collections were taken at 25 ^{o}C and synchronized batches of hatched larvae were reared at 29 ^{o}C until dissection .All of my assays were done at 84 hours post egg laying. Also, since my experiments employ Hml, a validated driver for the plasmatocyte lineage of differentiated blood cells, any reference to differentiating blood cells or the CZ, indicates plasmatocytes.

CHAPTER 2

MATERIALS AND METHODS

2.1 VIRGIN SELECTION

The selection of virgin females for crosses is critical to the experiment. Females frequently mate with multiple males, and are capable of storing sperm from previous matings in a specialized structure known as Spermatheca. It is, therefore, essential that you pick females either before they hatch out of their pupal cases or shortly after hatching to ensure that the females you use for the cross are not carrying sperm from males of other unwanted genotypes, which could interfere with the experiment.

Selection of virgins can be done either during the pupal stage or after hatching out of the pupal case.

Adult virgins possess a dark patch in their abdomen, known as meconium, present for a few hours after eclosing. Meconium is the waste material accumulated during the pupal stage. Females are non-receptive for the first few hours after hatching, which coincides with the presence of meconium.

Virgin selection in adults:

a. Etherize the flies and place them under a microscope.

b. Observe the etherized flies for the presence of meconium.

c. Separate the virgins into another food vial.

Virgin selection in pupae is done by examining them for the presence of sex combs. Sex combs are tiny hairs present on the first pair of legs, exclusively in male flies.

Virgin selection in pupae:

a. Pick out the black pupae using a brush.

b. Transfer them into a small amount of water taken on a wooden slide placed under the microscope.

c. Observe the pupae for the presence of sex combs.

d. Separate the virgins into another food vial and wait for their eclosion.

2.2 SETTING UP A GENETIC CROSS

1) Take a food vial and scratch the surface using a needle to provide crevices for the female to dig its ovipositor into, to lay eggs.
2) Add 3-5 pellets of yeast to promote egg-laying.
3) Make a cone using a piece of Whatman filter paper to provide a surface for mating and to remove excess moisture.
4) Etherize the male and female flies required for the cross and transfer them into the vial. A ratio of about 1:3 male to female flies is maintained. NOTE: Do not keep the flies in ether for too long, as they could become sterile or die.
5) Flip the cross into a fresh vial with a scratched surface, yeast pellets, and a cone every two days for maintenance.

2.3 TAKING COLLECTIONS IN FOOD VIALS

Collections are essential to experiments as eggs laid by the females in the vial containing the cross are not synchronized. It will have eggs laid at different time points, increasing within-group diversity and making it difficult to draw conclusive results. During a collection, the flies are starved by transferring them into a foodless vial. The first response of females to starvation is laying all the eggs they have at the time.
1) Flip the desired cross into an empty vial without food and keep it at 25 ^{o}C for 1 hour.
2) Scratch the surface of a food vial, place a paper cone in it, and transfer the crosses in starvation into this to prompt the females to lay eggs. This is the discard batch. It will have eggs that were fertilized at various time points, making it undesirable for experiments. Keep it at 25 ^{o}C for 1 hour.
3) Take a food vial, scratch the surface using a needle, add 3-5 pellets of yeast, and put a cone. This is the collection vial. Transfer the crosses in the discard batch into the collection vial and keep it at 25 ^{o}C for 4 hours. Discard the discard vial.
4) After 4 hours, flip the crosses into a food vial. Count the number of eggs in the collection vial. If it is higher than 25, remove the excess using a spatula cleaned with 70 % ethanol.

5) Keep the collection vials at 29 ^{o}C for 84 hours before dissection.

2.4 MAKING RECOMBINANT LINES

2.4.1 For DadnRFP intensity analysis

To check whether or not Upd2 knockdown affected the Dpp signaling activity, we did an intensity analysis of DadnRFP. For this, we needed to bring the Hml driver in a DadnRFP background.

Lines in hand:
a. w; pColGFP/Cyo; DadnRFP/Tb
b. w; Hml>GFP/Hml>GFP;+/+
c. Sco/Cyo; MKRS/Tb

Required line for the experiment: w; Hml>GFP/Cyo; DadnRFP/Tb

CROSSES:

A. w; Hml>GFP/Hml>GFP;+/+ X Sco/Cyo; MKRS/Tb

Rear larvae from this cross and select larvae of the following genotype:

w; Hml>GFP/Cyo; +/Tb

Note: Cyo selection can only be done in adults. Select all Tb larvae positive for Hml>GFP, and once they eclose, select for Cyo.

B. w; Hml>GFP/Cyo; +/Tb X w; pColGFP/Cyo; DadnRFP/Tb

Rear larvae from this cross and select larvae of the following phenotype:

w; Hml>GFP/Cyo; DadnRFP/Tb

Note: Hml>GFP and pColGFP can be differentiated under the fluorescence microscope by their differential expression. Select RFP positive larvae to ensure that it is DadnRFP positive.

2.4.2 For tGPH intensity analysis

To check whether or not Upd2 knockdown affected the insulin-signaling activity, we did an intensity analysis of tGPH. For this, we needed to bring the Hml driver in a tGPH background.

Lines in hand:
a. w; tGPH/Cyo; MKRS/Tb
b. w; Hml>GFP.HmldsRed/Cyo;+/+
c. Sco/Cyo; MKRS/Tb

Required line for the experiment: **w; Hml>GFP.HmldsRed/Cyo,tGPH; MKRS/Tb**

CROSSES:
A. w; Hml>GFP.HmldsRed/Cyo;+/+ X w; tGPH/Cyo; MKRS/Tb

Rear larvae from this cross and select larvae of the following genotype:

w; Hml>GFP.HmldsRed/tGPH; +/Tb

B. w; Hml>GFP.HmldsRed/tGPH; +/Tb X Sco/Cyo; MKRS/Tb

Rear larvae from this cross and select larvae of the following genotype:

w; Hml>GFP.HmldsRed,tGPH/Cyo; MKRS/Tb

Note: Some larvae will be positive for Hml>GFP, HmldsRed and tGPH. Such larvae were obtained due to recombination during meiosis in the second chromosome of the parent with the

genotype w; Hml>GFP.HmldsRed/tGPH; +/Tb, that gave rise to a gamete of the genotype w; Hml>GFP.HmldsRed,tGPH; +(or Tb).

Cyo and MKRS selection can only be done in adults. Select all Tb larvae positive for Hml>GFP, HmldsRed, and tGPH (tGPH can be distinguished from Hml>GFP by their differential; expression). Once they eclose, select MKRS and Cyo.

2.5 DISSECTION, PROCESSING, AND MOUNTING THE LYMPH GLAND

1) Pick out larvae from the collection and transfer them into a cavity block containing distilled water.

2) Put a drop of 1xPBS on a slide and transfer the larvae into it after removing any food that might be sticking to it.

3) Place the slide under a microscope and place one needle just below the larvae's mouth hook and the other at a two-thirds length of the larva.

4) Pull the needle placed below the mouth hook slowly so that the lymph gland does not break off.

5) Transfer the pullouts into a cavity block containing 1xPBS kept on ice. Once all the larvae have been dissected and transferred into 1xPBS, aspirate the 1xPBS and add 1 ml of 10% PFA.

6) Incubate the tissues in 10% PFA for one hour on a shaker.

7) Replace the PFA with 1xPBS and wash for 5 minutes.

8) Incubate the pullouts in DAPI for 45 minutes. Aspirate the DAPI and wash twice with 1xPBS.

9) Take 10 μL of vectashield on a slide placed under the microscope and transfer the pullouts into it.

10) Isolate the brain from the rest of the pullout and detach the lymph gland from the brain at the ring gland by placing the needle just below the ring gland and slowly pulling it.

11) Place all the lymph glands in a line and remove the excess vectashield. Make a narrow line of vectashield next to the lymph glands and place the coverslip, anchoring it on this line.

12) Seal the coverslip using nailpolish.

2.6 IMMUNOHISTOCHEMISTRY FOR LYMPH GLAND

Immunohistochemistry or Immunostaining is a method used to verify the presence of a particular protein of interest in the tissues under consideration. This method employs a primary antibody to bind with the protein of interest and then uses a fluorophore-tagged secondary antibody against the primary to visualize the expression and localization of the protein. This process indicates the presence or absence of the protein of interest through the presence or absence of fluorescence, respectively.

Protocol:

All processes are done at room temperature unless stated.

1) Dissect the larvae in a drop of 1xPBS taken on a slide, remove the excess tissues such as fat bodies and cuticle, and transfer the pullouts into a cavity block containing 1xPBS kept on ice.
2) Once all the larvae have been dissected, aspirate the 1xPBS and incubate the pullouts in 5 % PFA (fixative) for 1 hour on a shaker.
3) Aspirate the fixative and wash the pullouts in 1xPBS for 5 minutes.
4) Wash the pullouts with 0.3 % PBT thrice for 15 minutes each.
5) Incubate the pullouts in 500 µL 10 % BSA in 0.3 % PBT for 45 minutes.
6) Transfer three pullouts into each well of a NUNC plate containing 8 µL of the primary antibody, and keep it at 4 degrees for 16-20 in a moist tissue box.
7) Transfer the tissues into a cavity block with 0.3 % PBT and wash thrice for 15 minutes each.
8) Incubate the pullouts in 500 µL of 5 % BSA in 0.35 PBT for 45 minutes.
9) Aspirate the block and incubate the pullouts in secondary antibody at 4 degrees for 16-20 hours in a moist tissue box.
10) Wash the tissues with 0.3 % PBT thrice for 15 minutes each.
11) Wash with 1xPBS for 5 minutes.
12) Incubate the pullouts in DAPI solution for 45 minutes.
13) Wash the pullouts twice with 1xPBS and mount the lymph glands in vectashield mounting medium.

2.7 RNA ISOLATION

1) Transfer the larvae into a cavity block containing distilled water and clean it properly to remove any food particles.

2) Dissect out the lymph glands in Schneider media and use a low retention pipette tip to transfer them into a 1.5 µL MCT containing 1 X PBS. Isolate and transfer about 50 lymph glands.

3) Spin it down at 5000 revolutions per minute (rpm) for 5 minutes at 4 ^{o}C and ensure that a pellet is formed.

4) Remove the 1 X PBS and add 500 µL Trizol.

5) Crush the pellet for a few minutes at regular intervals, keeping it on ice to prevent the temperature from rising.

6) Make up the volume of Trizol to 1 mL and keep it at room temperature for 5 minutes.

7) Add 500 µL chloroform and vortex it. Keep it at room temperature for 5 minutes.

8) Centrifuge at 12000 relative centrifugal force (rcf) for 15 minutes at a temperature of 4 ^{o}C.

9) Transfer the supernatant into a separate 1.5 mL MCT. To this, add an equal amount of 2-Propanol.

10) Add 1 µL of glycogen and store it at -80 ^{o}C overnight.

11) Take out the MCT kept at -80 ^{o}C and let it thaw on ice for 10 minutes.

12) Spin it down at 12000 rcf for 10 minutes at a temperature of 4 ^{o}C and carefully remove the 2-Propanol supernatant.

13) Add 75 % pre-chilled ethanol and spin it down at 7500 rcf for 5 minutes at a temperature of 4 ^{o}C.

14) Remove the supernatant taking care that the pellet is not disturbed.

15) Do a dry spin at 5000 rcf for 5 minutes at a temperature of 4 ^{o}C.

16) Keep the pellet in a laminar hood to let the rest of the ethanol evaporate.

17) Add molecular grade water and keep it at 20 ^{o}C overnight.

18) Take out the MCT and let it thaw in ice.

19) Estimate RNA concentration using NanoDrop.

2.8 PREPARATION OF cDNA

1) Make the following master mixes in microcentrifuge tubes:

	1 X Reaction	3X Reaction
cDNA	7 µL	21 µL
Primer mix	2 µL	6 µL
Enzyme	1 µL	1 µL

2) Calculate the amount of RNA required for the final concentration to be 1 µg, and add molecular grade water to make the volume up to 10 µL.

3) To this, add 10 µL of the reaction mixture such that the total volume becomes 20 µL.

4) Mix it well and run the cDNA protocol on the thermocycler:

Elongation: 45 minutes at 42 ^{o}C

Termination: 2 minutes at 92 ^{o}C

5) Store overnight at -20 ^{o}C.

2.9 qPCR

1) Take out the MCTs and keep them in ice for them to thaw.

2) Run qPCR protocol which is as follows:

a. 3 minutes at 95 ^{o}C

b. 30 seconds at 95 ^{o}C

c. 30 seconds at 55 ^{o}C

d. 1 minute at 72 ^{o}C

e. 10 minutes at 72 ^{o}C

f. at 4 ^{o}C for ∞ time

Cycle steps b through f 40 times.

3) Store at -20 ^{o}C.

PRIMERS USED

Rp49, the housekeeping gene, was used as the positive control for the experiment.

mRNA: Upd2

Forward primer: 5'- ACC TTA AAC GCC AGC CAA CA -3'

Tm: 57.5 ^{o}C; GC content : 50 %

Reverse primer: 5'- AGA CCG CGA TAC GGA TTG AC -3'

Tm: 57.0 ^{o}C; GC content: 55

2.10 GEL ELECTROPHORESIS

1) Make 1.5 % Agarose gel by mixing 0.6 g agarose powder in 50 mL 1 X TAE buffer.

2) Heat the solution in an oven until it becomes clear and devoid of particles.

3) Level the gel holder on a leveling table and put a gel comb.

4) Let it cool by keeping it at room temperature.

5) Once the fumes stop coming out, add 2.5 µL of ethidium bromide to the solution and mix it by swirling the conical flask.

6) Pour this mixture into the gel holder and cast the bubbles to the side. Carefully remove the comb once the gel solidifies and transfer it to the gel electrophoresis apparatus along with the casting tray.

7) Take a piece of parafilm and put 4 drops each of 1.66 µL of loading dye in it, making sure to keep sufficient distance between the drops.

8) Mix 8.34 µL of the PCR product, mix it well with the loading dye using a pipette and load it onto the gel.

9) Load the 10 µL ladder(100bp ladder of 10ng/µl concentration).

10) Close the lid of the apparatus and connect the electrodes.

11) Take out the gel once the samples have run the desired distance and take the gel image.

2.11 STOCKS USED

Table 1. List of fly stocks used

Stock #	Genotype	Description
3605	W^{1118}	Wild type control : A partial deletion in the white gene locus induces a white eye phenotype
33949	w ; +/+ ; UAS-Upd2RNAi/UAS-Upd2RNAi	Expresses dsRNA for RNAi of Upd2 under UAS control. (BDSC)
32859	w; +/+ ; UAS-Upd3RNAi/UAS-Upd3RNAi	Expresses dsRNA for RNAi of Upd3 under UAS control (BDSC)
33637	w; +/+ ; UAS-STATRNAi/UAS-STATRNAi	Expresses dsRNA for RNAi of STAT92E under UAS control (BDSC)
Gifted by U. Banerjee	w; HmlGAL4-UASGFP/HmlGAL4-UASGFP ; +/+	Expresses GFP in tissues expressing Hml via the GAL4-UAS system
20915	w ; +/+; UAS-STAT/UAS-STAT	Contains an insertion of STAT92E (BDSC)
3703	Sco/Cyo; MKRS/Tb	This double balancer line is used to make stable lines for a homozygous lethal allele
Recombinant line	w; HmlGAL4. HmldsRed, tGPH/Cyo; +/+	This recombinant line marks Hml expressing cells in red through dsRed, expresses GAL4 under the control of Hml and has a GFP tagged PI3K(tGPH)
Recombinant line	w; HmlGAL4. HmldsRed, STAT92EGFP/Cyo; +/+	This recombinant line marks Hml expressing cells in red through dsRed, expresses GAL4 under the control of Hml driver and has STAT92 tagged with GFP
Recombinant line	HhGFP ; HmlGAL4/Cyo ; +/+	This is a recombinant line that has a transcriptional readout of Hh, and expresses GAL4 under the control of an Hml driver
Recombinant line	w; Hml>GFP/Cyo; DadnRFP/Tb	This line has an Hml driver coupled with an RFP tagged Dad

2.12 ANTIBODIES USED

Table 2. List of Antibodies used

	Antibody	Organism of Origin	Dilution used
PRIMARY ANTIBODY	Antp	Mouse	1:10
	EGFP	Rabbit	1:100
	Ance	Rabbit	1:500
SECONDARY ANTIBODY	mouseCy3	Goat	1:500
	RabbitCy3	Donkey	1:500
	Mouse647	Goat	1:500
	RabbitFITC	Donkey	1:500

2.13 BUFFERS AND REAGENTS

2.13.1 BUFFER: 1 X Phosphate Buffer Saline (PBS)

PBS is a buffer solution that is isotonic with the Drosophila tissues. It does not induce any stress within the tissues and is ideal for washing and storage. Being non-toxic to the cells is another positive for using PBS.

1. Dissolve the following amount of salts in 800 mL of distilled water:

   a. 8g NaCl

   b. 0.2g KCl

c. 1.44g Na_2HPO_4

d. 0.24g KH_2PO_4

2. Set the pH of the solution in a range between 7.2-7.4. If the pH is higher than 7.4, add a few drops of concentrated HCl to bring it down and if it is lower, add a few drops of high molarity NaOH to elevate it.

3. Make up the volume to 1 L.

4. Autoclave the solution at 121 ^{o}C and ensure that the pH is still in the desired range.

2.13.2 DETERGENT: 0.3% PBT

PBT is used as a detergent to permeabilize the cell membranes for immunostaining. PBT contains Triton-X 100 mixed with 1 X PBS, which functions as the detergent.

1. Take 40mL 1 X PBS in a 50 mL falcon tube.

2. Cut out the tip of a 200 µL micropipette tip and use it to measure 120 µL of Triton-X 100. The removal of the tip helps with pipetting the viscous Triton-X 100 solution.

3. Properly mix the solutions by initially shaking it thoroughly and then placing it on a shaker for 45-60 minutes**.**

2.13.3 FIXATIVE: 10% Paraformaldehyde (PFA)

PFA is a cross-linking fixative that acts by forming covalent bonds between the proteins in tissues.

1. Weigh 0.2 g PFA in a 2mL MCT.

2. Add 1.5 mL 1 X PBS to the MCT and mix it properly using a vortex.

3. Seal the MCT using a parafilm strip and keep it in a water bath maintained at 65 ^{o}C.

4. Flip the MCT every 15-20 minutes to ensure that the PFA dissolves properly.

5. Once completely dissolved, store the solution at -20 ^{o}C.

6. When ready to use, make up the volume to 2 mL.

2.13.4 BLOCK: 10 % Bovine serum albumin (BSA)

BSA is a block that aids in immunohistochemistry by preventing non-specific antibody binding. It binds non-specifically to all proteins, ensuring that the antibodies only bind to those proteins against which it has high affinity.

1. Weigh 0.1 g crystalline BSA in a 2 mL centrifuge tube.

2. Add 1 mL 0.3% PBT to the MCT and mix it using a vortex.

3. Place the solution on a nutating mixer for 15-20 minutes for proper mixing.

2.13.5 CELLULAR MARKERS USED

A) CORTICAL ZONE : Hemolectin (Hml): Hml>GFP, HmldsRed
B) MEDULLARY ZONE: Angiotensin converting enzyme (Ance)
C) POSTERIOR SIGNALING CENTRE: Daughters against Dpp (Dad): DadnRFP, Hedgehog: HhGFP, Antennapedia (Antp)

CHAPTER 3

RESULTS

3.1 Upd2 from the cortical zone is essential for PSC homeostasis and progenitor maintenance

Upd2 is a ligand of the JAK-STAT signaling cascade, and is a homolog of human protein leptin (Beshel *et al.* 2017). Upd2 is known to play critical roles in various processes in *Drosophila melanogaster*. Upd2 from the fat body is essential to maintain systemic growth and energy balance through the regulation of insulin secretion from the brain (Rajan *et al.* 2012). Upd2 is also important for survival post-septic injury. In response to septic injury, Upd2 released from the hemocytes promotes stem cell renewal in the gut, the inhibition of which increases susceptibility to septic injury (Chakrabarti *et al.* 2016). Upd2 from hemocytes is involved in activating the JAK-STAT pathway in muscles, which is required for a systemic immune response upon wasp parasitization (Yang *et al.* 2015). However, the role of Upd2 in the lymph gland is yet to be elucidated.

We crossed w; HmlGAL4>UAS-GFP/HmlGAL4>UAS-GFP;+/+ driver lines with w;+/+; UAS-Upd2RNAi/UAS-Upd2RNAi to specifically knockdown Upd2 from the Hml expressing differentiating cells of the cortical zone. We see that upon knockdown of Upd2, specifically from the differentiating cell population, the niche cell homeostasis is disrupted (Figure 8). Such lymph glands show an overproliferation of the niche cell population (number of niche cells in: Hml>GFP/+; UAS-Upd2RNAi/+ = 57 ± 6.843, Hml>GFP/+; +/+ = 28.928 ± 5.730) (Graph 1). The Upd2 knockdown lymph glands also exhibit a differentiation defect. They showed increased levels of differentiation compared to control lymph glands, indicating precocious differentiation in such lymph glands. Also, the differentiated blood cells of the Upd2 knockdown lymph glands peel off quicker (at about 85-86 hours) compared to wild type lymph glands. These characteristics lead to premature rupturing of the lymph gland.

Upd2 from the differentiating cells is therefore required for overall lymph gland hematopoiesis, playing an essential role in regulating niche cell number and prohemocyte differentiation. This project focuses on the characterization of the niche upon loss of Upd2 from the differentiating cells.

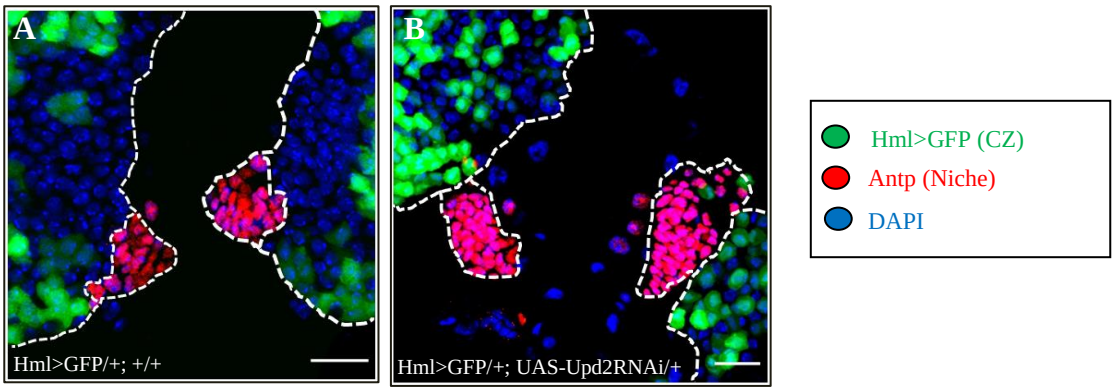
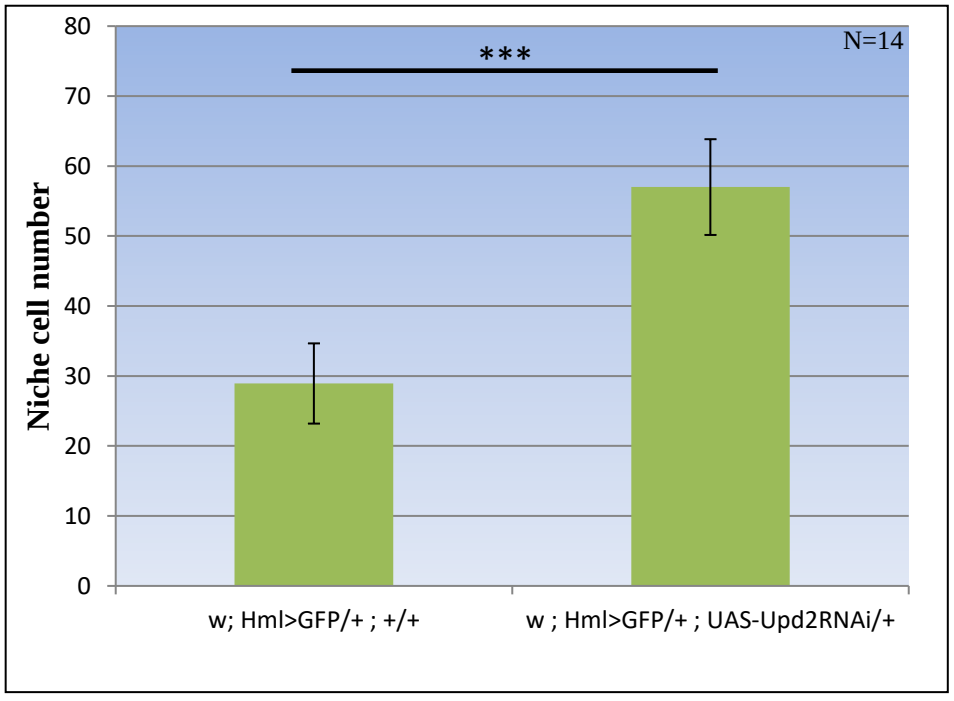


Figure 8. Upd2 knockdown lymph glands have lost normal lymph gland homeostasis.

A. Confocal image of the primary lobes of control lymph gland.

B. Confocal image of the primary lobes of Upd2 knockdown lymph gland.

The number of niche cells and overall differentiation in the Upd2 knockdown lymph gland is significantly higher compared to that of control lymph glands. These lymph glands have lost proper morphology due precocious differentiation and peeling off of the differentiated blood cells.



Graph 1. Niche cell numbers increase almost two-fold upon Upd2 downregulation in the differentiating blood cells. ($p<0.001$; student's t test; N=14).

3.2 The phenotype is due to loss of Upd2 from the lymph gland

We then wanted to ensure that the phenotypes obtained upon driving UAS-Upd2RNAi in the differentiating cell population are, in fact, due to the loss of Upd2 expression in these cells. For this, we isolated RNA from the lymph gland, prepared and amplified its cDNA, and performed gel electrophoresis. The gel image analysis showed that while Upd2 transcripts are seen in the control lymph glands, it is absent in the Upd2 knocked down lymph glands (Figure 9). This indicates that Upd2 is in fact expressed in the lymph gland, and that it takes place in the CZ. This result shows that the Upd2 loss from the Hml expressing population is the cause of the increased niche cell proliferation and precocious differentiation in the MZ.

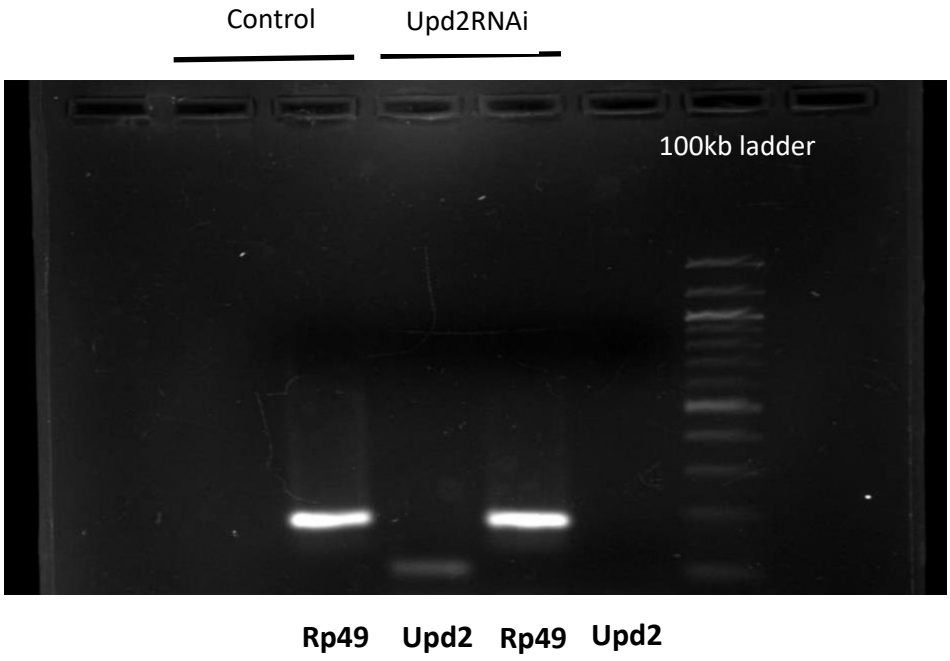


Figure 9. Upd2 transcripts are normally expressed in the lymph gland, and is not seen upon Upd2 knockdown from the differentiating cells, indicating CZ specific Upd2 expression. This shows that the Upd2 knockdown phenotype is due to loss of Upd2 expression in such lymph glands.

3.3 Control of niche cell proliferation is ligand-specific

Drosophila lymph glands, in addition to Upd2, also express another JAK-STAT ligand, namely Upd3. Upd3 is known to have roles in hematopoiesis and hemocyte-mediated immune response (Morin-Poulard *et al.* 2013). So we wanted to check if Upd3 also played a part in the regulation of niche cell homeostasis. We used w; HmlGAL4>UAS-GFP/HmlGAL4>UAS-GFP driver lines to knock down Upd3 specifically from the differentiating cells by crossing it with the w; +/+; UAS-Upd3RNAi/UAS-Upd3RNAi line. The Upd3 knockdown lymph glands do not phenocopy the control lymph glands (Figure 10). The average niche cell number in the Upd3 knockdown lymph glands resembles that of the wild-type lymph glands and it does not show precocious differentiation (number of niche cells in: Hml>GFP/+; UAS-Upd3RNAi/+ = 31.5 ± 3.368, Hml>GFP/+; +/+ = 28.928 ± 5.730) (Graph 2).

This indicates that the regulation of niche cell proliferation is independent of Upd3. The control of niche cell homeostasis is, therefore, ligand-specific.

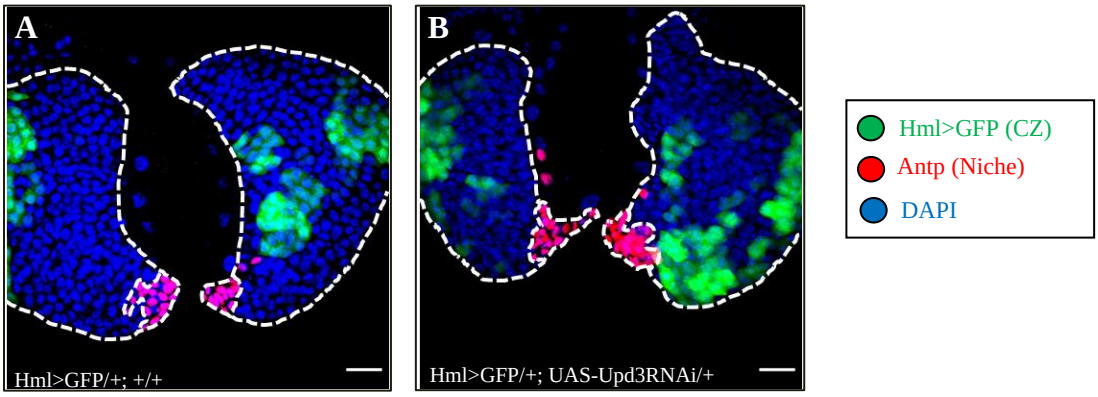
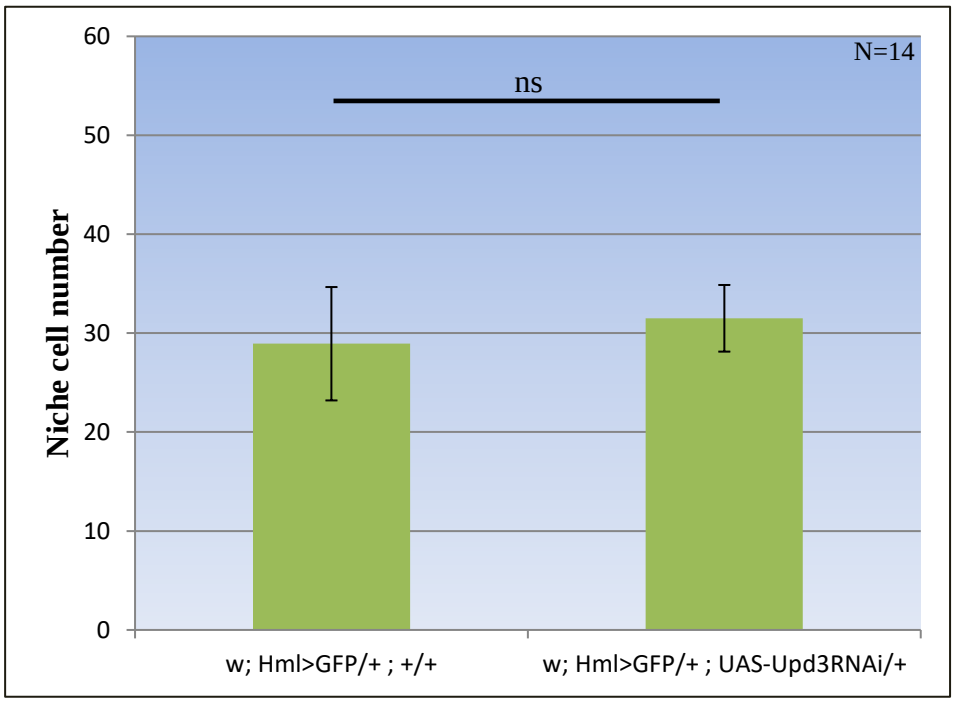


Figure 10. Upd3 knockdown from the CZ does not give a phenotype.

A. Confocal image of the primary lobes control lymph gland.

B. Confocal image of the primary lobes of Upd3 knockdown lymph gland.

Upd3 knockdown lymph glands do not significantly differ from control lymph glands in niche cell number and does not exhibit the precocious differentiation phenotype



Graph 2. Niche cell numbers in Upd3 knockdown lymph glands is not significantly different from that of the control lymph glands. ($p>0.05$; student's t test; N=14).

3.4 Upd2 activates the canonical JAK-STAT signaling pathway

We then wanted to check whether Upd2 was involved in activating the canonical JAK-STAT pathway or if its regulation of the niche cell size is through the non-canonical activation of some other pathway. To examine this, we knocked down STAT92E specifically from differentiating cell populations expressing Hml. This was done by crossing w; HmlGAL4>UAS GFP/HmlGAL4>UAS-GFP driver lines with w; +/+; UAS-STAT92ERNAi/UAS-STAT92ERNAi. We saw that upon knocking down STAT92E from the Hml expressing cells of the CZ, the lymph glands phenocopy the Upd2 knockdown lymph glands (Figure 11). The niche cell number in such lymph glands (average niche cell number=75.214 ± 11.696) was significantly different from control (average niche cell number= 28.928 ± 3.368) (Graph 3) and is even higher than the Upd2 knockdown lymph glands.

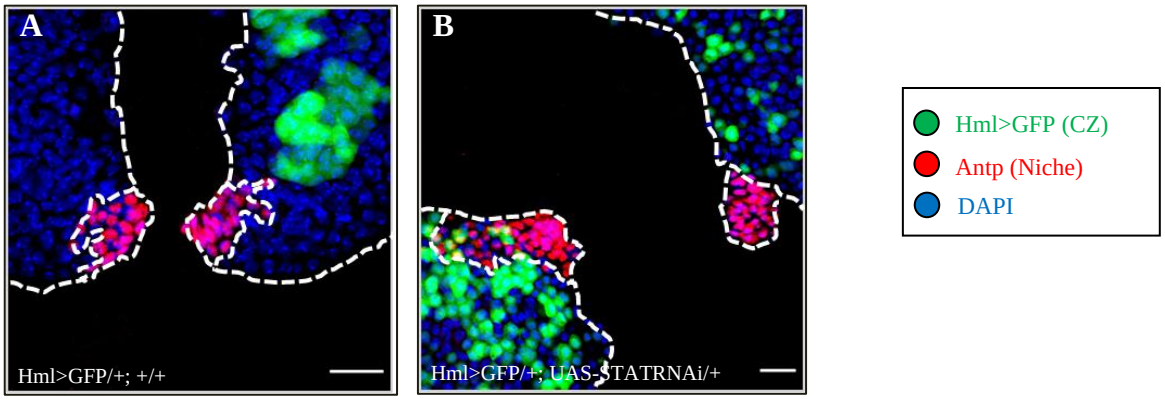
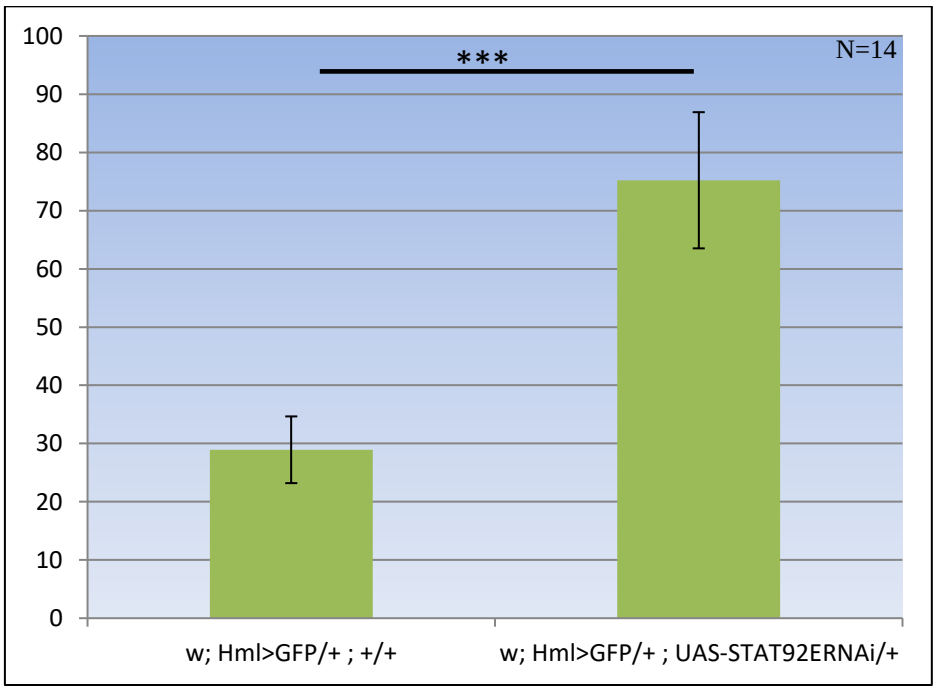


Figure 11. STAT knockdown lymph glands phenocopy Upd2 knockdown lymph glands.

A. Confocal image of the primary lobes of control lymph gland.

B. Confocal image of the primary lobes of STAT knockdown lymph gland.

STAT knockdown lymph glands show increased niche cell number, precocious differentiation and irregular morphology, similar to Upd2 knockdown lymph glands.



Graph 3. STAT knockdown from differentiating blood cells results in lymph glands with niche cell numbers that are 2.5 times that of control lymph glands ($p<0.001$; student's t test; N=14).

This increase in the niche cell number in STAT92E knockdown lymph glands compared to that of the Upd2 knockdown lymph glands could be that Upd2 is compensated for by other JAK-

STAT ligands, which was shown to happen by Hombria et al. 2005 (Hombría *et al.* 2005). Other research groups have also suggested that the knockdown of downstream components of the JAK-STAT pathway gives a greater phenotype compared to knockdown of the ligand. Another possibility is that this could be due to residual STAT92E expression in the CZ of such lymph glands due to the non-canonical activation by Pvf.

This result suggests that Upd2 activates the canonical JAK-STAT pathway to regulate niche cell homeostasis.

3.5 STAT activation takes place in the IZ as well as the MZ

The Upd ligands bind to the type I cytokine receptor Domeless (Dome). Dome is expressed by the progenitors of the medullary zone as well as the intermediate progenitors (Brown *et al.* 2001). To check whether Upd2 binds to Dome in the MZ, IZ, or both, we used a line that had a GFP tagged STAT92E in the background of HmldsRed and the HmlGAL4 driver (w; HmlGAL4.HmldsRed, STAT92EGFP/Cyo; +/+), to cross with w; +/+; UAS-Upd2RNAi/UAS-Upd2RNAi. The resulting progeny would have STAT92EGFP and HmldsRed in an Upd2 knockdown background, which would allow us to tell how STAT92E expression changes upon Upd2 knockdown. The intermediate progenitors would appear yellow, as they express both STAT92EGFP and HmldsRed, while MZ progenitors would be marked by STAT92EGFP expression.

The intensity of STAT92EGFP in the MZ is decreased upon Upd2 knockdown, suggesting that STAT92E expression is downregulated in the MZ. STAT92E expression is also downregulated in the IZ, which can be inferred from the decrease in the number of cells expressing both STAT92E and HmldsRed (Figure 12). This decreased number of such cells is due to the loss of STAT92E expression from these cells, thus only being marked by HmldsRed.

These results suggest that Upd2 activates STAT92E in the progenitors of the medullary zone as well as the intermediate progenitors to maintain niche cell homeostasis.

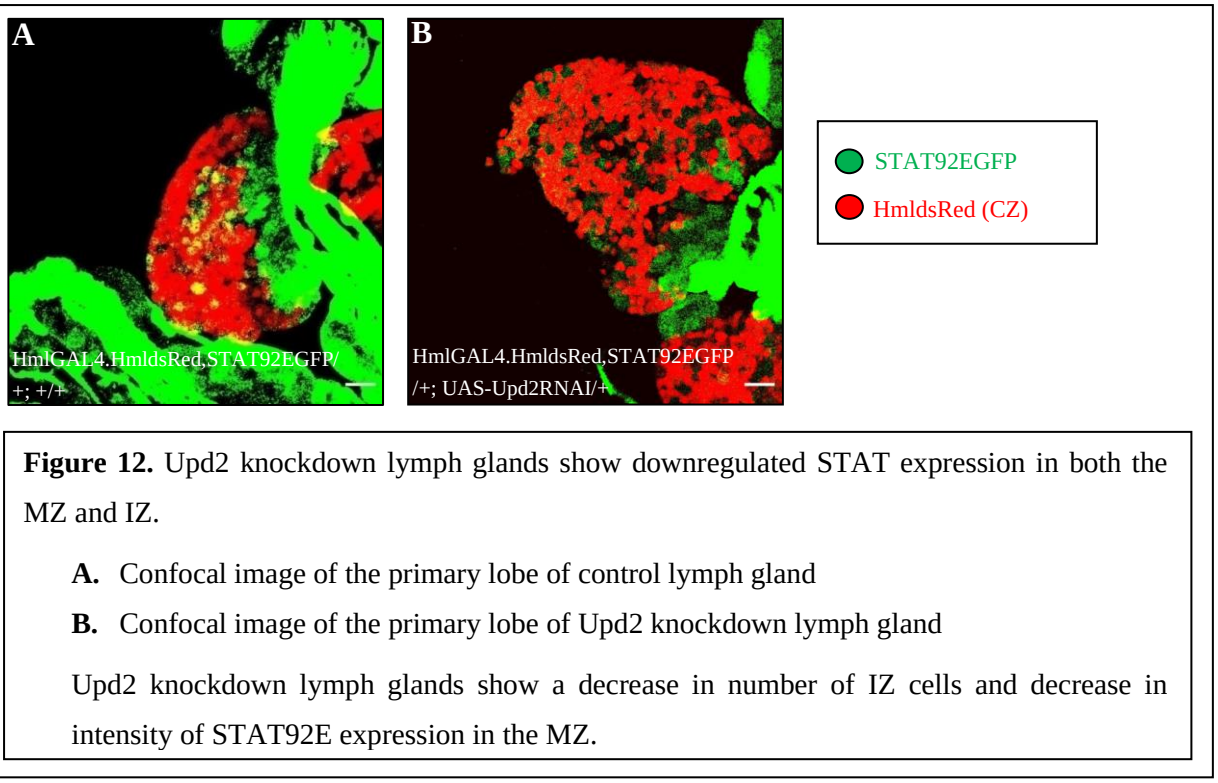


Figure 12. Upd2 knockdown lymph glands show downregulated STAT expression in both the MZ and IZ.

A. Confocal image of the primary lobe of control lymph gland

B. Confocal image of the primary lobe of Upd2 knockdown lymph gland

Upd2 knockdown lymph glands show a decrease in number of IZ cells and decrease in intensity of STAT92E expression in the MZ.

3.6 Dpp signaling is downregulated in the niche cells of Upd2 knocked down lymph glands

Dpp signaling in the niche has been shown to be involved in the regulation of niche cell proliferation through its regulation on Myc (Pennetier et al. 2012). Dpp negatively regulates Myc to control niche size (Pennetier et al. 2012). To understand the reason for niche cell proliferation upon Upd2 loss from differentiating cells, we explored the status of Dpp signaling in a Upd2 loss background. For this, we used a DadnRFP enhancer trap construct. Dad is a transcriptional target of Dpp signaling (Hamaratoglu *et al* 2014). So we compared the intensity per unit area of DadnRFP expression in the niche cells of the Upd2 knockdown lymph glands to that of the control. We dissected larvae obtained by crossing w; HmlGAL4>UAS-GFP/Cyo; DadnRFP/Tb with w; +/+; UAS-Upd2RNAi/UAS-Upd2RNAi. The niche cells of the Upd2 knockdown lymph glands show a significant decrease in DadnRFP expression intensity compared to those of the control (intensity per unit area in niche cells of: Upd2 knockdown lymph glands=61.24 ± 5.34, control lymph glands= 88.097 ± 10.46928) (Figure 13; Graph 4). The decreased DadnRFP

expression in Upd2 knockdown lymph glands indicates that Dpp signaling is downregulated in such lymph glands.

This result indicates that the increased niche cell proliferation upon Upd2 loss from differentiating cells is at least in part due to a downregulation of Dpp signaling.

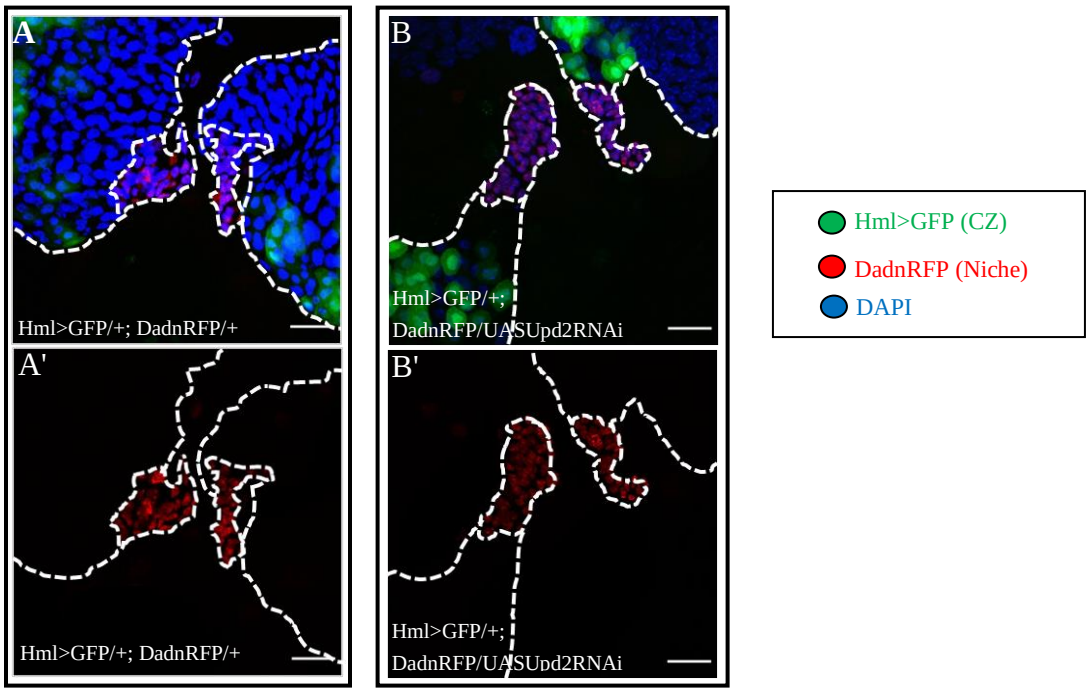


Figure 13. Dpp signaling activity is lower in Upd2 knockdown lymph glands.

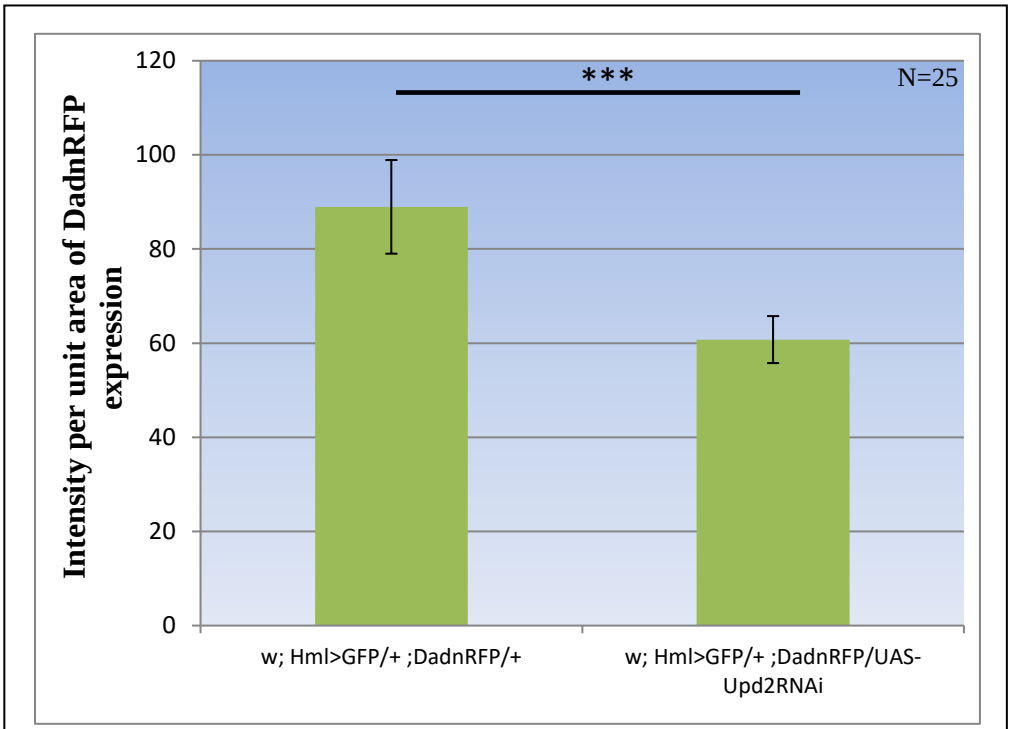
A. Confocal image of the primary lobe of control lymph gland.

A'. The niche of the control lymph gland

B. Confocal image of the primary lobe of Upd2 knockdown lymph gland.

B'. The niche of the Upd2 knockdown lymph gland

Upd2 knockdown lymph glands show reduced DadnRFP intensity suggesting a downregulation in Dpp signaling.



Graph 4. DadnRFP expression intensity is lower in Upd2 knockdown lymph glands compared to that of control ($p<0.001$; student's t test; N=25).

3.7 Insulin signaling is upregulated in the niche cells upon Upd2 loss from the differentiating cell population

The insulin signaling pathway has been shown to regulate niche cell proliferation positively (Benmimoun *et al.* 2012). The next step was therefore to check whether the insulin signaling pathway is affected in a HmlGAL4 > UAS-Upd2RNAi lymph gland. For this purpose, we used a tGPH construct. The tGPH construct has GFP fused to the pleckstrin homology(PH) domain of Grp1 placed under the control of the constitutive α-Tub84B promoter (Schmitt *et al.* 2015). The PH domain of GRP1 binds to phosphatidylinositol-3,4,5-trisphosphate generated by PI3K upon the activation of insulin signaling, thus allowing the use of tGPH as an assay for insulin pathway activation (Schmitt *et al.* 2015). Upon activation of the insulin signaling pathway, generation of membrane-anchored PIP3 recruits the GFP-tagged PH domains to the membrane. The membrane to cytoplasmic ratio of tGPH intensity per unit area was used to analyze the extent of insulin

activity in the niche cells. We made a recombinant line having tGPH in the background of HmlGAL4 and HmldsRed to examine the status of tGPH in a Upd2 knockdown background. We dissected larvae obtained from the collections of w; HmlGAL4.HmldsRed, tGPH/Cyo; +/+ crossed with w; +/+; UAS-Upd2RNAi/UAS-Upd2RNAi.

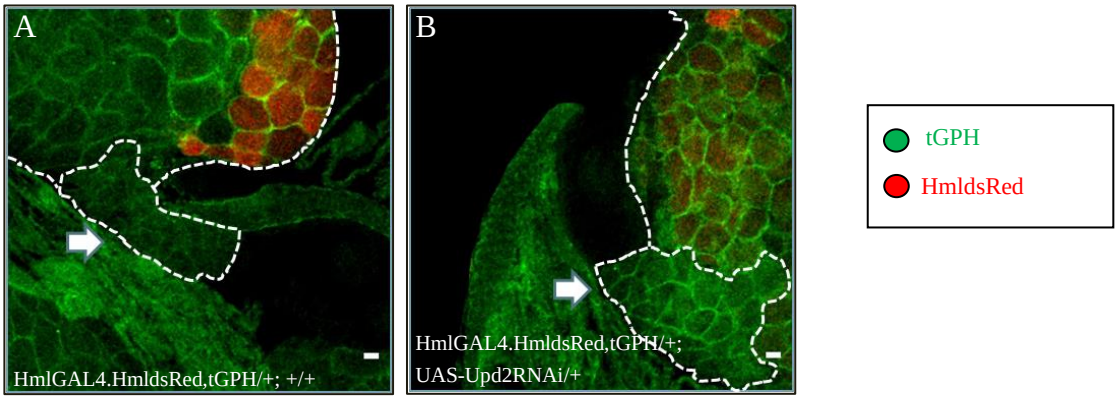


Figure 14. Insulin signaling in Upd2 knockdown lymph glands is upregulated.

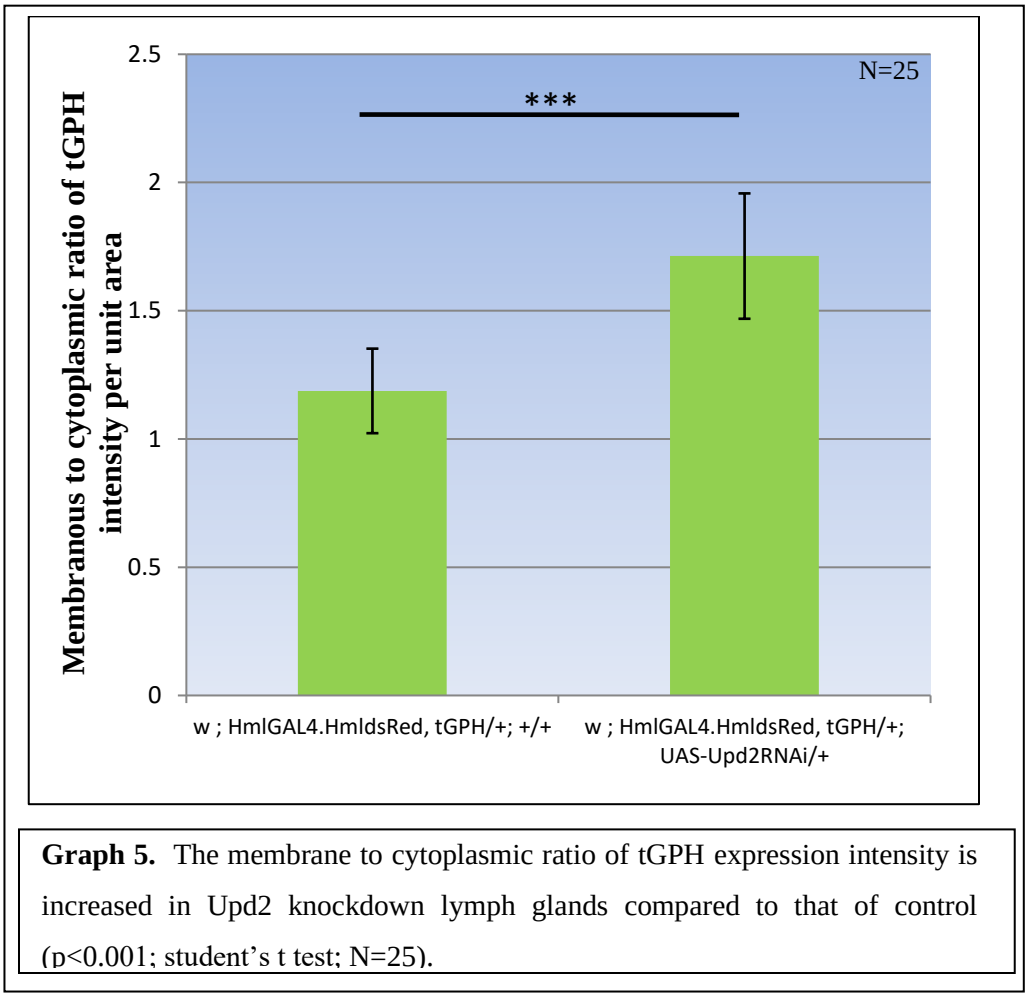
A. Confocal image of the niche of a control lymph gland

B. Confocal image of the niche of a Upd2 knockdown lymph gland

The increased membranous localization of tGPH in Upd2 knockdown lymph glands indicates an upregulated insulin signaling.

(Arrow(⇨) indicates the niche).

The niche cells of the Upd2 knockdown lymph glands show a significantly higher ratio of the membrane to cytoplasmic tGPH expression intensity (intensity per unit area in niche cells of: Upd2 knockdown lymph glands= 1.713 ± 0.244, control lymph glands= 1.187 ± 0.165) (Figure 14; Graph 5). This increased membranous localization of tGPH expression indicates a higher amount of membranous PIP3 in the niche cells of the Upd2 knockdown lymph glands, pointing to an increased PI3K activity, and by extension, an increased insulin signaling activity.



Graph 5. The membrane to cytoplasmic ratio of tGPH expression intensity is increased in Upd2 knockdown lymph glands compared to that of control ($p<0.001$; student's t test; N=25).

Our results thus show upregulated insulin signaling in the niche cells of Upd2 knockdown lymph glands. This suggests that the niche cell overproliferation could also be driven by the increased insulin signaling exhibited by such niches.

3.8 The forward signal from the PSC to MZ is downregulated in the Upd2 knockdown niche cells

Hedgehog, a morphogen secreted by the PSC cells, is a critical regulator of progenitor maintenance in the medullary zone (Mandal *et al.* 2007). Mandal et al. 2007 showed that the PSC cells extend thin processes into the MZ to maintain the progenitors in an undifferentiated state by maintaining the expression of Ci^{act} in these cells. We wanted to check whether the

precocious differentiation in the Upd2 knockdown lymph glands is due to a loss of Hh signal from the niche cells. We used a recombinant line with HhGFP (transcriptional reporter) (Torroja et al. 2004) and an HmlGAL4 driver (HhGFP; HmlGAL4/Cyo; +/+) to cross with w; +/+; UAS-Upd2RNAi/UAS-Upd2RNAi, and dissected larvae from the collections. We then compared the intensity per unit area of Hh expression in the niche cells of experimental and control groups. The niche cells of the Upd2 knockdown lymph glands show reduced HhGFP expression

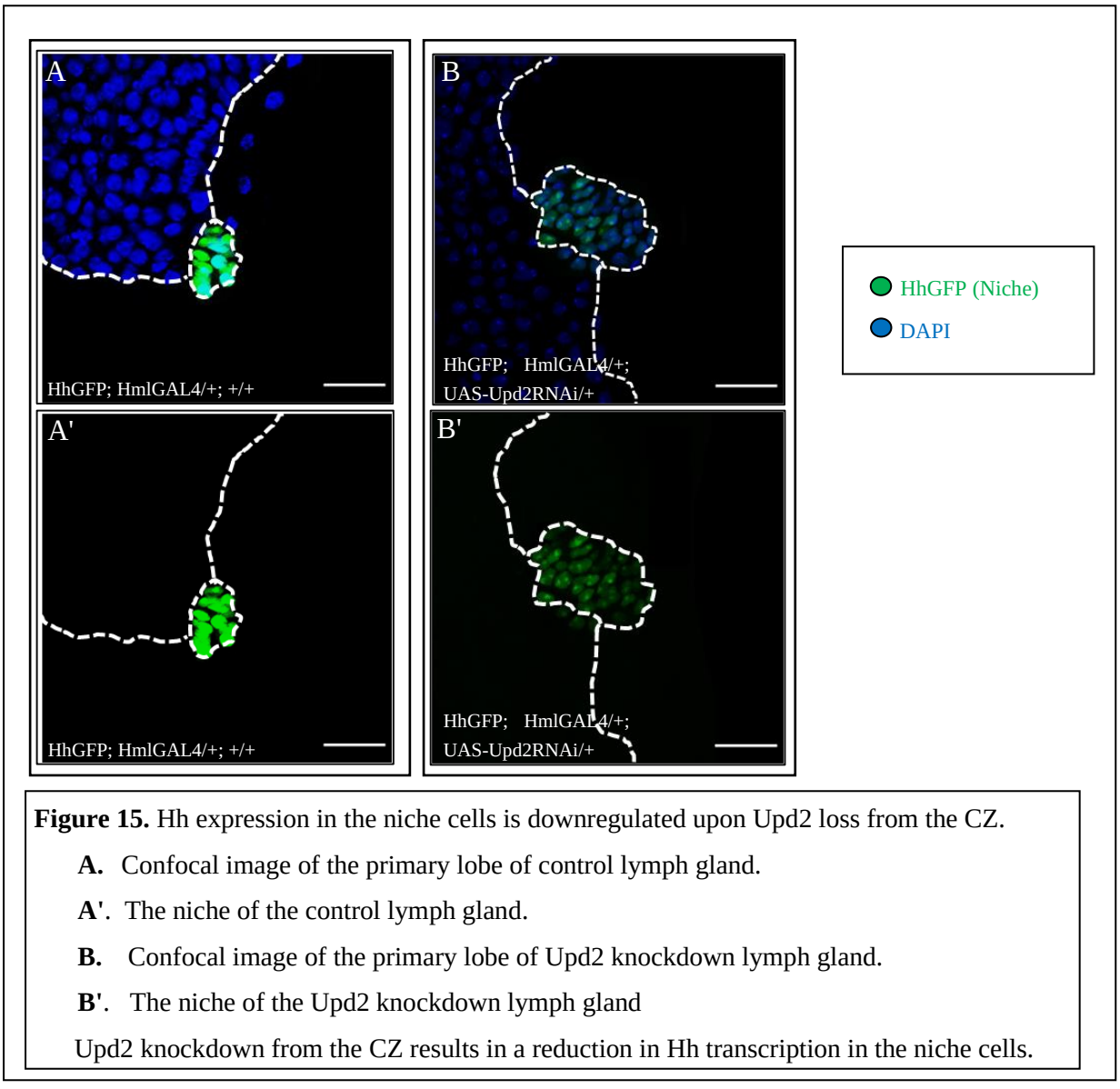


Figure 15. Hh expression in the niche cells is downregulated upon Upd2 loss from the CZ.

A. Confocal image of the primary lobe of control lymph gland.

A'. The niche of the control lymph gland.

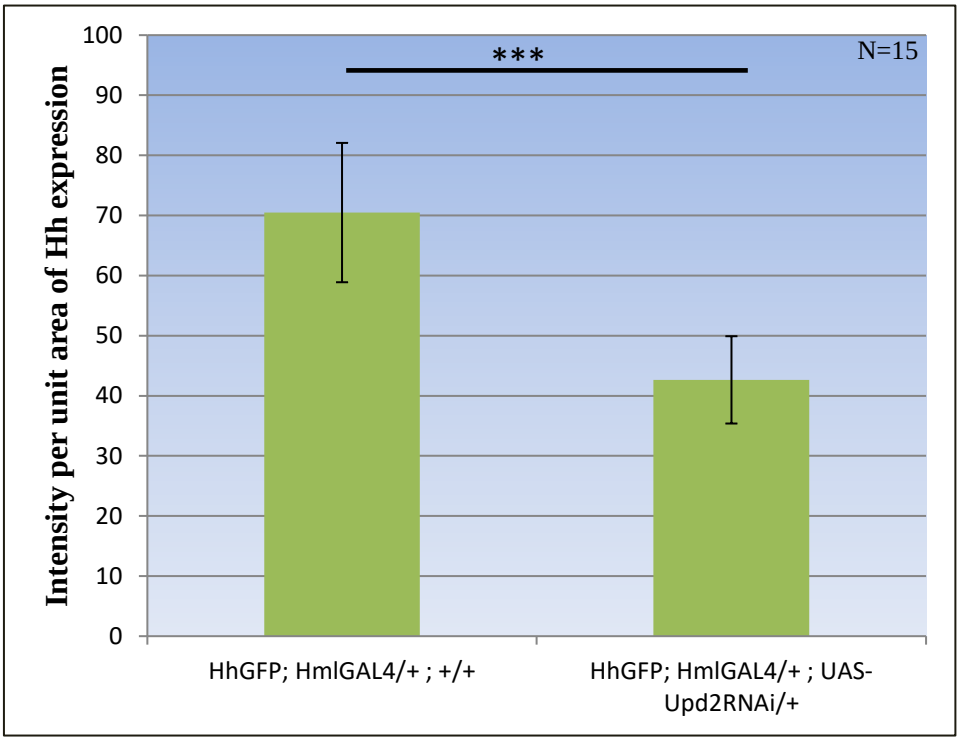
B. Confocal image of the primary lobe of Upd2 knockdown lymph gland.

B'. The niche of the Upd2 knockdown lymph gland

Upd2 knockdown from the CZ results in a reduction in Hh transcription in the niche cells.

compared to control niches indicating that Hh transcription is downregulated in the niche cells of such lymph glands (intensity per unit area in niche cells of: Upd2 knockdown lymph glands= 42.64 ± 7.261, control lymph glands= 70.478 ± 11.591) (Figure 15; Graph 6).

Our results show that Hh transcription is downregulated in the niche cells of the Upd2 knockdown lymph glands. This downregulation could be one of the reasons for the precocious differentiation phenotype seen in lymph glands that have Upd2 knocked down from the differentiating cell population.



Graph 6. Hh transcription intensity per unit area is lower in the Upd2 knockdown lymph glands compared to control ($p<0.001$; student's t test; N=15).

3.9 STAT92E overexpression in Hml positive cells does not affect niche size

We showed that loss of either of Upd2 or STAT92E from the differentiating cell population results in niche cell proliferation. So we wanted to check whether overexpressing STAT92E in the same population would give a phenotype in the niche. We dissected larvae obtained from collections of w; HmlGAL4>UAS-GFP/HmlGAL4>UAS-GFP; +/+ crossed with w; +/+ UAS-STAT92E/UAS-STAT92E. We saw that the niche cell numbers of the STAT overexpression lymph glands are comparable to that of control lymph glands (number of niche cells in: Hml>GFP/+; UAS-STAT92E/+ = 36.400 ± 9.070, Hml>GFP/+; +/+ = 34.2 ± 7.178) (Graph 7).

However, STAT92E overexpression lymph glands showed an apparent decrease in lymph gland size (Figure 16). The confocal images also suggested a reduction in differentiation. But the apparent reduction in differentiation could also be due to the reduced size.

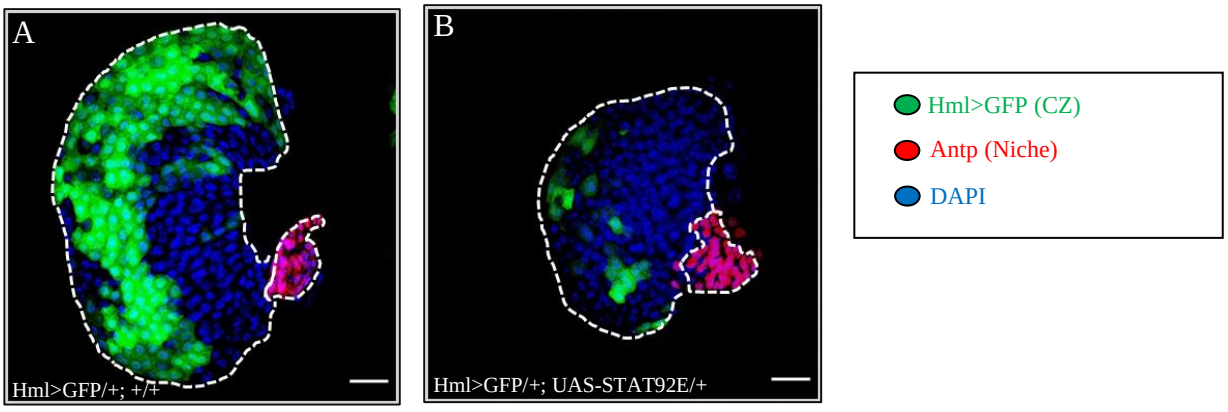
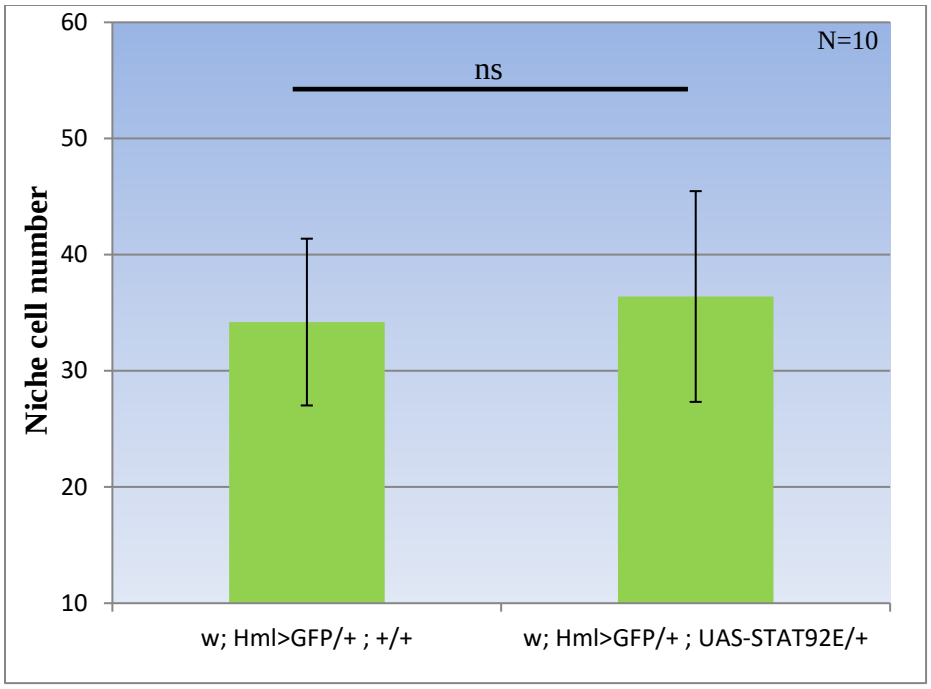


Figure 16. STAT92E overexpression lymph glands do not give phenotype in the niche.

A. Confocal; image of the primary lobe of control lymph gland.

B. Confocal image of the primary lobe of STAT92E overexpression lymph gland.

STAT92E overexpression results in a smaller lymph gland with no niche phenotype.



Graph 7. Niche cell number in STAT92E overexpression lymph gland is comparable to control lymph glands ($p>0.05$; student's t test; N=10).

3.10 STAT92E overexpression from Hml positive cells reduces differentiation and promotes progenitor maintenance

To check the status of the progenitor population upon STAT overexpression in Hml expressing cells, we looked at the progenitor index. The progenitor index would conclusively tell us of the existence of a differentiation defect in such lymph glands, and confirm that it is not a byproduct of the overall reduction in lymph gland size. We used Ance antibody to mark the medullary zone and looked at the ratio of MZ size to the size of the whole lymph gland. The STAT92E overexpression lymph glands show a higher MZ to lymph gland ratio (Progenitor index of : Hml>GFP/+; UAS-STAT92E/+ = 0.6954 ± 0.0867, Hml>GFP/+; +/+ = 0.5276 ± 0.0288) (Figure17; Graph8).

Our results suggest that STAT92E overexpression from Hml expressing cells leads to increased progenitor maintenance.It also follows that such lymph glands have lowered levels of differentiation compared to control.

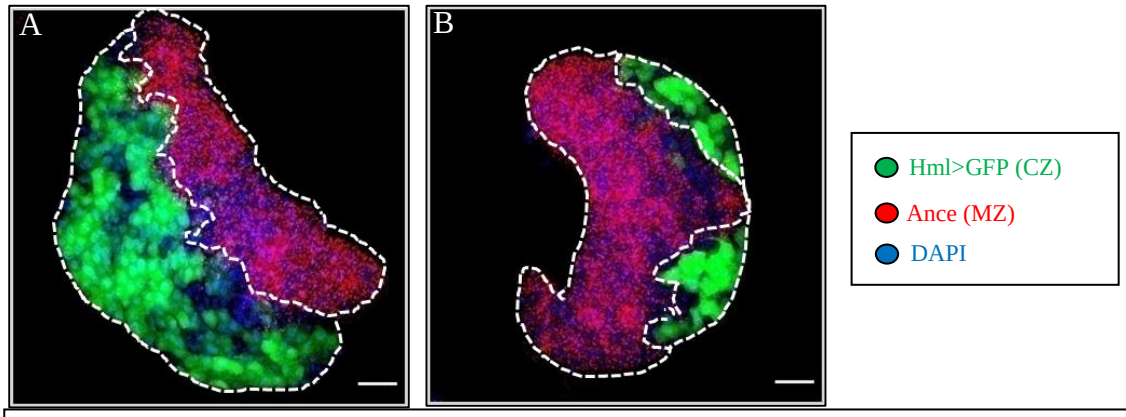
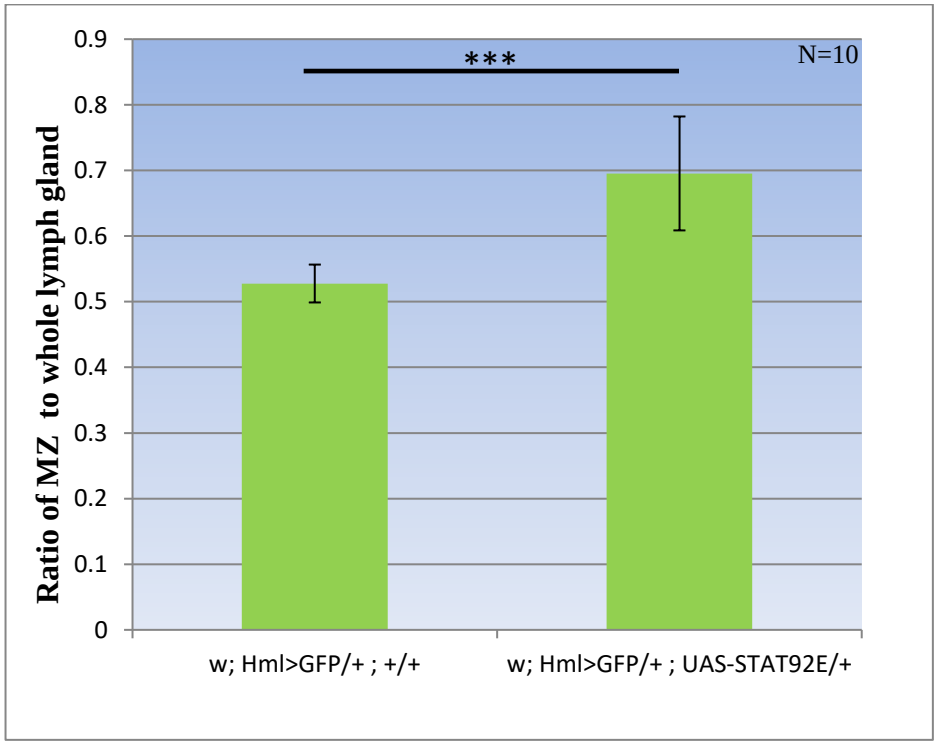


Figure 17. STAT92E overexpression lymph glands exhibit a differentiation defect.

A. Confocal image of the primary lobe of control lymph gland.

B. Confocal image of the primary lobe of STAT92E overexpression lymph gland

STAT92E overexpression lymph glands are smaller in size and show a reduced differentiation phenotype.



Graph 8. The progenitor index is higher in STAT92E overexpression lymph glands compared to control ($p<0.001$; student's t test; N=10).

CHAPTER 4

DISCUSSION AND FUTURE DIRECTIONS

Cytokines are pleiotropic proteins or small glycoproteins with molecular masses of less than 30 kDa. Cytokines have been long known to be involved in the regulation of hematopoiesis and immunity (Gulati *et al.* 2016). Cytokines activate the JAK-STAT signaling pathway, which shares several conserved functions in vertebrates and *Drosophila melanogaster*. Activated by a variety of cytokines and growth factors, different STAT proteins in vertebrates function to regulate immune response, T-cell specification and proliferation, B-cell development, and fetal erythropoiesis (Dorritie *et al.* 2014). *Drosophila* possesses a much simpler cascade with respect to JAK-STAT signaling, having only three activating cytokines, one receptor, and one STAT protein (Hombría *et al.* 2002). The role of Upd3 in hematopoiesis is known (Gao *et al.* 2009). However, Upd2 has not yet been designated a role in the same. Here, we show that Upd2 in the lymph gland plays a critical role in hematopoiesis.

The loss of Upd2, specifically from the differentiating cells on the periphery of the lymph gland, results in severe impairment of normal definitive hematopoiesis. The knockdown of Upd2 from the Hml expressing cells leads to niche cell numbers that are almost twice that of normal lymph glands. We also observed precocious differentiation and an early peeling off of the lymph gland hemocytes. This role of cytokines is ligand-specific and is not exhibited by Upd3, as Upd3 loss from the differentiating cells shows normal lymph gland hematopoiesis. We also show that Upd2 binds to the Dome receptor in the IZ and MZ to activate canonical JAK-STAT signaling. How JAK-STAT signaling regulates niche cell homeostasis and what genes it activates in this scenario remains to be explored. However, we show that downstream of this cascade, insulin and Dpp signaling pathways are regulated, as Upd2 loss from the differentiating cells affects the steady-state regulation of these pathways. Upregulation in insulin signaling and a decrease in Dpp signaling in niche cells of Upd2 knockdown lymph glands most likely contribute to the overproliferation phenotype that is seen. Wingless signaling is another known cascade involved in the regulation of niche cell numbers. The status of Wg signaling in a Upd2 knockdown background remains to be explored (Sinenko *et al.* 2009). Although Upd2 from the fat body has been shown to regulate insulin signaling in the *Drosophila* brain (Rajan *et al.* 2012), it is the first time that cytokine signaling has been shown to regulate the insulin levels in the lymph gland. Since dILPs are not produced in the lymph gland (Géminard et al. 2009), the altered response might be due to Upd2 loss from the circulating hemocytes.

Ecdysone signaling regulates hemocyte motility and dispersal during metamorphosis and upon infection, and during the latter, also induces a mitotic burst in the lymph gland hemocytes (Regan *et al.* 2013*;* Sorrentino *et al.* 2002). It will be interesting to look at the status of ecdysone signaling in a Upd2 loss background as it could potentially give us answers on why there is an early dispersal of hemocytes in such lymph glands and could also be a contributing factor to the over-differentiation phenotype. The over differentiation phenotype could also be a consequence of the downregulated Hh transcription in the niche cells of the Upd2 knockdown lymph glands.

Our results indicate that overexpressing STAT92E using an Hml driver is not sufficient to induce a phenotype in the niche, although it causes a reduction in overall lymph gland size and differentiation. The reduced lymph gland size might be due to STAT driving precocious quiescence in the progenitor population. This hypothesis needs to be assessed through either an EdU assay or with the use of Dual-FUCCI. If the hypothesis were correct, the proliferating progenitors of the control lymph glands would incorporate EdU, while the quiescent progenitors of STAT92 overexpressed lymph glands would not. With the Dual-FUCCI lines, STAT92E overexpressed lymph glands should have progenitors arrested in the G1 phase at 72 hours post-egg-laying, while in control lymph glands, these cells would be in the S, G2, and M phases. The reduced differentiation is most likely an outcome of the increased backward signaling from the cortical zone, which contributes to progenitor maintenance under normal conditions (Mondal *et al.* 2011). Lack of a niche phenotype in such lymph glands could be due to the lack of sufficient levels of activated STAT. With the UAS-STAT92E construct, there is an overexpression of STAT, but not necessarily activated STAT. Another potential reason could be that the cascade activated by Upd2 acts as a gatekeeper, wherein it is required for the maintenance of the pathway, but overexpression does not have any effect.

While we show that the cascade involved in niche regulation is activated by Upd2 and initial transduction occurs through the JAK-STAT pathway, the downstream components are yet to be revealed. We also have shown the misregulation of several other pathways upon Upd2 loss from the differentiating cells. Jumaeu (Jumu), a forkhead family transcription factor, has been shown to be expressed in the lymph gland and regulates hematopoiesis. In the MZ, Jumu non-cell

autonomously regulates niche cell proliferation via dMyc regulation. The exact details of this regulation remain unknown (Hao *et al.* 2017). Unpublished data from the lab had shown that loss of the progenitor population results in niche cell overproliferation, suggesting the presence of a negative proliferation signal from the MZ onto the PSC. So a possible hypothesis is that Upd2 from the differentiating cells function to maintain progenitor multipotency and that the loss of Upd2 results in a loss of the progenitor population, and consequently that of Jumu. The loss of Jumu might be the ultimate cause for niche cell proliferation upon Upd2 loss. This is a potential experiment to be done in the future of this project.

Misregulation of JAK-STAT signaling causes several hematological and immunological defects in both humans as well as the fruit fly (O'Shea *et al.* 2015; Amoyel *et al.* 2014). In accordance with these results, our results indicate that loss of cytokine signaling in the lymph gland results in a defect in the definitive hematopoiesis. The precocious differentiation and early peeling-off phenotypes are seen in larvae upon bacterial infection (Khadilkar *et al.* 2017). It will be interesting to check how the immune response is affected in such larvae with Upd2 knocked down from the Hml expressing cells.

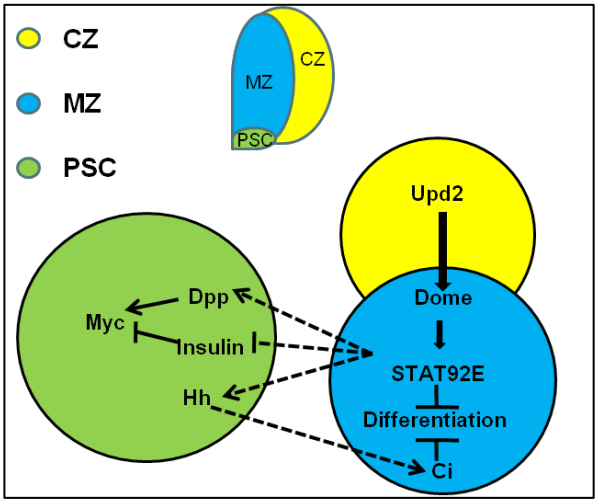


Figure 18. Proposed model based on experimental results, depicting a possible link between Upd2 and niche cell homeostasis (Banerjee *et al.* 2019).

CHAPTER 5

BIBLIOGRAPHY

1. Fernández-Moreno, M. A., Farr, C. L., Kaguni, L. S., & Garesse, R. (2007). Drosophila melanogaster as a model system to study mitochondrial biology. Methods in molecular biology (Clifton, N.J.), 372, 33–49.

2. Aranha, M. M., & Vasconcelos, M. L. (2018). Deciphering Drosophila female innate behaviors. Current opinion in neurobiology, 52, 139–148.

3. Parvathi V. D, Amritha A., Paul S. F. D (2009) Wonder Animal Model for Genetic Studies – Drosophila melanogaster – its life cycle and breeding methods – A review. Sri Ram J Med. 2: 33-38.

4. Demerec M, Kaufman P (1996). Drosophila Guide: Introduction to the genetics and cytology of Drosophila melanogaster. Cold Spring Harbor Laboratory ; pp3-8.

5. Economos, A. C., & Lints, F. A. (1986). Developmental temperature and life span in Drosophila melanogaster. I. Constant developmental temperature: evidence for physiological adaptation in a wide temperature range. Gerontology, 32(1), 18–27.

6. Zabihihesari, A., Hilliker, A. J., & Rezai, P. (2020). Fly-on-a-Chip: Microfluidics for Drosophila melanogaster Studies. Integrative Biology : quantitative biosciences from nano to macro, 11(12), 425–443.

7. Osterfield, M., Berg, C. A., & Shvartsman, S. Y. (2017). Epithelial Patterning, Morphogenesis, and Evolution: Drosophila Eggshell as a Model. Developmental cell, 41(4), 337–348.

8. Hales, K. G., Korey, C. A., Larracuente, A. M., & Roberts, D. M. (2015). Genetics on the Fly: A Primer on the Drosophila Model System. Genetics, 201(3), 815–842.

9. Jennings BH. Drosophila – a versatile model in biology & medicine (2011). Materials Today, 14(5), 190-195.

10. Mirzoyan et al. (2019). Drosophila melanogaster: A Model Organism to Study Cancer. Frontiers in genetics, 10, 51.

11. Allocca, M., Zola, S., & Bellosta, P. (2018). The Fruit Fly, Drosophila melanogaster: The Making of a Model (Part I). In Drosophila melanogaster - Model for Recent Advances in Genetics and Therapeutics. InTech.

12. Evans et al. (2009). G-TRACE: rapid Gal4-based cell lineage analysis in Drosophila. Nature methods, 6(8), 603–605.

13. Duffy J. B. (2002). GAL4 system in Drosophila: a fly geneticist's Swiss army knife. Genesis (New York, N.Y. : 2000), 34(1-2), 1–15.

14. Kakidani, H., & Ptashne, M. (1988). GAL4 activates gene expression in mammalian cells. Cell, 52(2), 161–167.

15. Halpern et al. (2008). Gal4/UAS transgenic tools and their application to zebrafish. Zebrafish, 5(2), 97–110.

16. Radoeva, T., Ten Hove, C. A., Saiga, S., & Weijers, D. (2016). Molecular Characterization of Arabidopsis GAL4/UAS Enhancer Trap Lines Identifies Novel Cell-Type-Specific Promoters. Plant physiology, 171(2), 1169–1181.

17. Brand, A. H., & Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. development, 118(2), 401-415.

18. Fischer, J. A., Giniger, E., Maniatis, T., & Ptashne, M. (1988). GAL4 activates transcription in Drosophila. Nature, 332(6167), 853–856.

19. Zeidler et al. (2004). Temperature-sensitive control of protein activity by conditionally splicing inteins. Nature biotechnology, 22(7), 871–876.

20. Cho K.S., Bang S.M. and Toh A. (2014) Lipids and Lipid Signaling in Drosophila Models of Neurodegenerative Diseases. In: Omega-3 Fatty Acids in Brain and Neurological Health(Watson RR, ed) pp327-336. Arizona: University of Arizona.

21. Banerjee, U., Girard, J. R., Goins, L. M., & Spratford, C. M. (2019). Drosophila as a Genetic Model for Hematopoiesis. Genetics, 211(2), 367–417.

22. Evans, C. J., Hartenstein, V., & Banerjee, U. (2003). Thicker than blood: conserved mechanisms in Drosophila and vertebrate hematopoiesis. Developmental cell, 5(5), 673–690.

23. Tremblay, M., Sanchez-Ferras, O., & Bouchard, M. (2018). GATA transcription factors in development and disease. Development (Cambridge, England), 145(20), dev164384.

24. de Bruijn, M. F., Speck, N. A., Peeters, M. C., & Dzierzak, E. (2000). Definitive hematopoietic stem cells first develop within the major arterial regions of the mouse embryo. The EMBO journal, 19(11), 2465–2474.

25. Orkin, S. H., & Zon, L. I. (2008). Hematopoiesis: an evolving paradigm for stem cell biology. Cell, 132(4), 631–644.

26. Holz, A., Bossinger, B., Strasser, T., Janning, W., & Klapper, R. (2003). The two origins of hemocytes in Drosophila. Development (Cambridge, England), 130(20), 4955–4962.

27. Fossett, N., & Schulz, R. A. (2001). Functional conservation of hematopoietic factors in Drosophila and vertebrates. Differentiation; research in biological diversity, 69(2-3), 83–90.

28. Bataillé, L., Augé, B., Ferjoux, G., Haenlin, M., & Waltzer, L. (2005). Resolving embryonic blood cell fate choice in Drosophila: interplay of GCM and RUNX factors. Development (Cambridge, England), 132(20), 4635–4644.

29. Fossett, N., Hyman, K., Gajewski, K., Orkin, S. H., & Schulz, R. A. (2003). Combinatorial interactions of serpent, lozenge, and U-shaped regulate crystal cell lineage commitment during Drosophila hematopoiesis. Proceedings of the National Academy of Sciences of the United States of America, 100(20), 11451–11456.

30. Zhang et al. (2003). Identification of the haematopoietic stem cell niche and control of the niche size. Nature, 425(6960), 836–841.

31. Pennetier et al. (2012). Size control of the Drosophila hematopoietic niche by bone morphogenetic protein signaling reveals parallels with mammals. Proceedings of the National Academy of Sciences of the United States of America, 109(9), 3389–3394.

32. Dey, N. S., Ramesh, P., Chugh, M., Mandal, S., & Mandal, L. (2016). Dpp dependent Hematopoietic stem cells give rise to Hh dependent blood progenitors in larval lymph gland of Drosophila. eLife, 5, e18295.

33. Bigas, A., & Espinosa, L. (2012). Hematopoietic stem cells: to be or Notch to be. Blood, 119(14), 3226–3235.

34. Duvic, B., Hoffmann, J. A., Meister, M., & Royet, J. (2002). Notch signaling controls lineage specification during Drosophila larval hematopoiesis. Current biology : CB, 12(22), 1923–1927.

35. Mandal, L., Banerjee, U., & Hartenstein, V. (2004). Evidence for a fruit fly hemangioblast and similarities between lymph-gland hematopoiesis in fruit fly and mammal aorta-gonadal-mesonephros mesoderm. Nature genetics, 36(9), 1019–1023.

36. Krzemien, J., Oyallon, J., Crozatier, M., & Vincent, A. (2010). Hematopoietic progenitors and hemocyte lineages in the Drosophila lymph gland. Developmental biology, 346(2), 310–319.

37. Amoyel, M., & Bach, E. A. (2012). Functions of the Drosophila JAK-STAT pathway: Lessons from stem cells. JAK-STAT, 1(3), 176–183.

38. Lan, W., Liu, S., Zhao, L., & Su, Y. (2020). Regulation of Drosophila Hematopoiesis in Lymph Gland: From a Developmental Signaling Point of View. International journal of molecular sciences, 21(15), 5246.

39. Ramond, E., Dudzic, J. P., & Lemaitre, B. (2020). Comparative RNA-Seq analyses of Drosophila plasmatocytes reveal gene specific signatures in response to clean injury and septic injury. PloS one, 15(6), e0235294.

40. Yu S., Luo F., Jin L.H. (2018). The Drosophila lymph gland is an ideal model for studying hematopoiesis. Developmental & Comparative Immunology, 83, 60-69.

41. Tung et al. (2013). Phosphatidylserine recognition and induction of apoptotic cell clearance by Drosophila engulfment receptor Draper. Journal of biochemistry, 153(5), 483–491.

42. Franc, N. C., Heitzler, P., Ezekowitz, R. A., & White, K. (1999). Requirement for croquemort in phagocytosis of apoptotic cells in Drosophila. Science (New York, N.Y.), 284(5422), 1991–1994.

43. Gold, K. S., & Brückner, K. (2015). Macrophages and cellular immunity in Drosophila melanogaster. Seminars in immunology, 27(6), 357–368.

44. Letourneau et al. (2016). Drosophila hematopoiesis under normal conditions and in response to immune stress. FEBS letters, 590(22), 4034–4051.

45. Shin, M., Cha, N., Koranteng, F., Cho, B., & Shim, J. (2020). Subpopulation of Macrophage-Like Plasmatocytes Attenuates Systemic Growth via JAK/STAT in the Drosophila Fat Body. Frontiers in immunology, 11, 63.

46. Charroux, B., & Royet, J. (2009). Elimination of plasmatocytes by targeted apoptosis reveals their role in multiple aspects of the Drosophila immune response. Proceedings of the National Academy of Sciences of the United States of America, 106(24), 9797–9802.

47. Vlisidou, I., & Wood, W. (2015). Drosophila blood cells and their role in immune responses. The FEBS journal, 282(8), 1368–1382.

48. Chen et al. (2012). Specific amino acids affecting Drosophila melanogaster prophenoloxidase activity in vitro. Developmental and comparative immunology, 38(1), 88–97.

49. Binggeli, O., Neyen, C., Poidevin, M., & Lemaitre, B. (2014). Prophenoloxidase activation is required for survival to microbial infections in Drosophila. PLoS pathogens, 10(5), e1004067.

50. Shrestha, R., & Gateff, E. (1982). Ultrastructure and Cytochemistry of the Cell Types in the Larval Hematopoietic Organs and Hemolymph of Drosophila Melanogaster: (drosophila/hematopoiesis/blool cells/ultrastructure/cytochemistry). Development, Growth & Differentiation, 24(1), 65-82.

51. Williams M. J. (2007). Drosophila hemopoiesis and cellular immunity. Journal of immunology (Baltimore, Md. : 1950), 178(8), 4711–4716.

52. Dudzic, J. P., Kondo, S., Ueda, R., Bergman, C. M., & Lemaitre, B. (2015). Drosophila innate immunity: regional and functional specialization of prophenoloxidases. BMC biology, 13, 81.

53. Jung, S. H., Evans, C. J., Uemura, C., & Banerjee, U. (2005). The Drosophila lymph gland as a developmental model of hematopoiesis. Development (Cambridge, England), 132(11), 2521–2533.

54. Mandal, L., Martinez-Agosto, J. A., Evans, C. J., Hartenstein, V., & Banerjee, U. (2007). A Hedgehog- and Antennapedia-dependent niche maintains Drosophila haematopoietic precursors. Nature, 446(7133), 320–324.

55. Gao, H., Wu, X., & Fossett, N. (2009). Upregulation of the Drosophila Friend of GATA gene U-shaped by JAK/STAT signaling maintains lymph gland prohemocyte potency. Molecular and cellular biology, 29(22), 6086–6096.

56. Mondal et al. (2011). Interaction between differentiating cell- and niche-derived signals in hematopoietic progenitor maintenance. Cell, 147(7), 1589–1600.

57. Gao, H., Wu, X., & Fossett, N. (2013). Drosophila E-cadherin functions in hematopoietic progenitors to maintain multipotency and block differentiation. PloS one, 8(9), e74684.

58. Sinenko, S. A., Mandal, L., Martinez-Agosto, J. A., & Banerjee, U. (2009). Dual role of wingless signaling in stem-like hematopoietic precursor maintenance in Drosophila. Developmental cell, 16(5), 756–763.

59. Sharma, S. K., Ghosh, S., Geetha, A. R., Mandal, S., & Mandal, L. (2019). Cell Adhesion-Mediated Actomyosin Assembly Regulates the Activity of Cubitus Interruptus for Hematopoietic Progenitor Maintenance in Drosophila. Genetics, 212(4), 1279–1300.

60. Benmimoun, B., Haenlin, M., & Waltzer, L. (2015). Haematopoietic progenitor maintenance by EBF/Collier: beyond the Niche. Cell cycle (Georgetown, Tex.), 14(22), 3517–3518.

61. Morin-Poulard et al. (2016). Vascular control of the Drosophila haematopoietic microenvironment by Slit/Robo signalling. Nature communications, 7, 11634.

62. Benmimoun, B., Polesello, C., Waltzer, L., & Haenlin, M. (2012). Dual role for Insulin/TOR signaling in the control of hematopoietic progenitor maintenance in Drosophila. Development (Cambridge, England), 139(10), 1713–1717.

63. Nässel, D. R., Liu, Y., & Luo, J. (2015). Insulin/IGF signaling and its regulation in Drosophila. General and comparative endocrinology, 221, 255–266.

64. Baldeosingh, R., Gao, H., Wu, X., & Fossett, N. (2018). Hedgehog signaling from the Posterior Signaling Center maintains U-shaped expression and a prohemocyte population in Drosophila. Developmental biology, 441(1), 132–145.

65. Zeidler, M. P., Bach, E. A., & Perrimon, N. (2000). The roles of the Drosophila JAK/STAT pathway. Oncogene, 19(21), 2598–2606.

66. Benmimoun, B., Haenlin, M., & Waltzer, L. (2015). Blood cell progenitor maintenance: Collier barks out of the niche. Fly, 9(4), 160–164.

67. Oyallon et al. (2016). Two Independent Functions of Collier/Early B Cell Factor in the Control of Drosophila Blood Cell Homeostasis. PloS one, 11(2), e0148978.

68. Hombría, J. C., & Brown, S. (2002). The fertile field of Drosophila Jak/STAT signalling. Current biology : CB, 12(16), R569–R575.

69. Arbouzova, N. I., & Zeidler, M. P. (2006). JAK/STAT signalling in Drosophila: insights into conserved regulatory and cellular functions. Development (Cambridge, England), 133(14), 2605–2616.

70. Braunstein J.,Brutsaert S., Olson R., Schindler C.(2003). STATs Dimerize in the Absence of Phosphorylation. Journal of Biological Chemistry, 278(36), 34133-34140.

71. Brown, S., Hu, N., & Hombría, J. C. (2001). Identification of the first invertebrate interleukin JAK/STAT receptor, the Drosophila gene domeless. Current biology : CB, 11(21), 1700–1705.

72. Rawlings, J. S., Rosler, K. M., & Harrison, D. A. (2004). The JAK/STAT signaling pathway. Journal of cell science, 117(Pt 8), 1281–1283.

73. Morris, R., Kershaw, N. J., & Babon, J. J. (2018). The molecular details of cytokine signaling via the JAK/STAT pathway. Protein science : a publication of the Protein Society, 27(12), 1984–2009.

74. Bousoik, E., & Montazeri Aliabadi, H. (2018). "Do We Know Jack" About JAK? A Closer Look at JAK/STAT Signaling Pathway. Frontiers in oncology, 8, 287.

75. Morin-Poulard, I., Vincent, A., & Crozatier, M. (2013). The Drosophila JAK-STAT pathway in blood cell formation and immunity. JAK-STAT, 2(3), e25700.

76. Herrera, S. C., & Bach, E. A. (2019). JAK/STAT signaling in stem cells and regeneration: from Drosophila to vertebrates. Development (Cambridge, England), 146(2), dev167643.

77. Avila, F. W., & Erickson, J. W. (2007). Drosophila JAK/STAT pathway reveals distinct initiation and reinforcement steps in early transcription of Sxl. Current biology : CB, 17(7), 643–648.

78. Luo, H., & Dearolf, C. R. (2001). The JAK/STAT pathway and Drosophila development. BioEssays : news and reviews in molecular, cellular and developmental biology, 23(12), 1138–1147.

79. Lee, J. H., Lee, C. W., Park, S. H., & Choe, K. M. (2017). Spatiotemporal regulation of cell fusion by JNK and JAK/STAT signaling during Drosophila wound healing. Journal of cell science, 130(11), 1917–1928.

80. Ghiglione et al. (2002). The Drosophila cytokine receptor Domeless controls border cell migration and epithelial polarization during oogenesis. Development (Cambridge, England), 129(23), 5437–5447.

81. Makki et al. (2010). A short receptor downregulates JAK/STAT signalling to control the Drosophila cellular immune response. PLoS biology, 8(8), e1000441.

82. Calò et al. (2003). STAT proteins: from normal control of cellular events to tumorigenesis. Journal of cellular physiology, 197(2), 157–168.

83. Luo et al. (1997). Mutation in the Jak kinase JH2 domain hyperactivates Drosophila and mammalian Jak-Stat pathways. Molecular and cellular biology, 17(3), 1562–1571.

84. Watford, W. T., Moriguchi, M., Morinobu, A., & O'Shea, J. J. (2003). The biology of IL-12: coordinating innate and adaptive immune responses. Cytokine & growth factor reviews, 14(5), 361–368.

85. Shelly, S., Lukinova, N., Bambina, S., Berman, A., & Cherry, S. (2009). Autophagy is an essential component of Drosophila immunity against vesicular stomatitis virus. Immunity, 30(4), 588–598.

86. Niwa, H., Burdon, T., Chambers, I., & Smith, A. (1998). Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. Genes & development, 12(13), 2048–2060.

87. de Cuevas, M., & Matunis, E. L. (2011). The stem cell niche: lessons from the Drosophila testis. Development (Cambridge, England), 138(14), 2861–2869.

88. Lin, G., Xu, N., & Xi, R. (2010). Paracrine unpaired signaling through the JAK/STAT pathway controls self-renewal and lineage differentiation of drosophila intestinal stem cells. Journal of molecular cell biology, 2(1), 37–49.

89. Panayidou, S., & Apidianakis, Y. (2013). Regenerative inflammation: lessons from Drosophila intestinal epithelium in health and disease. Pathogens (Basel, Switzerland), 2(2), 209–231.

90. Beshel, J., Dubnau, J., & Zhong, Y. (2017). A Leptin Analog Locally Produced in the Brain Acts via a Conserved Neural Circuit to Modulate Obesity-Linked Behaviors in Drosophila. Cell metabolism, 25(1), 208–217.

91. Rajan, A., & Perrimon, N. (2012). Drosophila cytokine unpaired 2 regulates physiological homeostasis by remotely controlling insulin secretion. Cell, 151(1), 123–137.

92. Chakrabarti, S., Dudzic, J. P., Li, X., Collas, E. J., Boquete, J. P., & Lemaitre, B. (2016). Remote Control of Intestinal Stem Cell Activity by Haemocytes in Drosophila. PLoS genetics, 12(5), e1006089.

93. Yang, H., Kronhamn, J., Ekström, J. O., Korkut, G. G., & Hultmark, D. (2015). JAK/STAT signaling in Drosophila muscles controls the cellular immune response against parasitoid infection. EMBO reports, 16(12), 1664–1672.

94. Hombría, J. C., Brown, S., Häder, S., & Zeidler, M. P. (2005). Characterisation of Upd2, a Drosophila JAK/STAT pathway ligand. Developmental biology, 288(2), 420–433.

95. Hamaratoglu, F., Affolter, M., & Pyrowolakis, G. (2014). Dpp/BMP signaling in flies: from molecules to biology. Seminars in cell & developmental biology, 32, 128–136.

96. Schmitt, S., Ugrankar, R., Greene, S. E., Prajapati, M., & Lehmann, M. (2015). Drosophila Lipin interacts with insulin and TOR signaling pathways in the control of growth and lipid metabolism. Journal of cell science, 128(23), 4395–4406.

97. Torroja, C., Gorfinkiel, N., & Guerrero, I. (2004). Patched controls the Hedgehog gradient by endocytosis in a dynamin-dependent manner, but this internalization does not play a major role in signal transduction. Development (Cambridge, England), 131(10), 2395–2408.

98. Gulati, K., Guhathakurta, S., Joshi, J., Rai, N., & Ray, A. J. M. I. (2016). Cytokines and their role in health and disease: a brief overview. Moj Immunol, 4(2), 1-9.

99. Dorritie, K. A., McCubrey, J. A., & Johnson, D. E. (2014). STAT transcription factors in hematopoiesis and leukemogenesis: opportunities for therapeutic intervention. Leukemia, 28(2), 248–257.

100. Géminard, C., Rulifson, E. J., & Léopold, P. (2009). Remote control of insulin secretion by fat cells in Drosophila. Cell metabolism, 10(3), 199–207.

101. Regan et al. (2013). Steroid hormone signaling is essential to regulate innate immune cells and fight bacterial infection in Drosophila. PLoS pathogens, 9(10), e1003720.

102. Sorrentino, R. P., Carton, Y., & Govind, S. (2002). Cellular immune response to parasite infection in the Drosophila lymph gland is developmentally regulated. Developmental biology, 243(1), 65–80.

103. Hao, Y., & Jin, L. H. (2017). Dual role for Jumu in the control of hematopoietic progenitors in the Drosophila lymph gland. eLife, 6, e25094.

104. O'Shea, J. J., Schwartz, D. M., Villarino, A. V., Gadina, M., McInnes, I. B., & Laurence, A. (2015). The JAK-STAT pathway: impact on human disease and therapeutic intervention. Annual review of medicine, 66, 311–328.

105. Amoyel, M., Anderson, A. M., & Bach, E. A. (2014). JAK/STAT pathway dysregulation in tumors: a Drosophila perspective. Seminars in cell & developmental biology, 28, 96–103.

106. Khadilkar, R. J., Vogl, W., Goodwin, K., & Tanentzapf, G. (2017). Modulation of occluding junctions alters the hematopoietic niche to trigger immune activation. eLife, 6, e28081.