Curriculum Vitae

Rajkumar Baldeosingh
rajkumar.baldeosingh@umaryland.edu

EDUCATION

M.S. Molecular Medicine, University of Maryland, Baltimore. May 2016
Thesis: Hedgehog Signaling Regulates Friend of GATA to Maintain Hematopoietic Progenitor Cells in *Drosophila melanogaster.*

B.S. Biology, Stevenson University. May 2013
Minor: Chemistry

HONORS/AWARDS

Dean's List, Stevenson University (Spring 2013, Fall 2012, Spring 2012, Fall 2010)

Beta Beta Beta National Biological Honor Society Member, Stevenson University (November 2012)

Recipient of Dean's Scholarship, Stevenson University (March 2009)

RAMS Academy Scholastic Award (June 2006)

Outstanding Academic Achievement Award (September 2005)

U.S. President's Award for Gifted and Talented Students (June 2005)

RESEARCH INTERESTS

Regenerative Medicine; Cell and Developmental Biology; Stem Cell Biology; Cellular Differentiation, Dedifferentiation and Transdifferentiation; Signal Transduction Network and Epigenetic Regulation of Stem Cell Differentiation, Proliferation and Self-Renewal; Hedgehog Signaling Pathway; GATA:FOG Transcriptional Complex; microRNAs; Hematopoiesis; Vasculogenesis and Angiogenesis; Hematological Diseases; Degenerative Diseases; Cancer; Diabetes Mellitus; Ophthalmology.

RESEARCH EXPERIENCE

During my undergraduate education, I worked with Dr. Wendy Kimber investigating the various roles of the ubiquitin-conjugating enzyme, *Ube2a*, in embryonic stem cell differentiation. For these studies, I performed RNAi knockdown and cultured Embryonic Stem Cells and utilized techniques including PCR, Q-PCR, RNA interference, siRNA transfection, restriction enzyme analysis

and cDNA cloning in manipulation of gene expression. Dr. Kimber's lab is building on my initial observations with the anticipation of publishing this work.

More recently at the University of Maryland, I worked in the lab of Dr. Eckert, as a Graduate rotation student under the supervision of Dr. Candice Kerr. I investigated the effects of the compound Sulforaphane in melanoma cells in vitro. Sulforaphane is found in cruciferous vegetables such as broccoli. I utilized techniques that include cell culturing, light microscopy, Bradford assay, scratch assay and western blotting for this project.

I then worked in Dr. Bruce Vogel's laboratory on a project investigating candidate genes that encode a possible transmembrane homing receptor, which interact with the secretion of an extracellular matrix protein, Hemicentin, in *C. elegans*. I worked on a bioinformatics screen for such candidate genes and performed RNAi screens of several genes of interest.

For my thesis, I worked in Dr. Nancy Fossett's lab, which investigates the roles of various signal transduction networks in hematopoiesis. The lab utilizes *Drosophila melanogaster* as a model organism for studies. My thesis project involved using fly genetics to identify factors that work with the GATA:FOG complex to block differentiation of hematopoietic progenitors. To accomplish this project, I conducted a Second Site Non-Complementation genetic screen, which is based on detecting the production of stress-responsive lamellocytes (a rare type of *Drosophila* blood cell). To date, we have identified at least nine potential candidates that act with the GATA:FOG transcriptional complex to block differentiation. One of the candidates is the G-Protein coupled receptor known as Smoothened (Smo). Smo is a factor in the Hedgehog (HH) signaling pathway.

HH is a conserved pathway that is essential for embryonic development and for the maintenance of adult stem cells in a variety of systems. Canonical HH signaling requires the binding of a Hedgehog family ligand to the Patched (Ptc) transmembrane receptor, resulting in the activation of Smo. Activated Smo will induce the nuclear translocation of transcriptionally active factors of the Gli transcription factor family.

We hypothesize that canonical HH signaling maintains U-shaped (Ush), the *Drosophila* homologue of FOG, and hematopoietic progenitors; Ush then blocks HH expression in progenitors through its interaction with GATA in a negative feedback loop. Thus, I performed loss of function and over-expression studies of Smo and the other factors of the HH pathway in *Drosophila* to further

determine its role with GATA:FOG during hematopoiesis with the anticipation of publishing this work.

Another project in Dr. Fossett's lab that I assisted with involves the *Drosophila* homologue of IκB, Cactus (Cact). Cact was another factor identified in our screen and is involved in Toll-like Receptor (TLR) signaling, which regulates hematopoietic progenitor differentiation. In comparison to HH signaling, TLR regulation of hematopoietic progenitor differentiation is conserved. However, in contrast to HH signaling, which blocks differentiation, TLR signaling promotes differentiation. For this project, we hypothesized that Cact most likely maintains Ush by antagonizing the activity of Dorsal, *Drosophila* homologue of NFκB. We observed that TLR signaling controls progenitor differentiation, in part, by regulating the level of Ush by showing that Dorsal down-regulates Ush to promote differentiation, whereas Cactus maintains Ush levels to block differentiation. The manuscript detailing our work on this project is currently in press for publication. For both projects, I carefully dissected the appropriate tissue from *Drosophila* larvae under a dissecting microscope, stained the tissue with appropriate antibodies, prepared slides of the stained tissue for examination, and then analyzed data using florescence microscopy and Microsoft Excel.

WORK EXPERIENCE

Master's Thesis Student/Graduate Research Assistant, University of Maryland, PI: Dr. Nancy Fossett. Baltimore, MD.
May 2014-May 2016

Graduate Rotation Student, University of Maryland, PI: Dr. Bruce Vogel. Baltimore, MD.
February 2014-April 2014

Graduate Rotation Student, University of Maryland, PI: Dr. Richard Eckert. Baltimore, MD.
December 2013- February 2014

Undergraduate Research Assistant, Stevenson University, PI: Dr. Wendy Kimber. Stevenson, MD.
January 2013-May 2013

Laboratory Safety Inspector, Stevenson University. Stevenson, MD.
September 2012-December 2012

Volunteer, Sinai Hospital: Pediatric Intensive Care Unit. Baltimore, MD.
May 2012-July 2012

Open Anatomy Lab Moderator, Stevenson University. Stevenson, MD.
January 2012-May 2012

LABORATORY SKILLS

Molecular Biology and Biochemistry lab techniques such as RT-PCR, Agarose Gel-Electrophoresis, Genomic DNA and RNA isolation, RNA Transfection, RNA interference, Affinity Chromatography, Cell Culturing, Cell Passaging, Western Blotting and Bacterial Transformation**.**

Developmental Biology and Genetic techniques involving *Drosophila melanogaster*, such as RNAi and gain of function mutagenesis through the UAS-GAL4 system technique, larval dissection, and antibody staining of organs, in addition to basic fly genetics, fly stock maintenance and breeding.

Microbiology lab techniques including Colony Isolation and Gram-Staining**.**

Organic and Inorganic Chemistry lab techniques including Infrared Spectroscopy, Nuclear Magnetic Resonance analysis, Extraction, Fractional distillation, Titration**,** Thin layer Chromatography and Gas Chromatography/Mass Spectrometry**.**

Skillful in various Microscopy techniques, including fluorescence microscopy.

SKILLS

Basic computer skills in Microsoft Office (Word, Excel, PowerPoint), Adobe Photoshop, Adobe Illustrator, Image J, Logger Pro, Linux/Unix, Spartan and Chimera**.**

The ability to interpret and analyze an electrocardiograph.

Proficient Statistical Analysis skills.

PUBLICATIONS

H. Gao, **R. Baldeosingh**, X. Wu, N. Fossett. "The Friend of GATA transcriptional co-regulator, U-shaped, is a downstream antagonist of Dorsal-driven prohemocyte differentiation in *Drosophila*" *PLoS ONE* (In Press).

CONFERENCE PRESENTATIONS

R. Baldeosingh, H. Gao, X. Wu, N. Fossett. Regulators of Hematopoietic Progenitor Differentiation. Poster presentation delivered at the 2014 Stem Cell Center Retreat and the 2015 Graduate Research Conference at the University of

Maryland, Baltimore, Baltimore, MD, December 2014 & March 2015.
http://hdl.handle.net/10713/4479

R. Baldeosingh, J. Grynovicki, W. Kimber. Determination of the role of ubiquitin conjugation enzyme *Ube2a* in Mouse Embryonic Stem Cell Differentiation. Poster presentation delivered at the School of Sciences Conference at Stevenson University, Stevenson, MD, May 2013.

R. Baldeosingh. The Demographics of Diabetic Children at Highest Risk for Diabetic Ketoacidosis. Poster presentation delivered at the School of Sciences Conference at Stevenson University, Stevenson, MD, December 2012.

Abstract

Hedgehog Signaling Regulates Friend of GATA to Maintain Hematopoietic Progenitor Cells in *Drosophila melanogaster*.

Hematopoietic stem cells must be capable of both self-renewal and production of progenitor cells that give rise to all of the blood cell types, the two basic principles of stem cell biology. These unique stem cell properties have generated vast interest, due to the potential of stem cell therapies to treat various types of blood diseases. In this study, fly genetics were used to identify factors that act with the GATA:Friend Of GATA (GATA:FOG) complex to maintain hematopoietic progenitor cells. The Hedgehog signaling factor, Smoothened, was identified as one of those factors. We have determined that canonical Hedgehog signaling regulates U-shaped, the *Drosophila* homologue of FOG, to maintain hematopoietic progenitors. Furthermore, we propose a model in which we outline how Hedgehog signaling maintains FOG and hematopoietic progenitors. Our results may identify conserved regulatory mechanisms whereby signals from the microenvironment control progenitor differentiation.

Hedgehog Signaling Regulates Friend of GATA to Maintain Hematopoietic Progenitor Cells in *Drosophila melanogaster*.

By

Rajkumar Baldeosingh

Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, Baltimore in partial fulfillment
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of Master of Science
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I would like to express my gratitude to my research mentor, Dr. Nancy Fossett, whose knowledge, perception and patience added considerably to my graduate experience. Her guidance helped me throughout my time of conducting research in her laboratory and writing of this thesis. I could not have imagined having a better advisor and mentor for my Master's study.

In addition to my mentor, I would like to thank the rest of my thesis committee: Dr. Tami Kingsbury, Dr. Bruce Vogel, Dr. Steven Zhan, and Dr. Jim Du, for their intuitive comments and support.

I also give thanks to Dr. Richard Eckert, Dr. Candice Kerr and once again to Dr. Bruce Vogel, who provided me with an opportunity to join their lab as a graduate rotation student, and who gave access to their laboratory and research facilities.

I would also like to thank Dr. Wendy Kimber, who provided me with an opportunity to join her lab as an undergraduate research student and for enlightening me the first glance of conducting scientific research.

I would further like to thank my program director, Dr. Frank Margolis, for his guidance.

I thank my fellow labmates, Dr. Hongjuan Gao and Xiaorong Wu for helping me conduct my research for this study. Without their support and assistance I would not have generated the results presented in my thesis. I also thank my friends at the University of Maryland, Baltimore.

Last but not least, I would like to thank my family, most notably my parents, for supporting me throughout writing this thesis and in my life in general.

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List of Abbreviations

Antp: Antennapedia

AGM: Aorta, gonad and mesonephros

Babo: Baboon

Ci: Cubitus Interruptus

CRM: Cis-Regulatory Module

Cyo: Curly of Oster

CZ: Cortical Zone

Dhh: Desert hedgehog

Dome: Domeless

EC: Endothelial Cell

Eya: Eyes Absent

FOG: Friend of GATA

Fu: Fused

HH: Hedgehog

HHLT: Hand-Hemolectin Lineage Traced

HSC: Hematopoietic stem cell

HSPCs: Hematopoietic stem and progenitor cells

Ihh: Indian hedgehog

LT-HSC: Long-term hematopoietic stem cell

MSN-C: Misshapen mCherry

MZ: Medullary Zone

Odd: Odd-skipped

PBS: Phosphate-buffered saline

PSC: Posterior signaling center

Ptc: Patched

Shh: Sonic hedgehog

Smo: Smoothened

Srp: Serpent

SSNC: Second Site Non-Complementation

ST-HSC: Short-term hematopoietic stem cell

Sufu: Suppressor of fused

Tep: TepIV

UAS: Upstream-activating sequence

Ush: U-shaped

Chapter 1: Introduction

1.1 Hematopoiesis: An Overview

Hematopoiesis is the lifelong process in which blood cells are produced from hematopoietic stem cells (HSCs). This process is tightly regulated by various factors that promote cell diversification (Orkin, 2000). During mammalian embryogenesis, HSCs develop from the mesoderm by a complex process that begins in the yolk sac of the embryo, and then shifts to the aortic region, and sequentially to the placenta, fetal liver and adult bone marrow (Orkin and Zon, 2008; Wilson and Trumpp, 2006). The initial wave of blood production in the mammalian yolk sac is known as primitive hematopoiesis, in which red blood cells are produced that facilitate tissue oxygenation as the embryo undergoes rapid growth (Muller et al., 1994; Orkin and Zon, 2008; Wilson and Trumpp, 2006). HSCs and endothelial cells (ECs) arise from the hemangioblast of the yolk sac (North et al., 1999; North et al., 2002; Orkin and Zon, 2008). Primitive hematopoiesis is temporary and is rapidly replaced by definitive hematopoiesis, which begins in the embryo and continues throughout the life of the organism (Ciau-Uitz et al., 2000; Orkin and Zon, 2008). The region of definitive hematopoiesis is the aorta, gonad and mesonephros (AGM) (Ciau-Uitz et al., 2000; Muller et al., 1994; Orkin and Zon, 2008). It has been proposed that the AGM forms hemogenic ECs in the ventral wall of the aorta that bud off HSCs (North et al., 1999; North et al., 2002; Orkin and Zon, 2008). Subsequent definitive hematopoiesis involves the colonization of HSCs that compose the fetal liver, thymus, spleen, and ultimately the bone marrow (Ciau-Uitz et al., 2000; Orkin and Zon, 2008).

One in every 10,000 to 15,000 of cells in the bone marrow is thought to be a stem cell (Coulombel, 2004). The "gold-standard" for identifying stem-cells in the bone marrow is the serial transplantation assay, a functional assay conducted in mice. Mice are sub-lethally irradiated in order to kill hematopoietic cells and then bone marrow cells from a donor mouse are transplanted into the irradiated mice. HSCs from the donor will replace the hematopoietic system lost in the irradiated mouse, during a time-span of several weeks. After several weeks, a second set of mice are irradiated and their hematopoietic cells, that were lost, are replaced by the bone marrow cells obtained from the first irradiated mouse. Thus, the cells from the first donor mouse include long-term HSCs (LT-HSCs), which are stem cells that are capable of self-renewal throughout the life span of the organism (Coulombel, 2004). Studies that utilize this technique have revealed that there are two types of hematopoietic stem cells: LT-HSCs, which can self-renew throughout long periods of time, and short-term HSCs (ST-HSCs), multipotent progenitor cells that arise from LT-HSCs and have the capability of differentiating into all of the different lineages of hemocytes, but cannot self-renew over long periods of time (Coulombel, 2004; Orkin, 2000; Orkin and Zon, 2008). Furthermore, in contrast to LT-HSCs, ST-HSCs are not capable of completely repopulating the bone marrow (Coulombel, 2004).

ST-HSCs produce common myeloid progenitors and common lymphoid progenitors (Orkin, 2000) (Figure 1). In vertebrates, the common myeloid and lymphoid progenitors have been identified on the basis of cell surface markers and developmental potential (North et al., 2002; North et al., 2004; Orkin and Zon, 2008). The myeloid progenitor produces the monocytes/macrophages, neutrophils, eosinophils, erythrocytes,

megakaryocytes, and mast cells (Akashi, 2005; Iwasaki and Akashi, 2007; North et al., 2004; Orkin, 2000), whereas T and B lymphocytes develop from the common lymphoid progenitor (Akashi, 2005; North et al., 2004) (Figure 1). Together, these cells carry out life sustaining processes including oxygen transport, blood coagulation, phagocytosis of foreign invaders and cellular debris, and humeral and cellular immunity (North et al., 2004; Orkin, 2000).

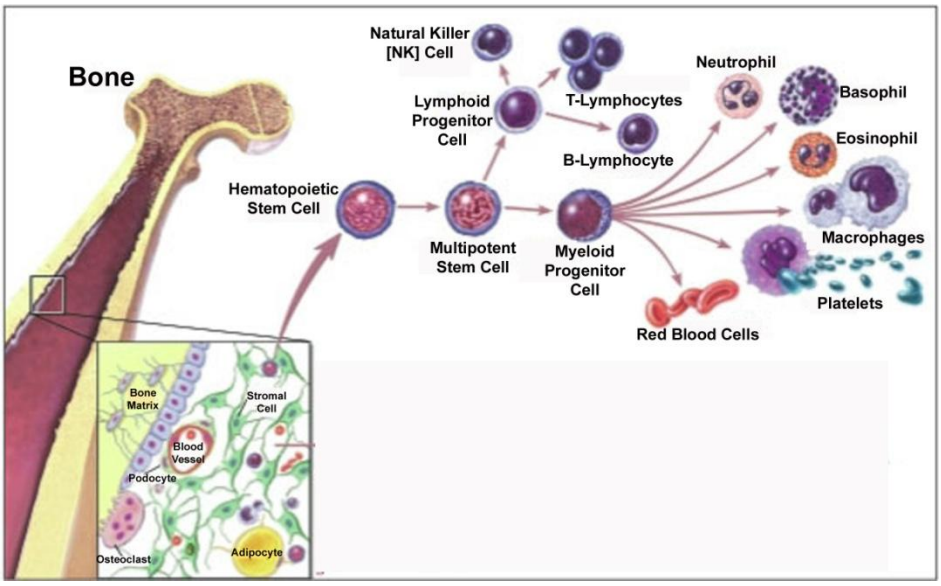

Figure 1. Overview of Hematopoiesis in Bone Marrow of Mammals. LT-HSCs can either self renew throughout the lifespan of the organism or differentiate into multipotent ST-HSCs (hematopoietic progenitors). These multipotent stem cells can then differentiate into either myeloid progenitors or lymphoid progenitors. Myeloid progenitors differentiate into red blood cells, platelets, macrophages, eosinophils, basophils, and neutrophils. Lymphoid progenitors differentiate into Natural Killer cells, B- and T-lymphocytes. Image © 2001 Terese Winslow assisted by Lydia Kibiuk and Caitlin Duckwall.

As stated above, HSCs self renew and give rise to all blood cell types (Orkin, 2000). Thus, their properties have generated vast interest, due to possible stem-cell based therapies for degenerative diseases and cancers (Mimeault and Batra, 2012). It is therefore important to further investigate the differentiation process of stem cells in order

to better understand stem cells in response to stress and in maintenance of homeostasis because this knowledge will be crucial for developing more effective stem-cell based therapies. The differentiation of hematopoietic stem cells is controlled by complicated gene networks that have yet to be completely identified. Investigations into these networks can be conducted using model organisms such as *Drosophila melanogaster*, commonly known as the fruit fly (Fossett, 2013; Gao et al., 2011; Gao et al., 2013; Muratoglu et al., 2007).

1.2 *Drosophila melanogaster*: Model Organism to Investigate Hematopoiesis *in vivo*.

The hematopoietic system in *Drosophila* is ideally suited to rapidly identify regulatory pathways that control cell fates during steady-state conditions and in response to changes in external stimuli, such as stress (Fossett, 2013; Gao et al., 2013). A short generation time in *Drosophila* and powerful genetic methods ensure swift identification of interactions between hematopoietic regulators *in vivo* (Gao et al., 2013; Muratoglu et al., 2007). The simplicity of *Drosophila* facilitates the identification of gene functions that are often obscured in a mammalian model system (Rieder and Larschan, 2014). There are multiple similarities between mammalian HSCs and *Drosophila* hematopoietic progenitors, such as quiescence, multipotency and niche dependence (Fossett, 2013; Gao et al., 2011; Gao et al., 2013; Ghosh et al., 2015; Mandal et al., 2007; Muratoglu et al., 2007). These similarities contribute to the suitability of *Drosophila* as a model organism to study hematopoiesis. Several of the signaling pathways, such as Notch, Jak/Stat, BMP, and particularly Hedgehog (HH), which is the focus of our study, are involved in

differentiation of mammalian HSCs into blood cells, and also involved in the differentiation of hematopoietic progenitors into blood cells in *Drosophila* (Fossett, 2013; Frandsen et al., 2008; Gao et al., 2009). Since these essential pathways are conserved in mammals, they can be studied using a simple and more cost effective model organism, such as *Drosophila.*

In contrast to vertebrates, *Drosophila* hemocytes consist of three main classes (Akashi, 2005; Fossett, 2013) that to some degree are functionally analogous to the myeloid lineage of the vertebrate system (Figure 2), providing an innate immune response against invaders and a system for the removal of apoptotic cells. Two of the most common types of hemocytes are plasmatocytes and crystal cells (Figure 2). Plasmatocytes, which constitute 95% of circulating hemocytes, most closely resemble the vertebrate monocytes/macrophages, whereas crystal cells, which comprise ~5% of circulating hemocytes, are necessary for wound healing and encapsulation of foreign invaders and have no known vertebrate counterpart (Fossett, 2013; Gao et al., 2009). The third and rarest type of hemocyte in *Drosophila* is known as the lamellocyte (Figure 2). Lamellocytes, which comprise of <1% of the circulating hemocytes, are only detected during the larval stage of development and differentiate as a defense against large invaders that are unable to be phagocytized by plasmatocytes and in response to various types of stress, including both oxidative and nutritional stress (Fossett, 2013; Tokusumi et al., 2009). *Drosophila* hematopoietic progenitors give rise to all three types of *Drosophila* blood cells; thus, studies of the regulatory systems that limit differentiation of progenitors into lamellocytes may identify factors that protect the progenitor pool against the negative impact of stress.

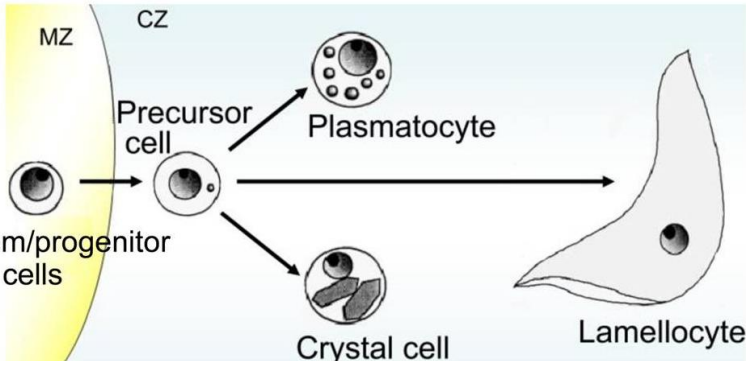

Figure 2. Hematopoiesis in *Drosophila.* Progenitor cells in the medullary zone (MZ) of the *Drosophila* lymph gland form precursor cells which can differentiate into three different hemocyte lineages: plasmatocytes, crystal cells or lamellocytes, in the cortical zone (CZ) of the lymph gland.

Similar to vertebrates, there are two waves of hematopoiesis in *Drosophila*. Primitive hematopoiesis occurs in the embryonic head mesoderm of the fly, while definitive hematopoiesis is initiated during the larval stage in a specialized organ: the lymph gland, which is functionally analogous to the mammalian bone marrow (Fossett and Schulz, 2001; Fossett, 2013; Tokusumi et al., 2009). This organ is formed during embryogenesis and persists until the late third instar stage of larval development. Prior to pupariation, the lymph gland degenerates and releases the hemocytes into circulation. This occurs in anticipation of the phagocytic removal of the apoptotic cells generated during the histolysis and remodeling of larval tissues during metamorphosis (Fossett and Schulz, 2001; Fossett, 2013). In adult fruit flies, hemocyte development takes place in the dorsal abdominal clusters (Ghosh et al., 2015). The presence of progenitors and differentiated blood cells embedded in a functional network of Laminin A and Pericardin within this hematopoietic hub, projects it as a simple version of the bone marrow in vertebrates (Ghosh et al., 2015)

1.3 Specific Class of Hematopoietic Regulators: GATA:FOG Complex

Previous studies have identified the GATA:FOG complex as a major regulator of hematopoiesis across taxa (Patient and McGhee, 2002). GATA factors recognize the (A/G)GATA(A/T) sequence of genes and function as transcriptional activators or repressors (Newton et al., 2001) (Figure 3). In mammals, there are six members of the GATA factor family (Chlon and Crispino, 2012). Three members of the GATA family, GATA-1, GATA-2, and GATA-3, are required for hematopoiesis (Patient and McGhee, 2002; Sorrentino et al., 2005). GATA-2 regulates proliferation and promotes the survival of hematopoietic stem and progenitor cells (HSPCs) (Patient and McGhee, 2002), while GATA factors 1 and 3 control lineage commitment and differentiation into specific blood cell types (Patient and McGhee, 2002; Muratoglu et al., 2007; Newton et al., 2001; Sorrentino et al., 2005). Meanwhile, GATA-4, GATA-5 and GATA-6 play a role in heart and gut development and are also known as endodermal GATA factors (Chlon and Crispino, 2012; Laverriere et al., 1994).

Friend of GATA (FOG) transcriptional regulators are multitype zinc-finger proteins that activate or repress the GATA-directed transcription by binding to the N-terminal zinc finger of GATA (Figure 3) (Fossett, 2013; Muratoglu et al., 2007; Zhou et al., 2001). In mammals, there are two FOG proteins: FOG-1 and FOG-2 (Chlon and Crispino, 2012). FOG-1 functions mainly in the hematopoietic system through its interaction with a cognate hematopoietic GATA factor 1 or 3 to form the corresponding GATA:FOG complex (Fossett, 2013; Patient and McGhee, 2002; Gao et al., 2009;

Muratoglu et al., 2007; Sorrentino et al., 2005). Meanwhile, FOG-2 interacts mainly with GATA-4 and has been found to be expressed in the developing nervous system, as well as the heart, lungs and liver in mammals (Chlon and Crispino, 2012; Hoene et al., 2009).

GATA:FOG complexes regulate the development of a number of blood lineages and inhibit differentiation of granulocytes, eosinophils, mast cells and helper T-cells (Fossett, 2013; Zhou et al., 2001). The GATA:FOG complex also blocks blood cell differentiation in flies; therefore this function is conserved between mammals and flies (Fossett, 2013; Patient and McGhee, 2002; Muratoglu et al., 2007; Zhou et al., 2001).

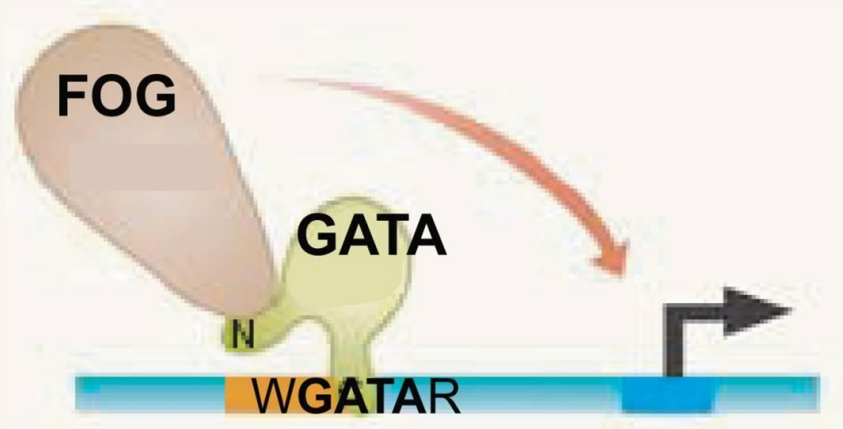

Figure 3. GATA:FOG mediated transcription. FOG (Friend of GATA) transcriptional regulators are multitype zinc-finger proteins that activate or repress the GATA-directed transcription by binding to the N-terminal zinc finger of GATA. GATA factors recognize the GATA sequence of genes and function as transcriptional activators. W=A/G. R=A/T.

The identification of factors that interact with the GATA:FOG complex may benefit translational research. GATA is generally considered to be a poor drug target since it is a DNA binding protein (Zheng and Blobel, 2010). However, GATA factors work in combination with other DNA binding proteins and co-regulatory molecules to regulate transcription of GATA target genes, such as *myc* and *cyclin D1* (Zheng and

Blobel, 2010). Therefore, factors that interact with the GATA:FOG transcriptional complex could be targeted during therapeutic approaches for GATA factor related diseases, such as Parkinson's disease and many form of cancers, such as leukemia, neuroblastoma and breast cancer (Kurzawski et al., 2010; Zheng and Blobel, 2010).

In *Drosophila*, five GATA factors have been identified (Sorrentino et al., 2005). One of the *Drosophila* GATA factors, Serpent (Srp), functions in a wide variety of processes. Srp acts analogously to GATA-2 in that it is required to maintain the hematopoietic progenitor cell pool (Muratoglu et al., 2007). Srp also directs blood lineage commitment and differentiation, analogous to GATA-1 and -3 (Muratoglu et al., 2007). One of the isoforms of Srp in *Drosophila* is SrpNC. This isoform binds to the FOG homolog in *Drosophila*, U-shaped (Ush) (Muratoglu et al., 2007). Loss of the Ush function arrests embryonic development. Previous studies have shown that *ush* is required to maintain hematopoietic progenitor multipotency by blocking differentiation (Fossett, 2013; Muratoglu et al., 2007). During lymph gland hematopoiesis, Ush most likely interacts with SrpNC to form the GATA:FOG homologue complex (Srp:Ush) that maintains hematopoietic progenitor multipotency and controls lineage choice (Fossett et al., 2003). Furthermore, a decrease in the level of Ush frees SrpNC to act with lineage-specific factors to drive commitment and differentiation (Fossett et al., 2003).

The relative level of Ush also regulates the choice between maintenance of hematopoietic progenitor multipotency and differentiation of lamellocytes, crystal cells and plasmatocytes (Fossett, 2013; Gao et al., 2009; Fossett et al., 2003). Two functional copies of *ush* are needed for progenitor maintenance; loss of one functional copy of *ush* leads to plasmatocyte and crystal cell differentiation; loss of more than one functional

copy of *ush* leads to lamellocyte differentiation (Fossett, 2013; Muratoglu et al., 2007; Fossett et al., 2003).

In our study, fly genetics was used to identify factors that act with the GATA:FOG (Srp:Ush) complex to block lamellocyte differentiation. A *smoothened (smo)* mutant was identified, which resulted in strong lamellocyte differentiation when carried as a transheterozygote with *ush* (in a double heterozygous mutant background). Smo is a G-Protein coupled receptor that is a part of the HH signaling pathway.

1.4 Role of Hedgehog Signaling in Regulating Hematopoietic Progenitor Maintenance

HH is a conserved signal transduction pathway that is essential for embryonic development and for the maintenance of adult stem cells in a variety of systems (Fossett, 2013; Huangfu and Anderson, 2006). The HH family of secreted proteins regulates many developmental processes in both vertebrates and invertebrates (Fossett, 2013; Huangfu and Anderson, 2006). The *hh* gene was first identified in *Drosophila* because of its role in embryonic segmentation (Nusslein-Volhard and Wieschaus, 1980) and was later shown to act in other aspects of *Drosophila* development, such as patterning of the imaginal discs (Basler and Struhl, 1994; Tabata et al., 1992; Mohler, 1988). Vertebrate homologs of HH were identified in chick and mouse, and were implicated in patterning of the limb and the neural tube (Roelink et al., 1994; Echelard et al., 1993; Riddle et al., 1993). In contrast to mammals, in which there are three HH homologues (*sonic hedgehog* (*shh*), *indian hedgehog* (*ihh*) and *desert hedgehog* (*dhh*)), there is only one homologue of HH in *Drosophila* (Fossett, 2013).

Canonical HH signaling requires the binding of a HH family ligand to the Patched (Ptc) transmembrane receptor resulting in the activation of Smo (Figure 4) (Huangfu and Anderson, 2006). Activated Smo will induce the nuclear translocation of the transcriptionally active factor Cubitus Interruptus (Ci). Full-length Ci can be modified by HH signaling to become a transcriptional activator. In the absence of HH ligand, Ci is truncated into a shorter form (CiR) that acts as a transcriptional repressor of HH target genes (Huangfu and Anderson, 2006). Both the truncation and the nuclear translocation of Ci are tightly regulated processes that involve a protein complex containing the atypical kinesin protein: Costal 2 (Cos2); the serine threonine kinase: Fused (Fu); and the novel protein: Suppressor of fused (Sufu) (Huangfu and Anderson, 2006) (Figure 4). The mammalian homologues of Ci are the Gli transcription factor family─ Gli1, Gli2 and Gli3 (Huangfu and Anderson, 2006). Gli1 and Gli2 act primarily as activators, while Gli3 acts both as an activator and repressor, analogous to Ci (Huangfu and Anderson, 2006) (Figure 4).

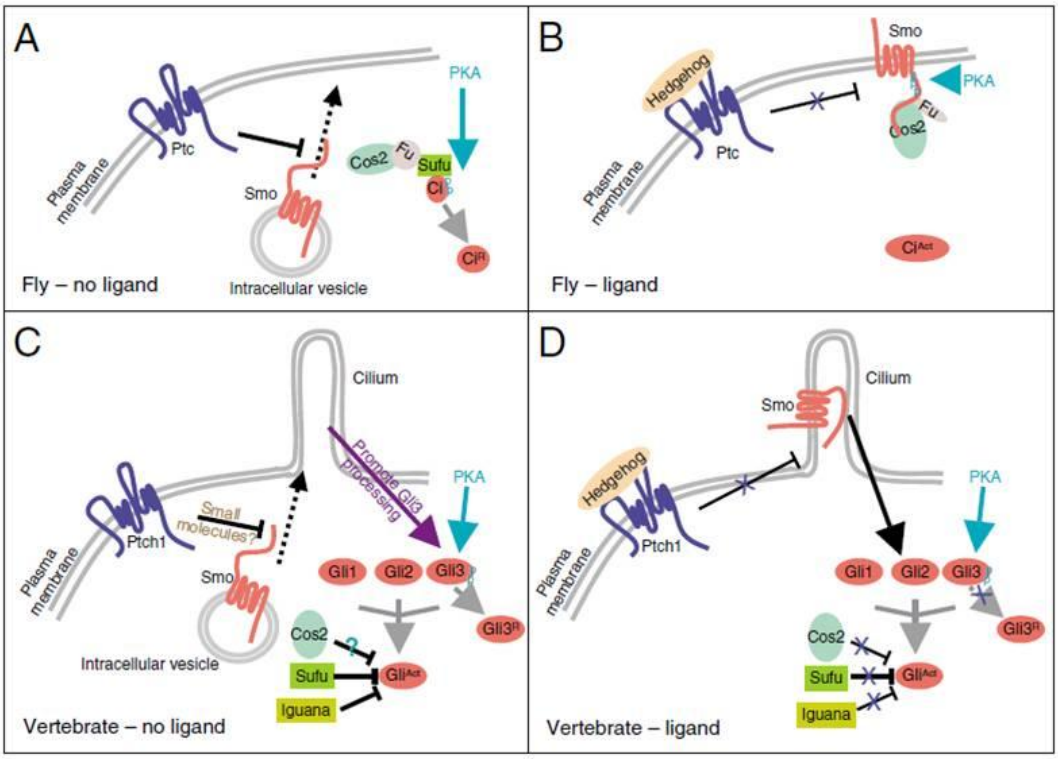

Figure 4. Comparison between Hedgehog signaling in *Drosophila* and vertebrates. **(A, B)** HH signaling in *Drosophila*. **(C, D)** HH signaling in vertebrates. **(A, C)** HH signaling in *Drosophila* and vertebrates in the absence of HH ligand. **(B, D)** HH signaling in *Drosophila* and vertebrates in the presence of HH ligand. **(A)** In *Drosophila*, when HH is absent, Ptc prevents the cellular translocation of Smo towards the cellular membrane. Ci forms a complex with Cos2, Fu and Sufu, which targets Ci for truncation into its repressor form (CiR). **(B)** In *Drosophila*, HH ligand binds to Ptc relieving its inhibition of Smo. Smo then translocates to the membrane and forms a complex with Cos2 and Fu through its C-terminal tail; Ci becomes activated. **(C)** In vertebrates when HH is absent, Patched-1 (Ptch1) inhibits the translocation of Smo to the cilia of the cell. Gli3 is processed into its repressor form (Gli3R). The activation of all proteins in the Gli family is inhibited by the proteins Sufu, Iguana (in zebrafish) and Cos2. **(D)** HH binds to and inhibits Ptch1. Smo is targeted to cilia and activates Gli proteins in a cilia-dependent manner. Gli3 processing is also inhibited. P=phosphorylation; PKA= protein kinase A (Huangfu and Anderson, 2006).

Abnormal HH signaling is known to result in tumor development and hematological malignancies (Ingham and McMahon, 2001; Xie et al., 1998). Thus, factors of HH signaling have become therapeutic targets for various types of cancer and

malignancies. For example, antagonists targeting Smo, such as Vismodegib (Erivedge®), have been developed for basal cell carcinoma therapy (Atwood et al., 2014). As mentioned in the previous section, GATA transcription factors are typically poor drug targets (Zheng and Blobel, 2010). Therefore, if a connection between the GATA:FOG transcriptional complex and HH signaling could be established then it raises the possibility that improved drug therapies for GATA related diseases could be developed through targeting HH signaling factors, such as Smo.

In our study, we determined that HH signaling regulates Ush and may maintain hematopoietic progenitors in *Drosophila* by showing that the factors of Hedgehog signaling (HH, Ptc, Smo and Ci) regulate Ush expression as well as the progenitor marker Odd-skipped (Odd). Since HH signaling and GATA:FOG are conserved, these molecular interactions may also be conserved. Finally, our studies may identify conserved regulatory mechanisms whereby external signals from the microenvironment, regulate intrinsic pathways to control progenitor and stem-cell differentiation.

Chapter 2: Materials and Methods

2.1 *Drosophila* Strains

The w^{1118} strain served as the wild-type stock for these studies. The following strains were obtained from the Bloomington Stock Center: *Upstream-Activating Sequence (UAS)-smo*^{RNAi}, *UAS-hh*^{RNAi}, *UAS-ci*^{RNAi}, as well as the strains carrying multi-locus deficiencies and single gene mutations corresponding to the left arm of Chromosome 2 (2L). The *Hand-Hemolectin Lineage Traced-Gal4 (HHLT-Gal4)* pan lymph gland driver was obtained from C. Evans, (UCLA). The following strains were generous gifts from colleagues: *domeless-Gal4 (Dome-Gal4)* from M. Crozatier (University Paul Sabatier); *TepIV-Gal4 (Tep-Gal4), and MSNF9mo-DsRed* from T. Tokusumi and R. A. Schulz (University of Notre Dame); *Antennapedia-Gal4 (Antp-Gal4)* from S. Govind (City College of New York). The following stocks have been previously described: *y* w^{67c23}*; ush*^{vx22}*/CyO* y^{+}, *y* w^{67c23}; *ush*^{R24}*/CyO* y^{+}, *y w; UAS-ush* (Gao et al., 2009; Muratoglu et al., 2007).

A recombinant strain of *Drosophila* with the genotype of *yw; ush*^{VX22}*, MSN-C/CyO* y^{+} was created that enables us to use a one-generation cross to rapidly screen for factors that interact with *ush* to regulate hematopoiesis. This stock carries the *ush*^{VX22}, null allele and the *misshapen-mCherry (MSN-C)* fluorescent reporter gene on the same chromosome. *MSN-C* is a marker for lamellocyte differentiation (Tokusumi et al., 2009). Larvae with increased numbers of lamellocytes were rapidly identified using fluorescent microscopy. This recombinant strain was made using standard recombination techniques described as follows: Female flies that carry *ush*^{VX22} over a *Curly of Oster* (*CyO*) balancer (genotype: *yw; ush*^{VX22}*/CyO* y^{+}) were crossed to male flies with a genotype of *w; MSN-C*

(w+). This F1 generation produced a strain of offspring with a genotype: *yw/w; ush*^{VX22} / *MSN-C (w+) Cy+ w+*. Virgin female flies of this strain were then collected since there is no recombination in males, then crossed with male flies containing *ush*^{VX22}, carried over a *CyO* balancer (genotype: *yw; ush*^{VX22} */ CyO y*$^{+}$) to undergo mass mating. In the progeny from this F2 cross, recombinant and non-recombinant strains have the same phenotype: curly wings and red eyes. Thus, the flies that were selected were of the genotype *ush*^{VX22}, *MSN-C (w+)/ CyO y*$^{+}$ or *MSN-C (w+)/CyO y*$^{+}$. To identify the recombinant flies, each fly was then individually mated to *ush*^{VX22} / *CyO y*$^{+}$ males to create an F3 generation. From the progeny of these crosses only the recombinant strains were kept, which were flies containing only curly (*Cy*) wings (genotype: *ush*^{VX22}, *MSN-C (w+)/ CyO y*$^{+}$). The recombinant strains were kept as a stock to be used in our genome-wide screen for factors that act with Ush to block lamellocyte differentiation. The flies that did not have curly wings, which was the non-recombinant strain, were discarded.

2.2 Genomic Functional Screen

We conducted a genetic screen which was based on the principle of Second Site Non-Complementation (SSNC); this occurs when two genes act in the same pathway or network. When genes are recessive for a particular phenotype, loss of one allele produces the wild type phenotype while loss of both copies of a gene produces a mutant phenotype. In complementation, loss of one functional copy of two different genes would still result in the wild type phenotype. But in SSNC, loss of one functional copy of each gene produces a mutant phenotype. Loss of one copy of *ush* does not produce lamellocyte differentiation (Hawley and Gilliland, 2006). In this study, we screened for candidate

genes that do not complement the loss of a functional copy of *ush* and result in increased lamellocyte differentiation when a functional copy of that candidate gene is lost in addition to a functional copy of *ush*. Thus, the gene identified in the screen likely acts in the same network as *ush*.

The *Drosophila* genome contains ~14,600 genes (Adams et al., 2000). Thus, we conducted a genetic screen of deficiencies in which multiple genes were deleted (multi-locus deficiencies), which allows one to rapidly screen 98% of the *Drosophila* genome, without bias. This reduced the number of crosses needed to screen the entire fly genome from ~14,600 to ~500. In order to conduct this screen, we used the Bloomington Stock Center Deficiency Kit, which is a series of 468 overlapping multi-locus deficiencies that collectively delete 98% of the *Drosophila* genome. This kit is subdivided into *Drosophila* stocks that correspond to each of the four *Drosophila* chromosomes. We have screened all of the deletions that map to the left arm of Chromosome 2 (2L); 100 stocks─ covering 20% of the *Drosophila* genome.

The first step of the SSNC functional screen was to conduct the multi-locus genetic deficiency screen. Virgin female flies from the recombinant strain (genotype: *yw; ush^{VX22}, MSN-C/CyO y^{+}*) as described in the section above were crossed to male flies carrying large multi-locus genomic deficiencies. These crosses were then stored at room temperature for about 8 days until a significant number of third-instar wandering larvae were seen (Figure 5). Larvae were collected at this developmental stage, and lamellocyte differentiation was then observed (Tokusumi et al., 2009; Weigmann et al., 2003).

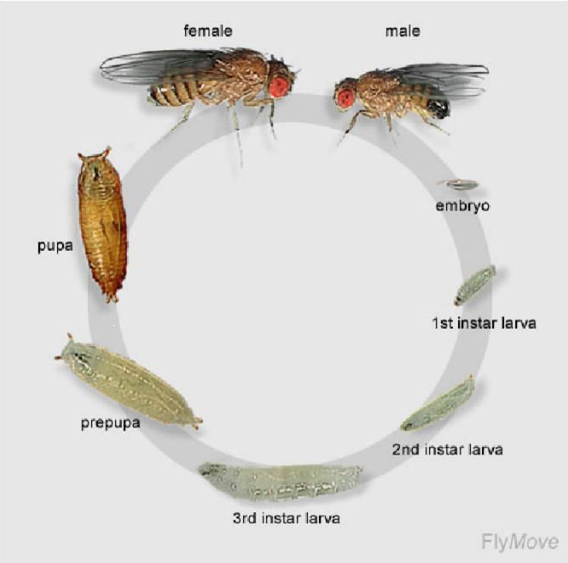

Figure 5. Developmental cycle of *Drosophila melanogaster.* The embryo develops into the first-instar lavae in ~22–24 hours at 25°C. After another 25 hours, the first-instar larva molts into the second instar larva, which is greater in size than the first-instar. After about 24 hours, the second-instar larva molts into the third instar larva. This is the largest of the larval forms. In addition, this is the stage where the lymph gland reaches maturity (Jung et al., 2005). The third instar then molts into a pupa after 30 hours. The pupal stage lasts for 3–4 days, after which the adult fly emerges from the pupal case (Weigmann et al., 2003).

Between 10 and 25 larvae were sampled from each cross. Initially 10 larvae were sampled. However, 10 larvae is not a sufficient sample size to come to a conclusion about the strength of lamellocyte differentiation for each larval population. For this reason, we increased the sample size to 25. Larvae were placed on a slide with a drop of Phosphate-buffered saline (PBS) and covered gently with a cover slip. The slides were then placed on ice to partially immobilize the larvae. The larvae were then assayed using fluorescent microscopy. MSN-C was used as a fluorescent reporter to identify the presence of lamellocytes, which are rarely observed under normal conditions. MSN-C is constitutively active in the muscle tissue (Tokusumi et al., 2009) and serves as the marker for the *ush/MSN-C* chromosome. Therefore, 50% of the progeny should carry *MSN-C*,

and ~50% of the progeny carrying MSN-C should be trans-heterozygous (Figure 6), which will allow us to identify progeny that have lamellocytes.

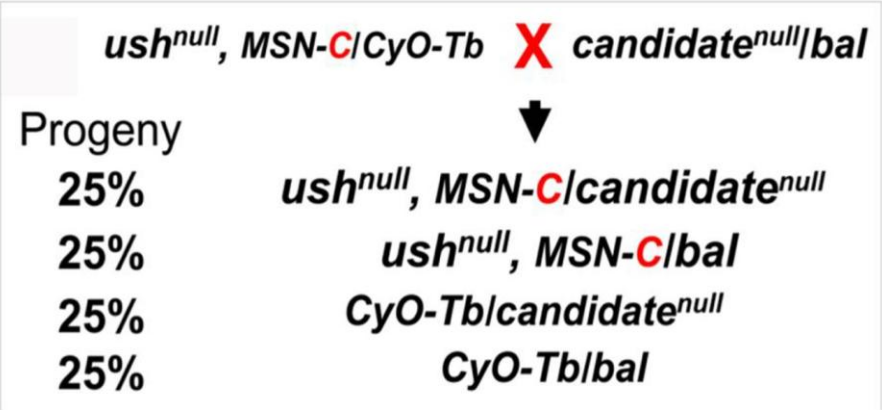

Figure 6. Genetic cross used in the deficiency screen. Virgin female flies with a chromosome carrying a null allele of *ush* with an m-Cherry marker *(C)* over a balancer chromosome were crossed with male flies carrying a chromosome with the null allele of possible candidate genes over a balancer chromosome to give the following progeny with the listed genotypes. The m-Cherry reporter is used as a marker for lamellocyte differentiation. About half of the progeny inherit the marker and approximately half of the progeny with the marker is expected to have the null allele of *ush* and the null allele of the candidate gene. 0.25 was expected ratio of progeny of each genotype.

If lamellocytes were present, then the larvae were further analyzed to determine the degree of lamellocyte differentiation that ranged from weak to strong. Individual larvae were classified as having negative, weak, weak-medium, medium, medium-strong, or strong lamellocyte differentiation (Figure 7). Crosses, which contained larvae scored as positive for strong lamellocyte differentiation, were repeated to confirm our data.

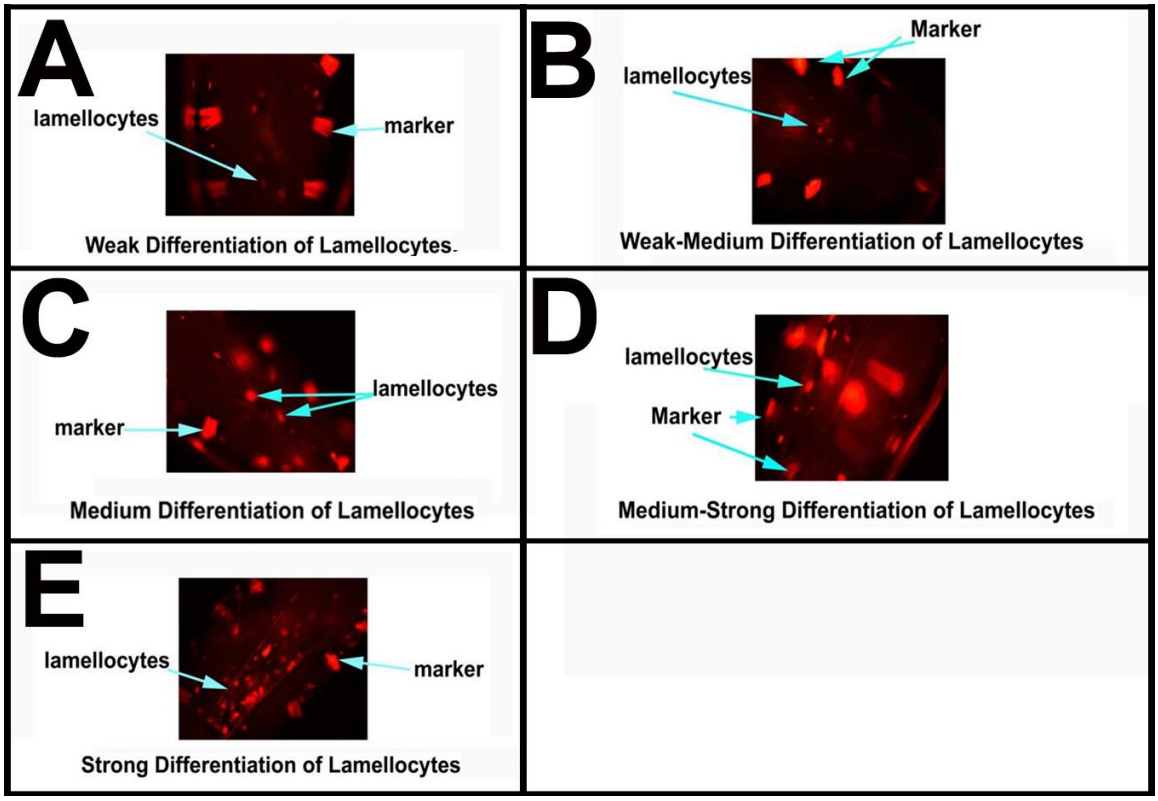

Figure 7. Third-instar larvae expressing various levels of lamellocyte differentiation. Lamellocyte differentiation was classified as **(A)** weak, **(B)** weak-medium, **(C)** medium, **(D)** medium-strong, and **(E)** strong. Larvae were exposed under fluorescent microscopy. Larvae exposed under 10X fluorescent microscopy. MSN-C marker and circulating lamellocytes are red.

The multi-locus deficiencies with the strongest differentiation were selected for further study (Table 1). Genes that map to each of these deficiencies were then screened during the next step of the SSNC functional screen: the single-gene mutation screen. Virgin female flies from the recombinant strain described above (genotype: *yw; ush*^{VX22}, *MSN-C/CyO* y^{+}) were crossed to male flies carrying selected single-gene mutations (Table 1 & Figure 8). In addition, as with our multi-locus deficiency screen, genes that exhibited a strong lamellocyte differentiation when carried as trans-heterozygotes with *ush* were repeated to confirm the results.

Table 1. Multi-locus deficiencies from Chromosome 2L identified as having a strong lamellocyte differentiation phenotype when crossed to the null allele of *ush*

Genomic insertions/breakpoints of Multi-locus deficiencies in Chromosome 2L
21B3-21B7
218B-21D1
22F4-23C3
25D5-26E6
26D3-26F7
31C-32E
31F5-32B4
35E1-35F1
38F1;39D2

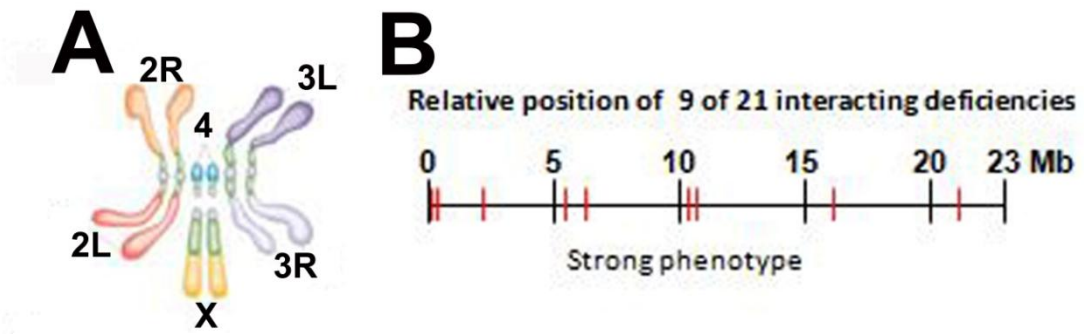

Figure 8. Genomic map of nine of the twenty-one interacting deficiencies. A) The four chromosomes of *Drosophila.* We have screened the entire left arm of Chromosome 2 (2L). **B)** Relative genomic location of nine of the twenty-one interacting deficiencies identified in our screen on Chromosome 2L. The deficiencies resulted in the strong phenotype for lamellocyte differentiation when crossed with the null allele of *ush.*

For both screens, fly strains that showed strong lamellocyte production when carried with the *ush*^{vx22}, *MSN-C* genes were crossed with the parental *MSN-C* strain to determine if the mutated allele of a candidate gene and *ush*^{vx22} both produced lamellocytes or if the mutated allele of the candidate gene produced lamellocytes on its own. This would further establish if the wild type allele of both the candidate gene and *ush* are required to block lamellocyte differentiation or if the wild-type allele of the candidate gene blocked lamellocyte differentiation on its own.

2.3 Functional Analyses of Genes that interact with *ush*.

Genes identified as positive candidates for SSNC with *ush* were selected for further study based on the following criteria: 1) genes that are conserved across taxa and 2) availability of tools for further analysis. We identified nine candidate genes and began testing these genes to determine if they block lamellocyte differentiation and maintain the hematopoietic progenitor cell population in a *ush* wild-type background. Functional analyses were conducted using lymph glands from mid-third instar larvae (collected 96 to 104 hours after egg laying) or late-third instar larvae (collected 112 to 120 hours after egg laying). All control and experimental samples were age-matched and cultured on standard media at 23°C.

The lymph gland of *Drosophila* larvae is anatomically accessible. Furthermore, the niche, hematopoietic progenitor cells and differentiating cells are compartmentalized within the organ are easily identifiable (Jung et al., 2005). Progenitor cells are located in the medullary zone (MZ) of the primary lobe of the lymph gland, whereas differentiated cells are restricted to the cortical zone (CZ) (Figure 9). The posterior signaling center

(PSC) is located at the base of the primary lobe (Figure 9), and serves as a stem cell niche regulating the balance between multipotent progenitors and cells undergoing differentiation (Jung et al., 2005).

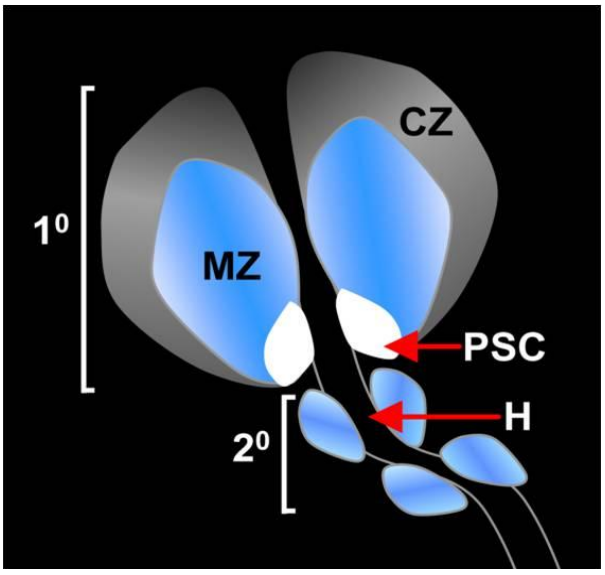

Figure 9. Schematic of the *Drosophila* lymph gland. The anterior primary lobe is labeled 1^0, containing the medullary zone, which is labeled MZ and the cortical zone is labeled CZ. The posterior secondary lobes are labeled 2^0.The posterior signaling center (niche) is labeled PSC and represents a group of about 30 cells which secretes several signaling molecules. The heart is labeled H.

The genes we tested in our study are expressed in multiple types of tissues in the fly. Furthermore, in our screen all the genes were assayed as trans-heterozygotes, so the level of expression was decreased in all tissues which express a particular gene. Thus, a gene of interest could be acting in different hematopoietic compartments or even in non-hematopoietic tissues to block lamellocyte differentiation. Since we were interested in investigating how these genes function to regulate hematopoietic progenitor maintenance for our study, we wanted to perform further functional analyses specifically in hematopoietic progenitor cells. Thus, the UAS/Gal4 binary system (Brand and Perrimon,

1993) was used to express transgenes for knockdown or over-expression in a hematopoietic progenitor-specific manner (Figure 10).

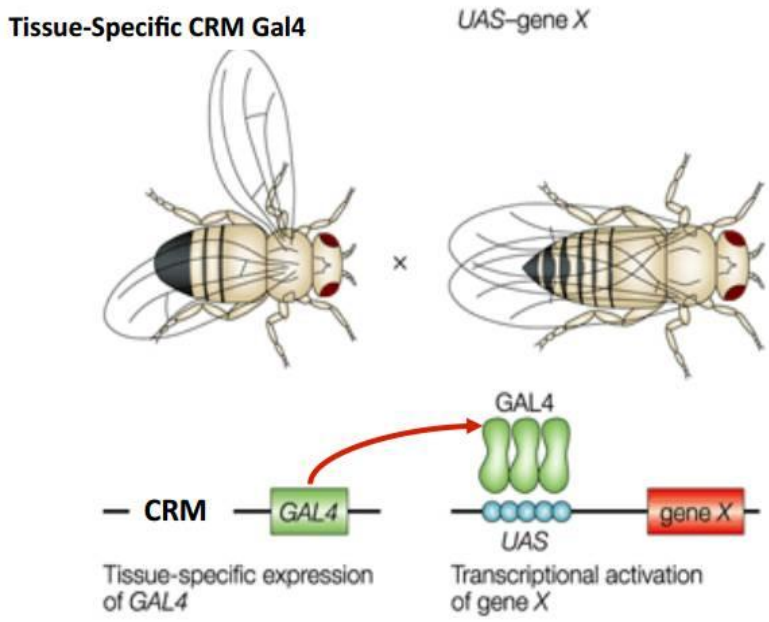

Figure 10. UAS/Gal4 system binary system. Male strain containing cis-regulatory module (CRM) driving tissue specific expression of the yeast transcription factor Gal4 is crossed with a female strain containing an upstream activation sequence (UAS) driving a "gene of interest." In the resulting progeny, Gal4 will bind to the UAS, which will result in activation and expression of the gene of interest in a tissue-specific pattern in the progeny.

The drivers used in the UAS/Gal4 binary system are tissue specific for progenitor cells located in the MZ of the lymph gland. The *Dome-Gal4* and *Tep-Gal4* drivers are specific for the hematopoietic progenitors that compose the MZ of the lymph gland. The *Hemolectin-Gal4 (hml-Gal4)* driver is tissue specific for the plasmatocytes and crystal cells that compose the CZ of the lymph gland. *Antp-Gal4* is a driver that is tissue specific for the niche of the lymph gland. The niche is specified early in the embryo by Antp and expresses HH.

2.4 Sample Processing and Immunoflourescent Data Analysis.

Lymph glands were dissected from third instar larvae in PBS, and then fixed in 4% Parafomaldehyde for 10 minutes. The lymph glands were then washed in PBT (which is a solution of 1X PBS with 0.2% bovine serum albumin (BSA) and 0.1% Triton-X) 4-5 times, and then stored in PBT at 4°C, until antibody staining. Antigens of interest located in lymph gland cells were stained using the following antibodies. Rabbit anti-Odd was a generous gift from J. Skeath (Washington University School of Medicine, (Ward and Skeath, 2000) and used at a 1:4,000 dilution. Mouse anti-Attila (L1) (Kurucz et al., 2007)was a generous gift from I. Ando (Biological Research Center of the Hungarian Academy of Sciences) and used at a 1:50 dilution. Rabbit anti-U-shaped was used at a 1:4,000 dilution (Fossett and Schulz, 2001). Alexafluor-555, -568, or -488-conjugated secondary antibodies directed against rabbit, mouse, or rat (Invitrogen) were used at a 1:2,000 dilution.

Fluorescence was captured, analyzed and recorded using Olympus confocal microscopy or Zeiss Axioplan optics. The relative expression of hematopoietic progenitor cell markers was determined from the densitometric mean values calculated for fluorescent antibody staining using Zeiss Axiovision software as previously described (Gao et al., 2009). Cell counts were divided by the total primary lobe area to normalize for differences in lymph gland size. Cell counts were analyzed using Zeiss Axioplan software as previously described (Gao et al., 2011). The statistical significance was evaluated using the student's t-test. In our hands, control lymph glands have an average of 1 lamellocyte per lymph gland lobe. However, lamellocytes can form large aggregates making it difficult to obtain accurate cell counts. For this reason, we scored primary

lymph gland lobes positive for aberrant lamellocyte differentiation when aggregates were greater than 300μm^2 or more than 5 individual lamellocytes were visible (Gao et al., 2011). Statistical significance was then evaluated using aberrant differentiation as a categorical variable for experimental and control samples in 2x2 contingency tables. P-values were calculated using Fisher's exact test. At least 20 primary lymph gland lobes were sampled, and each assay consisted of at least 10 control and 10 experimental samples.

Chapter 3: Results

3.1 Nine Potential Genes were Identified as Interacting Factors with the GATA:FOG (Srp:Ush) Complex in a SSNC Screen of *Drosophila* Chromosome 2L.

The deficiencies that mapped to Chromosome 2L (100 stocks) were screened using SSNC (see Chapter 2, section 2.2). We screened all of the deletions that map to the left arm of Chromosome 2 (2L), covering 20% of the *Drosophila* genome. Using this method we identified and confirmed twenty-one deficiencies that showed SSNC with Ush.

We selected nine deficiencies that repeatedly showed strong lamellocyte differentiation when crossed to the ush^{vx22}, MSN-C chromosome. We also showed that lamellocytes did not differentiate in animals that carried these deficiencies with two wild-type copies of the *ush* gene. Thus, the deficiencies did not cause lamellocyte differentiation independently, but did cause lamellocyte differentiation when carried as a transheterozygote with *ush.*

The genes uncovered by these deficiencies were then screened, using the methods described in Chapter 2, section 2.2, for factors that function with *ush* to block lamellocyte differentiation. From this screen for single genes, nine candidate genes were identified: 1) *smo,* is involved in the HH pathway; 2) *cactus,* is a Toll-like pathway inhibitor; 3) *ubc9,* is involved in sumolyation; 4) *eyes absent (eya),* a target of MLL; 5) *e2f2,* which is involved in the cell cycle; 6) $tbc1d1517^{d05023}$, a GATA-1 target gene; 7) *thioredoxin,* an

antioxidant; 8) *porin*, which encodes a membrane pore-forming protein; and 9) *intersectin*, an adaptor protein that interacts with RTKs and GTPases (Table 2).

Factors identified in the Ush SSNC Screen that Produced a Strong Lamellocyte Differentiation When Crossed to Animals Carrying a Hypomorphic Allele of *ush*.			
Factor	**2L Position**	**Associated Factors/Pathways**	**Role in Hematopoiesis**
Smoothened	21B7	Hedgehog Pathway effector	Regulates primitive hematopoiesis
Cactus/IκB	35F1	Toll-Pathway inhibitor	Regulates differentiation of a variety of blood cell types
Ubc9	21E2	Sumolyation	HSPC development in Zebrafish/Fly
Eyes Absent	26E1-2	MLL Target	Unregulated in Leukemia
E2F2	39A5-6	Cell Cycle/Tumor Suppression	GATA-1/pRb/E2F-2 tricomplex inhibits erythroid precursor proliferation
Tbc1d1517^{d05023}	21B4	GATA-1 target gene	Putative RabGAP expressed in T-Cells and hematopoietic cells
Thioredoxin	39A4	Antioxidant	Limits ROS Levels
Porin	32B1	Membrane-pore forming protein	Unknown
Intersectin	39A3-4	Multi-domain adaptor protein interacts with RTKs, GTPases	Unknown

Table 2. Nine factors that were identified in the Ush SSNC screen. These nine factors produced a strong lamellocyte differentiation when crossed to animals carrying a null allele of *ush*. The factors are listed with their genomic position, associated signal transduction factors/pathways, and their role in hematopoiesis.

3.2 HH Signaling Factor, Smo is One of the Nine Potential Factors that Interact with the GATA:FOG Complex.

The HH pathway is a conserved pathway that is essential for embryonic development and for the maintenance of stem cells in a variety of systems (Fossett, 2013; Huangfu and Anderson, 2006). Smo is a G-Protein coupled receptor and a part of the HH signaling pathway (Huangfu and Anderson, 2006). This factor was selected for further analysis based on the criteria that it may identify a link between HH signaling and the

GATA:FOG complex in the regulation of HSPC differentiation and multipotency. Ush is required to maintain hematopoietic progenitors and block lamellocyte differentiation (Gao et al., 2009). We hypothesized that the HH pathway interacts with Ush to maintain hematopoietic progenitors in the fly. Thus, we tested for interactions between Ush and members of the HH pathway. We also investigated how these different factors maintained progenitors and blocked lamellocyte differentiation.

3.3 Smo Acts in the Hematopoietic Progenitor Cells of the Lymph Gland to Block Lamellocyte Differentiation.

Our functional screen identified Smo as a factor that interacts with Ush to block lamellocyte differentiation. However, our screen assays *smo/ush* transheterozygotes in which both *smo* and *ush* are reduced in all tissues. Thus, we do not know if this potential interaction occurs specifically in hematopoietic progenitors. To determine if the interaction occurs in hematopoietic progenitors we used the UAS/Gal4 system to knock down Smo specifically in progenitors using the Dome-Gal4 driver and tested for lamellocyte differentiation. Dome driven Smo^{RNAi} resulted in a significant increase in lamellocyte differentiation compared to controls (Figure 11). However, in subsequent experiments we were unable to show that Dome-Gal4 driven Smo^{RNAi} resulted in a significant decrease of Ush expression and loss of progenitor cells. Therefore, further work is needed to conclude if Smo blocks the differentiation of progenitor cells into hemocytes or if Smo is acting in a canonical or non-canonical fashion to specifically block lamellocyte differentiation.

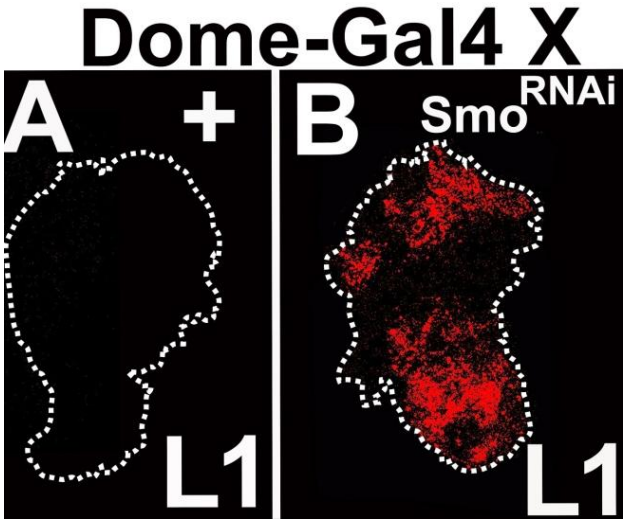

Figure 11. Knockdown of Smo in progenitor cells results in increased lamellocyte differentiation. (A, B) Dome-Gal4 drives the knockdown of Smo (Dome>Smo^{RNAi}) in the progenitor cells of mutant lymph glands. Lamellocyte differentiation was significantly increased in Smo^{RNAi} mutant lymph glands compared to controls (+). Red= L1 antibody that was used to identify lamellocytes. White dotted line outlines lymph gland primary lobe. Gene expression analyses were conducted using lymph glands from late-third instar larvae (collected 96 to 104 hours after egg laying).

3.4 Smo is Required to Maintain Odd-skipped, a Genetic Marker for Hematopoietic Progenitors.

Next, we investigated if Smo maintained hematopoietic progenitors. A previous study established that the zinc finger-protein Odd-skipped (Odd), is expressed specifically in progenitors and is required to block differentiation (Gao et al., 2011). Thus, Odd has been used as a marker to identify progenitor cells.

As discussed in the previous section, Smo was unable to be successfully knocked down using the UAS-Smo^{RNAi} stock during experiments that measured Odd or Ush expression. Therefore, we used a stock that contained a dominant-negative allele of Smo (UAS-Smo^{DN}) for our investigations. We expressed Smo^{DN} in the progenitors of lymph

glands using the Tep-Gal4 driver and assayed Odd expression. Tep-Gal4 driving Smo^{DN} resulted in significantly reduced levels of Odd expression in mutant lymph glands compared to controls (Figure 12a-c). To support our hypothesis that HH signaling interacts with Ush and maintains hematopoietic progenitors in the *Drosophila*, we then tested if over-expression of Smo in the progenitor cells of lymph glands increased the size of this cell population. As anticipated, we observed that Odd expression was significantly increased when Smo was over-expressed compared to controls (Figure 12d-f). Thus, we established that Smo was required to maintain expression of the progenitor marker, Odd.

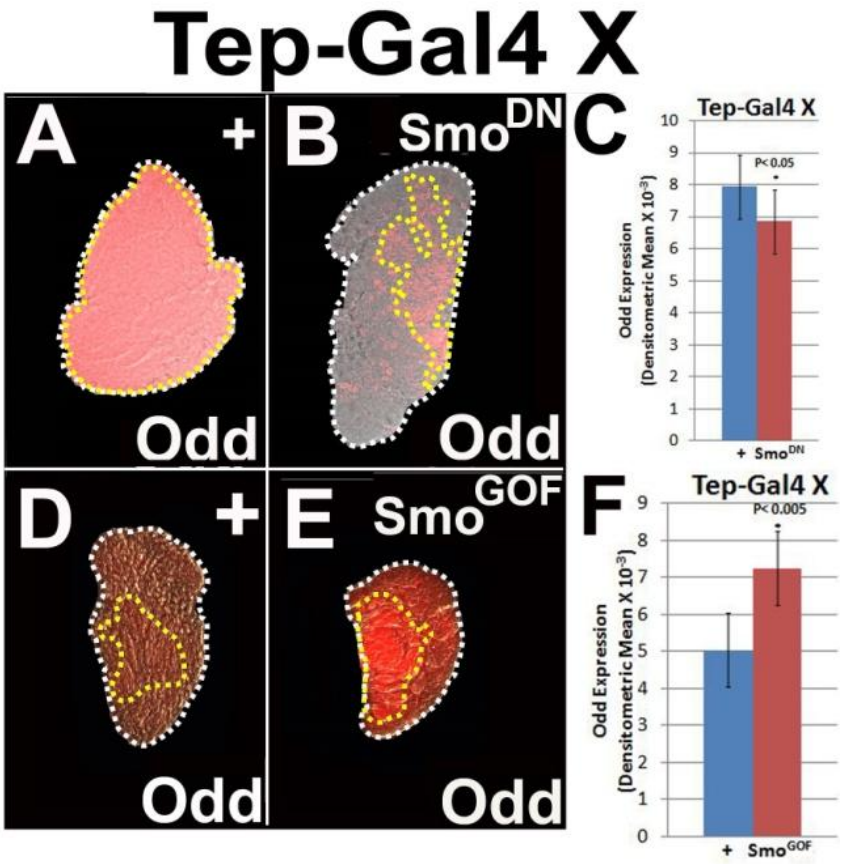

Figure 12. Smo maintains Odd expression, a marker for progenitors. Dominant negative (DN) allele of Smo results in a decrease of Odd expression. **(A, B)** Tep-Gal4 drives Dominant-Negative Smo (Tep>Smo^{DN}) in lymph gland progenitors. Odd (progenitor marker) expression was significantly decreased in Smo^{DN} mutant lymph glands when compared to controls. **(C)** Control (+) and mutant lymph gland (Smo^{DN}) Odd expression quantified. Student's t-test used to analyze statistical significance. Results are expressed as densitometric mean X 10^{-3}. Error bars show standard deviation. P-value as shown. N=20. Over-expression of Smo results in an increase of Odd expression. **(D, E)** Tep-Gal4 drives Smo over-expression (Tep>Smo^{GOF}) in the progenitor cells of mutant lymph glands. Odd expression was significantly increased in Smo^{GOF} mutant lymph glands when compared to controls. Red= Odd antibody that was used to identify progenitors. White dotted line outlines lymph gland primary lobe. Yellow dotted line outlines area of Odd expression. **(F)** Control (+) and mutant lymph gland (Smo^{GOF}) Odd expression quantified. Student's t-test used to analyze statistical significance. Error bars show standard deviation. P-value as shown. Results are expressed as densitometric mean X 10^{-3}. N=12. Gene expression analyses were conducted using lymph glands from mid-third instar larvae (collected 96 to 104 hours after egg laying).

3.5 Smo is Required to Maintain Ush.

Once we confirmed that Smo was required to maintain Odd expression, we began to investigate the relationship between Smo and Ush. Expression of Smo^{DN} in the progenitors significantly reduced the level of Ush expression in mutant lymph glands compared to controls (Figure 13a-c). To add further support for the notion that Smo maintains Ush expression, we tested if over-expression of Smo in the progenitors of lymph glands increased Ush expression. Over-expression of Smo resulted in a significant increase of Ush expression in mutants compared to controls (Figure 13d-f). Thus, the data showed that Smo is required to maintain Ush expression.

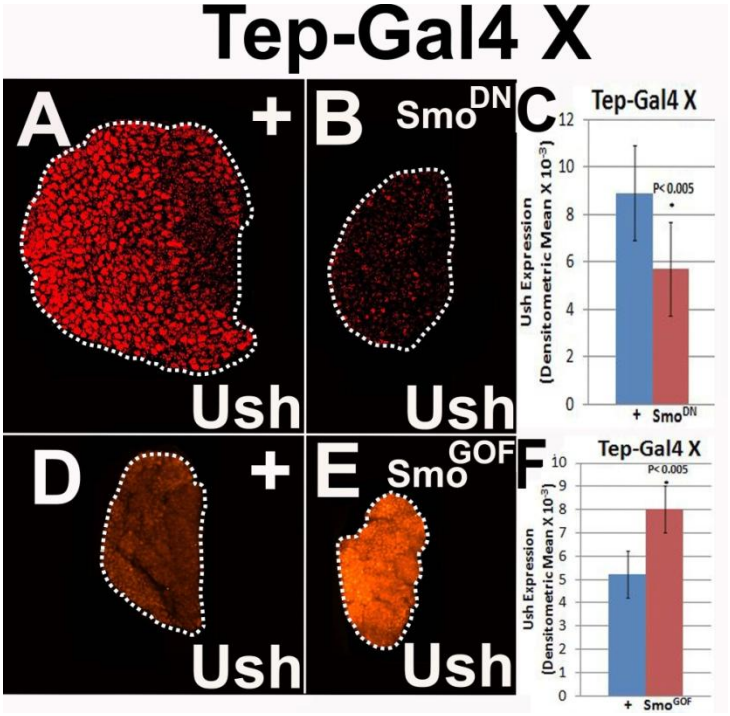

Figure 13. Smo maintains Ush. Dominant negative (DN) allele of Smo results in a decrease of Ush expression. **(A, B)** Tep-Gal4 drives dominant-negative Smo (Tep>Smo^{DN}) in progenitor cells of mutant lymph glands. Ush expression was significantly decreased in Smo^{DN} mutant lymph glands when compared to controls. **(C)** Control (+) and mutant lymph gland (Smo^{DN}) Ush expression quantified. Student's t-test used to analyze statistical significance. Results are expressed as densitometric mean X 10^{-3}. Error bars show standard deviation. P-value as shown. N=17. Over-expression of Smo results in an increase of Ush expression. **(D, E)** Tep-Gal4 drives Smo over-expression (Tep>Smo^{GOF}) in the progenitors of mutant lymph glands. Ush expression was significantly increased in Smo^{GOF} mutant lymph glands when compared to controls. Red= antibody was that was used to identify Ush. White dotted line outlines lymph gland primary lobe. **(F)** Control (+) and mutant lymph gland (Smo^{GOF}) Ush expression quantified. Student's t-test used to analyze statistical significance. Results are expressed as densitometric mean X 10^{-3}. Error bars show standard deviation. P-value as shown. N=16. Gene expression analyses were conducted using lymph glands from mid-third instar larvae (collected 96 to 104 hours after egg laying).

3.6 Ush is Not Required to Maintain Smo.

After determining that Smo maintains expression of Ush, we hypothesized that Smo acts upstream of Ush to maintain progenitor cells and that Ush is not required for Smo expression. To test this hypothesis, we measured Smo expression in lymph glands

from larvae that carried a hypomorphic allele of *ush* (*ush*$^{vx22/r24}$) and observed that Smo expression was not affected by loss of Ush function. We used a hypomorphic allele of *ush* as opposed to siRNA for *ush* because we wanted to use a system that can test loss of function in multiple types of tissues in the event that loss of *ush* in progenitor cells or differentiated cells leads to loss of Smo in progenitor cells (Gao et al., 2009). In addition, gene expression profiling showed that loss of function of Ush does not affect Smo expression (data not shown). Thus, it is unlikely that Ush activates Smo expression (Figure 14).

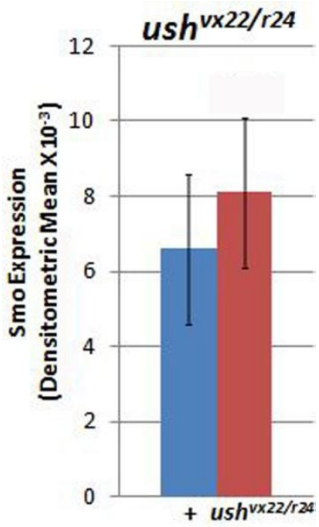

Figure 14. Ush does not maintain Smo. Loss of function of Ush does not affect Smo expression. A hypomorphic allele of Ush (*ush*$^{VX22/r24}$) was expressed in mutant lymph glands. Student's t-test used to analyze statistical significance. There was no significant difference in Smo expression in *ush*$^{VX22/r24}$ mutant lymph glands when compared to controls (+). Control and mutant lymph gland Smo expression quantified. Results are expressed as densitometric mean X 10^{-3}. Error bars show standard deviation. P-value as shown. N=12. Gene expression analyses were conducted using lymph glands from mid-third instar larvae (collected 96 to 104 hours after egg laying).

3.7 Additional Canonical Hedgehog Signaling Factors Regulate Ush Expression and Maintain Expression of the Progenitor Marker Odd-skipped.

A previous study shows that Smo activates small RhoA GTPases independently of Gli transcriptional activity, and thus can act in a non-canonical fashion to promote mouse fibroblast migration (Polizio et al., 2011). After establishing that Smo was required to maintain Ush and Odd expression in the fly, the question of whether Smo was working canonically, as a factor of the HH pathway, or non-canonically, remained unanswered. We hypothesized that Smo acts canonically as a part of the HH pathway to maintain Ush and hematopoietic progenitors. To test this hypothesis, we investigated whether other members of HH signaling pathway regulate Ush expression.

The PSC of the lymph gland functions as a hematopoietic niche, which is essential for the maintenance of hemocyte precursors in *Drosophila*. HH is a ligand released from the PSC to inhibit Ptc, thus activating the downstream signal transduction pathway. Therefore, to further test our hypothesis, we performed loss of function studies of HH in the PSC. Knockdown of HH expression in the PSC showed that there was a significantly lower expression of Ush in mutant lymph glands when compared to controls (Figure 15).

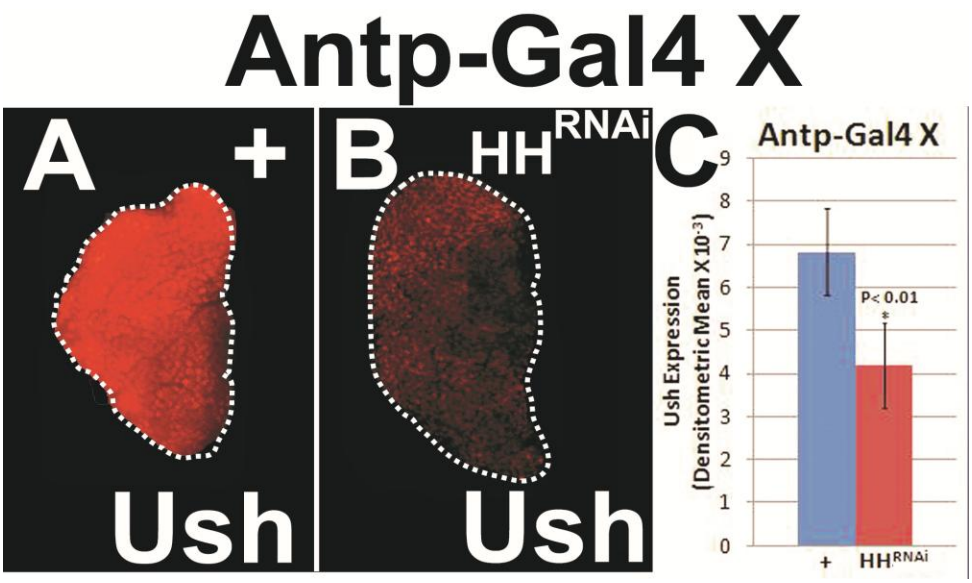

Figure 15. HH maintains Ush. Knockdown of HH decreases Ush expression. **(A, B)** Antp-Gal4 drives the knockdown of HH (Antp>HH^{RNAi}) in the PSC of mutant lymph glands. Expression of Ush was significantly decreased in HH^{RNAi} mutant lymph glands when compared to controls (+). Red=antibody was that was used to identify Ush. White dotted line outlines lymph gland primary lobe. **(C)** Control and mutant lymph gland (HH^{RNAi}) Ush expression quantified. Student's t-test used to analyze statistical significance. Results are expressed as densitometric mean X 10^{-3}. Error bars show standard deviation. P-value as shown. N= 20. Gene expression analyses were conducted using lymph glands from mid-third instar larvae (collected 96 to 104 hours after egg laying).

Furthermore, knockdown of HH resulted in a significant decrease of Odd expression in the progenitors of mutant lymph glands when compared to controls (Figure 16). These results support our hypothesis that HH maintains Ush expression and Odd expression, a marker for hematopoietic progenitors.

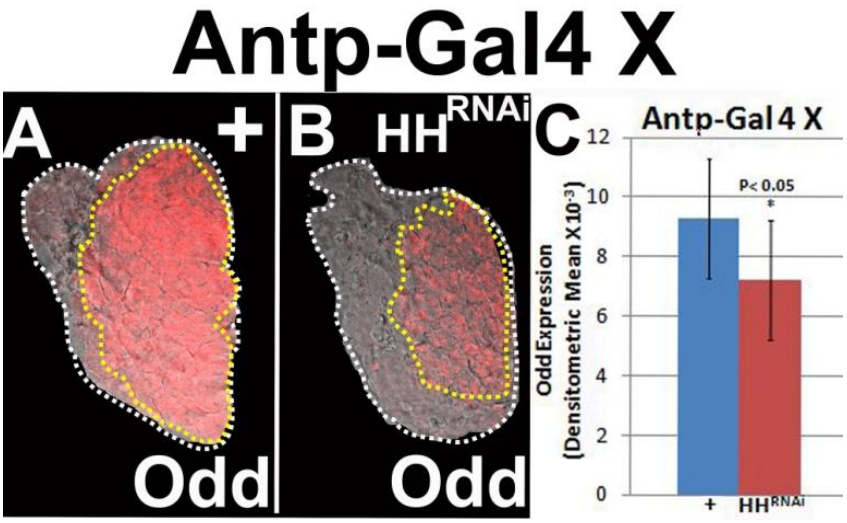

Figure 16. HH maintains Odd. Knockdown of HH results in the loss of Odd expression. **(A, B)** Antp-Gal4 drives the knockdown of HH (Antp>HH^{RNAi}) in the PSC of mutant lymph glands. Expression of Odd marker was significantly decreased in HH^{RNAi} mutant lymph glands when compared to controls. Red=Odd antibody that was used to identify progenitors. White dotted line outlines lymph gland primary lobe. Yellow dotted line outlines area of Odd expression. **(C)** Control (+) and mutant lymph gland (HH^{RNAi}) Odd expression quantified. Student's T-test used to analyze statistical significance. Results are expressed as densitometric mean X 10^{-3}. Error bars show standard deviation. P-value as shown. N=12. Gene expression analyses were conducted using lymph glands from mid-third instar larvae (collected 96 to 104 hours after egg laying).

We predicted that over-expression of Ptc, an inhibitor in the HH pathway, would result in a decrease of Ush expression in mutant lymph glands when compared to controls; our result supports this prediction (Figure 17). This result also provides further support to our hypothesis that Smo is acting as a canonical factor of HH signaling to maintain Ush and hematopoietic progenitors. We did not conduct knockdown studies of Ptc, because knockdown of Ptc appears to produce non-specific effects. Larvae from Ptc knockdowns were much smaller than controls and appeared to be developmentally delayed (Data not shown). This could impact Ush expression resulting in false negatives or false-positives.

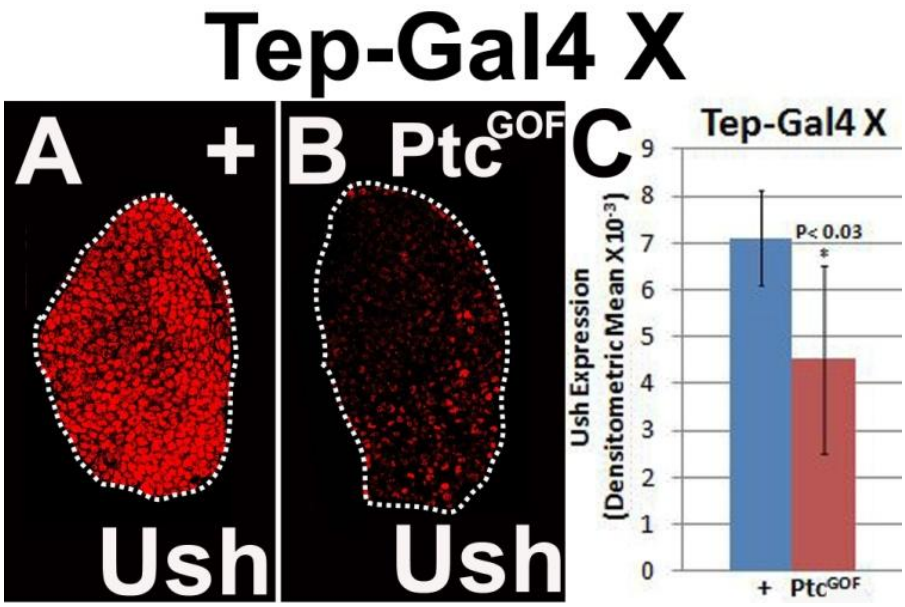

Figure 17. Ptc acts in hematopoietic progenitors of lymph gland to down-regulate Ush. Over-expression of the canonical HH pathway member Ptc decreases Ush expression. **(A, B)** Tep-Gal4 driving over-expression of Ptc (Ptc^{GOF}) in progenitor cells of the mutant lymph glands (*Tep>ptc^{GOF})* resulted in a significant decrease in Ush expression compared to controls. Red= antibody used to identify Ush. White dotted line outlines lymph gland primary lobe. **(C)** Control (+) and mutant lymph gland (Ptc^{GOF}) Ush expression quantified. Student's T-test used to analyze statistical significance. Results are expressed as densitometric mean X 10^{-3}. Error bars show standard deviation. P-value as shown. N=16. Gene expression analyses were conducted using lymph glands from mid-third instar larvae (collected 96 to 104 hours after egg laying).

Active Smo leads to the activation of the transcription factor Ci (Fossett, 2013; Huangfu and Anderson, 2006; Jiang et al., 2014; Mandal et al., 2007; Ingham and McMahon, 2001). We hypothesize that Ci is involved in maintaining Ush expression. Hence, we next tested the effect on Ush expression after knocking down Ci in the lymph gland. Knockdown of Ci expression in the lymph glands showed that the level of expression of Ush was significantly reduced in mutant lymph glands when compared to controls (Figure 18). From these results, we observed that Ci is required to maintain Ush

expression, giving further support to our hypothesis that canonical HH signaling maintains Ush.

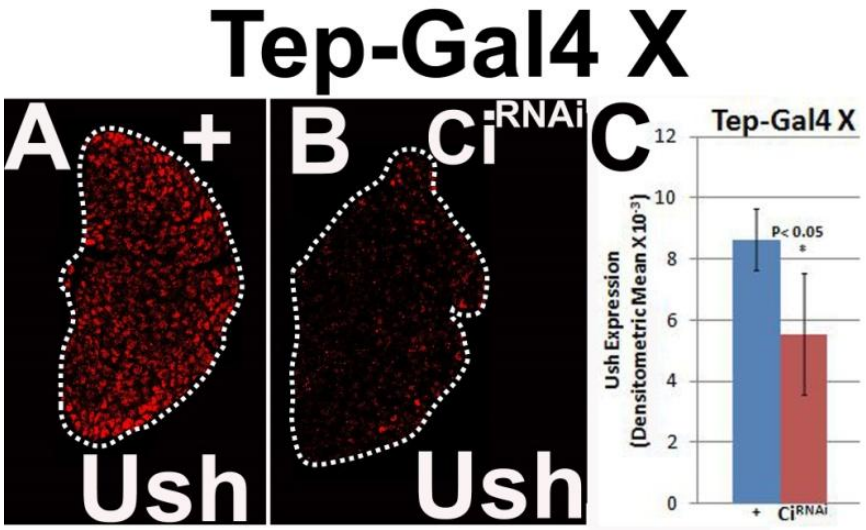

Figure 18. Ci acts in hematopoietic progenitors of lymph gland to maintain Ush. Knockdown of the canonical HH pathway member Ci decreases Ush expression. **(A, B)** Tep-Gal4 driving Ci knockout (Ci^{RNAi}) in progenitor cells (*Tep>ci*^{RNAi}) resulted in a statistically significant decrease in Ush expression compared to controls. Red= antibody used to identify Ush. White dotted line outlines lymph gland primary lobe. **(C)** Control (+) and mutant lymph gland (Ci^{RNAi}) Ush expression quantified. Student's T-test used to analyze statistical significance. Results are expressed as densitometric mean X 10^{-3}. Error bars show standard deviation. P-value as shown. N=16. Gene expression analyses were conducted using lymph glands from mid-third instar larvae (collected 96 to 104 hours after egg laying).

Overall, our results thus far support our hypothesis that canonical HH signaling is involved in maintaining Ush expression and hematopoietic progenitors in the *Drosophila* lymph gland. Our study also provides further evidence that one of the genes identified in our deficiency screen, *smo*, interacts with Ush and is required to block lamellocyte differentiation in *Drosophila*.

3.8 Baboon (Babo) Maintains Odd-Skipped, the Marker for Hematopoietic Progenitors and May Also be Involved in the Interaction between the HH Pathway and Ush.

A previous study describes the role of Babo, a conserved type I Activin receptor, in *Drosophila* development and characterizes aspects of the Babo intracellular signal-transduction pathway (Brummel et al., 1999). This study indicated that Babo signaling plays a role in regulating cell proliferation. Furthermore, another previous study showed that during head morphogenesis in *Drosophila*, Ptc interacts with Smo, leading to activation of Babo downstream and stimulation of cell proliferation in the eye-antennal disc (Shyamala and Bhat, 2002). Babo was also identified as a factor that is down-regulated in a *ush* mutant background during a previous microarray study by our lab (Data not shown). Therefore, we wanted to investigate if there was an interaction involving HH signaling, Babo and Ush in hematopoietic progenitors of *Drosophila.*

We hypothesize that Ush activates Babo to maintain hematopoietic progenitors. These observations suggested that Babo maintains hematopoietic progenitors in *Drosophila*. Babo was knocked down in mutant lymph glands and Odd expression was measured. Expression of Odd was significantly decreased in $Babo^{RNAi}$ mutant lymph glands when compared to controls (Figure 19). Our results support the notion that Babo is involved in the regulation of progenitor maintenance. Further studies are needed to provide support for our hypothesis that Ush activates Babo to maintain hematopoietic progenitors and to further establish the relationship between Babo and HH signaling in regards to hematopoiesis.

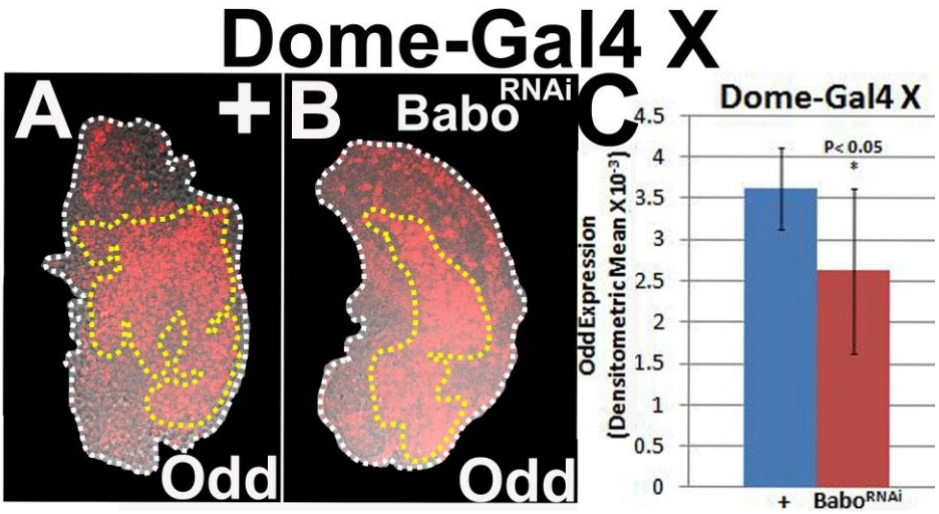

Figure 19. Babo maintains Odd. (A, B) Knockdown of Babo results in the loss of Odd expression. Dome-Gal4 drives the knockdown of Babo (Dome>Babo^{RNAi}) in the MZ of mutant lymph glands. Expression of Odd marker was significantly decreased in Babo^{RNAi} mutant lymph glands when compared to controls (+). Red=Odd antibody that was used to identify progenitor cells. White dotted line outlines lymph gland primary lobe. Yellow dotted line outlines area of Progenitor Expression. **(C)** Control (+) and mutant lymph gland (Babo^{RNAi}) Odd expression quantified. Student's t-test used to analyze statistical significance. Results are expressed as densitometric mean X 10^{-3}. Error bars show standard deviation. P-value as shown. N=16. Gene expression analyses were conducted using lymph glands from mid-third instar larvae (collected 96 to 104 hours after egg laying).

Chapter 4: Discussion

4.1 Nine Factors Identified in Genomic Functional Screen of *Drosophila* Chromosome 2L.

We identified and confirmed nine factors that interact with Ush to block lamellocyte differentiation. The factors identified were *smo,* which is involved in the HH pathway; *cactus*, which is a Toll-like pathway inhibitor; *ubc9,* which is involved in sumolyation; *eya*, a target of MLL; *e2f2,* which is involved in the cell cycle; *tbc1d1517*^{d05023}, a GATA-1 target gene; *thioredoxin,* an antioxidant; *porin,* which encodes a membrane pore-forming protein; and *intersectin*, an adaptor protein that interacts with RTKs and GTPases (Table 2). All of these factors, excluding *porin* and *intersectin*, have known roles in hematopoiesis.

Our screen showed that the principle of SSNC can be used to identify conserved hematopoietic regulators by identifying GATA:FOG homologue (Srp:Ush) network members at a rapid rate in the *Drosophila* model. Furthermore, our screen identified factors that are conserved across taxa, which not only regulate hematopoiesis, but also regulate the cell cycle and inflammatory reactions as well. Two notable genes identified were *e2f2* and *tbc1d1517*^{d05023}. These factors interact with GATA-1 during hematopoiesis in mammals (Kadri et al., 2009; Lewis et al., 2004; van den and Dyson, 2008), which demonstrated that our screen could identify conserved GATA:FOG network members. Finally, our screen can identify a diverse set of factors that act with *ush* to regulate hematopoiesis. Thus, information from our studies may identify important processes that regulate hematopoiesis.

Other notable factors identified were *smo* and *eya*. Eya is associated with a multi-functional protein family that has transactivation capacity and contains threonine and tyrosine phosphatase domains which mediate the interaction of Eya with a variety of key regulators of various biological processes (Zou et al., 2006). The mammalian homologue of Eya, Eya1, is expressed in HSPCs and up-regulated in ~25% of human leukemias (Wang et al., 2011). Thus, our screen is capable of identifying conserved factors that regulate hematopoiesis and are involved in leukemia.

Furthermore, as stated earlier, identifying factors that interact with the GATA:FOG complex is beneficial towards translational research. GATA, being a DNA binding protein, is generally considered to be a poor drug target (Zheng and Blobel, 2010). However, GATA factors work in combination with co-regulatory molecules to regulate transcription of GATA target genes (Zheng and Blobel, 2010). Thus, factors that interact with the GATA:FOG transcriptional complex could be targeted during therapeutic approaches for GATA factor related diseases, such as Parkinson's disease and many form of cancers (Zheng and Blobel, 2010).

In this study, we chose to investigate the interaction between Smo and Ush in greater depth. Smo is an activating factor of the HH signaling pathway. HH signaling is one of the primary regulators of animal development (Fossett, 2013; Huangfu and Anderson, 2006; Jiang et al., 2014; Mandal et al., 2007). This signaling pathway is conserved across taxa and is involved in embryogenesis and adult stem cell maintenance in humans (Fossett, 2013; Huangfu and Anderson, 2006; Mandal et al., 2007). Excess activation of the HH pathways can lead to tumor development in a variety of organs such as the brain, skin and prostate (Xie et al., 1998). A type of cancer that is commonly

associated with abnormal HH signaling is basal-cell carcinoma (Xie et al., 1998). Smo antagonists, such as Vismodegib (Erivedge®), have been developed to treat basal cell carcinoma (Atwood et al., 2014). Hence, if a connection between the GATA:FOG transcriptional complex and HH signaling could be established then it raises the prospect that more effective drug therapies for GATA related diseases could be developed that target HH signaling factors, such as Smo.

Further studies involving HH signaling and its targets are critical to basic and translational research involving cancers that are connected to abnormal signaling from HH and GATA factor related diseases. Finally, the advantages of utilizing *Drosophila*, as opposed to tissue culture in our screen, were that we were able to identify factors that can signal to other tissues. For example, our studies show that HH ligand, which signals from the niche, regulates Ush expression in hematopoietic progenitor cells.

4.2 The Stem Cell Niche Regulates Stem Cell Homeostasis in *Drosophila* through Canonical HH Signaling Interacting with Ush.

Smo can act canonically as a part of HH signaling and non-canonically as well, which was observed in the study that showed that Smo can act independently of Gli transcriptional activity to activate small RhoA GTPases that promote mouse fibroblast migration (Polizio et al., 2011). Therefore, we investigated how Smo interacts with Ush. We determined that Smo was required to maintain Ush expression (Figure 13); however Ush is not required for Smo expression (Figure 14). Our results showed loss of HH and Ci function also led to a down-regulation of Ush (Figures 15 & 18). Additionally, over-expressing the inhibitor of Smo, Ptc, led to a down regulation of Ush (Figure 17). These

results strongly suggest that Smo can maintain Ush expression and through canonical HH signaling. However, we cannot rule out that Smo also regulates Ush non-canonically. Further studies are needed to determine if Smo can maintain Ush through non-canonical processes. Moreover, we hypothesized that since Ci is a transcriptional factor; it likely interacts with Ush on the transcriptional level. However, further studies are needed to test this hypothesis by determining the level of regulation (transcriptional, translational or post-translational) between Ci and Ush.

Ush is required to maintain the progenitor pool. Thus we investigated how Smo and HH regulate hematopoietic progenitors. We used Odd as a marker for hematopoietic progenitors. Loss of Smo function significantly decreased Odd expression (Figure 12), while over-expression of Smo significantly increased Odd expression. Loss of HH function also led to a decrease in Odd expression (Figure 16). Further studies are needed to conclude whether Smo and HH are maintaining hematopoietic progenitors or just merely maintaining Odd expression. These studies will test the effect of loss of function of HH pathway members on the expression of E-Cadherin (E-Cad) and other markers specific for hematopoietic progenitor cells (Gao et al., 2013). However, it should be noted that loss of Ush or Odd leads to progenitor loss, which supports our hypothesis that HH signaling maintains progenitors by maintaining Ush. In addition, to further establish that the hematopoietic progenitor cells themselves are being regulated, a rescue study of HH loss of function by over-expressing Ush should be performed and hematopoietic progenitor population should be observed. Nevertheless, our study outlines a process of how canonical HH signaling regulates FOG to maintain hematopoietic progenitor multipotency.

4.3 Babo May Be a Downstream Target of HH signaling and Ush that Maintains Hematopoietic Progenitors.

Results from a previous study showed that Ptc interaction with Smo leads to the activation of Babo downstream, and stimulation of cell proliferation in the eye-antennal disc (Shyamala and Bhat, 2002). Results from our previous microarray study identified *babo* as a gene that is down-regulated in a *ush* mutant background (Data not shown). Thus, we hypothesized that Ush activates Babo to maintain hematopoietic progenitors. Our results support the notion that Babo is involved in the regulation of progenitor maintenance (Figure 19). Further studies are needed to confirm progenitor maintenance and establish a relationship between Babo, HH signaling and Ush.

4.4 GATA (Srp), which is Essential for HH Activation in Niche Cells, may be Inhibited by Stress.

Results from a previous study revealed that the GATA factor Serpent (Srp) is essential for HH activation in niche cells (Tokusumi et al., 2010). We hypothesized that stress inhibits expression of Srp which would also inhibit HH signaling and progenitor maintenance, leading to lamellocyte differentiation. Preliminary data from our lab shows that after inducing oxidative stress by knocking down ND75 (a key protein involved in the electron transport chain in *Drosophila*) in the PSC of mutant lymph glands, expression of Srp was significantly decreased when compared to controls (Data not shown). Preliminary data from our lab suggests that expression of HH is also decreased when ND75 is knocked down (Data not shown). These preliminary data, in addition to

the results of our study, suggest the possibility that an external stimulus, such as stress, can lead to the down-regulation of GATA in the stem cell niche. This would lead to the down-regulation of HH signaling, which would result in progenitor differentiation. However, further studies are needed to investigate this possibility, which would outline a molecular process of how stress can affect the microenvironment to regulate stem cell differentiation.

4.5 Proposed Model for HH Regulation of FOG and Hematopoietic Progenitor Maintenance.

We propose a model which outlines how canonical HH signaling activates FOG to maintain hematopoietic progenitors (Figure 20). Previous work has shown that HH is secreted as a ligand from the PSC/niche of the lymph gland. HH translocates to the progenitors of the lymph gland where it binds and inhibits the activity of its membrane receptor, Ptc (Fossett, 2013; Huangfu and Anderson, 2006; Mandal et al., 2007; Martinez-Agosto et al., 2007). When HH is not bound to Ptc, Ptc will inhibit translocation of Smo to the cell membrane. However, when Ptc activity is inhibited due to HH, Smo will be free to translocate to the membrane and become active (Huangfu and Anderson, 2006; Mandal et al., 2007; Martinez-Agosto et al., 2007). Active Smo leads to the activation of Ci, a transcription factor (Huangfu and Anderson, 2006; Jiang et al., 2014; Mandal et al., 2007; Martinez-Agosto et al., 2007). We have shown in this study that Ci is required for Ush in *Drosophila*. Our data also suggests that Smo and HH are required for hematopoietic progenitor maintenance. We hypothesize that Ci is a transcriptional activator of *ush*, but this has yet to be determined. Further studies are

needed to determine the mechanism of regulation between Ci and Ush: transcriptional, translational, or post-translational.

Our proposed model also outlines a hypothesized role for Babo. We hypothesize that Babo maintains hematopoietic progenitors as a downstream target of the HH pathway and Ush (Figure 20). Furthermore, we propose an additional supplementary model which shows how the external stimulus of stress would inhibit GATA, which would indirectly repress Ush through canonical HH signaling (Figure 20b). Our models show that under normal conditions, the molecular interactions discussed in our study will maintain hematopoietic progenitors; however, under conditions of stress, this pathway will be inhibited, leading to HSPC differentiation.

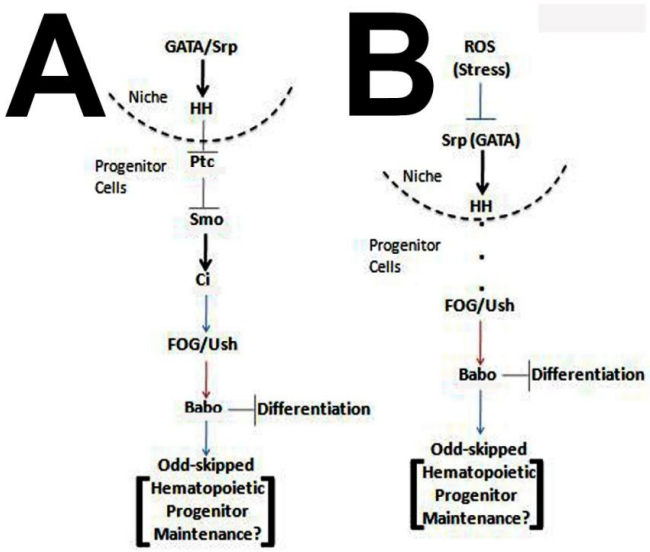

Figure 20. Proposed model for HH regulation of FOG and Hematopoietic Progenitor Maintenance. A) GATA (Srp) activates canonical HH signaling from the niche which maintains FOG (Ush) and Babo, blocking differentiation and maintaining hematopoietic progenitors. **B)** Stress inhibits GATA (Srp), which would also inhibit canonical HH signaling from the niche and its downstream effects. Thus, stress inhibits progenitor maintenance. Black thick arrows represent protein-protein interactions that have already been established in previous studies. Grey thin lines represent down-regulation. Blue arrows represent interactions confirmed in our study. Red arrows represent interactions that still need to be established for this proposed model.

4.6 Summary and Significance of Our Study.

Previous studies have shown that Srp:Ush (GATA:FOG) regulates HH (Mandal et al., 2007; Tokusumi et al., 2010). However, our study shows that canonical HH signaling maintains Ush expression. The results from our study suggest a negative feedback loop in which HH signaling up-regulates Ush expression; Ush then feeds back to block HH expression in progenitors through its interaction with Srp (GATA). Our study also outlines a molecular process of how the stem cell niche maintains stem cell homeostasis since HH signals from the niche. Thus, our screen is able to identify factors that can signal to other tissues, something that cannot be accomplished in an unbiased fashion using *in vitro* tissue culture studies. Since the HH pathway is conserved, it is possible that the relationship observed in our study between GATA:FOG and HH signaling in the *Drosophila* lymph gland may also be similar to the processes that regulate mammalian hematopoietic progenitor cell maintenance and hematopoietic stem-cell self renewal in the mammalian bone marrow. Our studies may identify conserved regulatory processes whereby signals from the microenvironment control progenitor and stem-cell differentiation, thus providing more information in regards to stem-cell biology which will be beneficial towards biomedical research relating to areas such as cancer therapy and regenerative medicine.

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