Title

Yorkie and Scalloped signaling regulates progenitor maintenance and differentiation in Drosophila hematopoiesis

Author

Ferguson, Gabriel Benjamin

Publication Date

2014

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

Yorkie and Scalloped signaling regulates progenitor maintenance and differentiation in *Drosophila* hematopoiesis

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Molecular Biology

by

Gabriel Benjamin Ferguson

2014

ABSTRACT OF THE DISSERTATION

Yorkie and Scalloped signaling regulates progenitor maintenance and differentiation in *Drosophila* hematopoiesis

by

Gabriel Benjamin Ferguson

Doctor of Philosophy in Molecular Biology Interdepartmental Ph.D Program

University of California, Los Angeles, 2014

Professor Julian Martinez-Agosto, Chair

Hematopoiesis is a highly complex developmental process that is remarkably conserved at the molecular level across higher order eukaryotes. An organ termed the lymph gland resides within the *Drosophila* larva which is the site of the majority of hematopoietic processes in the organism. The lymph gland contains a population of differentiating blood cells in addition to a pool of quiescent hematopoietic progenitors that are maintained by a small niche. While there has been a number of signaling pathways and mechanisms characterized in the regulation of lymph gland homeostasis, questions still remain unanswered in regards to the nature and requirement of progenitor cells and the mechanisms that promote differentiation in the lymph gland.

In this dissertation, we use genetic approaches to characterize the roles of the Hippo Pathway effectors Yorkie and Scalloped in regulating the maintenance and differentiation of hematopoietic progenitors through modulation of conserved signaling pathways. We show that differentiation of the crystal cell lineage of hemocytes is reliant on Yorkie and Scalloped dependent regulation of the Notch ligand Serrate in lineage specifying cells. Furthermore, Scalloped regulates organ size and differentiation of progenitors through modulation of the secreted factor PVF2 and its receptor PVR, respectively. Finally, we utilized an immune challenge model to interrogate the function of Yorkie and Scalloped in the cell fate decisions that are incurred during the cellular immune response induced by wasp parasitizaion. Using this immune challenge model, we demonstrated that wasp parasitization induces unique changes to Yorkie and Scalloped expression in the lymph gland. We also report that Serrate down-regulation in lineage specifying cells is critical for the cellular immune response, leading to a loss in crystal cell differentiation. Finally, we demonstrate a unique mechanism where-in Scalloped expression is enforced in the Posterior Signaling Center (PSC) of the lymph gland upon immune challenge by wasp parasitization as Scalloped function is required for maintenance of the PSC and differentiation of lamellocytes in the cellular immune response.

The dissertation of Gabriel Benjamin Ferguson is approved.

Volker Hartenstein

Amander Clark

Julian Martinez-Agosto, Committee Chair

University of California, Los Angeles

TABLE OF CONTENTS

LIST OF FIGURES

Chapter 2

Chapter 3

Appendix

LIST OF ABBREVIATIONS

4EBP:	translation initiation factor 4E-binding protein
ADGF:	Adenosine deaminase-related growth factor
AdoR:	Adenosine Receptor
Antp:	Antennapedia
Bc:	Black cells
CC:	Crystal cell
CCP:	Crystal cell progenitor
Col:	Collier
CZ:	Cortical Zone
Diap1:	*Drosophila* inhibitor of apoptosis
Dome:	Domeless
ECM:	extra-cellular matrix
Ex:	Expanded
EGF:	Epidermal Growtn Factor
EGFR:	Epidermal Growth Factor Receptor
FGF:	Fibroblast Growth Factor

FGFR:	Fibroblast Growth Factor Receptor
GFP:	Green Fluorescent Protein
Hh:	Hedgehog
HipK:	Homeodomain interacting protein kinase
HLT:	Hand Lineage Tracing
Hml:	Hemolectin
Hop:	Hopscotch
Hpo:	Hippo
Htl:	Heartless
IP:	intermediate progenitor
JAK/STAT:	Janus Tyrosine Kinase/Signal Transducers and Activators of Transcription
JNK:	c-Jun N-terminal kinases
LG:	Lymph Gland
LSC:	Lineage Specifying Cell
LT:	lineage traced
Lz:	Lozenge
MARCM:	mosaic analysis with a repressible cell marker

MZ: Medullary Zone

N: Notch

N^{Act}: activated Notch

N^{DN}: Notch dominant negative

p4EBP: phosphorylated-4EBP

PH: prohemocyte

pH3: phosphorylated Histone H3

PKA: Protein kinase A

PL: Plasmatocyte

ProPO: Prophenoloxidase

PSC: Posterior Signaling Center

PTEN: Phosphatase and Tensin homolog

PVF: Platelet Derived Growth Factor/Vascular Endothelial Growth Factor

PVR: Platelet Derived Growth Factor/Vascular Endothelial Growth Factor Receptor

Pxn: Peroxidasin

ROS: Reactive Oxygen Species

Sd: Scalloped

Sd^{Hypo}: Scalloped hypomorph

Ser: Serrate

Ser^{DN}: Serrate dominant negative

Srp: Serpent

STAT: Signal Transducers and Activators of Transcription

$STAT^{Act}$: activated STAT

TEAD: TEA domain

TOR: Target of Rapamycin

Trol: Terribly reduced optic lobes

TSC: Tuberous Sclerosis Complex

UAS: Upstream Activation Sequence

Ush: U-shaped

Wg: Wingless

WT: wild-type

Wts: Warts

YAP: Yes-associated protein

Yki: Yorkie

Yki^{Act}: activated Yorkie

Yki^{WT}: wild-type Yorkie

ACKNOWLEDGEMENTS

I would like to first thank my mentor, Julian Martinez-Agosto. His continued guidance, support, and incredible patience have allowed me to become the scientist that I am today. He has always been available to answer question or to spend long hours working together on writing a paper. His intelligence and passion for research have been an inspiration throughout my time as a graduate student. He has always been willing to listen to my ideas and allow me to steer my own projects, even through the toughest challenges, while providing me with the expertise and criticism necessary to progress. No crazy idea or experiment was ever out of question, and for that I am extremely grateful.

I would also like to thank my committee members, Volker Hartenstein, Amander Clark, Gay Crooks, and Albert Courey for their continued advice and guidance. It has been a long process, and they have always been patient and supportive of my projects.

I am also extremely grateful to my fellow lab-mates over the years, not only for their advice and willingness to assist in the lab, but also for making the laboratory environment a fun experience. I would particularly like to thank Michelle Dragojlovic and Steve Klein who have always been available to give advice, answer questions, or provide assistance.

I also need to thank the Banerjee lab, especially Suchandra Ghosh, for providing countless reagents, and I would also like to thank Lisa and John Fessler for providing the Peroxidasin antibody which has been used in a vast number of experiments.

Finally, I am incredibly grateful to my parents for their constant support of my pursuits in science. Even when I sometimes doubted myself, they have always had full confidence in my ability.

Chapter 2 is the final version of the manuscript: “Yorkie and Scalloped signaling regulates Notch dependent lineage specification during *Drosophila* hematopoiesis”, which has been accepted at *Current Biology* and is currently awaiting publication.

VITA

2003-2007	B.S. Molecular and Cellular Biology Minor in Psychological and Brain Sciences Johns Hopkins University Cumulative GPA: 3.32
Fall 2013	Teaching Assistant, Developmental Biology (MCDB 138) Department of Molecular, Cell and Developmental Biology
2009-2012	Cellular and Molecular Biology (CMB) Training Grant (UCLA)
Spring 2010	Teaching Assistant, Molecular Biology (MCDB 144) Department of Molecular, Cell and Developmental Biology
Winter 2009	Teaching Assistant, Molecular Biology (MCDB 144) Department of Molecular, Cell and Developmental Biology
2007-2009	Eugene V. Cota Robles Fellowship (UCLA)
2007	Tri Beta Biology Honors Society
Spring, Fall 2005	Dean's List
Spring 2004	Dean's List

PUBLICATIONS

Ferguson, Gabriel B., Martinez-Agosto, J.A. Yorkie and Scalloped signaling regulates Notch dependent lineage specification during *Drosophila* hematopoiesis, in press *Current Biology*.

Chapter 1:

Introduction

Drosophila melanogaster has been utilized as a developmental model for decades, and as our knowledge of the intricacies of development expand, the high level of conservation between *Drosophila* and higher organisms has become increasingly more apparent. This conservation is not lost in the hematopoietic system of the fruit fly. Conserved factors like GATA and Runx are required for the earliest specification and differentiation of *Drosophila* blood cells while specification of the *Drosophila* hematopietic organ, termed the lymph gland, is dependent on highly conserved signaling pathways such as Notch and Wingless. The genetic tools available in *Drosophila* also greatly facilitate the ability to identify and characterize the functions of the molecular machinery that underlie developmental processes. Utilization of the *gal-4/UAS* system allows for spatially and temporally controlled over-expression or knockdown with RNA interference to interrogate the function of novel genes. In the lymph gland, populations of hematopoietic progenitors, signaling cells, and mature blood lineages can all be independently manipulated by this system. In addition, other genetic tools can be utilized for lineage tracing analysis or to generate mutant clones. In total, the *Drosophila* lymph gland is an ideal system to explore the mechanisms which regulate signaling, progenitor maintenance, and differentiation in a highly relevant developmental model. In the following review, we address the origins of hemocyte specification, and describe the advancements that have been made in understanding the mechanisms which regulate *Drosophila* hematopoiesis.

Part I: Hematopoiesis in *Drosophila*: Specification and description of hematpoietic tissues and hemocyte lineages.

A. Two distinct waves of hematopoiesis are observed in *Drosophila*

In *Drosophila*, there are two waves of hematopoiesis which occur independently in the developing organism. In the first wave, hemocytes (*Drosophila* blood cells) are specified in the head mesoderm of the late stage 10 embryo (Tepass et al., 1994). By late stage 11, about 700 total hemocytes are observed in the embryo and will differentiate into macrophage-like plasmatocytes (Tepass et al., 1994; Lebestky et al., 2000) and crystal cells (Rizki and Rizki, 1959) which aid in wound healing and the immune response (Rizki and Rizki, 1984; Galko and Krasnow, 2004). By embryonic stage 12, the majority of hemocytes begin migrating away from the head mesoderm while differentiating into plasmatocytes. Hemocytes differentiating into crystal cells do not migrate, and remain in the head mesoderm. The undifferentiated progenitors, termed prohemocytes (Shrestha and Gateff, 1982), are specified by the GATA factor Serpent which is essential for *Drosophila* hematopoiesis (Rehorn et al., 1996). Hemocytes specified in the embryo, will populate the embryo and circulate throughout the hemolymph of the developing larva (Holz et al., 2003).

The second wave of hematopoiesis occurs in the cardiogenic mesoderm of the late embryo where a small cluster of *Odd-skipped* expressing cells turn on Serpent, specifying them to a hematopoietic fate (Mandal et al., 2004). This cluster of Srp+/Odd+ cells make up the early lymph gland, which is the hematopoietic organ in *Drosophila.* The lymph gland will eventually proliferate and expand into a multi-lobed organ that has primary, secondary, and tertiary lobes that develop parallel along either side of the dorsal vessel (Shrestha and Gateff,

1982). Expression of *Odd* and subsequent formation of the lymph gland is tightly regulated in the dorsal (cardiogenic) mesoderm. Loss of *tinman*, *dpp* (homolog Transforming growth factor B), *heartless* (homolog for Fibroblast growth factor receptor), or *wingless* (homolog of Wnt) severely perturb *Odd* expression (Mandal et al., 2004). Conversely, Notch signaling negatively regulates cardiogenic development early in the dorsal mesoderm, but is later required for lymph gland specification as loss of Notch results in a severely underdeveloped lymph gland and increase in cardioblast numbers, while activation of Notch significantly increases lymph gland cell numbers (Mandal et al., 2004).

The majority of the cells in the early lymph gland are prohemocytes, but there is a separate population of cells located at the posterior tip of the primary lobe that is termed the Posterior Signaling Center or PSC (Lebestky et al., 2003). The PSC is also specified in the late embryo by the Hox genes *Homothorax* and *Antennapedia* (Antp) (Mandal et al., 2007), and expression of the latter is maintained in the PSC throughout larval development. This cluster of cells also expresses the Notch ligand Serrate (Ser) (Lebestky et al., 2003)and the transcription factor Collier (Col) (Crozatier et al., 2004). *col* mutant larvae do not express Ser and display a phenotype where prohemocytes are depleted at the expense of differentiating plasmatocytes and crystal cells (Crozatier et al., 2004). Notch signaling through Ser also functions to maintain the PSC as over-expression of a dominant negative form of Ser specifically in the PSC inhibits *col* expression, and induces a loss of prohemocytes although there is not a strong increase in terminally differentiated plasmatocytes (Krzemien et al., 2007).

B. *Drosophila* hemocyte lineages.

Prohemocytes of the lymph gland proliferate through the first 2 instars until they become quiescent in the third instar, forming a pool of multi-potent progenitors termed the Medullary Zone (Jung et al., 2005). These prohemocytes of the Medullary Zone are densely packed together and express high levels of the E-Cadherin protein Shotgun, the receptor tyrosine kinase *domeless* (dome) (Jung et al., 2005), and *tepIV*, a Thioester containing protein (Irving et al., 2005). While there is still some debate about the nature of prohemocytes, it has been definitively demonstrated that *dome+* cells of the Medullary Zone give rise to the differentiated hemocytes found in the periphery of the lymph gland termed the Cortical Zone (Jung et al., 2005). A recent study demonstrated that MARCM clones generated at different developmental time points in the first instar lymph gland have varying sizes and encompass differing populations of cells. Clones generated in the late embryo and early first instar larva are generally larger and span both the Medullary Zone and Cortical Zone, while clones generated in the late first instar or later were generally smaller and more restricted to the Cortical Zone. These findings suggest that not all prohemocytes have the same developmental capacity and that a bonafide hematopoietic stem cell may be present in a mixed prohemocyte population (Minakhina and Steward, 2010). The same study identified Zfrp8 as a potential regulator of prohemocyte potential as *Zfrp8* mutant clones were smaller and contained far fewer Medullary Zone cells (Minakhina and Steward, 2010).

In the wild type organ, the first differentiating hemocytes are observed at the periphery of the lymph gland about midway through the second instar (Jung et al., 2005). This population of differentiating cells expresses the extracellular proteins Hemolectin (Hml) and Peroxidasin (Pxn), and expands throughout the third instar forming the Cortical Zone.

Differentiating hemocytes also express high levels of the PDGF and VEGF related Receptor (PVR) (Jung et al., 2005), but PVR is not expressed in the PSC. An intermediate population of cells has also been identified in the lymph gland (Krzemien et al., 2010; Dragojlovic-Munther and Martinez-Agosto, 2012). These Intermediate Progenitors express both markers for prohemocytes (*dome*+) and differentiating hemocytes (Pxn+) (Dragojlovic-Munther and Martinez-Agosto, 2012). Intermediate progenitors were first characterized as a population of cells that was not terminally differentiated, but undergo active cell-division (Krzemien et al., 2010), forming a quasi-transient amplifying population. Intermediate progenitors will eventually terminally differentiate into plasmatocytes expressing Nimrod (P1 antigen) or crystal cells in the WT lymph gland.

Two types of mature hemocytes populate the Cortical Zone: plasmatocytes and crystal cells, the same lineages that differentiate in early embryonic hematopoiesis. Plasmatocytes comprise the vast majority (~95%) of mature hemocytes in the lymph gland (Rizki, 1957; Rizki and Rizki, 1984; Lanot et al., 2001), and are identified by expression of *Hml*, *Pxn*, *eate*r (Kroeger et al., 2012), *and Nimrod* (P1 antigen)(Kurucz et al., 2007). *Hml*, *Pxn*, and *eater* are early differentiation markers while P1 labels terminally differentiated hemocytes. Plasmatocytes are small phagocytic cells whose primary function is to eat cellular debris caused by apoptosis. This function is most notable during metamorphosis as widescale remodeling of the organism's body plan occurs requiring degradation of larval constructs. Plasmatocytes also secrete extracellular matrix proteins like Peroxidasin, Laminin, and Collagens (R E Nelson, 1994; Murray et al., 1995; Yasothornsrikul et al., 1997) that are deposited on the Basement Membrane. The phagocytic function of plasmatocytes also aids in the innate immune response as they can devour smaller invading pathogens while

also secreting small anti-microbial peptides (C Samakovlis, 1990; Dimarcq et al., 1997; Roos et al., 1998). The transcription factor Glial cells missing (gcm)is expressed in plasmatocytes and is required for their differentiation in the embryo downstream of Srp (Valérie and Giangrande, 1997). Forced expression of *gcm* in crystal cell progenitors blocks crystal cell maturation and instructs these cells into the plasmatocyte lineage demonstrating that *gcm* is both necessary and sufficient to induce plasmatocyte differentiation (Lebestky et al., 2000).

Crystal cells make up the other 5% of mature hemocytes populating the Cortical Zone, with about 30-50 crystal cells observed in the primary lobe of the WT lymph gland (Lanot et al., 2001). Crystal cells are specified by the transcription factor Lozenge (Lz), the *Drosophila* homolog of Runx1, which is essential for their development(Lebestky et al., 2000). Lz labels both crystal cell progenitors and mature crystal cells, and is required for activation of the gene *Black-cells* which encodes Prophenoloxidase A1 (Gajewski et al., 2007). Mature crystal cells are identified by the crystalline inclusions containing Prophenoloxidase (ProPO) melanizing enzymes that are readily observed in the cytoplasm (Rizki and Rizki, 1980; Shrestha and Gateff, 1982; Lanot et al., 2001; Gajewski et al., 2007). The ProPOs (ProPO I and ProPO II)expressed in crystal cells are released and then cleaved to make phenoloxidases which are critical for the wound healing process (Galko and Krasnow, 2004) and also help the innate immune system combat invading pathogens (Nappi et al., 2009). Cleavage of ProPOs produces reactive oxygen species which are thought to be toxic to many bacteria. Indeed, the presence of ProPOs is required for survival and proper immune response to multiple strains of gram positive bacteria and fungi (Binggeli et al., 2014).

Lamellocytes are a third type of mature hemocyte that are only observed in the larval stages (Lanot et al., 2001). These large, flat, adherent cells surround and encapsulate invading pathogens too large to be phagocytosed (Nappi, 1975; Rizki and Rizki, 1992). Lamellocytes are identified by their unique morphology, increased levels of F-actin, and by expression of *misshapen* (Lanot et al., 2001)and L1 (Asha et al., 2003). While these cells are rarely observed in circulation or in lymph glands of WT animals, an innate immune response is mounted by the *Drosophila* larva when infected by various pathogens or parasites. A common method for inciting this immune response is infestation by the parasitic wasp *L. Boulardi* (Rizki and Rizki, 1992). Large numbers of lamellocytes differentiate both in circulation and within the lymph gland to combat the eggs which are laid in the larvae by *L. Boulardi* (Lanot et al., 2001; Sorrentino et al., 2002). This cellular immune response model has been implemented as a means to identify various signaling pathways and genes that have roles in regulating hemocyte differentiation and proliferation in *Drosophila.*

Part II. Multiple elements regulate the maintenance and differentiation of *Drosophila* hemocytes in the lymph gland.

A. Growth signaling pathways maintain a pool of hematopoietic progenitors

It has been demonstrated that a functional PSC is required for the maintenance of lymph gland prohemocytes (Crozatier et al., 2004). Interestingly, expansion of the PSC by over-expression of *Antp* leads to a similarly expanded Medullary Zone at the expense of plasmatocytes and crystal cells. Antp expression is absent in *col* mutants, and *Antp* hypomorphic lymph glands display a similar increase in differentiation further demonstrating the role of Col in PSC maintenance (Crozatier et al., 2004; Mandal et al., 2007). It was also demonstrated that *Hedgehog* (Hh) is specifically expressed in the PSC and *Hh* mutant larvae have fully differentiated lymph glands while still expressing Antp, suggesting Hh is not required for PSC specification. The Hh receptor Patched (Ptc) is expressed in the Medullary Zone and activated Cubitus Interuptus (Ci) is also observed in this population, demonstrating active Hh signaling. These results describe a model in which the PSC acts as a hematopoietic niche that secretes Hh required for the maintenance of lymph gland prohemocytes (Mandal et al., 2007).

A recent report has expanded upon the mechanism regulating Hh expression in the PSC, as Srp was shown to directly promote Hh expression in the lymph gland, while Supressor of Hairless (Su(H)) functions to inhibit Hh (Tokusumi et al., 2010). In this study it was demonstrated that *hh* expression is completely ablated when Srp is depleted in the PSC, although PSC identity is not lost as Antp expression is still observed. *Su(H)* mutant lymph glands display increased expression of *hh* and ectopic *hh* is observed in the Medullary Zone

where it is not present in WT lymph glands. Srp also functions with the Friend of Gata protein U-shaped (Ush) to inhibit *hh* outside of the PSC. Over-expression of *ush* in the PSC blocks *hh* expression, and *ush* mutant lymph glands display ectopic *hh* expression throughout the lymph gland, similar to Su(H) mutants (Tokusumi et al., 2010).

Another report has demonstrated the necessity for Col in maintaining Hh, as depletion of *col* in the PSC completely ablates *hh* expression (Pennetier et al., 2012). Furthermore, Col was shown to regulate expression of the heparin sulfate proteoglycan, Dally-like (Dlp) in the PSC. Dlp is strongly expressed in the WT PSC, and loss of *dlp* causes a significant increase in PSC size that is also observed upon knockdown of *col*. Loss of *col* also inhibits BMP signaling in the PSC, as indicated by decreased *Daughters against dpp* reporter activity. Inhibition of BMP signaling by over-expression of the Type IV BMP receptor also causes a significant increase in PSC size due to increased proliferation of PSC cells, accompanied by expansion of prohemocytes at the expense of mature hemocytes in the lymph gland. Dlp is known to regulate BMP signaling, so these findings demonstrate a mechanism where Col controls PSC size through Dlp and BMP signaling. BMP signaling also regulates PSC size, by inhibiting expression of *Drosophila* Myc (dMyc). dMyc is normally expressed throughout the lymph gland, but is excluded from the PSC. Inhibition of BMP signaling causes ectopic dMyc expression in the PSC, and over-expression of dMyc in the PSC causes a robust increase in PSC size. The PSC size increase induced by inhibition of BMP signaling can also be rescued by knockdown of *dm*, the gene encoding dMyc, demonstrating the epistatic relationship of dMyc and BMP. Finally, PSC size is also regulated by Wingless (Wg) (Sinenko et al., 2009). Over-expression of *wg* in the PSC causes a significant increase in PSC

size that can also by rescued by depletion of *dm*, suggesting that Wg mediates PSC size through dMyc (Pennetier et al., 2012).

In addition to the Hh signal that emanates from the PSC, there is autonomous expression of Wg amongst prohemocytes which signals through its receptor Frizzled to help maintain progenitor identity. Disruption of this signaling cascade significantly reduces Shotgun expression and allows for increased differentiation of hemocytes although the medullary zone as a whole is not lost (Sinenko et al., 2009). Activation of Wg signaling in prohemocytes does expand the Medullary Zone resulting in a lymph gland that is almost completely lacking any differentiating hemocytes. Therefore, Hh signaling is requisite for prohemocyte maintenance while over-expression of *hh* in the medullary zone does not expand (Tokusumi et al., 2010) the prohemocyte population. Conversely, Wg signaling is not necessary for prohemocyte maintenance, but enforced Wg signaling in the medullary zone is sufficient to block prohemocyte differentiation.

It has been recently reported that Bag of Marbles (Bam), a germ line differentiation factor, also regulates prohemocyte maintenance (Tokusumi et al., 2011). Bam is expressed specifically in the Medullary Zone of the lymph gland, and *bam* mutants display precocious differentiation of plasmatocytes, crystal cells, and lamellocytes at the expense of *dome+* prohemocytes. Conversely, over-expression of *bam* in the Medullary Zone leads to expansion of *dome+* prohemocytes while terminally differentiated plasmatocytes are absent. Bam also regulates prohemocytes cell-autonomously as the PSC is not affected by specific down-regulation of *bam* in the Medullary Zone although excessive differentiation of plasmatocytes is still observed (Tokusumi et al., 2011). Bam has previously been shown to be a target of mir-7 regulation (Pek et al., 2009), and *mir-7* expression is also observed in the Medullary

Zone indicating a similar negative regulatory function may be present in the lymph gland. However, *mir-7* mutant lymph glands have reduced numbers of *dome+* prohemocytes similar to *bam* mutants, and also display increased differentiation of plasmatocytes and lamellocytes. Prohemocyte specific over-expression of *mir-7* greatly expands the Medullary Zone at the expense of differentiating plasmatocytes and *mir-7;bam* heterozygotes closely resemble *mir-7* or *bam* homozygotes indicating a cooperative function. Furthermore, Bam expression is increased in the lymph gland upon Medullary Zone specific over-expression of *mir-7* indicating a different relationship than that observed in the germ line (Tokusumi et al., 2011).

A previous study had demonstrated that over-expression of an activated form of the ETS factor *yan* with *hemese-gal4,* a pan-hemocyte driver, gives rise to increased numbers of circulating hemocytes, including crystal cells and lamellocytes (Zettervall et al., 2004). Given that *mir-7* negatively regulates *yan* in the eye, it became a likely candidate as a downstream effector of Bam and mir-7 in the lymph gland. Indeed, Yan is expressed in the lymph gland, and is observed most highly in a subset of cells that express neither prohemocyte nor differentiation markers suggesting it labels a population of intermediate progenitors (Tokusumi et al., 2011). *yan* mutant lymph glands have phenotypes opposite of *bam/mir-7* displaying expansion of *dome+* prohemocytes throughout the organ. Conversely, overexpression of *yan* in the Medullary Zone causes differentiation of the entire lymph gland consistent with the previous report (Zettervall et al., 2004; Tokusumi et al., 2011). *mir-7* and *bam* mutants have increased levels of Yan observed throughout the organ, while over-expression of *mir-7* or *bam* represses Yan expression. These findings demonstrate an important regulatory function for Bam and mir-7 in prohemocyte maintenance by restricting expression of Yan.

An important role for Sumoylation has recently been identified for promoting prohemocyte quiescence and maintaining them in their undifferentiated state within the lymph gland. Larvae mutant for the U2 SUMO-conjugating enzyme, *Ubc9,* display a significant loss of d*ome+* prohemocytes in addition to detachment and hypertrophy of both primary and secondary lymph gland lobes (Kalamarz et al., 2011). *Ubc9* mutant lymph glands also display increased differentiation of plasmatocytes, lamellocytes and expansion of hemocytes expressing the GFP trap line *ZCL2897*. Importantly, microtumors observed in the hemolymph also express *ZCL2897* demonstrating that they arise from the lymph gland. Loss of function mutants in other Sumoylation enzymes *Aos1* and *PIAS*, similarly display increased differentiation of lamellocytes, expansion of *ZCL2987* expression, and tumors in the hemolymph. *Dome+* progenitors are also lost in backgrounds where E1 subunits *Aos1* and *Uba2* are downregulated in prohemocytes confirming the requirement for the SUMO pathway in regulating prohemocyte maintenance. Furthermore, *Ubc9* mutants display substantial overproliferation of progenitors in the secondary lobes, and primary lobes have significantly reduced levels of the CDK inhibitor Dacapo. Over-expression of *Dacapo* or the human cell cycle inhibitor *p21* leads to strong reduction of prohemcoyte number, indicating sensitivity to cell cycle inhibitors in this population of cells, and *p21 or Dacapo* over-expression rescues the *Ubc9* mutant phenotype (Kalamarz et al., 2011). Taken together, Sumoylation confers quiescence and resistance to tumor formation in prohemeocytes of the lymph gland by regulating cell cycle entry through the CDK inhibitor *Dacapo.*

Signaling pathways in the lymph gland have also been shown to regulate prohemocyte maintenance by responding to extrinsic factors like availability of nutrients. A recent report demonstrated increased differentiation of hemocytes in the lymph gland at the

expense of prohemocytes during starvation conditions (Shim et al., 2012). There is a marked increase in the numbers of plasmatocytes and lamellocytes in the lymph gland as well as in circulation. Furthermore, prohemocytes express the Insulin Receptor (InR), and are sensitive to levels of *Drosophila* Insulin Like Peptides 2 (dILP2) secreted from the brain. Depletion of InR specifically in prohemocytes causes increased differentiation similar to the starvation phenotype. Additionally, depletion or inhibition of the downstream effectors AKT and TOR also cause increased differentiation, while over-expression of Rheb expands the progenitor population, even under starvation conditions (Shim et al., 2012). Essential amino acids which are found in the hemolymph are also directly sensed by prohemocytes. Increased differentiation in starved larvae was rescued when supplemented by essential amino acids, but not when supplemented by non-essential amino acids or sucrose. Finally, it was determined that Insulin Receptor signaling through TOR directly modulates levels of Wg in prohemocytes and over-expression of Wg is able to rescue both the starved and InR depletion phenotypes (Shim et al., 2012).

Two parallel publications took a more in depth look at the role of Insulin and TOR signaling in the lymph gland. One study examined PTEN loss of function and its effect on the PSC in regulating prohemocyte maintenance (Benmimoun et al., 2012). PTEN mutants displayed increased differentiation of hemocytes at the expense of prohemocytes, while also causing an increase in the number of PSC cells. Activation of Insulin signaling by depletion of PTEN or InR or over-expression of activated PI3K specifically in the PSC all cause an increased number of PSC cells, while TSC1/TSC2 or PTEN over-expression severely constricts PSC size (Benmimoun et al., 2012). Interestingly, depletion of TSC1 does not alter PSC cell number, but does cause increase in cell size. These changes to PSC size also affect

hemocyte differentiation as numbers of both plasmatocytes and crystal cells are increased (Benmimoun et al., 2012).

The second study closely examined the roles of PTEN and TSC amongst prohemocytes in regulating growth and differentiation. Depletion of PTEN or TSC2 lead to significant increases in lymph gland size coupled with significantly higher levels of proliferation through the 2nd and early 3rd instars, while PTEN knockdown specifically causes a significant expansion of *dome*+ pxn+ intermediate progenitors (Dragojlovic-Munther and Martinez-Agosto, 2012). Both PTEN and TSC2 knockdown cause a significant increase in p4EBP levels, a downstream effector of TOR signaling. Interestingly, there is a strong correlation in second instar lymph glands between p4EBP+ cells and phH3+ cells, in both WT and PTEN or TSC2 depleted lymph glands, although the percentage of double positive cells is increased upon PTEN or TSC2 depletion. By the mid-3rd instar, high levels of p4EBP and TOR activity are observed specifically along the border of the Cortical Zone and Medullary Zone, perhaps labeling the intermediate progenitor population. The number of p4EBP+ cells expands greatly in PTEN or TSC2 deficient backgrounds correlating with an expanding population of *dome*+Pxn+ Intermediate Progenitors. Interestingly, by the late 3rd instar, lymph glands with prohemocyte specific knockdown of PTEN and TSC2 are significantly larger. While p4EBP levels are still increased there is a divergence in where TOR activity is observed. In PTEN depleted lymph glands p4EBP is observed in the cortical zone, but in TSC2 depleted lymph glands p4EBP is seen in the prohemocytes of the medullary zone, suggesting there may be non-autonomous activation upon PTEN depletion (Dragojlovic-Munther and Martinez-Agosto, 2012).

This divergence between TSC and PTEN is also observed when characterizing other hemocyte lineages in lymph gland. In the late third instar lymph gland TSC2 depletion in prohemocytes causes the population of Intermediate Progenitors to expand throughout what should be the entire medullary zone, consistent with p4EBP levels observed in this background. Additionally, there is a marked differentiation of lamellocytes observed upon TSC2 knockdown (Dragojlovic-Munther and Martinez-Agosto, 2012). Conversely, PTEN knockdown does not expand Intermediate Progenitors or induce lamellocyte differentiation, but a massive increase in crystal cells is observed while there is a decrease in crystal cells upon TSC2 depletion. This dichotomy between crystal cell and lamellocyte differentiation further complicates the question as to whether or not a common progenitor gives rise to these lineages, and whether or not cell fate decisions are competitive (does one preclude the other) or simply dependent on receiving different signals.

Finally, growth phenotypes observed upon depletion of PTEN or TSC2 in prohemocytes are significantly ameliorated by inhibition of TOR through Rapamycin or environmental stresses like hypoxia and starvation, further establishing the role of Insulin signaling specifically through TOR. Furthermore, phenotypes observed upon TOR activation through depletion of PTEN or TSC2 is specifically dependent on 4EBP as inhibition of this TOR effector rescues increased growth and differentiation. Conversely, activation of S6K, another effector of TOR signaling has no phenotype and inhibition of S6K does not rescue PTEN or TSC2 depletion phenotypes.

As previously described, PVR is highly expressed specifically in the Cortical Zone, while only low levels are observed in the Medullary Zone. Depletion of this receptor specifically in differentiating hemocytes leads to proliferation of prohemocytes coupled with

complete differentiation of the lymph gland, suggesting a critical role for PVR in maintaining lymph gland homeostasis (Mondal et al., 2011). Closer examination of the PVR ligands, indicated that both PVF1 and PVF2 are expressed in the lymph gland, but a third ligand, PVF3, is not. PVF1 is expressed most highly in the PSC, but punctate staining is observed throughout the lymph gland. PVF2 is more tightly regulated, as its expression is restricted to the most medial cells of the Medullary Zone, while also showing diminished expression as lymph gland development progresses (Mondal et al., 2011). Depletion of PVF2 in dome-expressing prohemocytes does not affect progenitor differentiation (Mondal et al., 2011), while over-expression of PVF2 does induce a striking differentiation phenotype (Dragojlovic-Munther and Martinez-Agosto, 2013), suggesting that PVF2 is not required for maintenance of progenitors but is sufficient to induce differentiation. Conversely, depletion of PVF1 in the PSC where it is most highly expressed, induces differentiation of progenitors in the same manner as depletion of PVR in the Cortical Zone (Mondal et al., 2011). It should be noted that depletion of PVR in the Medullary Zone does not have a significant effect on progenitor maintenance as expression of *dome* is not altered , but it does cause a decrease in Shotgun expression.

Further interrogation of this requirement for PVR, demonstrated that the Adenosine Growth Factor A (ADGF-A) is downstream of PVR signaling as ADGF-A mutants display similar differentiation phenotypes and over-expression of ADGF-A rescues PVR knockdown (Mondal et al., 2011). This differentiation phenotype is also observed when STAT is inhibited in differentiating hemocytes and the differentiation induced by PVR depletion can also be rescued by over-expression of activated STAT. ADGF-A functions as a scavenger of extracellular Adenosine, indicating that perturbations of this molecule can influence the

differentiation of lymph gland prohemocytes. Depletion of the Adenosine Receptor (AdoR) in the Medullary Zone leads to an increased number of progenitors and decreases differentiation, while over-expression of AdoR causes increased progenitor differentiation demonstrating the sensitivity of prohemocytes to adenosine levels. Furthermore, depletion of AdoR or the downstream effector PKA in the Cortical Zone also causes complete differentiation of the lymph gland which can be rescued by over-expression of ADGF-A. This finding demonstrates that differentiating cells of the Cortical Zone act as a sensor for levels of Adenosine and modulate its availability through PKA and ADGF-A (Mondal et al., 2011).

In addition to its role in Adenosine signaling, PKA also acts downstream of Hh by regulating Ci. Inhibition of PKA leads to an expanded Medullary Zone reminiscent of phenotypes observed upon increased PSC signaling. Conversely, over-expression of constitutively active PKA causes an overly differentiated lymph gland. Furthermore, inhibition of PKA is sufficient to rescue differentiation observed in ADGF-A mutants (Mondal et al., 2011). Activated Ci is present in prohemocytes of the medullary zone, and importantly increased levels of activated Ci are observed in Flip out clones over-expressing AdoR RNAi demonstrating that Adenosine signaling modulates Ci through PKA in conjunction with the Hh signal from the PSC. Therefore, two different factors secreted from the PSC mediate prohemocyte maintenance through independent signaling pathways while eventually intersecting at the common modulator PKA.

A recent study has elegantly demonstrated a novel mechanism for how olfaction regulates prohemocyte maintenance via secretion of GABA from the brain (Shim et al., 2013). The authors observed high levels of intracellular calcium specifically in the

preohemocyte population of the lymph gland. Disruption of Ca2+ release into the cytosol or depletion of Calmodulin, which facilitates Calcium signaling, causes a marked increase in prohemocyte differentiation. Conversely, increasing intracellular Ca2+ levels or downstream Calcium signaling, strongly inhibits differentiation and expands the medullary zone to encompass nearly the entire organ. The metabatropic receptor GABAbR, which is expressed in the lymph gland, was next identified as a regulator of Calcium signaling in the lymph gland. Depletion of GBR strongly decreases levels of intracellular calcium in the lymph gland and also induces a differentiation phenotype similar to that observed upon blocking Calcium signaling directly. Furthermore, over-expression of the Calmodulin-dependent kinase, which promotes calcium signaling, rescues the differentiation phenotype observed upon GBR knockdown (Shim et al., 2013).

GABA was found to be present in the lymph gland, specifically in the prohemocyte population. However, knockdown of Gad1, the enzyme which synthesizes GABA, in the medullary zone has no phenotypic effect, suggesting GABA must be synethesized and secreted from another source. Indeed, GABA can be detected and measured in the hemolymph. Knockdown of Gad1 using a pan-brain driver, not only decreases levels of circulating GABA and GABA immunoreactivity in the lymph gland, but also invokes a strong differentiation phenotype within in the lymph gland. GABA expression and synthesis in the brain was narrowed down to a small cluster of Kur6 neurons near the subesophageal ganglion region. Inhibiting GABA synthesis specifically in these neurons decreases GABA immunoreactivity specifically in the Kur6 neurons as well as in circulation and in the lymph gland, resulting in the same hemocyte differentiation phenotype (Shim et al., 2013).

The authors next identified the common odorant receptor as a regulator of GABA synthesis in the Kur6 neurons. Odorant receptor mutants display a lymph gland phenotype that is remarkably similar to those observed upon loss of GABA, and decreased levels of GABA are observed in Kur6 neurons, in circulation and in the lymph gland. Furthermore, disruption of Projection Neurons (PN), which function downstream of Odorant Receptor Neurons (ORN), also blocks GABA synthesis, results in a potent differentiation phenotype in the lymph gland. This same effect is also observed upon activation of Inhibitory Neurons which interfere with the ability of the ORNs to communicate with the PNs. Importantly, the differentiation phenotype in the lymph gland induced by inhibiting PNs can be rescued by enforced synthesis of GABA in Kur6 neurons. Finally, it was demonstrated that inhibition specifically of the OR42 causes the lymph gland differentiation phenotype accompanied by loss of GABA in circulation and in the lymph gland. Rearing larvae on synthetic food that did not have odorant molecules also incurred a loss of GABA and lymph gland phenotype that can be rescued either by activation of PNs or by the addition of small odorant molecules into the larval environment (Shim et al., 2013). Taken together these findings demonstrate an exciting and novel mechanism for how environmental cues can regulate blood development.

A role for cellular endocytosis has also been identified in regulation of lymph gland homeostasis. The endocytic protein Asrij is expressed in all hemocytes and *asrij* mutants display several hematopoietic defects (Kulkarni et al., 2011). There is a notable hypertrophy and numeration of secondary lobes of the lymph gland in *asrij* mutants that is accompanied by increased proliferation. In the primary lobes, there is no difference in proliferation observed, but there is increased differentiation of mature plasmatocytes and crystal cells, accompanied by a reduction in the Shotgun expression. There is also a significant reduction

in the number of Col+ cells of the PSC, however, there is no change in Antp+ cell numbers. This PSC affect could influence observed changes in differentiation, but it was not reported if there were any alterations in Hh signaling in *asrij* mutants. Finally, it was demonstrated that the subcellular localization of the Notch Intracellular domain was altered in these mutant lymoh glands, suggesting a possible mechanism for increased crystal cell differentiation (Kulkarni et al., 2011).

It was further demonstrated that the GTPase Arf1 interacts with Asrij in hemocyte cell lines and colocalization of the two proteins is observed in circulating hemocytes and in the lymph gland. Knockdown of *arf1* in the lymph gland produces very similar phenotypes to those observed in *asrij* mutant larvae (Khadilkar et al., 2014). Over-proliferation is observed in both the primary and secondary lobes of the lymph gland that is associated with increased differentiation of prohemocytes into both plasmatocytes and crystal cells. There is also a significant decrease in the number of Antp+ PSC cells which is also observed upon knockdown of the *Drosophila* GEF or over-expression of the *Drosophila* GAP, demonstrating that the GTPase function of ARF1 is required. ARF1 was also shown to be required for trafficking of the Notch intracellular domain leading to increased crystal cell differentiation. In addition, ARF1 functions downstream of PVR signaling, as over-expression of *arf1* is capable of rescuing the PVR depletion phenotype (Khadilkar et al., 2014). Presumably, excess ARF1 is able to recycle PVR more efficiently allowing for increased PVR signaling even in a background with depleted levels of PVR.

B. Hemocyte differentiation is induced by specific signaling pathways in the lymph gland.

While there are numerous signaling pathways and molecules that have been demonstrated to regulate preohemocyte maintenance, there have been fewer signaling pathways identified which instruct the differentiation of specific hemocyte lineages. One of the best established roles for a signaling pathway, is the requirement for Notch signaling in crstyal cell differentiation.(Duvic et al., 2002).

Notch is expressed throughout the lymph gland (Small et al., 2014) in the third instar larva providing any differentiating hemocyte with the capacity to become crystal cells if supplied with an activating signal. Genetic approaches have demonstrated that a loss of Notch is not compatible with crystal cell formation. Similarly, mutations to Supressor of Hairless, the downstream effector of Notch, inhibits crystal cell differentiation (Duvic et al., 2002). While there are two Notch ligands in *Drosophila,* Ser and Delta, only loss of Ser has been shown to inhibit crystal cell formation in the lymph gland, while ectopic expression of either *Ser* or *Delta* are capable of driving crystal cell differentiation (Duvic et al., 2002). The transcription factor Lz, which is essential for crystal cell formation (Lebestky et al., 2000), has been shown to be downstream of Notch signaling in the crystal cell differentiation program (Lebestky et al., 2003). Lz positive are absent in flip out clones in the lymph gland over-expressing a dominant negative form of Notch. Finally, over-expression of an activated form of Notch causes a very striking up-regulation of crystal cells in the lymph gland demonstrating that Notch signaling by itself is sufficient to induce crystal cell differentiation (Duvic et al., 2002).

One of the prevailing questions surrounding crystal cell differentiation in the lymph gland involves how these cells are specified. The Notch ligand Ser has been demonstrated to be the ligand responsible for Notch activation in the lymph gland, and Ser expression has

been well characterized in the PSC (Lebestky et al., 2003). However, the first crystal cells to differentiate in the lymph gland are observed in the Cortical Zone, which is not in close proximity to the Ser expressing cells of the PSC. Given that cell-cell contact is required for Notch signaling, this provides an interesting conundrum as to how the Notch ligand is presented to Notch expressing cells differentiating in the Cotical Zone. Furthermore, *col* mutant lymph glands that lose *Ser* expression in the PSC are still populated by crystal cells (Crozatier et al., 2004; Krzemien et al., 2007; Mandal et al., 2007). Perhaps, a clue can be taken from the earlier study as there is still a small number of *Ser*+ cells observed scattered in the lymph gland apart from the PSC even in *col* mutants (Crozatier et al., 2004). The etiology or requirement for these *Ser*+ cells was not characterized, but they have been hypothesized to be responsible for crystal cell differentiation. Additionally, it has been recently reported that stage specific depletion of *Ser* in differentiating hemocytes blocks crystal cell formation only in the early 3rd instar before a mature Cortical Zone has formed. This report went on to demonstrate that the *Drosophila* HIF1Alpha protein *sima* stabilizes Notch in crystal cell progenitors that have already been specified (Mukherjee et al., 2011). These findings suggest that scattered *Ser* expressing cells present amongst Notch+ differentiating hemocytes are capable of specifying crystal cell differentiation in the lymph gland. However the definitive function or nature of these cells has not yet been determined.

Similar to the specific requirement for Notch in crystal cell differentiation, JAK/STAT signaling has also been identified as an important regulator of lamellocyte differentiation. Activating mutations in the JAK kinase *Hopscotch* have been shown to cause melanotic tumors in *Drosophila* larvae correlating with a massive increase in lamellocyte numbers (H Luo, 1995), while inhibition of STAT largely blocks this effect (Luo et al.,

1997). Interestingly, it has been demonstrated that STAT signaling has to be downregulated in the lymph gland for proper immune response. Expression of *dome-GFP* and the LacZ reporter *dome-MESO,* which can be used as a reporter for STAT activity (Hombría et al., 2005; Krzemien et al., 2007), is strongly repressed in the lymph glands of immune challenged larvae (Krzemien et al., 2007; Makki et al., 2010). As previously described, *dome* is also a marker for prohemocytes in the lymph gland, so this decrease in *dome* expression suggests these cells could be differentiating. It has also been demonstrated that lamellocyte differentiation exhausts the population of prohemocytes in the Medullary Zone as a burst of proliferation is observed in lymph glands post wasp parasitization (Sorrentino et al., 2002; Krzemien et al., 2010). In addition, a recent study identified the short receptor *Latran* as an inhibitor of JAK/STAT signaling by forming a dimer with *dome*. *Latran* mutant larvae cannot properly down-regulate STAT signaling in the lymph gland of immune challenged larvae, blocking the cellular immune response as there is no differentiation of lamellocytes observed (Makki et al., 2010).

Lamelloctye differentiation induced by activation of Hop can also be blocked by over-expression of *U-shaped* , and *ush* mutants exhibit increased numbers of lamellocytes, suggesting a tumor suppressive role (Sorrentino et al., 2007). A closer examination of *ush* expression in the lymph gland identified the Friend of GATA protein as a potential regulator of differentiation, beyond just the tumor suppressor role associated with inhibition of lamellocyte differentiation. *ush* is expressed highly in the Medullary Zone of the lymph gland and is only observed at low levels in differentiating plasmatocytes and crystal cells (Gao et al., 2009). Interestingly, *ush* heterozygotes have significant increases in differentiation of both crystal cells and plasmatocyes, while there is no observable change to lamellocyte

numbers in this genetic background. However, *ush* trans- heterozygotes carrying two different *ush* mutant alleles display massive lamellocyte differentiation while the other mature hemocyte lineages are significantly reduced. Levels of Medullary Zone markers Shotgun and Patched are both severely diminished in either genetic background. Finally, *ush* expression was shown to be under direct control of STAT signaling as *ush* expression is lost in STAT mutants, and *ush* reporter activity is ablated when a putative STAT binding site is mutated. Therefore, *ush* acts downstream of STAT in the lymph gland to regulate differentiation of prohemocytes into mature hemocytes (Gao et al., 2009).

While lamellocyte induction has long been attributed solely to wasp parasitization, it has also been demonstrated that a cellular immune response can be mounted in response to bacterial infection as well (Frandsen et al., 2008). In this model, *Salmonella* was used to infect *Drosophila* larvae through the digestive tract, where they first colonize the gut and then spread through the rest of the animal. An immune response characterized by increased numbers of circulating hemocytes, including lamellocytes, was observed. Bacteria were even observed within plasmatocytes and lamellocytes, indicating infection. This report also identified BMP signaling as a regulator of *Drosophila* hematopoiesis and more specifically, the cellular immune response. *dpp* mutant larvae display enlarged lymph glands with increased levels of *ush*. Upon *Salmonella* infection there is a significant increase in the numbers of circulating hemocytes, however, a distinct lack of lamellocytes is observed in the *dpp* mutants which coincides with increased levels of *ush*. (Frandsen et al., 2008)

It has also been reported that the transcription factor Col, which is expressed specifically in the PSC, is required for lamellocyte differentiation. *col* mutant larvae are not capable of mounting a cellular immune response, and over-expression of *col* using a *srpD*-

gal4 driver which is expressed in both hemocytes and prohemocytes, induces lamellocyte differentiation in circulating hemocytes without wasp parasitization (Crozatier et al., 2004). This finding suggests an instructive role for the PSC in the differentiation of lamellocytes. Further evidence for the requirement and role of the PSC in this process was characterized in a recent report describing how oxidative stress in the PSC is regulatory factor in the cellular immune response (Sinenko et al., 2012) . Increased levels of reactive oxygen species (ROS) induced by depletion of *ND75*, a component of the electron transport chain, specifically in the hematopoietic niche drives massive lamellocyte differentiation both in the lymph gland and in circulation (Sinenko et al., 2012) . Over-expression of the anti-oxidant enzyme *SOD2* or *FoxO* which regulates SOD2, blocks this response. It was further demonstrated that wasp infection induces increased levels of ROS which promotes secretion of Spitz, the *Drosophila* EGFR ligand. Depletion of Spitz in the PSC strongly blocks lamellocyte differentiation induced by wasp infection or oxidative stress from ND75 depletion (Sinenko et al., 2012). Furthermore, over-expression of activated EGFR in the lymph gland promotes lamelloctye differentiation and inhibition in all hemocytes of the larva blocks the immune response upon wasp parasitization. These findings demonstrate that EGFR signaling is both necessary and sufficient for the cellular immune response.

In addition to its role in regulating Spitz and the cellular immune response from the PSC, changes to ROS levels in the lymph gland greatly impact differentiation of hemocytes. In wild type lymph glands, high levels of ROS are observed specifically in prohemocytes of the medullary zone (Owusu-Ansah and Banerjee, 2009) . Reducing ROS levels by over-expression of anti-oxidant proteins *Gtpx1* or *catalase* reduces hemocyte differentiation, while *Sod2* mutant larvae display increased differentiation (Owusu-Ansah and Banerjee, 2009).

Further interrogation revealed that oxidative stress induced by ND75 depletion in prohemocytes causes precocious differentiation of plasmatocytes, crystal cells and lamellocytes in the primary and tertiary lobes of lymph glands while this affect can be nullified by over-expression of Gtpx1. In addition, oxidative stress activates JNK signaling and inhibition of Drosophila JNK, *basket*, blocks the differentiation observed upon ND75 depletion. Over-expression of FoxO, which acts downstream of JNK, was also able to induce hemocyte differentiation into plasmatocytes and crystal cells, but not lamellocytes (Owusu-Ansah and Banerjee, 2009). JNK also mediates Polycomb repression, and knockdown of PcG components was able to induce lamellocyte differentiation, suggesting that JNK acts through both FoxO and Polycomb to regulate ROS incuced hemocyte differentiation (Owusu-Ansah and Banerjee, 2009).

Interestingly, increased levels of ROS have also been reported in the lymph gland when Notch is depleted specifically in the PSC. This surprising result also leads to lamellocyte differentiation (Small et al., 2014), although it was not determined if this was also a product of altered Polycomb repression via activated JNK. Additionally, itt was observed that depletion of *Notch* in flip out clones targeted to the cortical zone led to lamellocyte formation, although it was not reported if there is an alteration in ROS levels in this background. Accordingly, there is a sharp decrease in Su(H) reporter activity and ProPO+ crystal cells when larvae are infested by *L. Boulardi* (Small et al., 2014), suggesting that Notch activity must be depressed for a proper cellular immune response. This observation is in agreement with an earlier study that demonstrated a loss of both ProPO+ and *lz*+ cells upon wasp parasitization (Krzemien et al., 2010), but is contrary to earlier observations, that cited increased numbers of crystal cells observed in parasitized larvae

(Sorrentino et al., 2002). These findings suggest that a population of common progenitors for both crystal cells and lamellocytes (and plasmatocytes?) resides within the lymph gland. It is important to note that lamellocyte differentiation induced by oxidative stress does not block crystal cell differentiation, suggesting that not all stressors have the same functional consequences in the lymph gland.

A recent study has also identified FGFR signaling as a potent inductor of differentiation in the lymph gland (Dragojlovic-Munther and Martinez-Agosto, 2013). The *Drosophila* FGF receptor *heartless* is highly expressed in the lymph gland throughout larval development, but by the 3rd instar is restricted specifically to the prohemocytes of the Medullary Zone. Two FGF ligands, Thisbe and Pyramus, are similarly expressed in the lymph gland and are restricted to non-differentiating cells of the Medullary Zone. Inhibition of FGFR signaling in prohemocytes by over-expression of a dominant negative *heartless* construct or depletion of FGF ligands causes a marked expansion of *dome*+ prohemocytes at the expense of differentiating hemocytes. Conversely, over-expression of *thisbe*, *pyramus*, or an activated form of *heartless* leads to complete differentiation of the lymph gland. Interestingly, over-expression of *thisbe* but not *pyramus* induces differentiation of lamellocytes (Dragojlovic-Munther and Martinez-Agosto, 2013).

It was next determined that Ras-MAPK signaling functions downstream of FGFR in the lymph gland. Inhibition of Ras in the Medullary Zone inhibits differentiation, while activation or Ras induces a differentiation phenotype (Dragojlovic-Munther and Martinez-Agosto, 2013). Potential downstream effectors Pointed and Ush also display similar differentiation phenotypes, as depletion of either completely blocks differentiation while over-expression induces differentiation. Epistasis experiments firmly demonstrate that both

Pointed and Ush function downstream of FGFR signaling, and perhaps most interestingly, Ush is absolutely required for differentiation in the lymph gland (Dragojlovic-Munther and Martinez-Agosto, 2013). Activation of FGFR signaling by over-expression of *thisbe* or activated heartless is not capable of inducing differentiation when *ush* is depleted. Furthermore, activated Pointed which rescues the loss of differentiation observed upon FGF inhibition is not capable of rescuing the loss of differentiation incurred by *ush* depletion (Dragojlovic-Munther and Martinez-Agosto, 2013). It would be interesting to determine if Ush is required for any or all of the differentiation phenotypes induced by disruption of various signaling pathways in the lymph gland.

The lamellocyte differentiation induced by *thisbe* over-expression is reminiscent of the phenotype observed upon TOR activation by *tsc2* depletion (Dragojlovic-Munther and Martinez-Agosto, 2012). Interestingly, over-expression of *thisbe* or activated *heartless* in prohemocytes does in fact induce high levels of p4EBP in the lymph gland, indicating that TOR signaling is activated by FGFR. However, this effect is not observed upon *pyramus* over-expression, providing further evidence that these two FGF ligands have divergent functions. Furthermore, the differentiation phenotype induced by *thisbe* or activated *heartless* can be blocked by treating larvae with Rapamycin, while it does not affect differentiation induced by *pyramus* over-expression. Treatment with Rapamycin or over-expression of dominant negative *4EBP* also blocks the lamellocyte differentiation observed upon *thisbe* or activated *heartless* over-expression (Dragojlovic-Munther and Martinez-Agosto, 2013).

Given the expression patterns of *heartless* and the FGF ligands in the lymph gland, there must be some mechanism by which the ligands are appropriated to the prohemocytes or there would be precocious differentiation in the WT organ. Heparan sulfate proteoglycans

have been identified as a regulator of FGF signaling, and the *Drosophila* Perlecan homolog, Terribly Reduced Optic Lobes (Trol), is expressed highly in the extracellular matrix surround the prohemocytes of the Medullary Zone (Grigorian et al., 2011; Grigorian et al., 2013) . Importantly, *trol* mutant larvae display a differentiation phenotype similar to that observed with FGFR activation (Grigorian et al., 2013), and depletion of *trol* specifically in prohemocytes also yields a potent differentiation phenotype (Dragojlovic-Munther and Martinez-Agosto, 2013). Importantly, this phenotype can be rescued by depletion of FGF ligands or inhibition of heartless. This result demonstrates a novel role for Trol in regulating differentiation of hemocytes in the lymph gland, likely by sequestering FGF ligands.

In addition to Trol's function in regulating FGF signaling, it also has other important roles in regulating lymph gland development. The Trol protein contains two domains which specifically interact with extracellular matrix (ECM) proteins (Whitelock et al., 2008), and Trol is co-expressed very strongly in conjunction with the ECM proteins Laminin A and Collagen IV in the lymph gland (Grigorian et al., 2013). A closer examination of Trol expression indicates that "chambers" of proliferating cells are partitioned by Trol throughout the lymph gland, and *trol* mutants have significant defects proliferation. Proliferative defects lead to a much smaller organ, and there is also a precocious differentiation phenotype that is observed in *trol* mutants. Abnormal and early onset differentiation can be partially ascribed to release of FGF ligands, but it was also demonstrated that there is a defect in Hh signaling in these mutants. Patched and Ci are both down-regulated in the Medullary Zone of *trol* mutants, and *Hh* overexpression can partially rescue early differentiation. Finally, Trol helps facilitate the organization of the lymph gland. *trol* mutants have "holes" in the lymph gland where no cells are present. These holes are filled instead by a patchwork of ECM proteins

that form a very different 3D structure than WT ECM in the lymph gland. There is also abnormal localization of differentiated hemocytes, spread seemingly randomly throughout the organ (Grigorian et al., 2013). Taken together, Trol plays an important role in regulating differentiation through Hh and FGF signaling in addition to maintaining organization and structure of the lymph gland.

Part III. Conclusions

While our understanding of the mechanisms which regulate lymph gland homeostasis and blood development continue to increase, it becomes more and more apparent that there is much left to be uncovered. What appears to be a simple system at first glance is actually incredibly complex and nuanced as the number of signaling pathways and mechanisms which regulate progenitor maintenance and differentiation continue to increase. Extrinsic factors such as odorant sensing(Shim et al., 2013) and nutrient (Shim et al., 2012) or oxygen availability (Dragojlovic-Munther and Martinez-Agosto, 2012) have been demonstrated to influence lymph gland development. Studies have described critical functions for endocytic machinery in the trafficking of signals required for maintenance and differentiation of progenitors (Kulkarni et al., 2011; Khadilkar et al., 2014), and post translational modifications like Sumoylation (Kalamarz et al., 2011) have critical roles in regulating lymph gland homeostasis.

In addition, there are also still questions surrounding the nature of the prohemocyte in *Drosophila*. While these cells are classically identified by their location and expression of specific markers, it remains unclear if all prohemocytes have the same capacity to differentiate or self-renew. Clonal analysis of prohemocytes have described the heterogeneous nature of these progenitors as variable sized clones are observed that can give rise to different cell types in the lymph gland (Minakhina and Steward, 2010). Additionally, transplantation experiments have not been conducted which would verify the presence of a bona fide hematopoietic stem cell in *Drosophila.* It also remains unclear if there are any progenitors present in the larva outside of the lymph gland. Hemocytes which are present in the sessile tissue of the larva have been demonstrated to give rise to lamellocytes upon

immune challenge (Honti et al., 2010), but it has not been determined if these cells were already differentiated into plasmatocytes or if an intermediate or lamellocyte specific progenitor is present in this population.

The recently identified intermediate progenitors of the lymph gland are also an intriguing cell population that needs to be better characterized. These cells have been shown to express markers for both progenitors and differentiating hemocytes (Dragojlovic-Munther and Martinez-Agosto, 2012), while they do not express terminal differentiation markers. Intermediate progenitors have also been shown to be mitotically active (Krzemien et al., 2010) and express p4EBP (Dragojlovic-Munther and Martinez-Agosto, 2012), a marker for active TOR signaling. These cells have been postulated to give rise to plasmatocytes, crystal cells, and lamellocytes although no lineage tracing analysis specifically from this population has been performed.

Finally, the mechanisms which promote differentiation in the lymph gland have not yet been fully determined. EGF (Sinenko et al., 2012), FGF (Dragojlovic-Munther and Martinez-Agosto, 2013) and Notch (Duvic et al., 2002) ligands have all been shown to be sufficient to induce differentiation in the third instar lymph gland, but no studies have sufficiently addressed what signal triggers the initiation of the differentiation program in the second instar when *Pxn* expressing hemocytes are first observed.

Future investigation of *Drosophila* hematopoiesis should address these questions while also taking a closer look at the gene expression signature of the different populations of hemocytes in the larva. Common techniques utilized in mammalian research like Fluorescence Activated Cell Sorting (FACS) and RNA sequencing could reveal a wealth of

information about the nature of different hemocyte lineages and signaling populations. Identification of unique expression profiles within these populations of cells could potentially go a long way towards understanding the heterogeneity of progenitors and potential plasticity observed in differentiating hemocytes. The roles of novel genes could easily be dissected with readily available mutant stock and RNA interference collections, and new *gal-4* lines may help label cell populations or provide insight through lineage tracing analyses. Perhaps most importantly, conservation of the underlying mechanisms which govern hematopoiesis allows for novel discoveries in *Drosophila* to potentially translate to higher organisms like zebrafish, mice, and humans. Therefore, advancements in our understanding of lymph gland development could prove to be highly important for future progress in the field of hematopoiesis.

References:

Asha, H., Nagy, I., Kovacs, G., Stetson, D., Ando, I. and Dearolf, C. R. (2003) 'Analysis of Ras-Induced Overproliferation in Drosophila Hemocytes', *Genetics* 163(1): 203-215.

Benmimoun, B., Polesello, C., Waltzer, L. and Haenlin, M. (2012) 'Dual role for Insulin/TOR signaling in the control of hematopoietic progenitor maintenance in Drosophila', *Development* 139(10): 1713-1717.

Binggeli, O., Neyen, C., Poidevin, M. and Lemaitre, B. (2014) 'Prophenoloxidase Activation Is Required for Survival to Microbial Infections in Drosophila', *PLoS Pathog* 10(5): e1004067.

C Samakovlis, D. A. K., P Kylsten, A Engström, and D Hultmark (1990) 'The immune response in Drosophila: pattern of cecropin expression and biological activity.', *The Embo Journal* 9(9): 2969–2976.

Crozatier, M., Ubeda, J.-M., Vincent, A. and Meister, M. (2004) 'Cellular Immune Response to Parasitization in *Drosophila* Requires the EBF Orthologue Collier', *PLoS Biol* 2(8): e196.

Dimarcq, J.-L., Imler, J.-L., Lanot, R., Alan B. Ezekowitz, R., Hoffmann, J. A., A. Janeway, C. and Lagueux, M. (1997) 'Treatment of l(2)mbn Drosophila tumorous blood cells with the steroid hormone ecdysone amplifies the inducibility of antimicrobial peptide gene expression', *Insect Biochemistry and Molecular Biology* 27(10): 877-886.

Dragojlovic-Munther, M. and Martinez-Agosto, J. A. (2012) 'Multifaceted roles of PTEN and TSC orchestrate growth and differentiation of Drosophila blood progenitors', *Development* 139(20): 3752-3763.

Dragojlovic-Munther, M. and Martinez-Agosto, J. A. (2013) 'Extracellular matrix-modulated Heartless signaling in Drosophila blood progenitors regulates their differentiation via a Ras/ETS/FOG pathway and target of rapamycin function', *Developmental Biology* 384(2): 313-330.

Duvic, B., Hoffmann, J. A., Meister, M. and Royet, J. (2002) 'Notch Signaling Controls Lineage Specification during Drosophila Larval Hematopoiesis', *Current Biology* 12(22): 1923-1927.

Frandsen, J. L., Gunn, B., Muratoglu, S., Fossett, N. and Newfeld, S. J. (2008) 'Salmonella pathogenesis reveals that BMP signaling regulates blood cell homeostasis and immune responses in Drosophila', *Proceedings of the National Academy of Sciences* 105(39): 14952-14957.

Gajewski, K. M., Sorrentino, R. P., Lee, J. H., Zhang, Q., Russell, M. and Schulz, R. A. (2007) 'Identification of a crystal cell-specific enhancer of the black cells prophenoloxidase gene in drosophila', *genesis* 45(4): 200-207.

Galko, M. J. and Krasnow, M. A. (2004) 'Cellular and Genetic Analysis of Wound Healing in Drosophila Larvae', *PLoS Biol* 2(8): e239.

Gao, H., Wu, X. and 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.

Grigorian, M., Liu, T., Banerjee, U. and Hartenstein, V. (2013) 'The proteoglycan Trol controls the architecture of the extracellular matrix and balances proliferation and differentiation of blood progenitors in the Drosophila lymph gland', *Developmental Biology* 384(2): 301-312.

Grigorian, M., Mandal, L. and Hartenstein, V. (2011) 'Hematopoiesis at the onset of metamorphosis: terminal differentiation and dissociation of the Drosophila lymph gland', *Development Genes and Evolution* 221(3): 121-131.

H Luo, W. P. H., C R Dearolf (1995) 'An amino acid substitution in the Drosophila hopTum-l Jak kinase causes leukemia-like hematopoietic defects.', *The Embo Journal* 14(7): 1412-1420.

Holz, A., Bossinger, B., Strasser, T., Janning, W. and Klapper, R. (2003) 'The two origins of hemocytes in Drosophila', *Development* 130(20): 4955-4962.

Hombría, J. C.-G., Brown, S., Häder, S. and Zeidler, M. P. (2005) 'Characterisation of Upd2, a Drosophila JAK/STAT pathway ligand', *Developmental Biology* 288(2): 420-433.

Honti, V., Csordás, G., Márkus, R., Kurucz, É., Jankovics, F. and Andó, I. (2010) 'Cell lineage tracing reveals the plasticity of the hemocyte lineages and of the hematopoietic compartments in Drosophila melanogaster', *Molecular Immunology* 47(11–12): 1997-2004.

Irving, P., Ubeda, J.-M., Doucet, D., Troxler, L., Lagueux, M., Zachary, D., Hoffmann, J. A., Hetru, C. and Meister, M. (2005) 'New insights into Drosophila larval haemocyte functions through genome-wide analysis', *Cellular Microbiology* 7(3): 335-350.

Jung, S.-H., Evans, C. J., Uemura, C. and Banerjee, U. (2005) 'The Drosophila lymph gland as a developmental model of hematopoiesis', *Development* 132(11): 2521-2533.

Kalamarz, M. E., Paddibhatla, I., Nadar, C. and Govind, S. (2011) 'Sumoylation is tumor-suppressive and confers proliferative quiescence to hematopoietic progenitors in Drosophila melanogaster larvae', *Biology Open*.

Khadilkar, R. J., Rodrigues, D., Mote, R. D., Sinha, A. R., Kulkarni, V., Magadi, S. S. and Inamdar, M. S. (2014) 'ARF1–GTP regulates Asrij to provide endocytic control of Drosophila blood cell homeostasis', *Proceedings of the National Academy of Sciences* 111(13): 4898-4903.

Kroeger, P. T., Tokusumi, T. and Schulz, R. A. (2012) 'Transcriptional regulation of eater gene expression in Drosophila blood cells', *genesis* 50(1): 41-49.

Krzemien, J., Dubois, L., Makki, R., Meister, M., Vincent, A. and Crozatier, M. (2007) 'Control of blood cell homeostasis in Drosophila larvae by the posterior signalling centre', *Nature* 446(7133): 325-328.

Krzemien, J., Oyallon, J., Crozatier, M. and Vincent, A. (2010) 'Hematopoietic progenitors and hemocyte lineages in the Drosophila lymph gland', *Developmental Biology* 346(2): 310-319.

Kulkarni, V., Khadilkar, R. J., M. S, S. and Inamdar, M. S. (2011) 'Asrij Maintains the Stem Cell Niche and Controls Differentiation during Drosophila Lymph Gland Hematopoiesis', *PLoS ONE* 6(11): e27667.

Kurucz, É., Márkus, R., Zsámboki, J., Folkl-Medzihradszky, K., Darula, Z., Vilmos, P., Udvardy, A., Krausz, I., Lukacsovich, T., Gateff, E. et al. (2007) 'Nimrod, a Putative Phagocytosis Receptor with EGF Repeats in Drosophila Plasmatocytes', *Current Biology* 17(7): 649-654.

Lanot, R., Zachary, D., Holder, F. and Meister, M. (2001) 'Postembryonic Hematopoiesis in Drosophila', *Developmental Biology* 230(2): 243-257.

Lebestky, T., Chang, T., Hartenstein, V. and Banerjee, U. (2000) 'Specification of Drosophila Hematopoietic Lineage by Conserved Transcription Factors', *Science* 288(5463): 146-149.

Lebestky, T., Jung, S.-H. and Banerjee, U. (2003) 'A Serrate-expressing signaling center controls Drosophila hematopoiesis', *Genes & Development* 17(3): 348-353.

Luo, H., Rose, P., Barber, D., Hanratty, W. P., Lee, S., Roberts, T. M., D'Andrea, A. D. and Dearolf, C. R. (1997) 'Mutation in the Jak kinase JH2 domain hyperactivates Drosophila and mammalian Jak-Stat pathways', *Molecular and Cellular Biology* 17(3): 1562-71.

Makki, R., Meister, M., Pennetier, D., Ubeda, J.-M., Braun, A., Daburon, V., Krzemień, J., Bourbon, H.-M., Zhou, R., Vincent, A. et al. (2010) 'A Short Receptor Downregulates JAK/STAT Signalling to Control the Drosophila Cellular Immune Response', *PLoS Biol* 8(8): e1000441.

Mandal, L., Banerjee, U. and Hartenstein, V. (2004) 'Evidence for a fruit fly hemangioblast and similarities between lymph-gland hematopoiesis in fruit fly and mammal aorta-gonadal-mesonephros mesoderm', *Nat Genet* 36(9): 1019-1023.

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

Minakhina, S. and Steward, R. (2010) 'Hematopoietic stem cells in *Drosophila*', *Development* 137(1): 27-31.

Mondal, Bama C., Mukherjee, T., Mandal, L., Evans, Cory J., Sinenko, Sergey A., Martinez-Agosto, Julian A. and Banerjee, U. (2011) 'Interaction between Differentiating Cell- and Niche-Derived Signals in Hematopoietic Progenitor Maintenance', *Cell* 147(7): 1589-1600.

Mukherjee, T., Kim, W. S., Mandal, L. and Banerjee, U. (2011) 'Interaction Between Notch and Hif-α in Development and Survival of Drosophila Blood Cells', *Science* 332(6034): 1210-1213.

Murray, M. A., Fessler, L. I. and Palka, J. (1995) 'Changing Distributions of Extracellular Matrix Components during Early Wing Morphogenesis in Drosophila', *Developmental Biology* 168(1): 150-165.

Nappi, A., Poirié, M. and Carton, Y. (2009) Chapter 4 The Role of Melanization and Cytotoxic By-Products in the Cellular Immune Responses of Drosophila Against Parasitic Wasps. in P. Genevieve (ed.) *Advances in Parasitology*, vol. Volume 70: Academic Press.

Nappi, A. J. (1975) 'Inhibition by parasites of melanotic tumour formation in Drosophila melanogaster', *Nature* 255(5507): 402-404.

Owusu-Ansah, E. and Banerjee, U. (2009) 'Reactive oxygen species prime Drosophila haematopoietic progenitors for differentiation', *Nature* 461(7263): 537-541.

Pek, J. W., Lim, A. K. and Kai, T. (2009) 'Drosophila Maelstrom Ensures Proper Germline Stem Cell Lineage Differentiation by Repressing microRNA-7', *Developmental Cell* 17(3): 417-424.

Pennetier, D., Oyallon, J., Morin-Poulard, I., Dejean, S., Vincent, A. and Crozatier, M. (2012) 'Size control of the Drosophila hematopoietic niche by bone morphogenetic protein signaling reveals parallels with mammals', *Proceedings of the National Academy of Sciences* 109(9): 3389-3394.

R E Nelson, L. I. F., Y Takagi, B Blumberg, D R Keene, P F Olson, C G Parker, and J H Fessler (1994) 'Peroxidasin: a novel enzyme-matrix protein of Drosophila development.', *The Embo Journal* 13(15): 3438–3447.

Rehorn, K. P., Thelen, H., Michelson, A. M. and Reuter, R. (1996) 'A molecular aspect of hematopoiesis and endoderm development common to vertebrates and Drosophila', *Development* 122(12): 4023-4031.

Rizki, M. T. M. (1957) 'Alterations in the haemocyte population of Drosophila melanogaster', *Journal of Morphology* 100(3): 437-458.

Rizki, M. T. M. and Rizki, R. M. (1959) 'Functional Significance of the Crystal Cells in the Larva of Drosophila melanogaster', *The Journal of Biophysical and Biochemical Cytology* 5(2): 235-240.

Rizki, T. and Rizki, R. (1984) The Cellular Defense System of Drosophila melanogaster. in R. King and H. Akai (eds.) *Insect Ultrastructure*: Springer US.

Rizki, T. M. and Rizki, R. (1980) 'Properties of the larval hemocytes ofDrosophila melanogaster', *Experientia* 36(10): 1223-1226.

Rizki, T. M. and Rizki, R. M. (1992) 'Lamellocyte differentiation in Drosophila larvae parasitized by Leptopilina', *Developmental & Comparative Immunology* 16(2–3): 103-110.

Roos, E., Björklund, G. and Engström, Y. (1998) 'In vivo regulation of tissue-specific and LPS-inducible expression of the Drosophila Cecropin genes', *Insect Molecular Biology* 7(1): 51-62.

Shim, J., Mukherjee, T. and Banerjee, U. (2012) 'Direct sensing of systemic and nutritional signals by haematopoietic progenitors in Drosophila', *Nat Cell Biol* 14(4): 394-400.

Shim, J., Mukherjee, T., Mondal, Bama C., Liu, T., Young, Gloria C., Wijewarnasuriya, Dinali P. and Banerjee, U. (2013) 'Olfactory Control of Blood Progenitor Maintenance', *Cell* 155(5): 1141-1153.

Shrestha, R. and Gateff, E. (1982) 'Ultrastructure and Cytochemistry of the Cell Types in the Larval Hematopoietic Organs and Hemolymph of Drosophila Melanogaster', *Development, Growth & Differentiation* 24(1): 65-82.

Sinenko, S. A., Mandal, L., Martinez-Agosto, J. A. and Banerjee, U. (2009) 'Dual Role of Wingless Signaling in Stem-like Hematopoietic Precursor Maintenance in Drosophila', *Developmental Cell* 16(5): 756-763.

Sinenko, S. A., Shim, J. and Banerjee, U. (2012) *Oxidative stress in the haematopoietic niche regulates the cellular immune response in Drosophila*.

Small, C., Ramroop, J., Otazo, M., Huang, L. H., Saleque, S. and Govind, S. (2014) 'An Unexpected Link Between Notch Signaling and ROS in Restricting the Differentiation of Hematopoietic Progenitors in Drosophila', *Genetics* 197(2): 471-483.

Sorrentino, R. P., Carton, Y. and Govind, S. (2002) 'Cellular Immune Response to Parasite Infection in the Drosophila Lymph Gland Is Developmentally Regulated', *Developmental Biology* 243(1): 65-80.

Sorrentino, R. P., Tokusumi, T. and Schulz, R. A. (2007) 'The Friend of GATA protein U-shaped functions as a hematopoietic tumor suppressor in Drosophila', *Developmental Biology* 311(2): 311-323.

Tepass, U., Fessler, L., Aziz, A. and Hartenstein, V. (1994) 'Embryonic origin of hemocytes and their relationship to cell death in Drosophila', *Development* 120(7): 1829-1837.

Tokusumi, T., Tokusumi, Y., Hopkins, D. W., Shoue, D. A., Corona, L. and Schulz, R. A. (2011) 'Germ line differentiation factor Bag of Marbles is a regulator of hematopoietic progenitor maintenance during Drosophila hematopoiesis', *Development* 138(18): 3879-3884.

Tokusumi, Y., Tokusumi, T., Stoller-Conrad, J. and Schulz, R. A. (2010) 'Serpent, Suppressor of Hairless and U-shaped are crucial regulators of hedgehog niche expression and prohemocyte maintenance during Drosophila larval hematopoiesis', *Development* 137(21): 3561-3568.

Valérie, V. R. B. and Giangrande, A. (1997) 'glide/gcmIs Expressed and Required in the Scavenger Cell Lineage', *Developmental Biology* 191(1): 118-130.

Whitelock, J. M., Melrose, J. and Iozzo, R. V. (2008) 'Diverse Cell Signaling Events Modulated by Perlecan†', *Biochemistry* 47(43): 11174-11183.

Yasothornsrikul, S., Davis, W. J., Cramer, G., Kimbrell, D. A. and Dearolf, C. R. (1997) 'viking: identification and characterization of a second type IV collagen in Drosophila', *Gene* 198(1–2): 17-25.

Zettervall, C.-J., Anderl, I., Williams, M. J., Palmer, R., Kurucz, E., Ando, I. and Hultmark, D. (2004) 'A directed screen for genes involved in Drosophila blood cell activation', *Proceedings of the National Academy of Sciences of the United States of America* 101(39): 14192-14197.

Chapter 2:

Yorkie and Scalloped signaling regulates Notch dependent lineage specification during *Drosophila* hematopoiesis

Summary:

Cellular microenvironments established by the spatial and temporal expression of specific signaling molecules are critical for both the maintenance and lineage-specific differentiation of progenitor cells. In *Drosophila*, a population of hematopoietic progenitors, or prohemocytes, within the larval lymph gland (Lebestky et al., 2000) gives rise to three mature cell types: plasmatocytes, lamellocytes, and crystal cells. Removal of the secreted signaling molecules Hedgehog (Mandal et al., 2007) and PVF1 (Mondal et al., 2011) from the Posterior Signaling Center (PSC)(Lebestky et al., 2003; Krzemien et al., 2007; Mandal et al., 2007) which acts as a niche, is leads to a loss of progenitors and complete differentiation of the lymph gland. Here, we characterize a novel population of signaling cells within the lymph gland, distinct from the PSC, that are required for lineage specific differentiation of crystal cells. We provide evidence that Yorkie(Huang et al., 2005) and Scalloped(Wu et al., 2008), the *Drosophila* homologues of YAP and TEAD, are required in Lineage Specifying Cells to regulate expression of *Serrate*, the Notch ligand responsible for the initiation of the crystal cell differentiation program(Duvic et al., 2002) (Lebestky et al., 2003). Genetic manipulation of *yorkie* and *scalloped* in the lymph gland specifically alters *Serrate* expression and crystal cell differentiation. Furthermore, *Serrate* expression in Lineage Specifying Cells is eliminated in the Lymph Gland upon the immune response induced by wasp parasitization to ensure the proper differentiation of lamellocytes at the expense of crystal cells. These findings expand the roles for Yorkie/Scalloped beyond growth to encompass specific cell fate determination in the context of blood development. Similar regulatory functions may extend to their homologues in vertebrate progenitor cell niches that are required for specifying cell fate.

Experimental Procedures

Genetic analysis:

All crosses were reared at 29 degrees Celsius. Multiple *yorkie* (VDRC:104253 and NIG:4005R-1) and *scalloped* (VDRC: 101497 and NIG:8544R-3) RNAi constructs were tested and yielded similar phenotypes. In the case of *HLT* experiments, the *Hand-gal, UAS-2xEGFP,UAS-FLP; A5C-FRT-STOP-FRT-GAL4* genetic background was used to generate lymph gland specific clones expressing the UAS construct of interest. For *sd* knockdown experiments, *UAS-dicer2* was used in the background to enhance phenotypes. Inhibition of Serrate in hematopoietic progenitors of the Medullary Zone was achieved using the *dome-gal4, UAS-mCD8::GFP; gal80^{ts}* stock, with larvae reared at 18 degrees Celsius. After 48hr, larvae were then shifted to 29 degrees Celsius. *Notch^{ts}* larvae were raised at 29 degerees Celsius. *All* crosses involving *SerLacZ* reporter analysis were performed using two copies of *SerLacZ* in the background, except for the experiment involving over-expression of *UAS-yki^{WT}* or its corresponding control.

Immunohistochemistry:

Lymph glands (LGs) were dissected as previously described in 1xPBS and fixed in 3.7% paraformaldehyde for 20 minutes. LGs were then blocked for 30 minutes in 10% NGS in 0.4% TritonX/PBS (PBT). Antibodies were appropriately diluted in PBT and allowed to incubate with samples overnight at 4 degrees Celsius. LGs were washed 4x 15minutes in PBT and blocked again with 10% NGS in PBT for 30 minutes. Secondary antibodies were appropriately diluted in 10% NGS in PBT and allowed to incubate with samples overnight. LGs were then washed 4x15 minutes in PBT and mounted in Vectashield Mounting Medium. Samples were imaged using a

Carl Zeiss LSM 310 Laser Scanning Confocal Microscope. A middle section of a Z-stack was used in every image.

The following primary antibodies were used: mouse anti- $Notch^{ECD}$ (Developmental Studies Hybridoma Bank, DSHB) (1:5), mouse anti-Lozenge (concentrated, DSHB) (1:40), rabbit anti-Prophenoloxidase (gift from Mike Kanost) (1:200), mouse anti-Peroxidasin (gift from J. Fessler) (1:400), mouse anti-GFP (Abcam) (1:100), mouse anti-L1(gift from Istvan Ando) (1:20), mouse anti-β Galactosidase (Millipore) (1:20), rabbit anti-Yorkie (gift from Kenneth Irvine) (pre-absorbed in cuticle at 1:200). The following secondary antibodies were used: anti-Mouse Cy3 (Jackson Immunoresearch) (1:400), anti-Mouse Alexa Fluor648 (Jackson Immunoresearch) (1:400), anti-Mouse FITC (Jackson Immunoresearch) (1:100), anti-Rabbit Cy3 (Jackson Immunoresearch) (1:400), anti-Rabbit Alexa Fluor648 (Jackson Immunoresearch) (1:400).

TUNEL staining: We modified the *In Situ cell death detection kit, TMR red* (Roche) protocol as follows: Larvae were dissected in PBS and fixed in 3.7% formaldehyde for 20 minutes at room temperature. LGs were then permeabilized in 0.4% PBT with 4 X 15 minute washes at RT. LGs were then further permeabilized in 100mM NaCitrate + 0.1% PBT (pH 6.0) on ice for 3 minutes and rinsed in PBS for 4X10 minutes. The TUNEL solution was made by combining Label Solution with Enzyme Solution at a 10:1 ratio. The plate containing tissues in TUNEL solution was incubated at 37°C for 1 hour and 15 minutes. At the end of incubation lymph glands were rinsed in 0.1% PBT for 6 X 5 minutes in glass wells, and mounted in Vectashield Mounting Medium.

MARCM clones: Female flies carrying the sd^{47M} mutation were mated to MARCM 19A males and allowed to lay eggs. 24 hours after egg-laying, the embryos were heat shocked at 29 degrees Celsius in a water bath for 2 hours. Larvae were then raised at RT and dissected at the wandering stage of the 3rd instar. Lymph Glands with control or *sd* clones in the Cortical Zone (Peroxidasin+) were scored based upon the presence or absence of crystal cells (ProPO+) within the clone. ProPO+ cells were observed in 9/20 control clones compared to 1/12 *sd* clones (pValue < .05).

All statistical analyses of crystal cell numbers were achieved using a Two-tailed Student's *t*-test. Lamellocyte quantification was determined by the percentage of Lymph Glands containing lamellocytes in WT (*dome* >) or enforced *Ser* expression (*dome* > *Ser*).

Wasp Parasitization:

8-10 female *L. boulardi* wasps and 5-6 male wasps were added to vials containing second instar *Drosophila* larvae, and were allowed to develop at 25°C. Wandering third instar larvae parasitized by wasp larvae were then dissected.

***Drosophila* stocks**:

The following stocks were used: *hml-gal4, UAS-2xEGFP; lz-gal4, UAS-mCD8::GFP ; Antp-gal4,UAS-2xEGFP* ; *Ser-gal4, UAS-GFP; G-TRACE; dome-gal4, UAS-2xEGFP; dome-gal4;gal80^{ts}; Hand-gal4,UAS-2xEGFP,UAS-FLP; A5C-FRT-STOP-FRT-GAL4* (*HLT) ; Notch^{ts}* (U. Banerjee); *UAS-FLP; A5C-FRT-STOP-FRT-LacZ; sd-gal4* (DGRC#113-596)*; UAS-yki IR(4005R-1); UAS-yki^{WT}; UAS-sd;UAS-2xEYFP; UAS-dicer2;UAS-Ser IR(#28713)* (Bloomington Stock Center); *UAS-yki IR; UAS-sd IR; diap1-GFP(3.5)*(J.Jiang)*; UAS-N^{Act}* (S.

Artavanis-Tsakonas); *UAS-Ser; UAS-Ser^{DN}* ; *SerLacZ* (E. Knust) ; *sd^{47M} FRT19A* (D. Pan)*;* and *hsflp, tubgal80 FRT19A/FM7; tub > mCD8::GFP/TM3, Ser* (B. Ohlstein), *Black cells-GFP* (Robert Schulz).

Results:

Yorkie and Scalloped are required for crystal cell formation in the lymph gland

Differentiating hemocytes in the lymph gland (LG) are restricted to the periphery or Cortical Zone (CZ) of the organ **(Fig. 1A).** These hemocytes originate from a population of progenitors termed prohemocytes (PH) located in the Medullary Zone (MZ, Fig 1A) that are maintained by the PSC **(Fig1A).** PHs transition through an intermediate progenitor (IP) (Krzemien et al., 2010) state **(Fig. 1A)** where they express both progenitor (*dome*$^{+}$) and early differentiation (Pxn^{+}) markers (Dragojlovic-Munther and Martinez-Agosto, 2012). These IPs will eventually fully mature into plasmatocytes (PL), crystal cells (CC) which are specified by Notch signaling (Duvic et al., 2002; Lebestky et al., 2003), or lamellocytes . CCs are marked by crystalline inclusions which contain Prophenoloxidase (ProPO) that is essential in the immune response (Lebestky et al., 2000). These cells mature from newly specified CC progenitors (CCP), which express Lozenge (Lz) (Lebestky et al., 2003), the *Drosophila* homolog of Runx1, into functional ProPO^{+} cells.

Scattered amongst differentiating cells, we observe a population of Yorkie (Yki) expressing cells **(Fig. 1B-D).** Similarly, Yki's binding partner Scalloped (Sd) is expressed in clusters of cells found throughout the CZ **(Fig. 1, E-G)** where it is co-expressed with Yki **(Fig. 1F, Arrows)**. In addition, Yki^{+} and *sd*$^{+}$ cells are observed adjacent to each other **(Fig. 1F, Arrowhead)**. Yki is also observed in 77% of Lz^{+} CCPs (Lebestky et al., 2003) **(Fig. 1C)** , but only in 8% of *sd*$^{+}$ cells. Similarly, only a small percentage of Lz^{+} cells express *sd* **(Fig. S1A, arrowheads)**. Yki is also present in *Black cells-GFP*$^{+}$cells **(Fig. 1D),** a marker of mature CCs (Fig S1B). A small number of *sd*$^{+}$ cells are also ProPO^{+} **(Fig. 1G, Arrow)** , while a subset of *sd*$^{+}$

cells is observed adjacent to mature CCs, but do not express CC markers **(Fig. 1G, Arrowheads)**. Furthermore, lineage tracing analysis with *sd-gal4, UAS-GFP,* identified ProPO^{+} traced cells which do not express GFP **(Fig. 1G, inset)**, suggesting that *sd* is only transiently expressed in this population of CCs. Notch is also observed in a subset of *sd*$^{+}$ cells **(Fig. S1C, Arrow)**, but the majority of Notch^{+} cells do not co-express *sd* but are located adjacent to *sd*$^{+}$ cells **(Fig. S1C, Arrowhead)**. These observations demonstrate that Yki and Sd are present both in CCs and in neighboring populations.

We next generated *sd* and *yki* mutant clones to interrogate their function in the LG. While *yki* clones are extremely small or absent in the LG (data not shown), we do observe a very striking absence of mature ProPO^{+} CCs in *sd* loss of function mutant clones **(Fig S1D-E)**, confirming a requirement for Sd in CC formation. To gain further insight into their role in CC differentiation, we manipulated *yki* and *sd* expression using the *Hand Lineage Tracing* (*HLT*) driver, which clonally expresses *gal-4* throughout the LG **(Fig. S1F-J correspond to Fig. 1H-L)**. We observe an increase of Lz^{+} CCPs **(Fig. 1H-I, Q)** upon LG specific over-expression of *yki*^{WT}.Conversely, depletion of *yki* **(Fig. 1J, Q)** or *sd* **(Fig. 1K, Q)**. causes a decrease in Lz^{+} cells. Importantly, depletion of *sd* blocks the increase in CCPs observed upon *yki*^{WT} over-expression **(Fig. 1L, Q)**, demonstrating that Sd is required for Yki's function in CC differentiation. The extent of CC loss in this background is milder compared to *sd* depletion alone **(Fig. 1Q)**, which could be explained bylow levels of remaining Sd interacting with an over-abundance of Yki.

Based on the pattern of expression **(Fig. 1E-G)** and the functional results upon *sd* depletion **(Fig. 1K-L)**, we further investigated the relationship between Yki and Sd in the context of CC differentiation by manipulating *yki* and *sd* levels with *sd-gal4*. We observe a significant increase in CCP numbers **(Fig. 1M-N, R)** when *yki*^{WT} is over-expressed in *sd*$^{+}$ cells. Similarly,

depletion of *yki* in sd^{+} cells causes a dramatic loss of Lz^{+} cells **(Fig. 1O, R)** as does *sd* down-regulation **(Fig. 1P, R)**. Importantly, manipulating levels of *yki* and *sd* with *sd-gal4* or *HLT* drivers does not significantly alter differentiation of plasmatocytes **(Fig. S1K-L)**. Taken together, these observations provide evidence of an integral role for both Yki and Sd specifically in CC differentiation.

While over-expression of *sd* using the CCP driver *lz-gal4*, increases CC numbers **(Fig. S1M, N-O)** ,over-expression of yki^{WT} does not affect CCs **(Fig. S1M, P)**. We do observe a remarkable decrease in mature CCs when both *sd* and *yki* are depleted in CCPs **(Fig. S1M, Q-R)**. In addition, we observe striking ectopic expression of Yki and Lz in early 2^{nd} instar LGs upon over-expression of an activated form of Notch **(Fig. S1S-T)**. Furthermore, while *Notch* mutant LGs do not express Yki **(Fig. S1V-W)**, we do observe Yki expression in scattered cells of the CZ in lz^{R15} mutant LGs **(Fig. S1U)**. These findings indicate that Yki is specifically upregulated by Notch signaling independent of Lz early in the CC differentiation program, and that Yki and Sd are required within CCPs to maintain normal CC numbers.

Yorkie and Scalloped promote Serrate expression in Lineage Specifying Cells

While over-expression of *yki* throughout the LG **(Fig. 1I)** or specifically in *sd* expressing cells **(Fig. 1N)** significantly increases CCP numbers, a similar increase in CCs is not observed when *yki* is over-expressed in CCPs that have already been specified **(Fig. S1P)**. This discrepancy suggests that Yki can promote CC formation independent of any effects within already committed CCPs, perhaps due to limited availability of Sd in these cells. This finding, along with the observation that sd^{+} cells are frequently observed adjacent to CCs **(Fig. 1G)**,

suggested that there may be a non-cell autonomous role for Yki in CC differentiation, possibly through regulation of the Notch ligand Serrate.

Serrate is highly expressed in the PSC **(Fig. 2A**, (Lebestky et al., 2003; Crozatier et al., 2004)), however, Serrate function in this compartment is not required for CC differentiation **(Fig. S2A-C)**, (Krzemien et al., 2007)). Interestingly, both CCs (Crozatier et al., 2004; Mandal et al., 2007) and *Ser*$^{+}$ cells (Crozatier et al., 2004) are still observed in LGs which lack the PSC, and *Ser*$^{+}$ cells have also been observed outside of the PSC (Lebestky et al., 2003). We confirmed the presence of *Serrate*$^{+}$ cells within the CZ of third instar LGs **(Fig. 2A Arrowhead)**. Inhibition of Serrate in differentiating hemocytes of the CZ **(Fig. S2 D-F)** or MZ prohemocytes **(Fig. S2G-I)** does not affect CC differentiation. However, LG-wide inhibition of Serrate significantly decreases CC differentiation **(Fig. S2 J-L)** demonstrating that Serrate function is required in a subset of cells that are distinct from the PSC, hematopoietic progenitors, or differentiating hemocytes.

Having demonstrated that Yki and Sd can regulate CC numbers within the LG, we asked if they are specifically required in *Ser*$^{+}$ cells for CC formation. Indeed, we observe a significant decrease in CC numbers upon depletion of *yki* or *sd* in these *Ser*$^{+}$ cells **(Fig. 2B-D, I)** demonstrating a requirement for Yki and Sd in these signaling cells which are also observed adjacent to CCs **(Fig. 2E)**. Depletion of *yki* or *sd* in the PSC using the *Antp-gal4* driver does not affect CC differentiation **(Fig. 2F-H, J)**. Therefore, Yki and Sd function is required specifically in *Ser*$^{+}$ cells independent of the PSC for proper CC differentiation.

To gain further insight into the identity of *Ser*$^{+}$ cells in the LG we performed a comprehensive analysis of hemocyte differentiation markers. Using a *LacZ* reporter of *Ser*

expression, we confirmed that the population of *Ser*$^{+}$ cells is located in the CZ **(Fig. 3A)**. These cells do not express markers of differentiating hemocytes **(Fig. 3B)**, but are observed in close proximity to both CCPs **(Fig. 3C)** and mature CCs **(Fig. 3D)**. Furthermore, *Ser*$^{+}$ cells in the CZ co-express *sd* **(Fig 3E)** and Yki **(Fig. S3A-A")**. It is important to reiterate that these Yki^{+} *sd*$^{+}$ *Ser*$^{+}$ cells do not express any other hemocyte markers **(Fig. 3B-D)**, and are lineage traced from a *sd*$^{+}$ cell **(Fig. S3B-B")**. We also observe a subset of *Ser*$^{+}$ cells that arise from a *dome*$^{+}$ precursor **(Fig. S3C-C", Arrowhead)**, but not all *Ser*$^{+}$ cells originate from this population **(Fig. S3C-C", Arrow)**. Importantly, *Ser* expressing cells do not contribute to the CC lineage **(Fig. S3D-D")**. These data demonstrate that this unique population of *Ser*$^{+}$cells expresses both *sd* and Yki and represents a dedicated signaling cell that is distinct from other cell types in the LG.

Similar to the requirement of Yki and Sd in *Ser*$^{+}$ cells, depletion or inhibition of Ser in *sd*$^{+}$ cells is sufficient to block CC differentiation **(Fig. 3F-H, P)**. This demonstrates that Ser is uniquely required in *sd*$^{+}$ cells and no other LG cell populations **(compare to Fig S2A-I)** for CC differentiation. The Yki-mediated increase in CC numbers previously observed **(Fig. 1N)** is blocked by over-expression of *Ser*^{DN} **(Fig. 3I, P)**, while over-expression of *Ser* rescues **(Fig. 3J, P)** the loss of CCs observed upon *yki* knockdown **(Fig. 1O)**. In addition, over-expression of *yki*^{WT} in the LG increases *Ser* expression in the CZ **(Fig. 3K-L)**. Similarly, down-regulation of *yki* or *sd* specifically in *sd*$^{+}$ cells causes a significant decrease in the number of *Ser*$^{+}$ signaling cells **(Fig. 3M-O, Q)** and a corresponding decrease in CCP numbers **(Fig. 1O-P, R)**. However, over-expression of either *yki*^{WT} **(Fig. S3E, G)** or *Ser* **(Fig. S3F, G)** specifically in *Ser*$^{+}$ cells, does not affect CC differentiation **(compare to Fig. 2B)**, suggesting that changes in CC number upon *yki*^{WT} over-expression are due to an increase in the number of *Ser*$^{+}$ cells **(Fig. 3L)**. These results demonstrate that Yki and Sd have definitive roles in CC specification by regulating *Ser*

expression in a distinct population of cells within the LG that we have termed Lineage Specifying Cells (LSCs).

Wasp parasitization triggers cell fate decisions required for the lymph gland immune response by altering Serrate expression

Larval parasitization by the wasp *Leptopilina boulardi* elicits a strong cellular immune response in the *Drosophila* LG REF 11 (Sorrentino et al., 2002) characterized by lamellocyte differentiation **(Fig. 4A)** which is rarely observed in WT LGs. These large, flat cells defend the larva by engulfing invading pathogens or parasites, such as the *L. boulardi* eggs. Upon wasp parasitization there is a robust increase in lamellocyte differentiation along with a corresponding decrease in CC differentiation (Krzemien et al., 2010). However, the mechanism by which this change in lineage fate decisions is regulated has not been definitively determined, although it has been recently shown that Notch signaling blocks lamellocyte formation **(Fig. 4A)** (Small et al., 2013).

A possible explanation for the loss of CCs in the LG upon wasp parasitization could be that expression of *Ser* in LSCs is down-regulated under these conditions as a requirement for lamellocyte differentiation. Indeed, we observe a significant decrease in the numbers of Ser^{+}LSCs in parasitized larvae **(Fig. 4B-C, D)** associated with an up-regulation of lamellocytes **(Fig. 4E', G')** and a decrease in CCs **(Fig. 4 E, F, G)** (Krzemien et al., 2010). To further verify that this down-regulation of *Ser* is required for a proper immune response, we ectopically expressed *Ser* in the LG and subjected these larvae to wasp parasitization. Unlike the WT parasitized control **(Fig. 4G-G'')**, we observe a significant increase in CCs when *Ser* is over-expressed either in prohemocytes and IPs **(Fig. 4H- I)** or ubiquitously by *HLT* **(Fig.4K- L, N)**.

Most strikingly, there is a significant inhibition of lamellocyte differentiation upon enforced expression of *Ser* **(Fig. 4G', I', J)**. These findings demonstrate that down-regulation of *Ser* is responsible for the decreased numbers of CCs observed in the LGs of wasp parasitized larvae and is essential for lamellocyte differentiation in the LG.

We next examined if the alterations in cell fate and observed changes in *Ser* expression upon immune challenge were due to changes in Yki and Sd function. Interestingly, both Yki and *sd* are strongly expressed in lamellocytes, while expression of Yki and *sd* in other cells of the LG is severely diminished upon wasp parasitization **(Fig. S4 A-A", B-B")**. Given the expression of *sd* in lamellocytes, we used *sd-gal4* to interrogate the function of Yki and Sd in these cells. Down-regulating levels of *y*ki or *sd* has no effect on lamellocyte differentiation **(Fig. S4C-E)**, and over-expression of yki^{WT} is not sufficient to rescue loss of CCs in immune challenged LGs **(Fig. 4M, N)**. These findings indicate that wasp parasitization regulates *Ser* expression in LSCs to allow for lamellocyte differentiation at the expense of CCs, while emphasizing the dynamic role for LSCs in maintaining LG homeostasis under normal and stress conditions.

Discussion:

Our findings demonstrate a novel role for Yki and Sd in the Notch dependent lineage specification of CCs. While expression of the Yki and Sd homologues, YAP1/TAZ and TEAD, has been previously described in mammalian Hematopoietic Stem Cells (HSCs) (Ramalho-Santos et al., 2002; Jansson and Larsson, 2012), no phenotypes have been observed upon manipulation of these factors in the HSC compartment (Jansson and Larsson, 2012). Alternatively, we propose that a conserved role for YAP and TEAD signaling may reside in a non-cell autonomous manner originating from lineage specifying or niche cells, such as stromal cells of the bone marrow, thymic epithelium, and other sites of differentiation such as the liver and spleen. . Here, we have provided evidence for a novel regulatory role for Yki and Sd in promoting *Ser* expression in LSCs of the *Drosophila* LG while demonstrating LSC plasticity in immune challenged larvae. Parasitization by the wasp *L. boulardi* necessitates a lineage switch from CCs to lamellocytes, that is achieved by down-regulating *Serrate*, allowing a common pool of hematopoietic progenitors to differentiate into lamellocytes.

Recently, it was shown that YAP regulates expression of Jagged1, the mammalian homolog of Serrate, in hepatocytes (Tschaharganeh et al., 2013; Yimlamai et al., 2014). Hippo pathway signaling through YAP regulates liver cell fate decisions(Yimlamai et al., 2014), while misregulation of YAP leads to increased Jagged1 expression in a TEAD-dependent manner, causing irregular activation of the Notch pathway and hepatocellular carcinoma (Tschaharganeh et al., 2013). In addition, a biphasic lineage specification mechanism involving Notch signaling is required in the specification of Megakaryocyte-Erythroid Progenitor into erythrocytes at the expense of megakaryocytes(Oh et al., 2013) under stress conditions. Given the presence of YAP and TEAD within mammalian hematopoietic compartments(Ramalho-Santos et al., 2002; Xing

et al., 2010; Jansson and Larsson, 2012; Seo et al., 2013), a similar requirement for these factors may be necessary for the regulation of Notch-dependent lineage specification.

We have described a similar role for Yki and Sd in regulating *Serrate*$^{+}$ LSCs in the *Drosophila* LG. Our results demonstrate a mechanism where a small number of Ser expressing LSCs are tightly regulated by limiting availability of Yki and Sd, as perturbations to either of these factors alters CC differentiation. Furthermore, over-expression of *Ser* in the CZ increases CC numbers significantly (Ferguson and Martinez-Agosto, unpublished results), demonstrating the sensitivity to changes in Notch ligand availability in the LG (Small et al., 2013). Our finding that *Ser* expression in LSCs is specifically down-regulated upon wasp parasitization further demonstrates that these signaling cells are in fact dynamically regulated within the LG. . In total, these mechanisms further expand our understanding of hematopoietic niches and the regulation of signaling molecules that characterize hematopoietic microenvironments.

Figures:

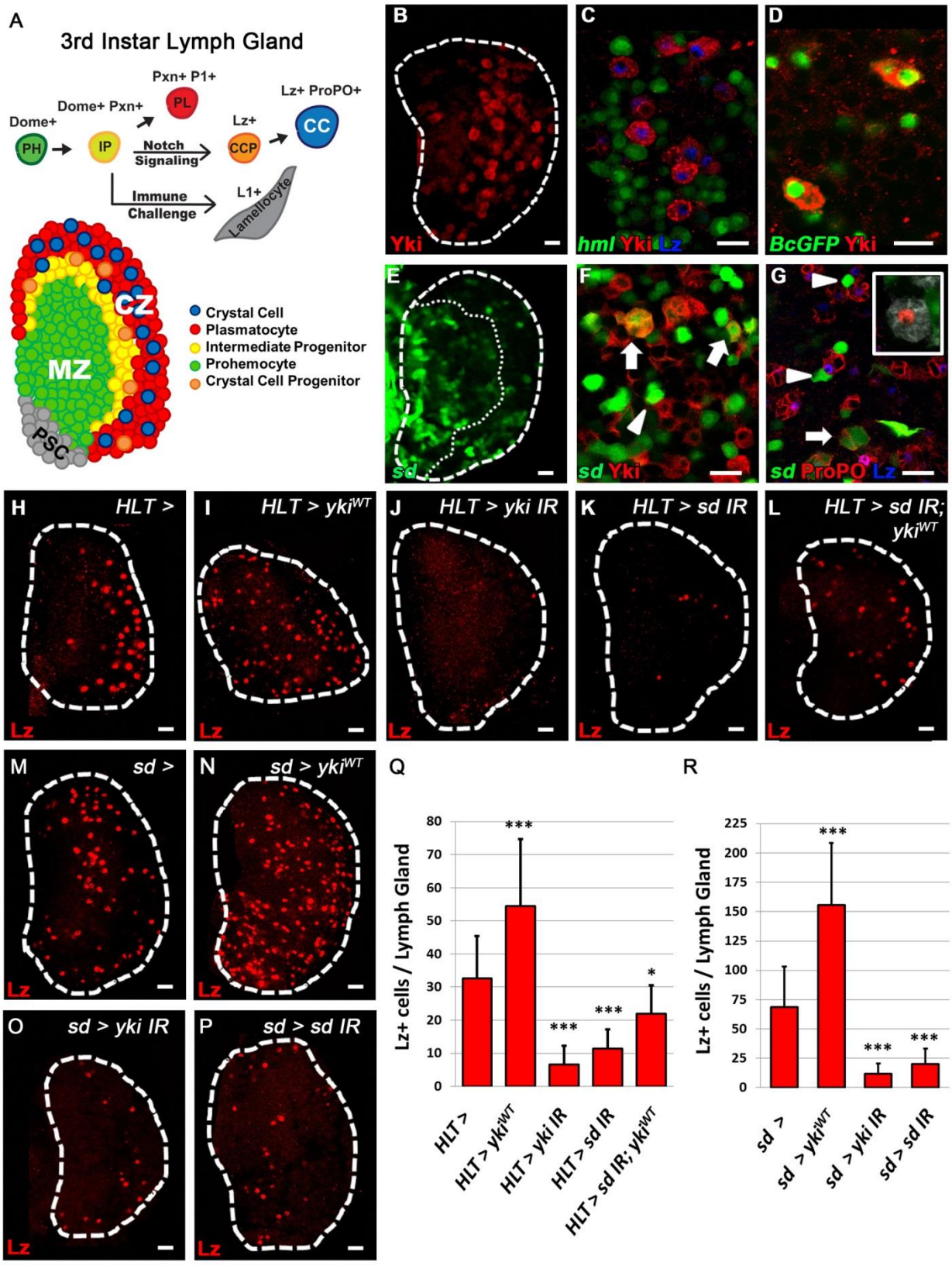
A
3rd Instar Lymph Gland
Pxn+ P1+
PL
Dome+ Pxn+
Dome+
PH
IP
Notch Signaling
Lz+
CCP
Lz+ ProPO+
CC
Immune Challenge
L1+
Lamellocyte
CZ
MZ
PSC
Crystal Cell
Plasmatocyte
Intermediate Progenitor
Prohemocyte
Crystal Cell Progenitor
B
Yki
C
hml Yki Lz
D
BcGFP Yki
E
sd
F
sd Yki
G
sd ProPO Lz
H
HLT >
I
HLT > yki^WT
J
HLT > yki IR
K
HLT > sd IR
L
HLT > sd IR; yki^WT
Lz
M
sd >
N
sd > yki^WT
O
sd > yki IR
P
sd > sd IR
Q
Lz+ cells / Lymph Gland
HLT >
HLT > yki^WT
HLT > yki IR
HLT > sd IR
HLT > sd IR; yki^WT
R
Lz+ cells / Lymph Gland
sd >
sd > yki^WT
sd > yki IR
sd > sd IR

Figure 2-1. Scalloped and Yorkie are required for proper crystal cell differentiation.

Crystal cell progenitors (CCP) are labeled with Lz (H-P, red). (A) Schematic of the 3rd Instar lymph gland and hemocyte differentiation. PSC in grey, prohemocytes (PH, green) of the MZ, intermediate progenitors (IP, yellow), plasmatocytes (PL, red) and crystal cells (CC, blue) in the CZ. (B) Yki (red) is expressed in scattered cells of the CZ in a 3rd instar lymph gland. (C) Yki (red) is observed in CCPs (Lz, blue) amongst differentiating hemocytes (*hml*, green) of the CZ. (D) Yki (red) is present in mature CCs labeled with *Black cells-GFP* (green). (E) *sd* (*sd-gal4 > UAS-2xEGFP*, green) is expressed in clusters of cells scattered throughout the lymph gland. CZ is demarcated by a dotted line. (F-G) *sd* (green) is present in a subset of Yki^+ cells (F, arrows) and mature CCs (G, arrows) and is also seen adjacent to Yki^+ cells (F, arrowhead) and CCs (G, arrowheads). (G, inset) Lineage traced (*sd-gal4, UAS-GFP > UAS-FLP, A5C-FRT-STOP-FRT-LacZ)* (red) mature CCs (ProPO, white) do not express *sd* (green). **H-L** For each panel, its corresponding pattern of *HLT> GFP* expression is demonstrated in Fig. S1F-J (H) WT lymph gland (I) Widespread over-expression of yki^{WT} in the lymph gland increases CCP numbers while (J) depletion of *yki* or (K) *sd* blocks CC formation. (L) *sd* knock-down blocks the increase of CCPs observed upon over-expression of yki^{WT}. (M) WT lymph gland. (N) Over-expression of yki^{WT} in *sd* expressing cells (*sd-gal4 >*) increases CCP numbers, while (O) depletion of *yki* or (P) *sd* strongly inhibits CC differentiation. (Q-R) Quantification of H-P (n=10). * p value <.05, *** p value < .001. Scale bar 10 μm. See also Fig. S1.

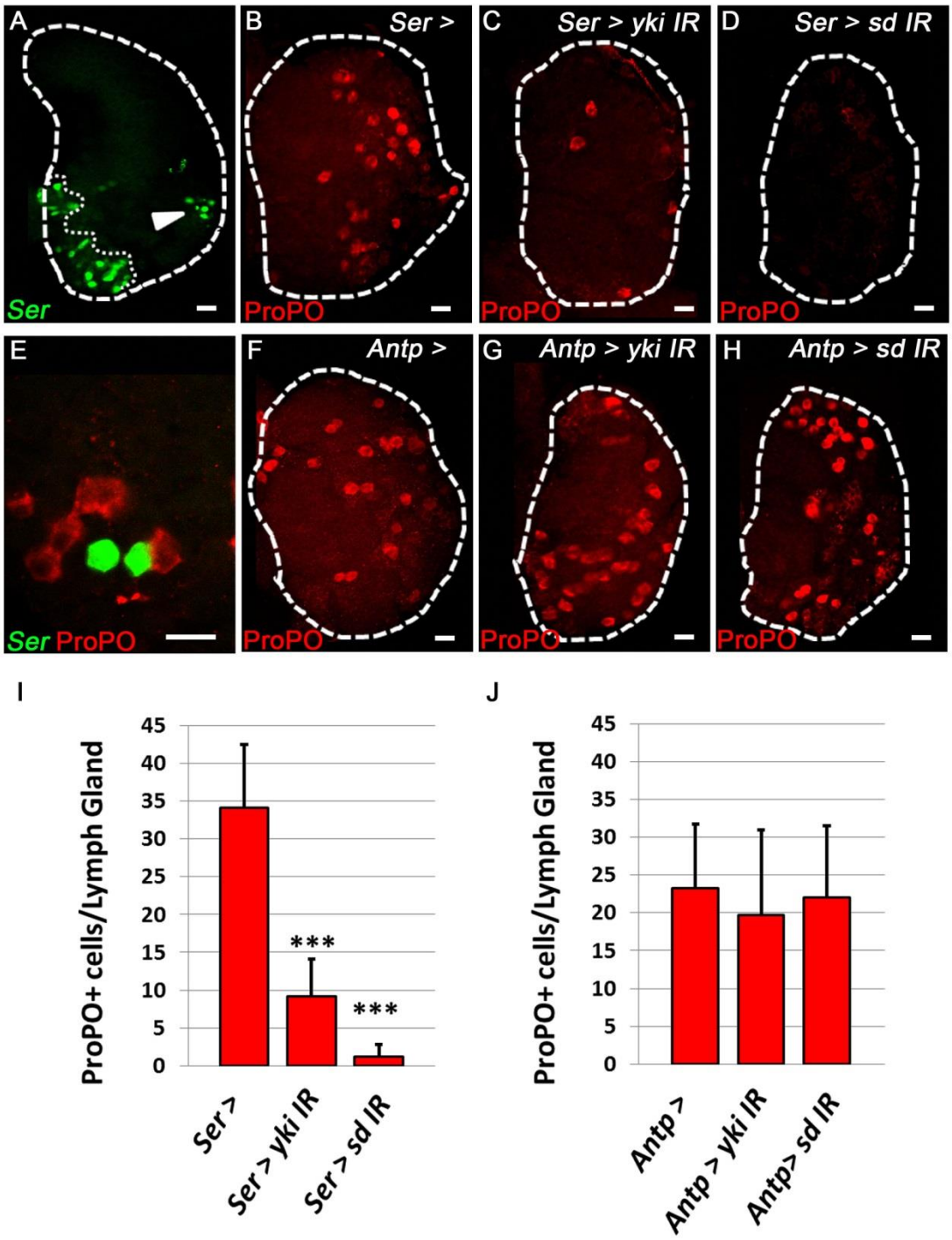

Figure 2-2. Yorkie and Scalloped are required specifically in *Serrate*-expressing cells for proper Crystal Cell differentiation. Green labels *Ser*$^{+}$ cells (*Ser-gal4, UAS-GFP*) (A, E) and red labels ProPO^{+} CCs. (A) Ser expressing cells (arrowhead) observed in the periphery of the

Cortical Zone, distinct from the PSC (outlined by a dotted line). (B) WT (C) Knockdown of *yki* or (D) *sd* in *Ser*$^{+}$ cells blocks CC formation. (E) *Ser*$^{+}$ cells observed in direct contact with CCs in the Cortical Zone. (F) WT (G) Knockdown of *yki* or (H) *sd* in the PSC (*Antp-gal4 >*) has no effect on CC formation. (I) Quantification of *yki* and *sd* Knockdown in *Ser*$^{+}$ cells. (J) Quantification of *yki* and *sd* Knockdown in the PSC. (n=10) *** indicates pValue <.001. Scale bar 10 µm. See also Fig. S2.

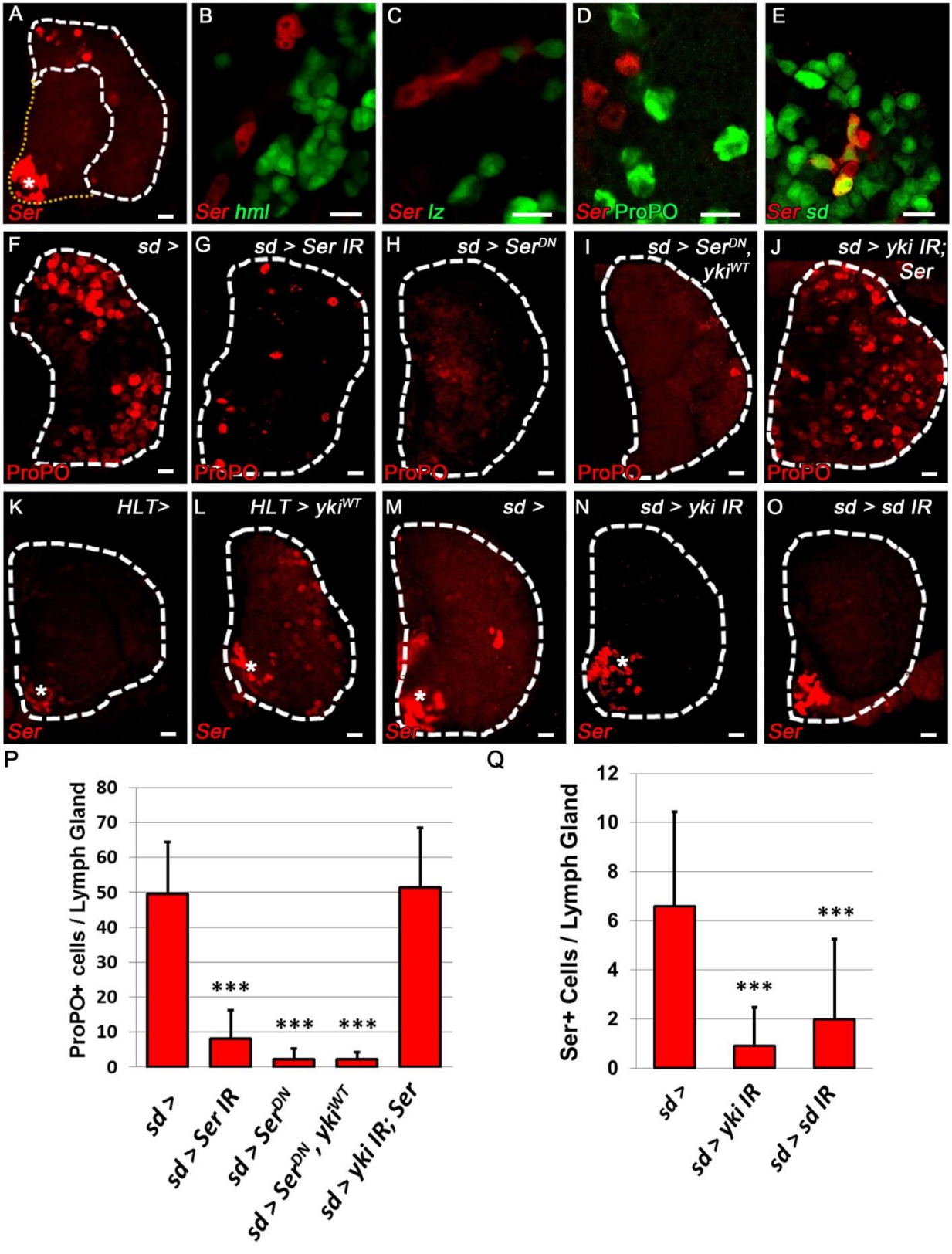
A
Ser
B
Ser hml
C
Ser lz
D
Ser ProPO
E
Ser sd
F
sd >
ProPO
G
sd > Ser IR
ProPO
H
sd > SerDN
ProPO
I
sd > SerDN, ykiWT
ProPO
J
sd > yki IR; Ser
ProPO
K
HLT>
Ser
L
HLT > ykiWT
Ser
M
sd >
Ser
N
sd > yki IR
Ser
O
sd > sd IR
Ser
P
ProPO+ cells / Lymph Gland
0
10
20
30
40
50
60
70
80
***
***
***
sd >
sd > Ser IR
sd > SerDN
sd > SerDN, ykiWT
sd > yki IR; Ser
Q
Ser+ Cells / Lymph Gland
0
2
4
6
8
10
12
***
***
sd >
sd > yki IR
sd > sd IR

Figure 2-3. Yorkie and Scalloped regulate *Serrate*-expressing lineage specifying cell (LSC) numbers. Red labels Ser^{+} LSCs (*SerLacZ,* A-E, K-O) and CCs (F-J). (A) *Ser* expressing cells are located in the CZ, outlined by white hatch marks (asterisk denotes PSC) (B) *Ser* does not co-localize with the PL marker *hml.* (C-D) Ser^{+} cells are observed adjacent to lz^{+} CCPs (C, green) and $ProPO^{+}$ mature CCs (D, green) but do not co-localize. (E) *sd* (green) is co-expressed with *Ser* (red) (F) WT lymph gland. (G) Depletion or (H) inhibition of *Ser* function in *sd* expressing cells (*sd-gal4>*) blocks CC differentiation. (I) CC differentiation is similarly blocked by inhibition of *Ser* after over-expression of yki^{WT}. (J) Loss of CCs observed upon *yki* depletion is rescued by over-expression of *Ser.* (K) *Ser* expression in larvae containing a single copy of *SerLacZ* is only observed in the PSC (asterisk). (L) Over-expression of yki^{WT} (*HLT >*)greatly increases *Ser* expression in lymph glands containing a single copy of *SerLacZ.* (M) WT lymph gland. (N) Depletion of *yki* or (O) *sd* in *sd* expressing cells (*sd-gal4>*) blocks *Ser* expression outside of the PSC (asterisk). (P-Q) Quantification of F-J and M-O (n=10). *** p value < .001. Scale bar 10 μm. See also Fig. S3.

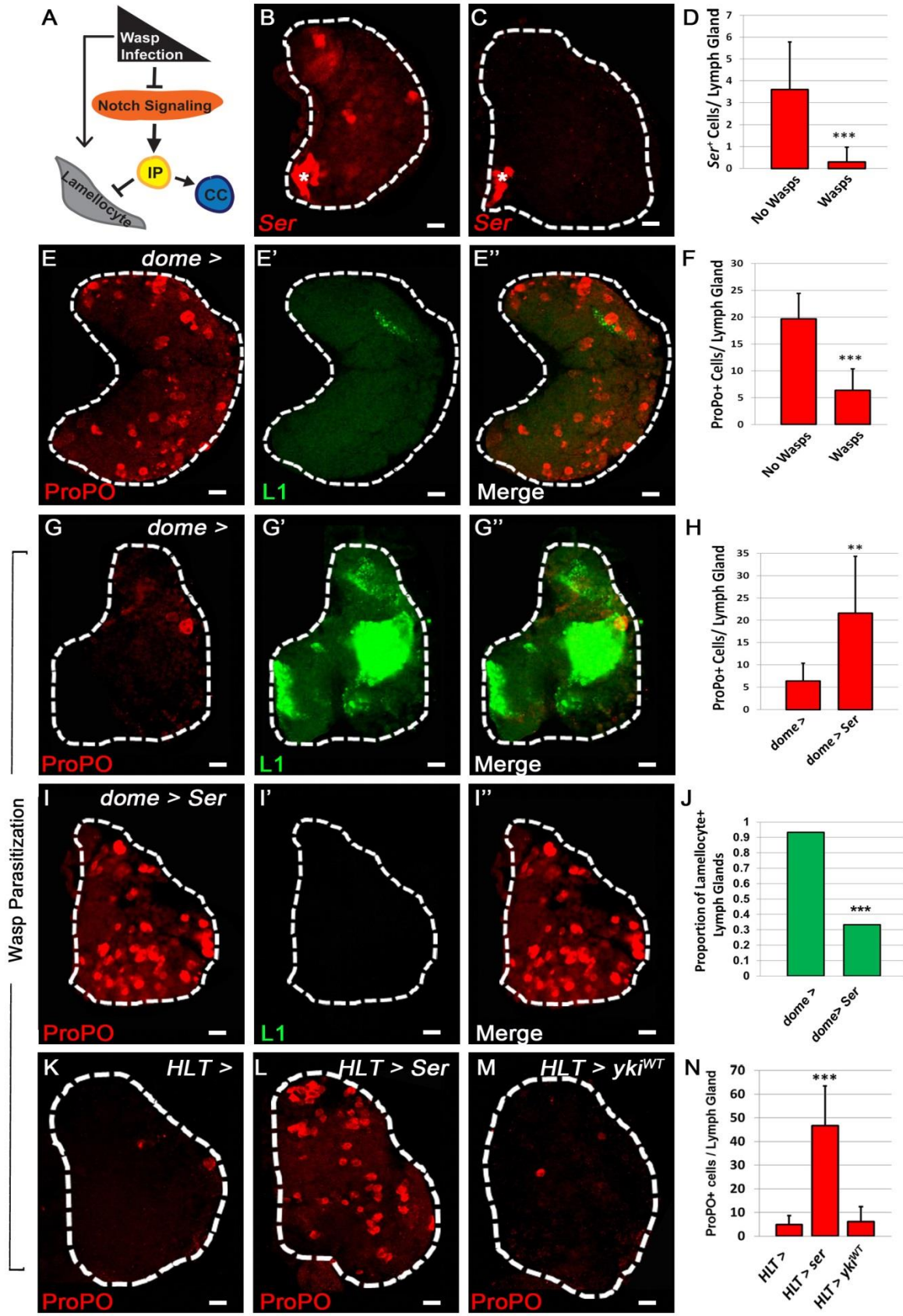
A
Wasp Infection
Notch Signaling
IP
CC
Lamellocyte
B
Ser
C
Ser
D
Ser+ Cells/ Lymph Gland
No Wasps
Wasps
***
E dome >
ProPO
E'
L1
E''
Merge
F
ProPo+ Cells/ Lymph Gland
No Wasps
Wasps
***
G dome >
ProPO
G'
L1
G''
Merge
H
ProPo+ Cells/ Lymph Gland
dome >
dome > Ser
**
Wasp Parasitization
I dome > Ser
ProPO
I'
L1
I''
Merge
J
Proportion of Lamellocyte+ Lymph Glands
dome >
dome> Ser
***
K HLT >
ProPO
L HLT > Ser
ProPO
M HLT > yki^WT
ProPO
N
ProPO+ cells / Lymph Gland
HLT >
HLT > ser
HLT > yki^WT
***

Figure 2-4. *Serrate* expression in LSCs is down-regulated in the lymph gland of immune challenged larvae. (A) Schematic representation of the immune response generated upon wasp infection. Notch signaling, which promotes crystal cell (CC) differentiation in the lymph gland, is blocked by wasp parasitization, allowing intermediate progenitors (IP) to differentiate into lamellocytes (Small et al., 2013). Asterisk denotes PSC (B-C). LSCs (*Ser*) are red in B-C while CCs (ProPO) are red in E, E", G, G", I, I", K-M. Lamellocytes are labeled by green (L1). (B) *Ser* expression in WT. (C) Loss of LSC *Ser* expression upon wasp parasitization. (D) Quantification of Fig.4B-C, (n= 10) (E-E") WT lymph gland contains CCs (E) and lacks lamellocytes (E'). (F) Quantification of Fig.4E, G (n=10). (G-G") Wasp parasitization eliminates CCs (G) and promotes lamellocytes (G'). (H) Quantification of Fig.4G, I (n=10) (I-I") Enforced expression of *Ser* upon wasp parasitization rescues CC loss (I), and inhibits lamellocyte formation (I'). (J) Quantification of Fig. 4G', I' (n= 10) (K) WT lymph gland. (L) Over-expression of *Ser* (*HLT* >) rescues CC numbers upon wasp parasitization, but (M) over-expression of yki^{WT} has no affect compared to WT. (N) Quantification of Fig.4 K-M. (n=10) ** pValue < .01, *** pValue < .001. Scale bar 10 μm. See also Fig. S4.

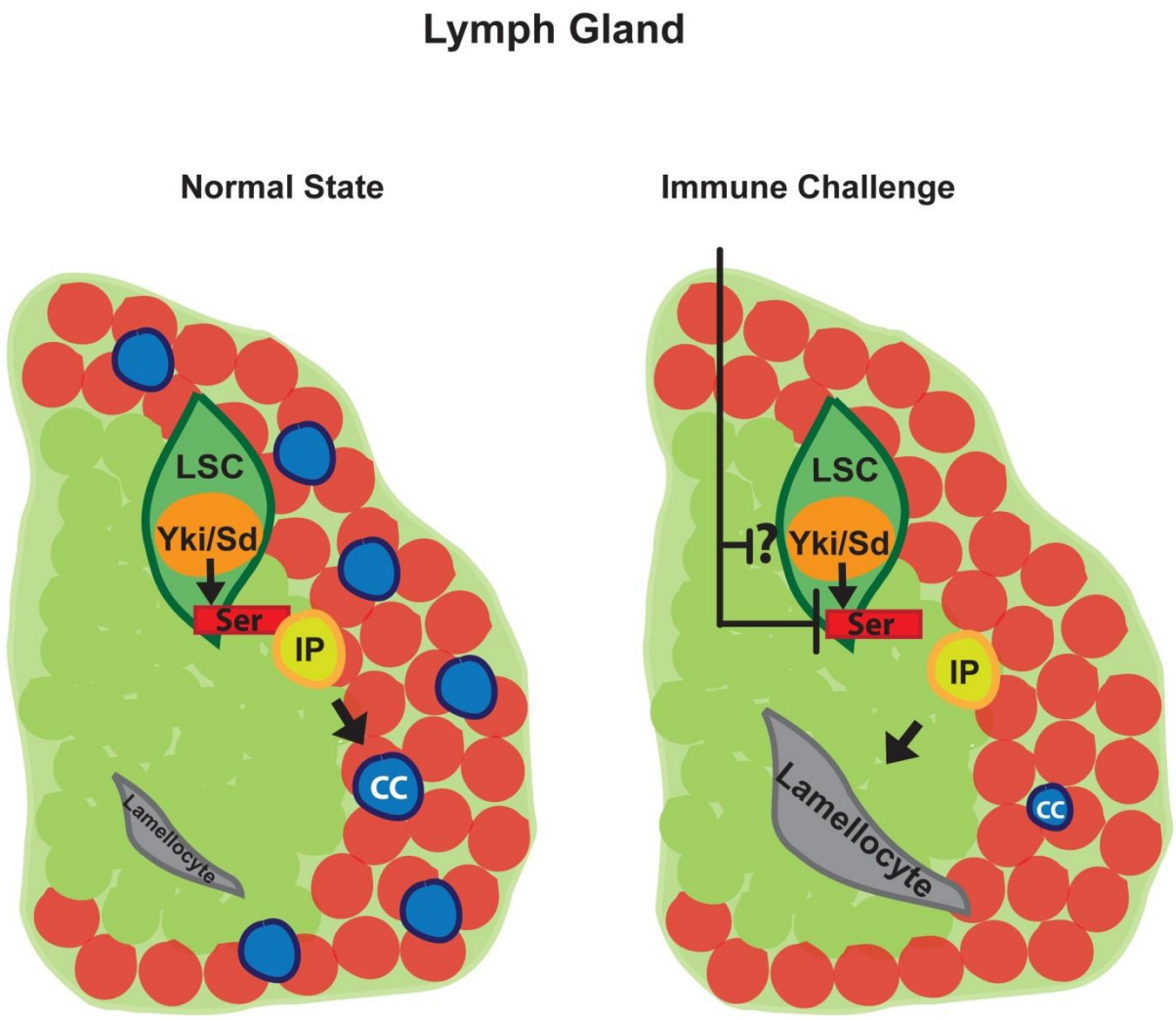

Figure 2-5. Graphical Abstract. Under normal conditions Yorkie and Scalloped function together to promote *Serrate* expression in Lineage Specifying Cells. Functional Yorkie, Scalloped, and Serrate are all required for proper crystal cell differentiation. Few lamellocytes are observed in WT lymph glands. Under immune challenge conditions, expression of Yorkie and Scalloped are diminished and *Serrate* expressing Lineage Specifying Cells are absent in the lymph gland. This inhibition of *Serrate* is required for the formation of lamellocytes in the cellular immune response which differentiate at the expense of crystal cells in the lymph gland.

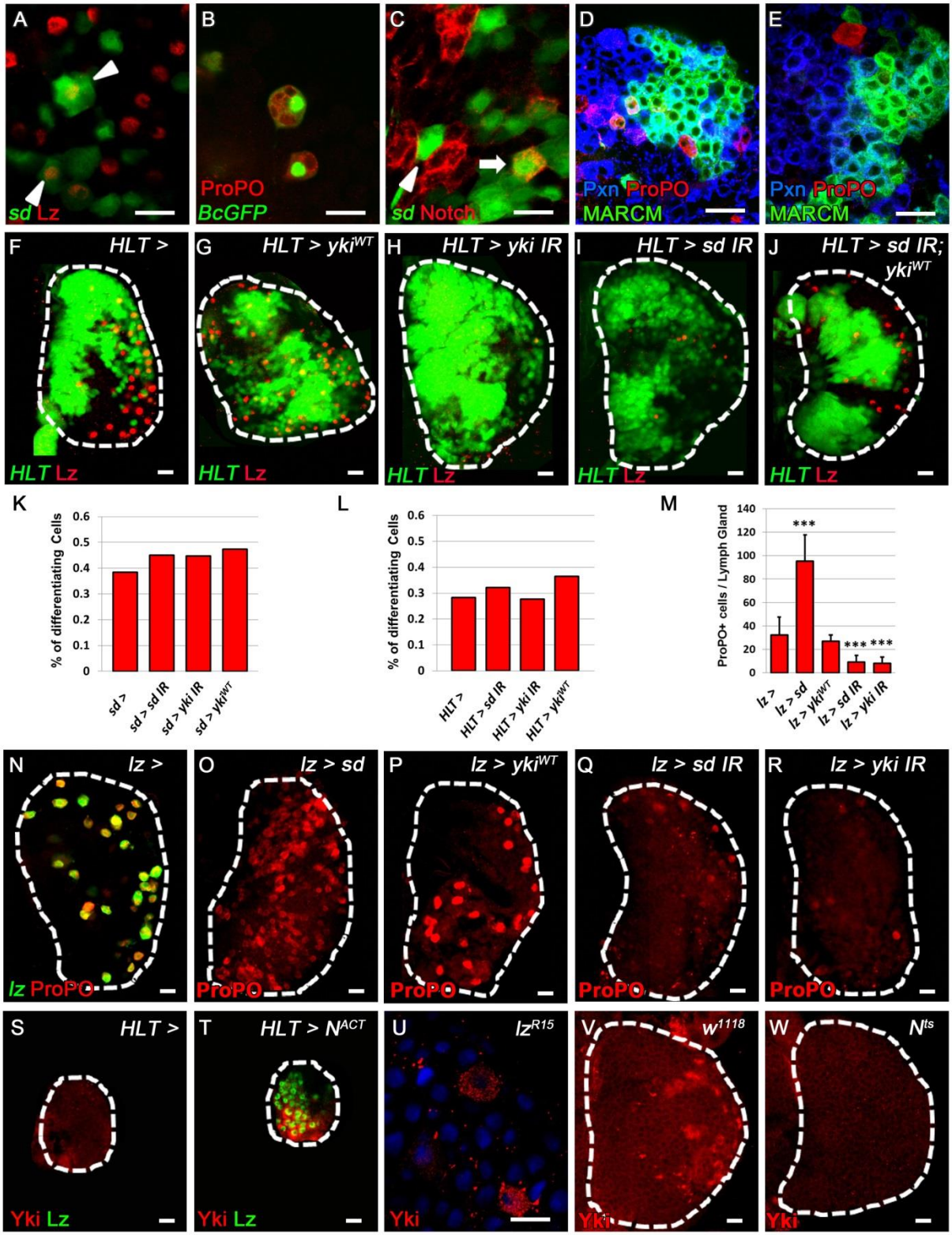
A
sd Lz
B
ProPO
BcGFP
C
sd Notch
D
Pxn ProPO
MARCM
E
Pxn ProPO
MARCM
F HLT >
HLT Lz
G HLT > yki^WT
HLT Lz
H HLT > yki IR
HLT Lz
I HLT > sd IR
HLT Lz
J HLT > sd IR; yki^WT
HLT Lz
K
% of differentiating Cells
0.6
0.5
0.4
0.3
0.2
0.1
0
sd >
sd > sd IR
sd > yki IR
sd > yki^WT
L
% of differentiating Cells
HLT >
HLT > sd IR
HLT > yki IR
HLT > yki^WT
M
ProPO+ cells / Lymph Gland
140
120
100
80
60
40
20
0
***
***
***
lz >
lz > sd
lz > yki^WT
lz > sd IR
lz > yki IR
N lz >
lz ProPO
O lz > sd
ProPO
P lz > yki^WT
ProPO
Q lz > sd IR
ProPO
R lz > yki IR
ProPO
S HLT >
Yki Lz
T HLT > N^ACT
Yki Lz
U lz^R15
Yki
V w^1118
Yki
W N^ts
Yki

Figure 2-6. Supplementary Figure 1. Yorkie and Scalloped promote crystal cell formation. (A) *sd* (green) and Lz (red) expression in the CZ of a 3rd instar lymph gland. (B) Mature CC marker ProPO (red) is co-expressed in CCs labeled with *Black-cells (Bc)GFP* (green) (C) *sd* (green) co-localizes with Notch (red, arrow), and in adjacent cells (arrowheads). (D) CCs (ProPO, red) are observed within control MARCM clones (green, overlap yellow) in the CZ (Peroxidasin, Pxn, blue) of 3rd instar lymph glands, but are not observed in (E) *sd* mutant clones. (F-J) *HLT* clonal expression in the lymph gland (green). CCPs are labeled with Lz (red) (K-L) Quantification of changes to differentiation upon *yki* and *sd* manipulation in the lymph gland with *sd-gal4* (K) and *HLT* (L) (M) Quantification of Fig. S1N-R (n=10) (N) WT lymph gland. *Lz-gal4 >UAS-GFP* (green) labels CCPs and ProPO (red) labels mature CCs. (O) Over-expression of *sd* in CCPs (*lz-gal4 >*) increases CC numbers while (P) over-expression of yki^{WT} has no effect. (Q) Depletion of *sd* and (R) *yki* leads to significant loss of mature CCs. (S) WT 2nd instar LG does not express crystal cell markers Yki (red) and Lz (green). (T) Over-expression of Notch Activated (*HLT > UAS-*N^{Act}) drives ectopic expression of Yki (red) and Lz (green). (U) Yki (red) is expressed in lz^{r15} mutant lymph glands, nuclear marker (blue). (V) Yki (red) expression in 3rd instar lymph gland. (W) Yki expression is not observed in N^{ts} mutant lymph glands raised at non-permissive temperature. Scale bar 10 µm.

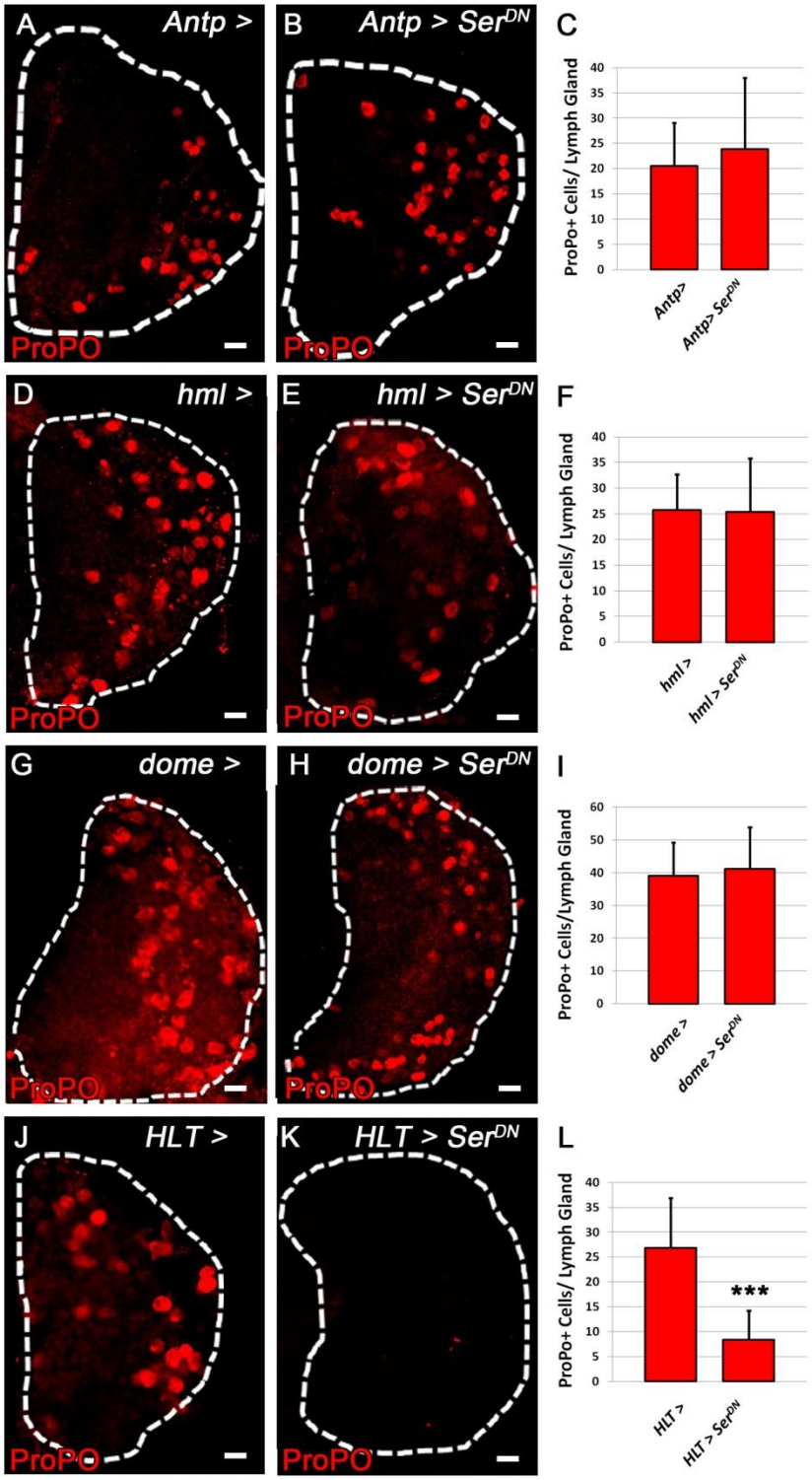

Figure 2-7. Supplementary Figure 2. Inhibition of Serrate in different populations of cells within the lymph gland. (A) WT lymph gland. (B) PSC specific inhibition of Serrate (*Antp-gal4*

> Ser^{DN}) does not block crystal cell differentiation. (C) Quantification of Serrate inhibition in the PSC. (n=10) (D) WT lymph gland. (E) Inhibition of Serrate (*hml-gal4* > Ser^{DN}) in differentiating hemocytes does not block crystal cell differentiation. (F) Quantification of Serrate inhibition in the CZ. (n=10) (G) WT lymph gland. (H) Inhibition of Serrate (*dome-gal4* > Ser^{DN}) in prohemocytes does not block crystal cell differentiation. (I) Quantification of Serrate inhibition in the Medullary Zone. (n=10) (J) WT LG. (K) Inhibition of Serrate throughout the lymph gland (*HLT* > Ser^{DN}) significantly blocks crystal cell differentiation. (L) Quantification of Serrate Inhibition. (n=10) *** indicates pValue <.001. Scale bar 10 μm.

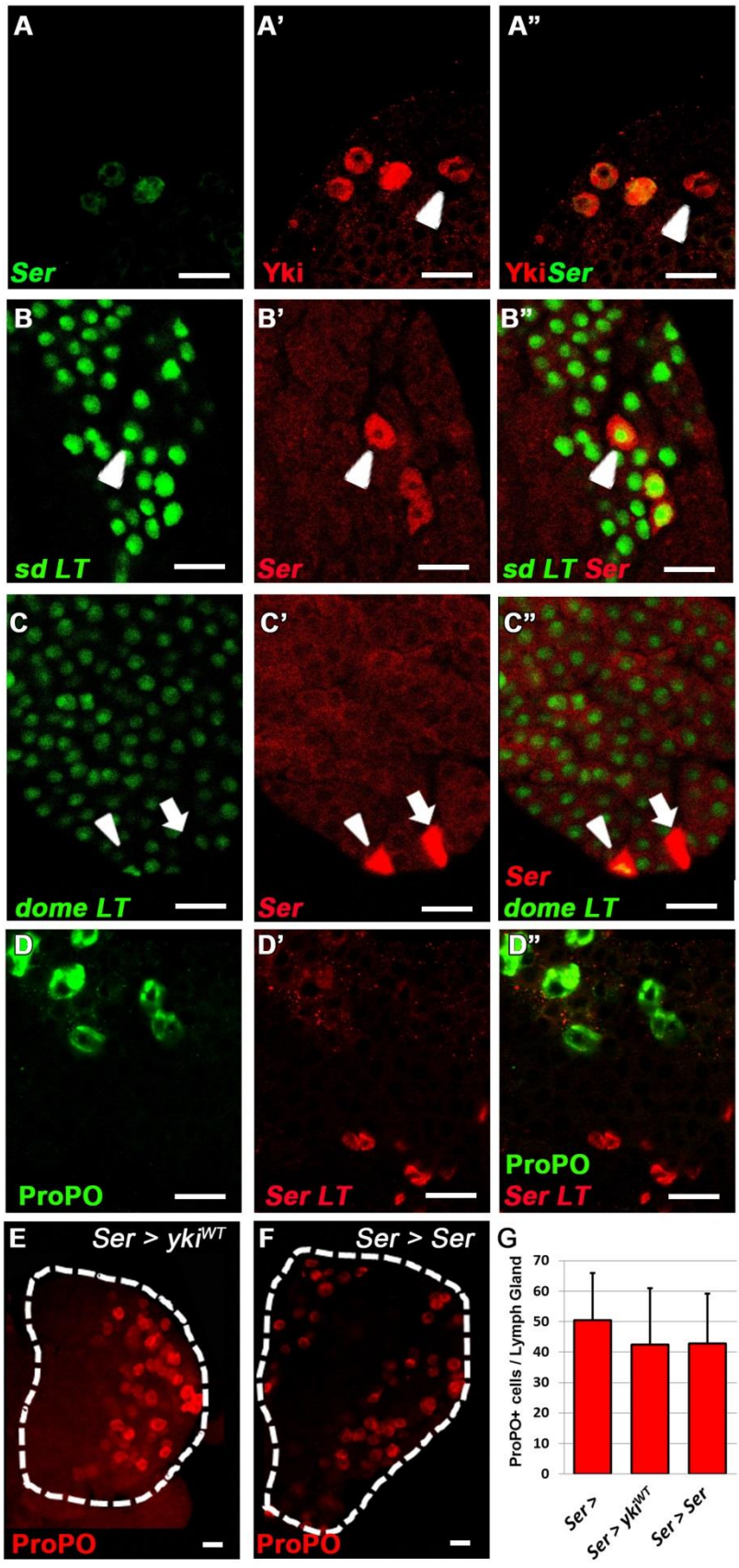
A
Ser
A'
Yki
A"
YkiSer
B
sd LT
B'
Ser
B"
sd LT Ser
C
dome LT
C'
Ser
C"
Ser
dome LT
D
ProPO
D'
Ser LT
D"
ProPO
Ser LT
E
Ser > yki^WT
ProPO
F
Ser > Ser
ProPO
G
ProPO+ cells / Lymph Gland
70
60
50
40
30
20
10
0
Ser >
Ser > yki^WT
Ser > Ser

Figure 2-8. Supplementary Figure 3. Characterization of Lineage Specifying Cells. (A-A") *Ser* (A, green) and Yki (A', red) co-localize (A", yellow), but not in all Yki^{+} cells (Arrowhead). (B-B") Lineage traced sd^{+} cells (B, green) and *Ser* (B', red) co-localize (B", yellow, Arrowhead). (C-C") Lineage traced $dome^{+}$ cells (B, green) and *Ser* (B', red) co-localize (B", yellow, Arrowhead), but not all Ser^{+} cells are traced from *dome* (Arrow). (D-D") $ProPO^{+}$ CCs (D, green) do not co-localize with lineage traced Ser^{+} cells (D', red). (E-G) Overexpression of (E) yki^{WT} or (F) *Ser* in *Ser+* cells (*Ser-gal4* >) does not affect crystal cell (ProPO, red) differentiation. (G) Quantification of Fig. S3E-F (n=10). Scale bar 10 μm.

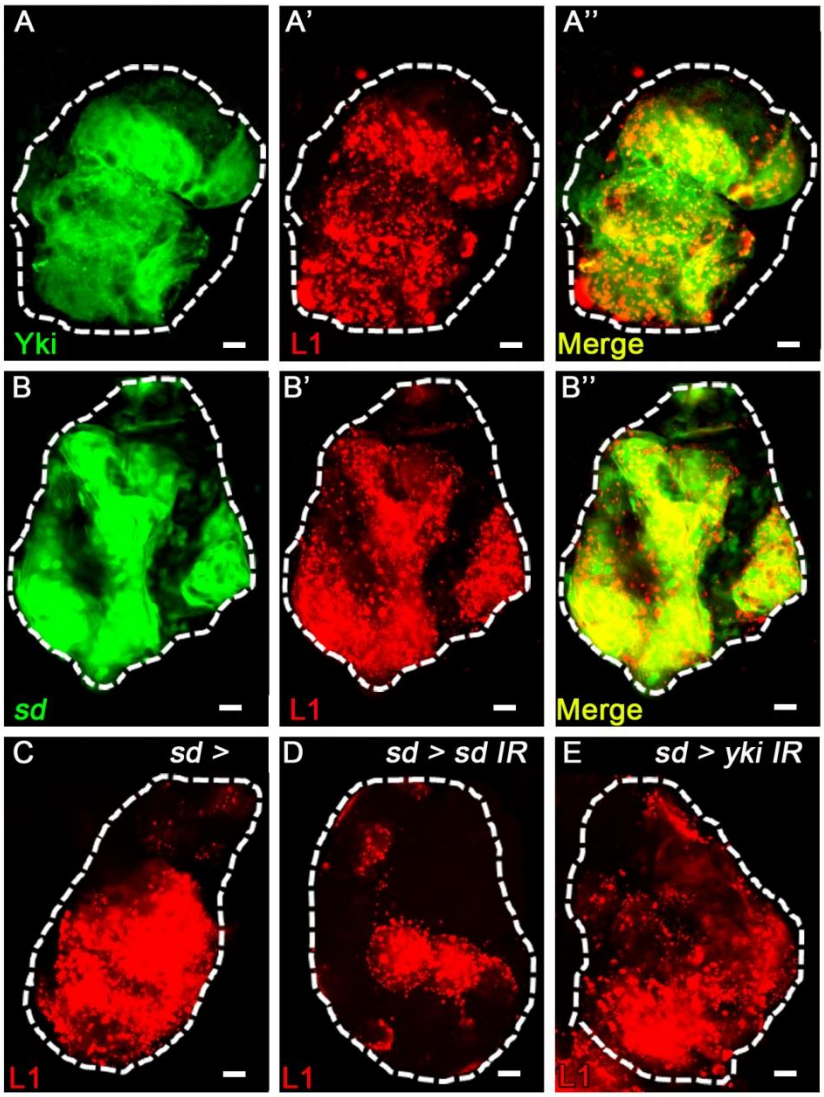

Figure 2-9. Supplementary Figure 4. Scalloped and Yorkie in lamellocytes. (A-A") Yki (A, green) is strongly expressed in lamellocytes labelled with L1 (A', red). (B-B") *sd* (B, green) is strongly expressed in lamellocytes labelled with L1 (B', red). (C-E) Lamellocytes labelled with L1 in wasp parasitized lymph gland. (C) WT lymph gland. (D) Depletion of *sd* or (E) *yki* with *sd-gal4* does not affect lamellocyte formation. Scale bar 10 µm.

References:

Crozatier, M., Ubeda, J.-M., Vincent, A. and Meister, M. (2004) 'Cellular Immune Response to Parasitization in *Drosophila* Requires the EBF Orthologue Collier', *PLoS Biol* 2(8): e196.

Dragojlovic-Munther, M. and Martinez-Agosto, J. A. (2012) 'Multifaceted roles of PTEN and TSC orchestrate growth and differentiation of Drosophila blood progenitors', *Development* 139(20): 3752-3763.

Duvic, B., Hoffmann, J. A., Meister, M. and Royet, J. (2002) 'Notch Signaling Controls Lineage Specification during Drosophila Larval Hematopoiesis', *Current Biology* 12(22): 1923-1927.

Huang, J., Wu, S., Barrera, J., Matthews, K. and Pan, D. (2005) 'The Hippo Signaling Pathway Coordinately Regulates Cell Proliferation and Apoptosis by Inactivating Yorkie, the Drosophila Homolog of YAP', *Cell* 122(3): 421-434.

Jansson, L. and Larsson, J. (2012) 'Normal Hematopoietic Stem Cell Function in Mice with Enforced Expression of the Hippo Signaling Effector YAP1', *PLoS ONE* 7(2): e32013.

Krzemien, J., Dubois, L., Makki, R., Meister, M., Vincent, A. and Crozatier, M. (2007) 'Control of blood cell homeostasis in Drosophila larvae by the posterior signalling centre', *Nature* 446(7133): 325-328.

Krzemien, J., Oyallon, J., Crozatier, M. and Vincent, A. (2010) 'Hematopoietic progenitors and hemocyte lineages in the Drosophila lymph gland', *Developmental Biology* 346(2): 310-319.

Lebestky, T., Chang, T., Hartenstein, V. and Banerjee, U. (2000) 'Specification of Drosophila Hematopoietic Lineage by Conserved Transcription Factors', *Science* 288(5463): 146-149.

Lebestky, T., Jung, S.-H. and Banerjee, U. (2003) 'A Serrate-expressing signaling center controls Drosophila hematopoiesis', *Genes & Development* 17(3): 348-353.

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

Mondal, Bama C., Mukherjee, T., Mandal, L., Evans, Cory J., Sinenko, Sergey A., Martinez-Agosto, Julian A. and Banerjee, U. (2011) 'Interaction between Differentiating Cell- and Niche-Derived Signals in Hematopoietic Progenitor Maintenance', *Cell* 147(7): 1589-1600.

Oh, P., Lobry, C., Gao, J., Tikhonova, A., Loizou, E., Manent, J., van Handel, B., Ibrahim, S., Greve, J., Mikkola, H. et al. (2013) 'In Vivo Mapping of Notch Pathway Activity in Normal and Stress Hematopoiesis', *Cell Stem Cell* 13(2): 190-204.

Ramalho-Santos, M., Yoon, S., Matsuzaki, Y., Mulligan, R. C. and Melton, D. A. (2002) '"Stemness": Transcriptional Profiling of Embryonic and Adult Stem Cells', *Science* 298(5593): 597-600.

Seo, E., Basu-Roy, U., Gunaratne, Preethi H., Coarfa, C., Lim, D.-S., Basilico, C. and Mansukhani, A. (2013) 'SOX2 Regulates YAP1 to Maintain Stemness and Determine Cell Fate in the Osteo-Adipo Lineage', *Cell Reports* 3(6): 2075-2087.

Small, C., Ramroop, J., Otazo, M., Huang, L. H., Saleque, S. and Govind, S. (2013) 'An Unexpected Link Between Notch Signaling and ROS in Restricting the Differentiation of Hematopoietic Progenitors in Drosophila', *Genetics*.

Sorrentino, R. P., Carton, Y. and Govind, S. (2002) 'Cellular Immune Response to Parasite Infection in the Drosophila Lymph Gland Is Developmentally Regulated', *Developmental Biology* 243(1): 65-80.

Tschaharganeh, D. F., Chen, X., Latzko, P., Malz, M., Gaida, M. M., Felix, K., Ladu, S., Singer, S., Pinna, F., Gretz, N. et al. (2013) 'Yes-Associated Protein Up-regulates Jagged-1 and Activates the NOTCH Pathway in Human Hepatocellular Carcinoma', *Gastroenterology* 144(7): 1530-1542.e12.

Wu, S., Liu, Y., Zheng, Y., Dong, J. and Pan, D. (2008) 'The TEAD/TEF Family Protein Scalloped Mediates Transcriptional Output of the Hippo Growth-Regulatory Pathway', *Developmental Cell* 14(3): 388-398.

Xing, W., Kim, J., Wergedal, J., Chen, S.-T. and Mohan, S. (2010) 'Ephrin B1 Regulates Bone Marrow Stromal Cell Differentiation and Bone Formation by Influencing TAZ Transactivation via Complex Formation with NHERF1', *Molecular and Cellular Biology* 30(3): 711-721.

Yimlamai, D., Christodoulou, C., Galli, Giorgio G., Yanger, K., Pepe-Mooney, B., Gurung, B., Shrestha, K., Cahan, P., Stanger, Ben Z. and Camargo, Fernando D. (2014) 'Hippo Pathway Activity Influences Liver Cell Fate', *Cell* 157(6): 1324-1338.

Chapter 3:

Scalloped regulates organ size, progenitor maintenance and niche competence in the *Drosophila* lymph gland

Summary:

Careful regulation of signaling pathways is required for the proper balance of proliferation and differentiation that pattern tissue and organ growth across all Metazoans. One of the best characterized organs for interrogating the functional interaction of a multitude of different signaling pathways in the highly relevant context of blood development is the *Drosophila* lymph gland. A plethora of well conserved pathways have been functionally characterized in this system, but there are still a number of questions which surround how these pathways are regulated. Here, we report that the *Drosophila* TEAD transcription factor Scalloped regulates the expression of the important signaling molecule PVR and its ligand PVF2 in the lymph gland, which are independently required for organ growth and progenitor maintenance. In addition we provide evidence that Scalloped is required for the cellular immune response by maintaining the identity of the Posterior Signaling Center of the lymph gland specifically during Immune Challenge conditions induced by wasp parasitization.

Introduction:

The *Drosophila* lymph gland is an ideal model for studying niche mediated maintenance of a stem/progenitor population. Signaling molecules secreted by a small cluster of cells called the Posterior Signaling Center (PSC) (Lebestky et al., 2003) maintain hematopoietic progenitors, or prohemocytes, of the Medullary Zone in a quiescent state (Mandal et al., 2007). These prohemoctyes are localized in the medial region of the lymph gland where they are identified by the expression of the E-Cadehrin Shotgun and the JAK/STAT receptor *domeless* (Jung et al., 2005). Midway through the second larval instar, prohemocytes begin differentiating into mature hemocyte lineages that are localized to the Cortical Zone at the periphery of the organ (Jung et al., 2005). Hedgehog (Hh) is one of factors secreted by the PSC that is required for progenitor maintenance. Prohemocytes expressing Patched receive the Hh signal and remain quiescent (Mandal et al., 2007). In addition to this signal from the PSC, prohemocytes also receive a signal emanating from the differentiating cells of the Cortical Zone in the form of Adenosine which signals through the seven transmembrane domain Adenosine receptor (AdoR) (Mondal et al., 2011). Elevated levels of adenosine cause increased signaling, leading to aberrant activation of PKA, phosphorylation and destabilization of Cubitus Interruptus, the downstream effector of Hh (Mondal et al., 2011). In this manner, prohemocyte quiescence is maintained by the regulation of Adenosine levels in the lymph gland. ADGF, which scavenges adenosine in the lymph gland, limits the activation of AdoR in prohemocytes. Maintaining proper levels of ADGF is therefore required for maintenance of prohemocytes. This is accomplished by activation of PDGF and VEGF related Receptor (PVR) by its ligand PVF1 which is also secreted from the PSC, as PVR promotes expression of ADGF through STAT signaling. Therefore, two independent signaling

pathways both sensitive to PSC secreted factors, converge to regulate prohemocyte maintenance (Mondal et al., 2011).

While this elegant mechanism to maintain hematopoietic progenitors in the lymph gland is well established, it is interesting to consider why this population of prohemocytes is necessary in the *Drosophila* larva. The duration of the three larval instars is roughly 4-5 days, at the end of which the entire lymph gland is dissolved, dispersing hemocytes throughout the animal upon pupation (Lanot et al., 2001). Crozatier et al. demonstrated that the transcription factor Collier (Col) maintains PSC identity and is also required for maintenance of prohemocytes (Crozatier et al., 2004). The cellular immune response, which is characterized by the differentiation of lamellocytes, does not occur in *col* mutant larvae (Crozatier et al., 2004). It was later determined that loss of Col and the PSC leads to complete differentiation of the lymph gland (Krzemien et al., 2007; Mandal et al., 2007). These findings suggest that the non-committed prohemocytes in the lymph gland must be available for differentiation of lamellocytes to occur in response to an immune challenge. However, it has not been definitively shown whether it is the loss of PSC or loss of progenitors that is responsible for the inability of lymph gland to respond to immune challenge. Furthermore, a recent report demonstrated that the EGFR ligand Spitz is secreted by the PSC in response to oxidative stress induced by immune challenge, and PSC specific depletion of *spitz* blocks lamellocyte differentiation (Sinenko et al., 2012). This finding suggests that it may be the maintenance of the PSC and not prohemocytes that is required for the cellular immune response.

Here, we demonstrate that Sd is paramount to the maintenance of lymph gland homeostasis by regulating important signaling molecules. We report that *sd* expression labels an unique population of hemocytes in the Medullary Zone of the lymph gland that do not express

domeless. Furthermore, Sd function is required to maintain proper levels of PVF2, another PVR ligand expressed in the lymph gland. We also demonstrate a previously uncharacterized requirement for PVF2 for determining lymph gland size. Additionally, depletion of *sd* in the Cortical Zone causes a swift and complete differentiation of the lymph gland. Sd functions together with its binding partner Yorkie (Wu et al., 2008), to regulate expression of PVR and PVR signal transduction. Finally, we asked if Sd mediated maintenance of prohmeocytes is required for the cellular immune response in the lymph gland. To our surprise, we report that prohemocyte maintenance is not required for the immune response; however Sd function is required for maintaining PSC identity and lamellocyte differentiation upon immune challenge.

Experimental Procedures:

Genetic analysis:

All crosses were reared at 29 degrees Celsius.

Larval Staging:

Eggs were layed on grape plates with yeast paste and maintained at room temperature until hatching. Larvae were removed immediately after hatching and transferred to food plates and were then permitted to grow at 29 degrees Celsius and dissected at the appropriate time point.

Circulating Hemocyte Analysis:

Single larvae were dissected in 20µL of PBS on a mini-well of a 12 multi-well glass slide. Circulating hemocytes were allowed to settle and attach to the surface of the slide for 30 minutes, and were then fixed and stained as previously described.

Immunohistochemistry:

Lymph glands were dissected, fixed, stained, and mounted as previously described. Samples were imaged using a Carl Zeiss LSM 310 Laser Scanning Confocal Microscope. A middle section of a Z-stack was used in every image.

Phalloidin Staining: Samples were fixed and blocked as previously described. Samples were then washed 2x in .4% PBT and incubated with Alexa fluor 546 phalloidin at a 1:30 dilution in 10% NGS in PBT for 30 minutes. Samples were then washed 4x in PBT and mounted.

The following primary antibodies were used: mouse anti-Peroxidasin (gift from J. Fessler) (1:400), mouse anti-β Galactosidase (Millipore) (1:20), mouse anti-P1 (gift from Istvan Ando), rat anti-PVR (gift from Ben Shilo). The following secondary antibodies were used: anti-Mouse Cy3 (Jackson Immunoresearch) (1:400), anti-Mouse Alexa Fluor648 (Jackson Immunoresearch) (1:400), anti-Mouse FITC (Jackson Immunoresearch) (1:100), anti-Rat Cy3 (Jackson Immunoresearch) (1:400).

All statistical analyses of cell numbers in the lymph gland were achieved using a Two-tailed Student's *t*-test. Lamellocyte quantification was determined by counting the number of lamellocytes attached to an individual miniwell of a multi-well slide.

Wasp Parasitization:

Wasp parasitization of larvae was conducted as previously described.

***Drosophila* stocks**:

The following stocks were used: *Hml-gal4, UAS-2xEGFP; Pxn-gal4, UAS-2xEGFP ; Antp-gal4,UAS-2xEGFP* ; *dome-gal4, UAS-2xEGFP;Hand-gal4, UAS-GFP; Hand-gal4,UAS-2xEGFP,UAS-FLP; A5C-FRT-STOP-FRT-GAL4* (*HLT)* (U. Banerjee) *;sd-gal4* (DGRC#113-596)*; domeMESO-LacZ;SerLacZ* (U. Banerjee); *Pvf2-LacZ* (U. Banerjee); *UAS-Pvf2* (U. Banerjee)*; UAS-STAT^{Act}; UAS-Adgf-A; UAS-yki IR(4005R-1); UAS-Pvr IR; UAS-yki^{WT}; UAS-sd;UAS-2xEYFP; UAS-dicer2; UAS-yki IR(VDRC 104523); UAS-sd IR (VDRC 101497);Pvf2^{d02444}, sd^{Etx4}*(*sd* hypomorph)*; sd^{47M} FRT19A* (D. Pan)*;* and *hsflp, tubgal80 FRT19A/FM7; tub > mCD8::GFP/TM3, Ser* (B. Ohlstein)

Results:

Scalloped is first observed in the lymph gland in the early 2nd instar in a small number of cells in the most medial region of the organ, near the Dorsal Vessel. As development progresses, the number of *sd*+ cells are observed more distally and steadily increase in number through the 2nd and 3rd instars. By the 3rd instar, *sd*+ cells populate a large percentage of the lymph gland and are found among both progenitors and differentiating hemocytes **(Fig. 1A)**. We have previously reported that *sd*+ cells give rise to crystal cells, *Ser*+ Lineage Specifying Cells, and is expressed in lamellocytes induced by wasp parasitization.

To interrogate what function Sd may have in other populations of the lymph gland, we depleted levels of sd in the Medullary Zone, PSC, and Cortical Zone. Knocking down *sd* in *dome*+ prohemocytes has little effect on the lymph gland, although a minor expansion of intermediate progenitors is observed **(Fig. 1B-C)**. Similarly, knockdown of *sd* in the PSC has no visible effect on differentiation or lymph gland size **(Fig. 1D-E)**. However, depletion of *sd* in differentiating hemocytes has a very potent effect, causing complete differentiation of the lymph gland **Fig. 1F-G)**. To parse these divergent phenotypes we took a closer look at the expression of *sd* in relation to *dome*+ prohemocytes over the course of lymph gland development. We were able to address this question by using the Jak/STAT reporter *domeMESO* that labels prohemocytes in conjunction with *sd-GFP*. Indeed, there are a number of cells within the Medullary Zone which do not express the *domeMESO* reporter **(Fig. 2 A', B', C', D')**. Strikingly, these cells are frequently populated by *sd*+ cells **(Fig. 2A", B", C", D")**. There are a small number of cells that express both *sd* and *domeMESO,* but the majority of *sd*+ cells do not express *dome.* This finding suggests *sd*+ cells may represent a unique population of cells within the lymph gland.

While *sd* is not strongly expressed in dome+ cells of the Medullary Zone, we observed that the *sd* expression pattern shares some similarities to that of *Pvf2,* a PVR ligand which is strongly expressed in the first 2nd and early 3rd instar lymph gland in the most medial region of the Lymph Gland close to the dorsal vessel (Mondal et al., 2011) (**Fig. 3A, A'**). Indeed, further expression analysis indicates a large percentage of *Pvf2*+ cells also co-express *sd* **(Fig. 3A")**. Furthermore, depletion of *Pvf2* in *sd*+ cells severely inhibits development of the lymph gland resulting in a much smaller organ **(Fig. 3B-D)**, while depletion of *Pvf2* with *dome-gal4* does not have the same effect **(Fig. 3E-G)**. Interestingly, a very similar decrease in lymph gland size is observed in both *sd* and *Pvf2* hypomorphic mutants **(Fig. 3H, K)** suggesting that PVF2 is required for maintaining proper organ size in a Sd dependent manner. Furthermore, expression of *Pvf2* is decreased in *sd* hypomorphic mutants **(Fig. 3L-M)**, and this phenotype can be rescued by overexpression of PVF2 **(Fig. 3I-J)**. Taken together these findings demonstrate a novel function for PVF2 in the lymph gland, and highlight the requirement for Sd in a unique population of cells for the regulation *Pvf2* expression.

Given the phenotype observed upon *sd* depletion in differentiating hemocytes, we more closely interrogated possible mechanisms behind this very poignant result. We first asked at what developmental stage this differentiation is first observed. Strikingly, we observe a massive differentiation of the lymph gland as early as 36hr after hatching, or mid-second instar. In the WT lymph gland, only a small number of differentiating hemocytes are present but depletion of Sd from these cells leads to an immediate and massive differentiation coupled with increased proliferation **(Fig. 4A-B)**. This phenotype is highly reminiscent of what is observed upon PVR depletion in differentiating hemocytes (Mondal et al., 2011). Therefore, we asked if *sd* depletion affects levels of PVR in the lymph gland. Interestingly, instead of a decrease in PVR, we observe

a very strong up-regulation of PVR levels **(Fig**. **5A-B)**. It has been previously reported that increasing PVR expression in differentiating hemocytes actually inhibits differentiation (Khadilkar et al., 2014), but a well characterized analysis of this background has not been reported. Conversely, increasing *Pvf2* levels does yield a phenotype where the lymph gland fully differentiates (Dragojlovic-Munther and Martinez-Agosto, 2013). Therefore, we reasoned that it could be possible that higher levels of PVR could lead to hypersensitivity to PVF2 and a subsequent increase in differentiation. However, it seems unlikely that knockdown of *sd* could affect levels of PVR rapidly enough to cause this differentiation phenotype. Indeed, by the end of the 2nd instar, levels of PVR have not been significantly affected **(Fig. 4 C-D)** suggesting that another mechanism for Sd regulation of differentiation and prohemocyte maintenance must be present.

Another possible mechanism could be that PVR signaling is somehow inhibited by a loss of Sd. It has been previously demonstrated that Sd's binding partner Yorkie (Yki) interacts with SMADs to facilitate shuttling from the cytoplasm to the nucleus (Oh and Irvine, 2011), and Yki has been shown to promote STAT signaling in the intestine (Ren et al., 2010). We therefore asked if Sd is required for STAT signal transduction downstream of PVR. Previous studies have demonstrated that over-expression of activated STAT or ADGF which PVR signaling regulates in the lymph gland, is capable of rescuing the differentiation phenotype observed upon loss of PVR (Mondal et al., 2011). Interestingly, over-expression of either activated STAT **(Fig. 5C)** or ADGF **(Fig. 5D)** both rescue the differentiation phenotype observed upon *sd* depletion. These results suggest that Sd is a critical factor in modulating PVR signaling in differentiating hemocytes of the lymph gland, perhaps by interacting with STAT which is required downstream of PVR **(Fig. 5E)**.

While these results place Sd downstream of PVR signaling, we were still intrigued by the observed increase in PVR levels when *sd* is depleted. This finding suggested that not only was Sd required downstream of PVR signaling, but perhaps it also regulated levels of PVR expression. We took a closer look at the PVR gene and found that it contains 6 putative binding sites for Sd which further suggests a direct regulatory role for Sd. To evaluate if Sd specifically regulates PVR expression, we generated *sd* mutant clones specifically in the lymph gland with the *Hand Lineage Trace* (*HLT*) driver. A very striking absence of PVR is observed in *sd* mutant clones compared to WT **(Fig. 6A, B)** definitively demonstrating that Sd regulates PVR. However, this finding does not explain how decreasing *sd* expression causes an increase in PVR levels. Interestingly, a recent report has demonstrated that Sd acts as a negative regulator in the absence of Yki (Koontz et al., 2013), which fits nicely into our observed results when Sd is depleted in the lymph gland. Decreasing levels of Sd could relieve its inhibitory function and allow for greater expression of a transcriptional target like PVR. To further validate this hypothesis, we over-expressed Sd in differentiating hemocytes and evaluated levels of PVR. In this background we observe a striking loss of PVR, coupled with a phenotype that is reminiscent of PVR depletion in differentiating hemocytes **(Fig. 6C)**. Furthermore, this differentiation phenotype can also be rescued by over-expression of activated STAT **(Fig. 6D)** or ADGF **(Fig. 6E)**. Finally, we wished to interrogate the function of Sd's binding partner Yki in regulating PVR. Knockdown of Yki causes a decrease in PVR **(Fig. 7A, B)** while over-expression of Yki causes an increase **(Fig. 7A, C)**, although neither result are as extreme as when Sd levels are manipulated. Importantly, co-depletion of *sd* and *yki* restores PVR levels close to WT **(Fig. 7A, D)**, demonstrating that normal levels of Yki and Sd are required for maintaining proper levels of

PVR in the lymph gland **(Fig. 7E)**. Taken together, these data establish a critical role for Sd in maintaining lymph gland homoestasis by regulating PVR expression and signaling.

It is apparent that Sd plays a critical role in regulating differentiation and maintenance of hematopoietic progenitors in the lymph gland through several different mechanisms. We therefore asked if the function of Sd in maintaining a pool of prohemocytes is necessary for the cellular immune response as previously postulated. To address this question we depleted *sd* in the different hematopoietic compartments of the lymph gland (*Hml-gal4, dome-gal4, and Antp-gal4*) in addition to a ubiquitous knockdown with *HLT*, and subjected these larvae to parasitization by the wasp *Leptopolina boulardi* to induce the cellular immune response (Rizki and Rizki, 1992). Knockdown of *sd* in the prohemocytes of the Medullary Zone **(Fig. 8A, B)** or differentiating hemocytes of the Cortical Zone **(Fig. 8C, D)** did not block lamellocyte differentiation. Similarly, *HLT* mediated depletion of *sd* had no effect on lamellocyte formation **(Fig. 8G, H)**. Interestingly, knockdown of *sd* in the PSC, which has no differentiation phenotype under normal conditions does have a noticeable decrease in lamellocyte differentiation although there are still some lymph glands with lamellocytes present. Previous studies have reported that maintaining a pool of undifferentiated progenitors in the lymph gland is required for mounting the cellular immune response (Krzemien et al., 2007); however lymph glands that have completely differentiated because of *sd* depletion in the Cortical Zone were still capable of forming lamellocytes **(Fig. 8D)**. We also promoted lymph gland differentiation by over-expressing *sd* or depleting *Pvr* in differentiating hemocytes. Both of these backgrounds display complete differentiation phenotypes, but do not inhibit the cellular immune response **(Fig. 8E, F)**. This suggests that the lack of lamellocytes observed in *col* mutant larvae is most likely due to

the absence of the PSC and loss of the EGF ligand Spitz, instead of a loss of undifferentiated prohemcoytes.

To further investigate if Sd function is necessary in the lymph gland for proper immune response, we subjected *sd* hypomorphic larvae to *L. boulardi* wasp parasitization. We first observed a remarkable lack of melanzied tumors in the hemolymph of *sd* hypomorphs that are readily apparent in WT larvae **(Fig. 9A, B)**. 100% of WT larvae displayed small melanotic spots while 80% had large melanotic capsules (n=25). While a majority of *sd* hypomorphs (76%) displayed at least 1 small melanotic spot, there were 0 larvae that had large capsules (n=25) **(Fig. 9C)**. We next asked if this loss of tumor formation was due to decreased numbers of lamellocytes. Indeed, there is a remarkable loss of lamellocytes in wasp parasitized mutant lymph glands compared to WT **(Fig. 9D, E)**. We also examined the numbers of lamellocytes in circulation in *sd* mutants compared to WT parasitized controls and observe a dramatic absence of lamellocytes similar to non-parasitized controls **(Fig. 9 F-I)**.

A closer examination of *sd* mutant lymph glands revealed some very striking differences. There are large numbers of lamellocytes observed in WT lymph glands that are often bursting or falling apart as lamellocytes disperse. Large numbers of lamellocytes can also be observed in expanding secondary lobes. In *sd* hypomorphs lamellocytes are rarely observed, and the few lymph glands that do contain lamellocytes have significantly fewer and are much smaller suggesting they are immature (Honti et al., 2010). Furthermore, the bursting phenotype associated with the cellular immune response is never observed in *sd* hypomorphs and lamellocytes are not present in the hypotrophic secondary lobes of the mutant lymph glands. Given that depletion of *sd* in the PSC has a noticeable phenotype and the requirement for the PSC in the cellular immune response, we asked if PSC identity is compromised in *sd*

hypomorphs. Interestingly, the PSC as detected by the presence of Antp, is still present in *sd* hypomorphs in normal conditions **(Fig. 9J)**, but when parasitized by *L. boulardi* the PSC is completely eliminated in these mutants **(Fig. 9K)**. We also observe a substantial increase in *sd* expression specifically in the PSC of WT wasp parasitized larvae **(Fig. 9L, M)**, suggesting that *sd* is normally upregulated upon immune challengeto help maintain PSC identity. These findings demonstrate a unique requirement for Sd in the maintenance of the PSC under stress conditions **(Fig. 9N)**.

Discussion:

We have previously demonstrated that Sd is required in addition to Yki for *Serrate* expression in Lineage Specifying Cells, which are responsible for crystal cell differentiation in the lymph gland. In this report we have described multiple different mechanisms in which Sd regulates signaling in the lymph gland required for growth, progenitor maintenance, and the cellular immune response. We have described a novel role for the PVR ligand PVF2 in the regulation of growth and organ size in the lymph gland, and identified PVR as a target of Sd regulation in the lymph gland. Furthermore, we described a role for Sd in STAT signal transduction downstream of PVR. Finally, we characterized a novel requirement for Sd in the maintenance of the PSC under immune challenge conditions.

One of the questions that we attempted to address pertained to the requirement for a pool of hematopoietic progenitors in the lymph gland. While our findings identified Sd as an important regulator of prohemocyte maintenance they also definitively demonstrated that maintaining this pool of progenitors is not required for the cellular immune response. However, we cannot rule out the possibility that a population of non-terminally differentiated hemocytes or intermediate progenitors (Krzemien et al., 2010; Dragojlovic-Munther and Martinez-Agosto, 2012) are still present in the differentiated lymph glands we studied. It has been demonstrated that Lat, an inhibitor of Domeless and JAK/STAT signaling is required in the lymph gland for the cellular immune response (Makki et al., 2010), so perhaps this intermediate progenitor population which express *Pxn* and low levels of *domeless* is *the* cell-type that gives rise to lamellocytes in the lymph gland.

Further insight into this quandary may be available by closer examination of TOR signaling in the lymph gland. Our lab has previously demonstrated that activation of TOR by depletion of *Tsc2* induces increased lamellocyte differentiation, which is accompanied by expansion of intermediate progenitors identified by low *dome* and Pxn co-expression in addition to high levels of p4EBP (Dragojlovic-Munther and Martinez-Agosto, 2012). Furthermore, TOR activation by over-expression of the FGF ligand *thisbe* or the FGFR $heartless^{Act}$ also induces lamellocyte differentiation (Dragojlovic-Munther and Martinez-Agosto, 2013) suggesting that TOR signaling downstream of *heartless* may prime intermediate progenitors for differentiation into the lamellocyte lineage. Investigating levels of p4EBP in wasp parasitized larvae may provide more insight into whether TOR activation and an intermediate transition from progenitor to terminally differentiated lamellocyte is essential.

We have also characterized an intriguing requirement for Sd in the PSC of immune challenged larvae. Previously, we have described the changes to *sd* expression in the lymph gland upon immune parasiztization as *sd* expression is dramatically reduced. In addition, we depleted *sd* specifically in *sd* expressing cells (including lamellocytes) and still observed lamellocyte differentiation upon wasp parasitization leading us to believe *sd* was not required in lamellocyte differentiation. However, we have more recently observed the inability of *sd* hypomorphic larvae to mount an immune response to wasp parasitization. While these findings appear to be contradictory, it is possible that RNAi dependent knockdown of *sd* is not powerful enough to fully inhibit Sd's function in the PSC, while *sd* hypomorphs have more significantly reduced *sd* function. This possibility is further supported by the fact that *sd* hypomorphic larvae display a severe lymph gland growth defect that is not observed with *sd* knockdown suggesting a greater loss of Sd function is present in the hypomorphic mutants. While our findings clearly

demonstrate that Sd is required in the PSC, it remains unclear the mechanism by which Sd functions to maintain this signaling center in immune challenge conditions.

Figures:

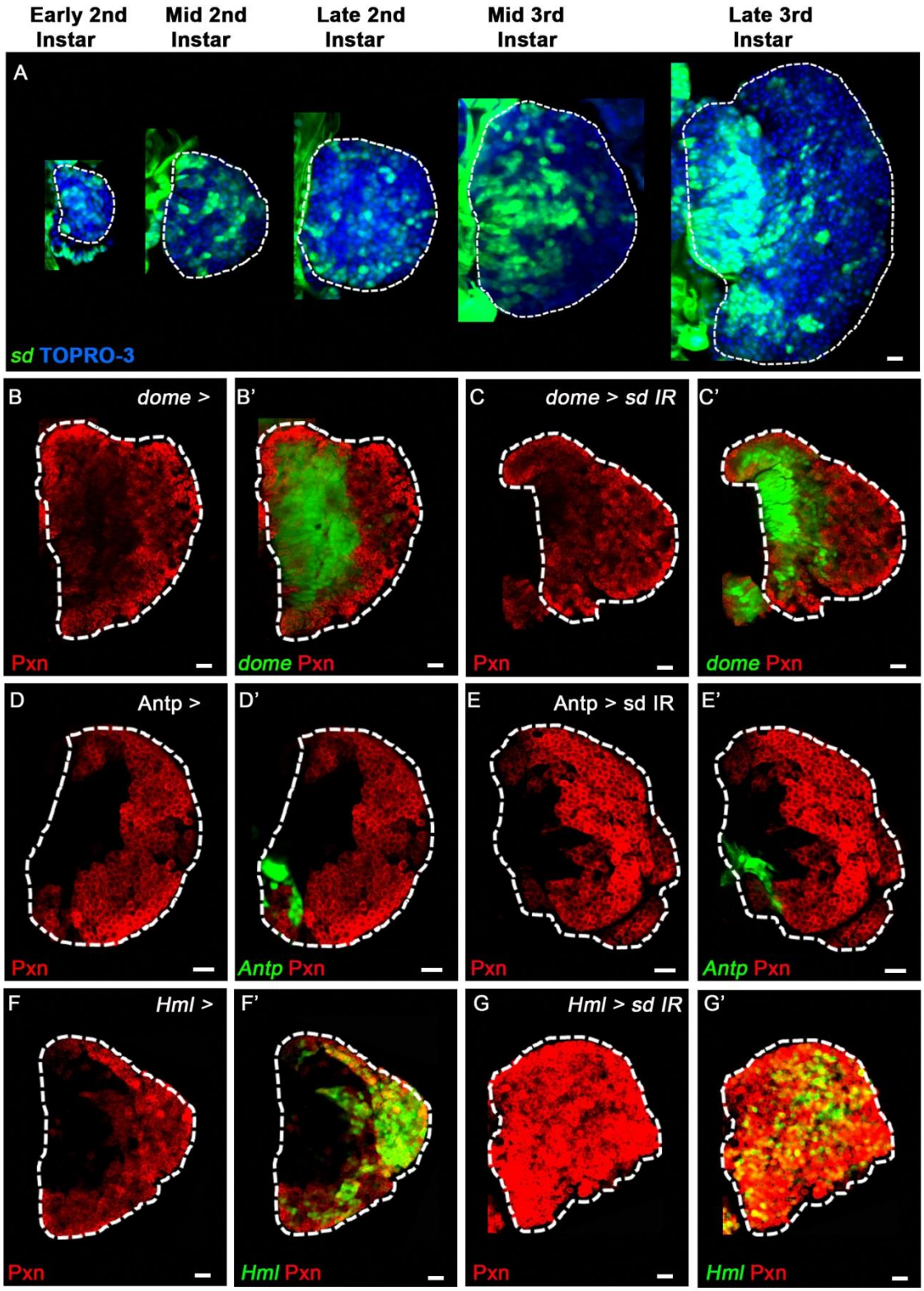
Early 2nd Instar
Mid 2nd Instar
Late 2nd Instar
Mid 3rd Instar
Late 3rd Instar
A
sd TOPRO-3
B
dome >
Pxn
B'
dome Pxn
C
dome > sd IR
Pxn
C'
dome Pxn
D
Antp >
Pxn
D'
Antp Pxn
E
Antp > sd IR
Pxn
E'
Antp Pxn
F
Hml >
Pxn
F'
Hml Pxn
G
Hml > sd IR
Pxn
G'
Hml Pxn

Figure 3-1. Scalloped expression and function in the lymph gland. (A) Expression of *sd-gal4, UAS-GFP* (green) in the lymph gland from the early 2nd instar to late 3rd instar. Topro-3 labels nuclei (blue). (B) Differentiating hemocytes labeled with Peroxidasin (Pxn, Red) in WT lymph gland and (B')prohemocytes are labeled with *dome-gal4, UAS-GFP* (Green). (B") Merge channels. (C) Depletion of *sd* in prohemocytes with *dome-gal4.* Differentiating hemocytes labeled with Pxn (Red)and (C') prohemocytes lableled with *dome* (Green). (C") Merge channels. (D) Differentiating hemocytes labeled with Pxn (Red) and (D') PSC labeled with *Antp-gal4, UAS-GFP* (Green). (D") Merge channels. (E) Depletion of *sd* in the PSC with *Antp-gal4.*Differentiating hemocytes labeled with Pxn (Red) and (E')PSC labeled with *Antp* (Green). (E") Merge channels. (F) Differentiating hemocytes are labeled with Pxn (Red) and (F') *Hml-gal4, UAS-GFP* (Green) in the WT lymph gland. (F") Merge channels. (G) Depletion of *sd* in differentiating hemocytes with *Hml-gal4* causes precocious differentiation. Differentiating hemocytes labeled with Pxn (Red) and (G')*Hml* (Green). (G") Merge channels. Scale bar 10 μm.

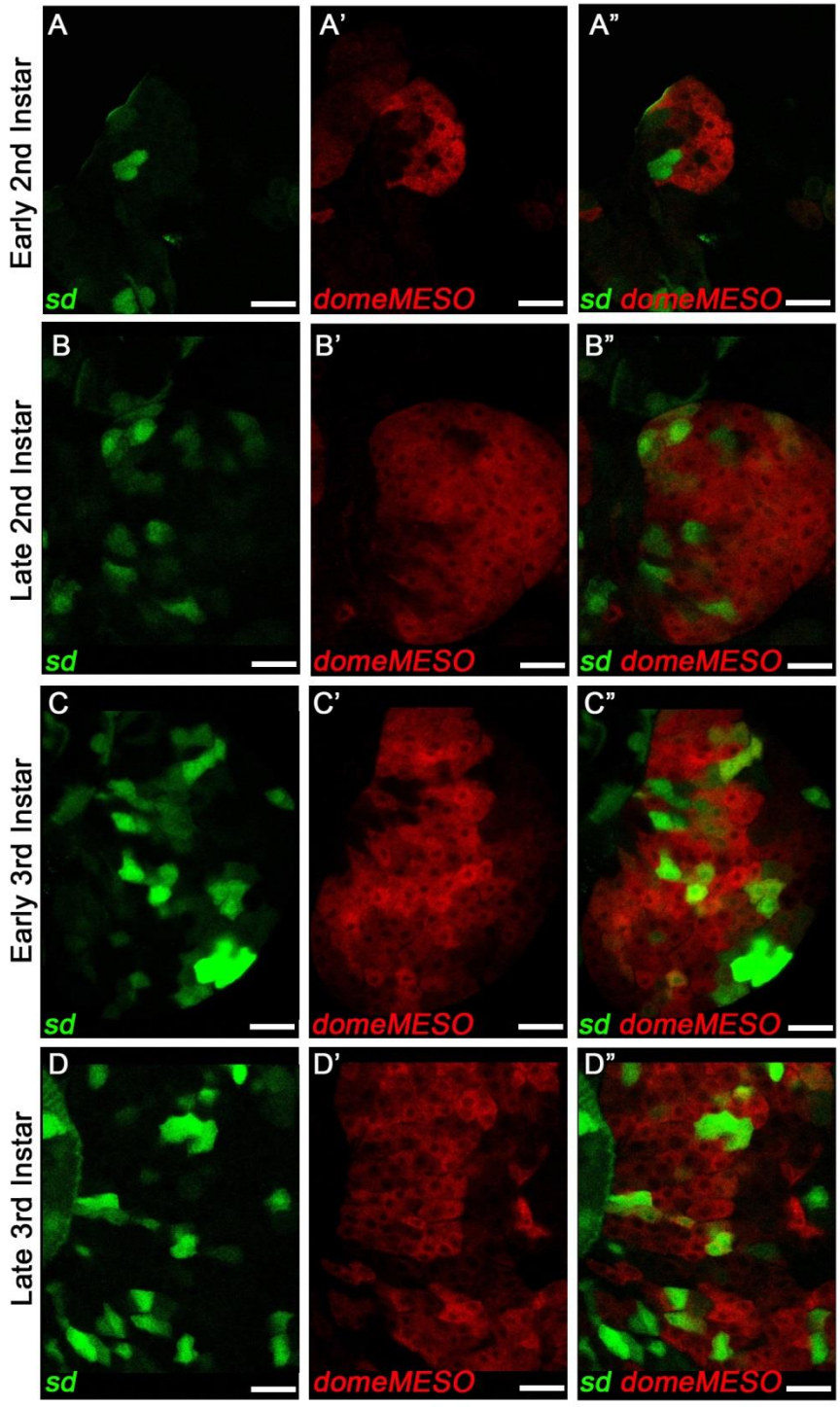

Figure 3-2. Staged expression of *scalloped* and *domeless* in the lymph gland. (A-A") *sd* (Green) expression is restricted to a small number of cells near the dorsal vessel in the early 2nd

instar lymph gland that do not express (A') *domeMESO* (Red).(A") Merge channels. (B-B") By the late 2nd instar, *sd* expression (Green) has expanded throughout the developing lymph gland, but is only seen in a small percentage of (B') *domeMESO* (Red) expressing cells. (B") Merge channels. (C-C") A similar expression pattern for *sd* (Green) and (C') *domeMESO* (Red) is observed in the early 3rd instar. (C") Merge channels. (D-D") By the late third instar, *sd* (Green) expression is readily observed throughout the lymph gland, but is still largely absent in (D') *domeMESO* (Red) expressing cells. (D") Merge channels. Scale bar 10 μm.

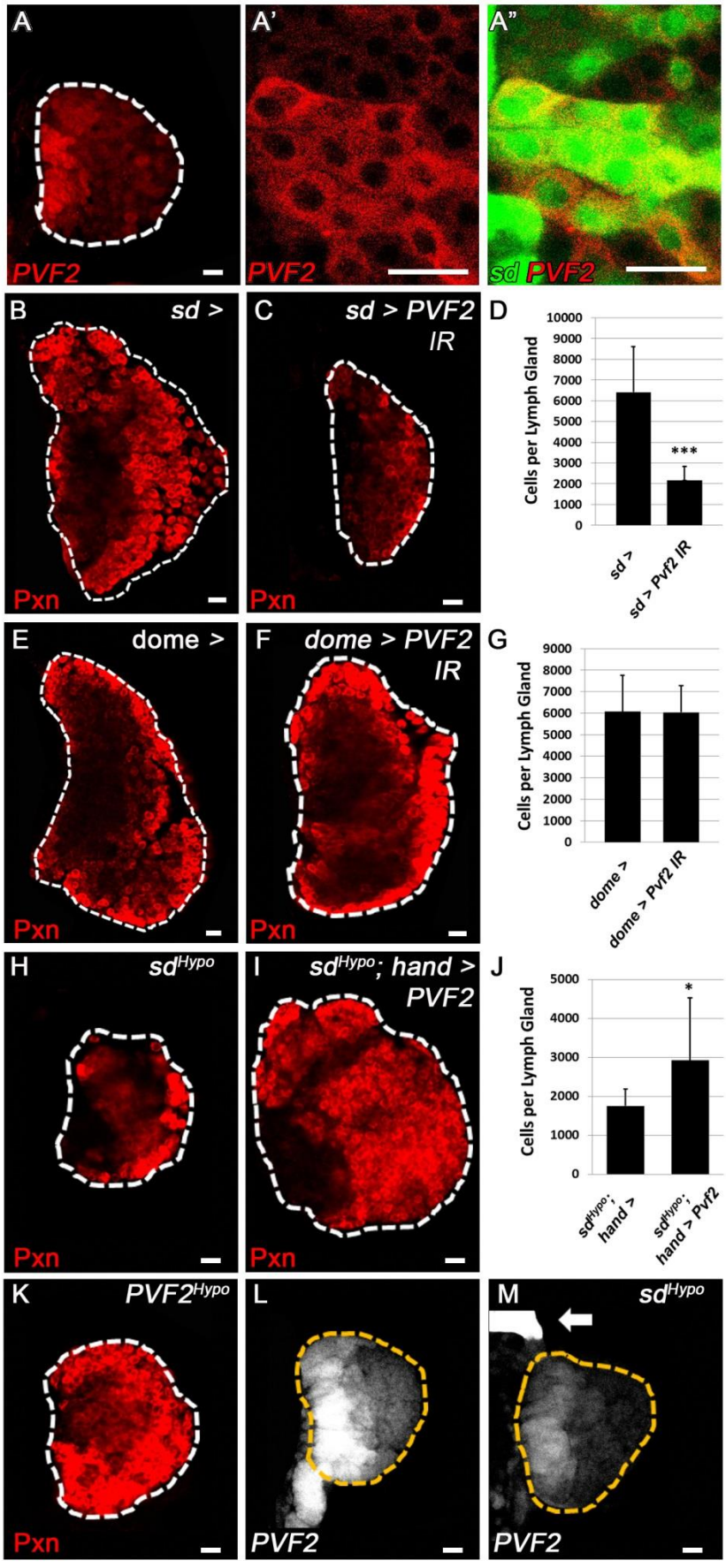
A
PVF2
A'
PVF2
A"
sd PVF2
B
sd >
Pxn
C
sd > PVF2 IR
Pxn
D
Cells per Lymph Gland
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
***
sd >
sd > Pvf2 IR
E
dome >
Pxn
F
dome > PVF2 IR
Pxn
G
Cells per Lymph Gland
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
dome >
dome > Pvf2 IR
H
sdHypo
Pxn
I
sdHypo; hand > PVF2
Pxn
J
Cells per Lymph Gland
5000
4000
3000
2000
1000
0
*
sdHypo; hand >
sdHypo; hand > Pvf2
K
PVF2Hypo
Pxn
L
PVF2
M
sdHypo
PVF2

Figure 3-3. Scalloped controls lymph gland size through regulation of Pvf2. (A)*Pvf2* expression in the early 3rd instar lymph gland. (A')*Pvf2* (Red) is co-expressed with (A") *sd* (Green). (B-K) Pxn (Red) labels differentiating hemocytes. (B)WT lymph gland. (C) *Pvf2* depletion with *sd-gal4.* (D) Quantification of *Pvf2* depletion in *sd* expressing cells (n=10). (E) WT lymph gland. (F) *Pvf2* depletion with *dome-gal4.* (G) Quantification of *Pvf2* depletion in prohemocytes (n=10). (H) Lymph gland of *sd* hypomorph. (I) Over-expression of *Pvf2* with *hand-gal4* rescues growth phenotype observed in *sd* hypomorphs. (J) Quantification of lymph gland size, (n=10). (K) Lymph gland of *Pvf2* hypomorph. (L) *Pvf2* expression in WT lymph gland. (M) *Pvf2* expression in *sd* hypomorph. Arrow is pointing towards the Ring Gland, internal control. Scale bar 10 μm.

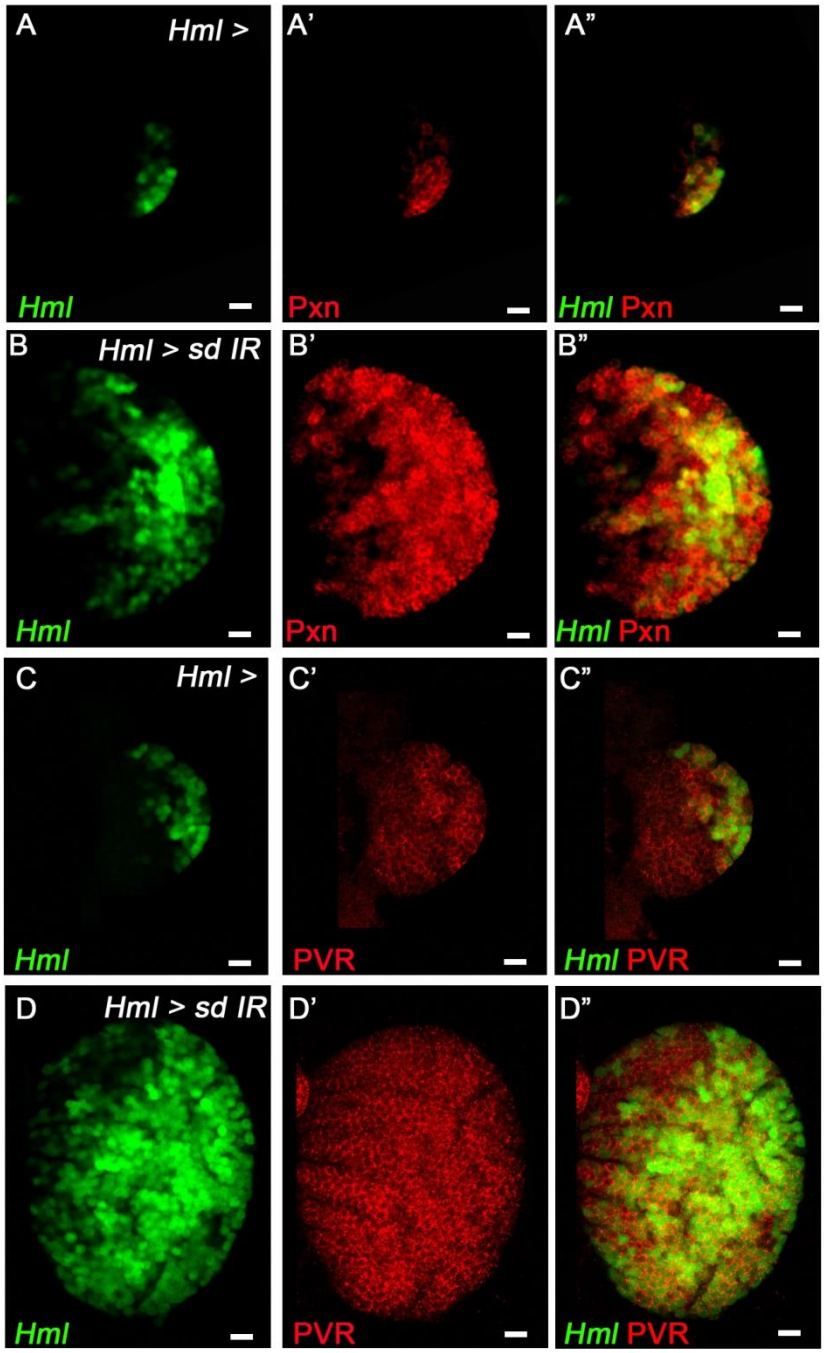

Figure 3-4. Depletion of *scalloped* in differentiating hemocytes induces rapid differentiation of the lymph gland. (A-A") Differentiating hemocytes expressing (A) *Hml* and (A') Pxn are observed in the 2nd instar WT lymph gland. (A") Merge channels. (B-B") Depletion of *sd* in differentiating hemocytes causes a robust increase in differentiation labeled by (B) *Hml* and (B')

Pxn. (B") Merge channels. (C-C") A modestly expanded Cortical Zone is labeled by (C) *Hml* and (C') PVR in the late 2nd instar lymph gland. (C") Merge channels. (D-D") Depletion of *sd* in differentiating cells of late 2nd instar lymph gland causes differentiation of entire organ labeled by (D) *Hml*, however changes to (D') PVR are not observed at this stage. (D") Merge channels. Scale bar 10 µm.

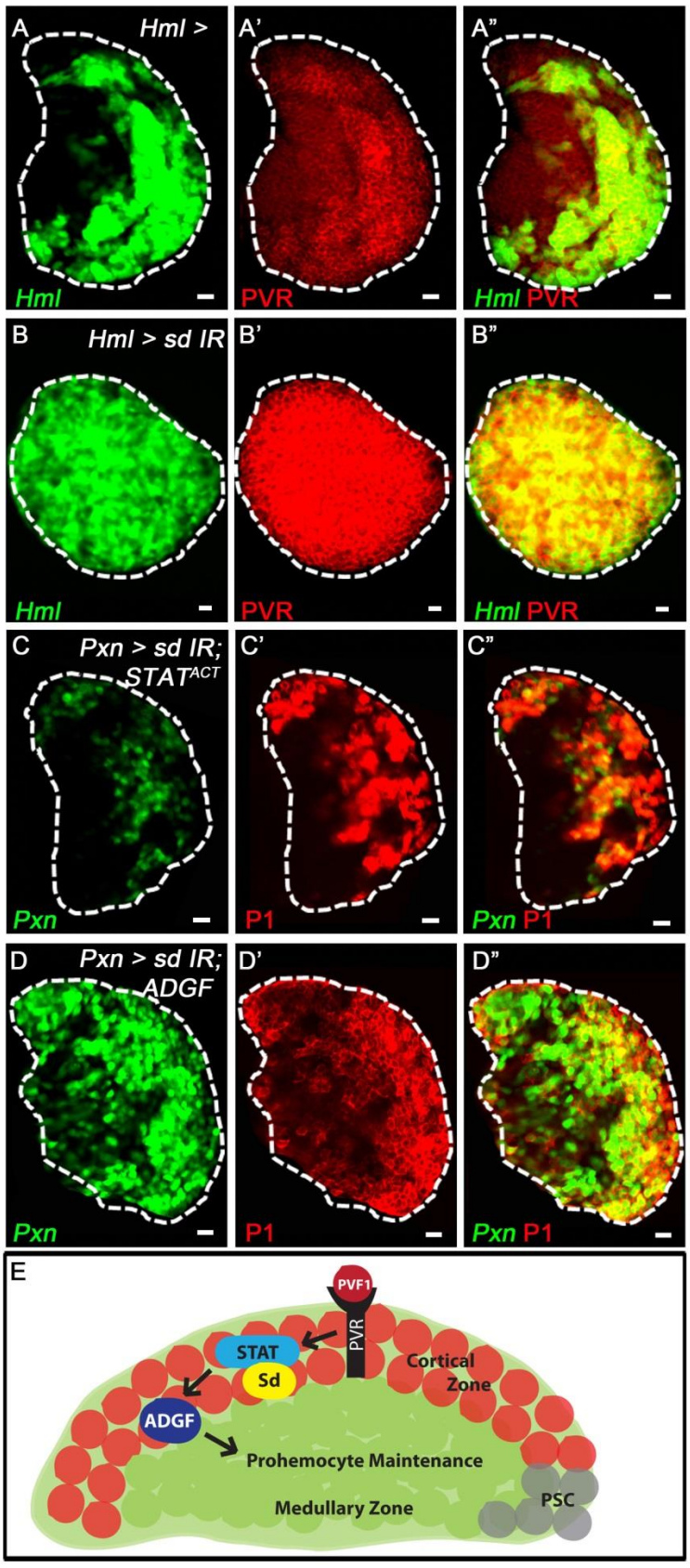
A
Hml >
Hml
A'
PVR
A"
Hml PVR
B
Hml > sd IR
Hml
B'
PVR
B"
Hml PVR
C
Pxn > sd IR;
STAT^ACT
Pxn
C'
P1
C"
Pxn P1
D
Pxn > sd IR;
ADGF
Pxn
D'
P1
D"
Pxn P1
E
PVF1
PVR
STAT
Sd
ADGF
Cortical
Zone
Prohemocyte Maintenance
Medullary Zone
PSC

Figure 3-5. Scalloped regulates prohemocyte maintenance through modulation of PVR signaling. (A-A") Differentiating hemocytes express *Hml* (Green) and (A') high levels of PVR (Red). (A") Merge channels. (B-B") Depletion of *sd* in differentiating hemocytes (*Hml*, Green) dramatically increases (B') PVR (Red) expression. (B") Merge channels. (C-C") Over-expression of $STAT^{Act}$ in (C) differentiating hemocytes (*Pxn*, Green) rescues differentiation induced by *sd* depletion. (C') Mature plasmatocytes are labeled with P1 (Red). (C") Merge channels. (D-D") Over-expression of *ADGF* in (D) differentiating hemocytes (*Pxn*, Green) rescues differentiation induced by *sd* depletion. (D') Mature plasmatocytes are labeled with P1 (Red). (D") Merge channels. (E) Schematic representation of Sd's function in maintenance of prohemocytes. Sd is required for proper STAT signaling and activation of ADGF downstream of PVR in the lymph gland. Scale bar 10 μm.

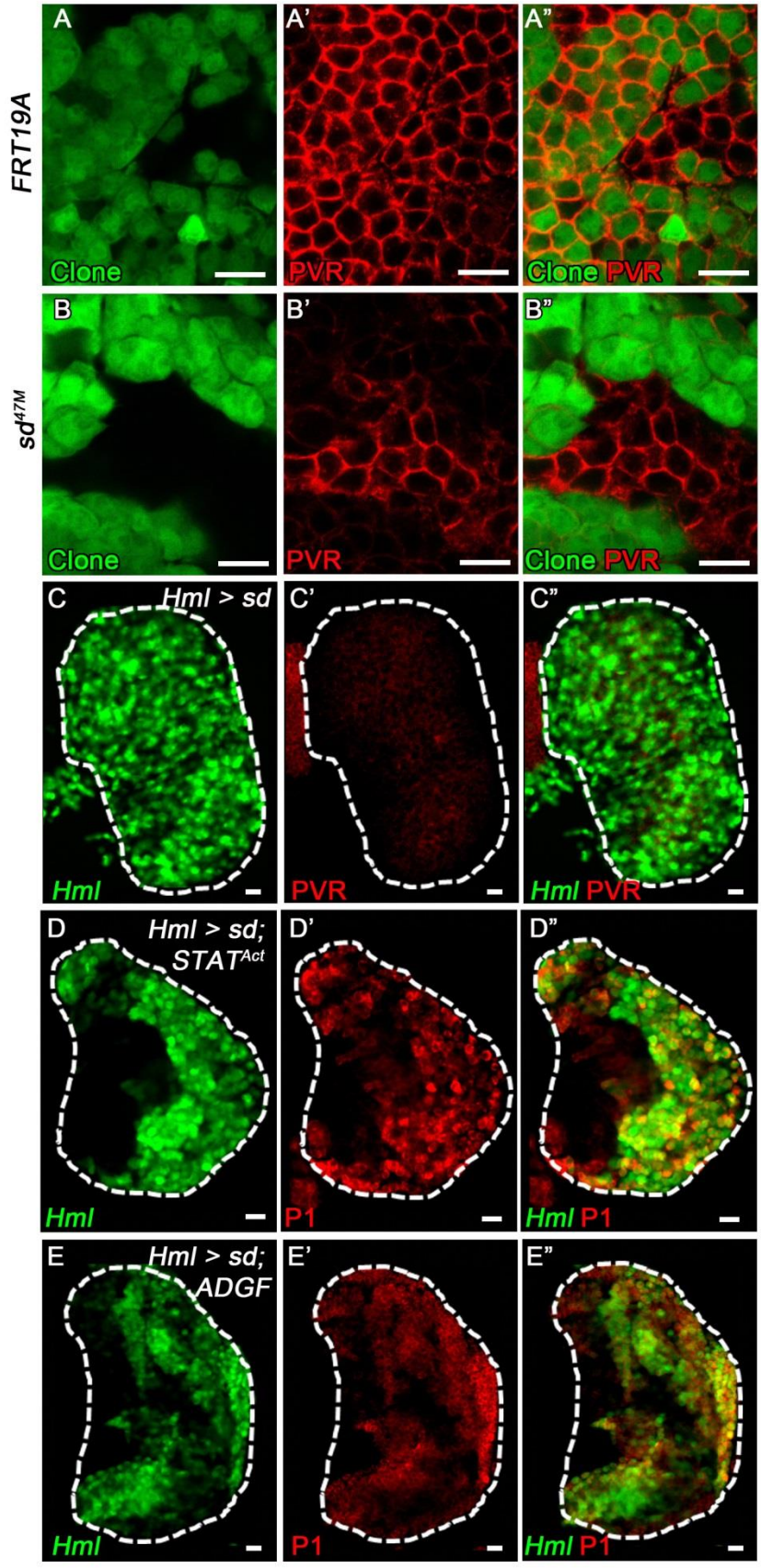
FRT19A
sd47M
A
A'
A"
Clone
PVR
Clone PVR
B
B'
B"
Clone
PVR
Clone PVR
C
Hml > sd
C'
C"
Hml
PVR
Hml PVR
D
Hml > sd;
STATAct
D'
D"
Hml
P1
Hml P1
E
Hml > sd;
ADGF
E'
E"
Hml
P1
Hml P1

Figure 3-6. Scalloped regulates PVR expression in the lymph gland. (A-A")PVR (Red) expression in the Cortical Zone of WT lymph glands with *HLT* clones (Green). (B-B") PVR (Red) is absent in *sd* mutant *HLT* clones (Green). (C-C") Over-expressionof *sd* in (C) differentiating hemocytes (*Hml*, Green) causes loss of (C') PVR (Red). (C") Merge channels. (D-D") Over-expression of $STAT^{Act}$ in (D) differentiating hemocytes (*Hml*, Green) rescues phenotype induced by *sd* over-expression. (D') P1 labels terminally differentiated plasmatocytes. (D") Merge channels. (E-E") Over-expression of *ADGF* in (E) differentiating hemocytes (*Hml*, Green) rescues phenotype induced by *sd* over-expression. (E') P1 labels terminally differentiated plasmatocytes. (E") Merge channels. Scale bar 10 μm.

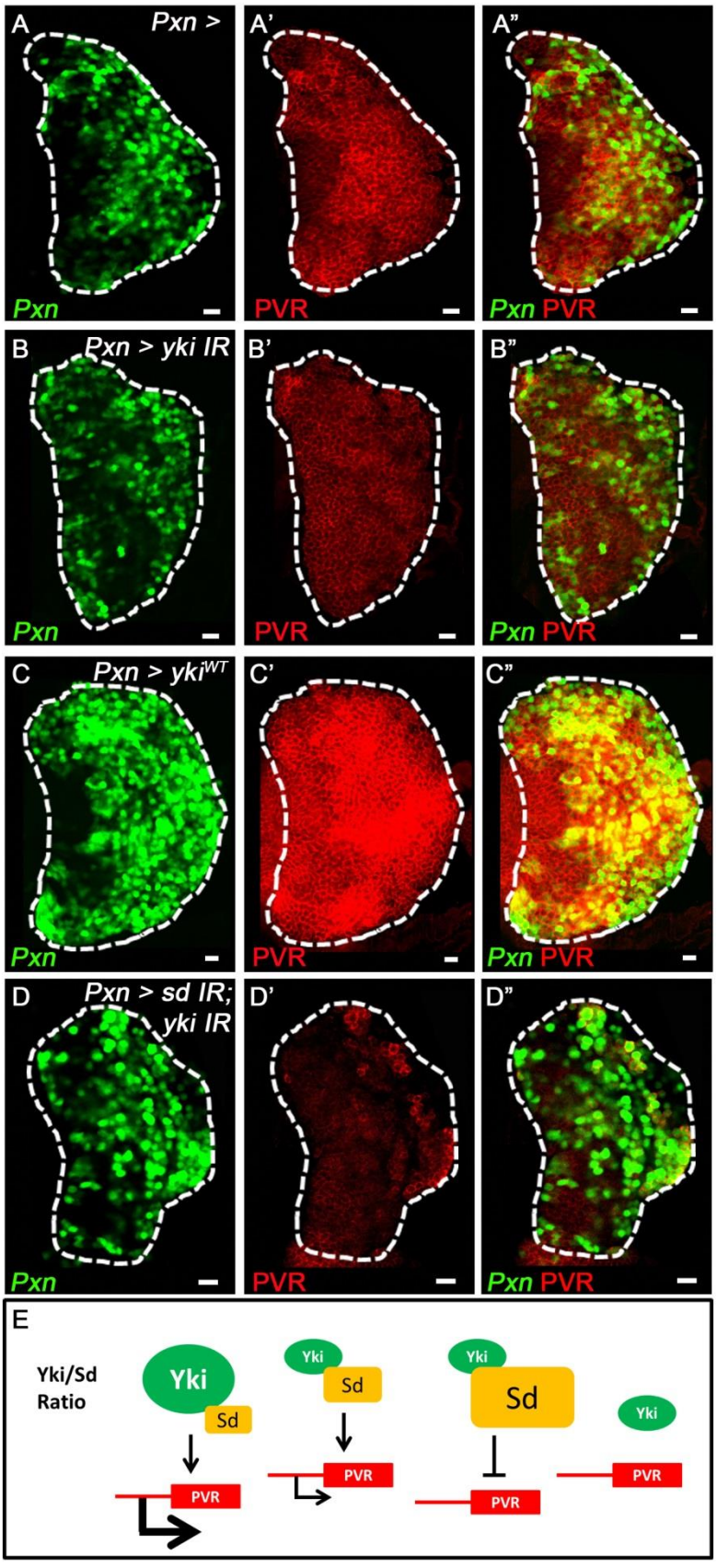
A
Pxn >
Pxn
A'
PVR
A"
Pxn PVR
B
Pxn > yki IR
Pxn
B'
PVR
B"
Pxn PVR
C
Pxn > yki^WT
Pxn
C'
PVR
C"
Pxn PVR
D
Pxn > sd IR;
yki IR
Pxn
D'
PVR
D"
Pxn PVR
E
Yki/Sd
Ratio
Yki
Sd
PVR
Yki
Sd
PVR
Yki
Sd
PVR
Yki
PVR

Figure 3-7. Yorkie functions with Scalloped to regulate PVR in the lymph gland. (A-A") Differentiating hemocytes in the WT lymph gland express *Pxn* (Green) and (A') high levels of PVR (Red). (A") Merge channels. (B-B") Depletion of *yki* in differentiating hemocytes (*Pxn*, Green) decreases levels of (B') PVR (Red). (B") Merge channels. (C-C") Over-expression of *yki* in differentiating hemocytes (*Pxn*, Green) increases levels of (C') PVR (Red). (C") Merge channels. (D-D") Depeltion of *yki* and *sd* in differentiating hemocytes (*Pxn*, Green) restores levels of (D') PVR (Red). (D") Merge Channels. (E) Schematic representation of Sd and Yki regulation of PVR. Depletion of Sd or increased levels of Yki leads to increased PVR, while increased levels of Sd or loss of Yki causes inhibition of PVR. PVR is not expressed in the absence of Sd. Scale bar 10 μm.

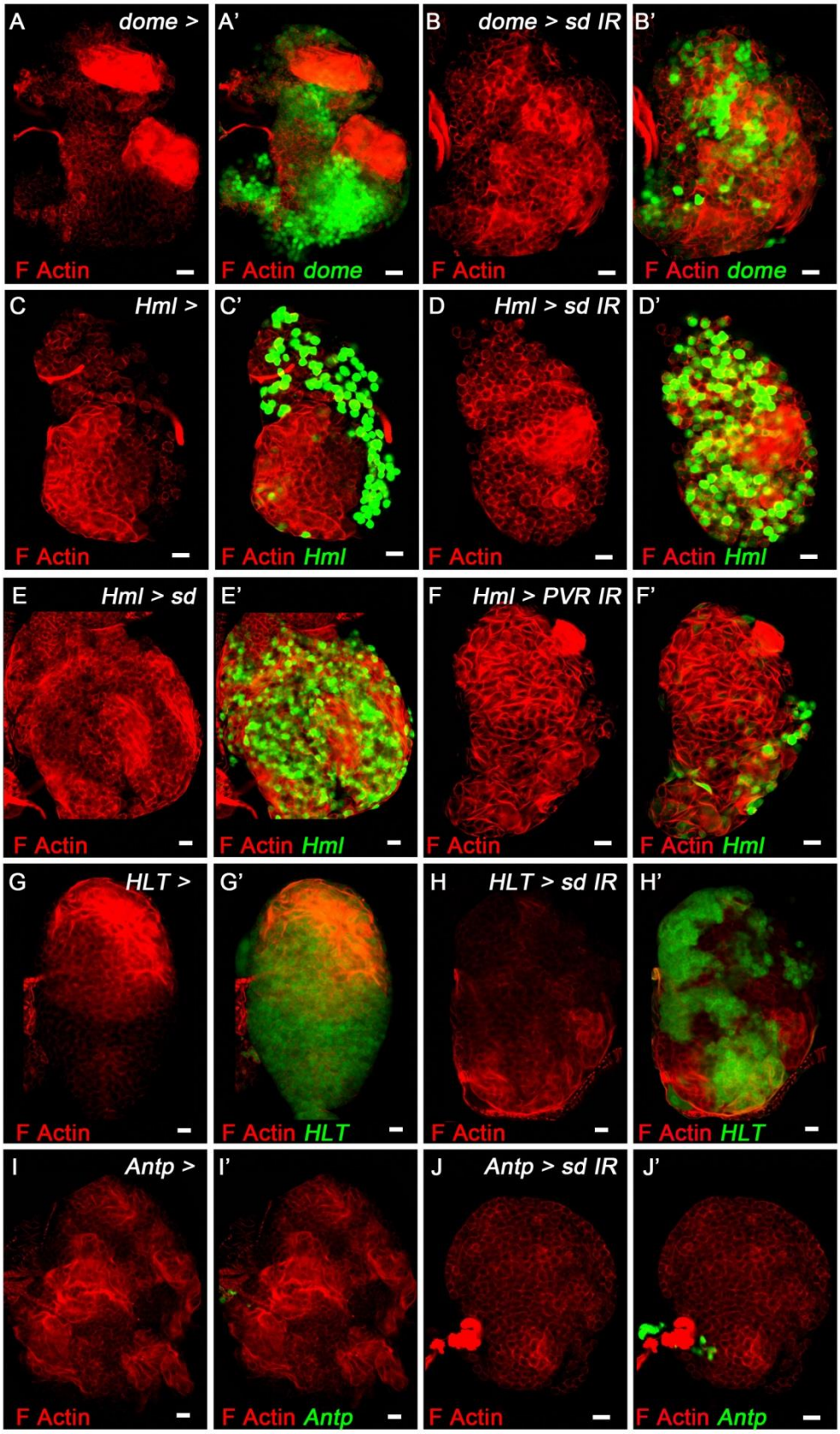
A
dome >
F Actin
A'
F Actin dome
B
dome > sd IR
F Actin
B'
F Actin dome
C
Hml >
F Actin
C'
F Actin Hml
D
Hml > sd IR
F Actin
D'
F Actin Hml
E
Hml > sd
F Actin
E'
F Actin Hml
F
Hml > PVR IR
F Actin
F'
F Actin Hml
G
HLT >
F Actin
G'
F Actin HLT
H
HLT > sd IR
F Actin
H'
F Actin HLT
I
Antp >
F Actin
I'
F Actin Antp
J
Antp > sd IR
F Actin
J'
F Actin Antp

Figure 3-8. Cellular Immune Response in the absence of Scalloped. (A-J) All larvae were subjected to wasp parasitization, and F-Actin (Red) is used to label lamellocytes. (A-A') WT lymph gland displays normal lamellocyte differentiation upon immune challenge. Prohemocytes are labeled by *dome* (Green). (B-B') Depletion of *sd* in prohemocytes (*dome,* Green) does not affect lamellocyte differentiation. (C-C') WT lymph gland displays normal lamellocyte differentiation upon immune challenge. Differentiating cells are labeled by *Hml* (Green). (D-D') Depletion of *sd* in differentiating hemocytes (*Hml,* Green) still increases differentiation, but does not affect lamellocyte differentiation. (E-E') Over-expression of *sd* in differentiating hemocytes (*Hml,* Green) induces full lymph gland differentiation, but does not inhibit lamellocyte differentiation. (F-F') Depletion of *Pvr* in differentiating hemocytes (*Hml,* Green) does not inhibit lamellocyte differentiation despite the differentiation phenotype. (G-G') WT lymph gland displays normal lamellocyte differentiation (*HLT,* Green). (H-H') Clonal depletion of *sd* in the lymph gland with *HLT* (Green) does not block lamellocyte differentiation. (I-I') Normal lamellocyte differentiation observed in WT lymph glands. PSC labeled by *Antp* (Green). (J-J') Depletion of *sd* in the PSC (*Antp,* Green) partially blocks lamellocyte differentiation, although small immature lamellocytes may still be present. Scale bar 10 μm.

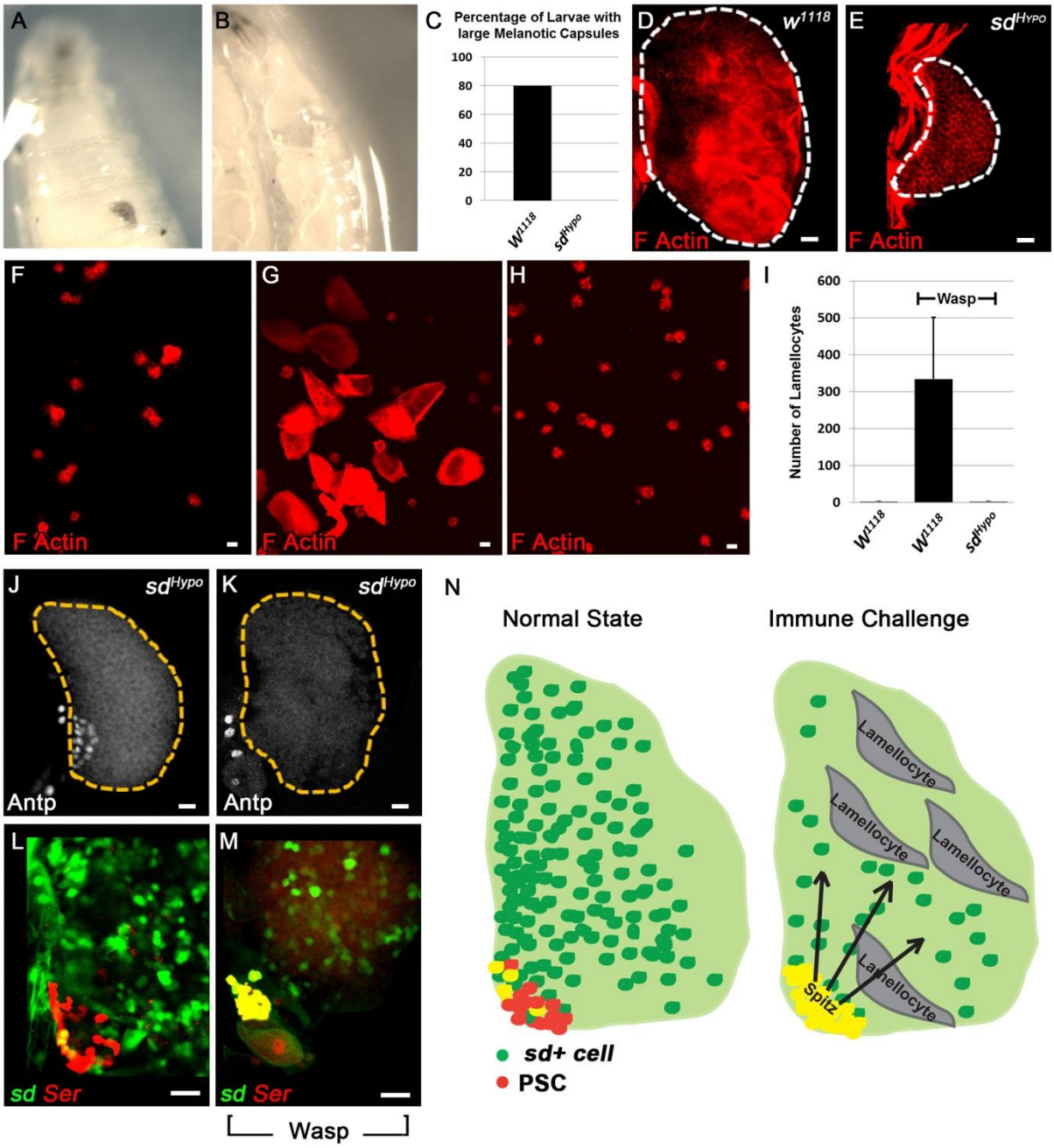

Figure 3-9. Cellular immune response is completely absent in *scalloped* hypomorphic larvae. (A) Large melanotic capsules are observed in WT larvae infested by *L. boulardi*. (B) *sd* hypomorphic larvae do not present melanotic capsules after wasp parasitization. (C) Quantification of frequency of large melanotic capsules in wasp parasitized larvae (n=25). (D)

Lamellocytes differentiate in WT lymph gland after wasp parasitization. (E) Lamellocytes are rarely observed in *sd* hypomorphic lymph glands. (F) Lamellocytes are rarely observed in circulation of non-infested larva. (G) Lamellocytes are readily observed in circulation of wasp parasitized WT larva. (H) Lamellocytes do not differentiate in *sd* hypomorphic larvae after wasp parasitization. (I) Quantification of lamellocyte numbers in circulation (n=10). (J) Antp is expressed in the PSC of *sd* hypomorphic lymph glands in normal conditions. (K) Antp is completely absent in the lymph glands of wasp parasitized *sd* hypomorphic larvae. (L) *sd* (Green) is observed in a small percentage of *Serrate*$^{+}$ (Red) PSC cells. (M) Wasp parasitzation induces a dramatic increase in *sd* (Green) expression in the PSC (*Serrate,* Red). (N) Schematic representation of changes to *sd* expression upon wasp parasitization. *Sd* (Green) expression is increased in the PSC (Red) to maintain PSC identity, allowing secretion of the EGF ligand Spitz to induce lamellocyte differentiation in the cellular immune response. Scale bar 10 μm.

References:

Crozatier, M., Ubeda, J.-M., Vincent, A. and Meister, M. (2004) 'Cellular Immune Response to Parasitization in *Drosophila* Requires the EBF Orthologue Collier', *PLoS Biol* 2(8): e196.

Dragojlovic-Munther, M. and Martinez-Agosto, J. A. (2012) 'Multifaceted roles of PTEN and TSC orchestrate growth and differentiation of Drosophila blood progenitors', *Development* 139(20): 3752-3763.

Dragojlovic-Munther, M. and Martinez-Agosto, J. A. (2013) 'Extracellular matrix-modulated Heartless signaling in Drosophila blood progenitors regulates their differentiation via a Ras/ETS/FOG pathway and target of rapamycin function', *Developmental Biology* 384(2): 313-330.

Honti, V., Csordás, G., Márkus, R., Kurucz, É., Jankovics, F. and Andó, I. (2010) 'Cell lineage tracing reveals the plasticity of the hemocyte lineages and of the hematopoietic compartments in Drosophila melanogaster', *Molecular Immunology* 47(11–12): 1997-2004.

Jung, S.-H., Evans, C. J., Uemura, C. and Banerjee, U. (2005) 'The Drosophila lymph gland as a developmental model of hematopoiesis', *Development* 132(11): 2521-2533.

Khadilkar, R. J., Rodrigues, D., Mote, R. D., Sinha, A. R., Kulkarni, V., Magadi, S. S. and Inamdar, M. S. (2014) 'ARF1–GTP regulates Asrij to provide endocytic control of Drosophila blood cell homeostasis', *Proceedings of the National Academy of Sciences* 111(13): 4898-4903.

Koontz, Laura M., Liu-Chittenden, Y., Yin, F., Zheng, Y., Yu, J., Huang, B., Chen, Q., Wu, S. and Pan, D. (2013) 'The Hippo Effector Yorkie Controls Normal Tissue Growth by Antagonizing Scalloped-Mediated Default Repression', *Developmental Cell* 25(4): 388-401.

Krzemien, J., Dubois, L., Makki, R., Meister, M., Vincent, A. and Crozatier, M. (2007) 'Control of blood cell homeostasis in Drosophila larvae by the posterior signalling centre', *Nature* 446(7133): 325-328.

Krzemien, J., Oyallon, J., Crozatier, M. and Vincent, A. (2010) 'Hematopoietic progenitors and hemocyte lineages in the Drosophila lymph gland', *Developmental Biology* 346(2): 310-319.

Lanot, R., Zachary, D., Holder, F. and Meister, M. (2001) 'Postembryonic Hematopoiesis in Drosophila', *Developmental Biology* 230(2): 243-257.

Lebestky, T., Jung, S.-H. and Banerjee, U. (2003) 'A Serrate-expressing signaling center controls Drosophila hematopoiesis', *Genes & Development* 17(3): 348-353.

Makki, R., Meister, M., Pennetier, D., Ubeda, J.-M., Braun, A., Daburon, V., Krzemień, J., Bourbon, H.-M., Zhou, R., Vincent, A. et al. (2010) 'A Short Receptor Downregulates JAK/STAT Signalling to Control the Drosophila Cellular Immune Response', *PLoS Biol* 8(8): e1000441.

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

Mondal, Bama C., Mukherjee, T., Mandal, L., Evans, Cory J., Sinenko, Sergey A., Martinez-Agosto, Julian A. and Banerjee, U. (2011) 'Interaction between Differentiating Cell- and Niche-Derived Signals in Hematopoietic Progenitor Maintenance', *Cell* 147(7): 1589-1600.

Oh, H. and Irvine, K. D. (2011) 'Cooperative Regulation of Growth by Yorkie and Mad through bantam', *Developmental Cell* 20(1): 109-122.

Ren, F., Wang, B., Yue, T., Yun, E.-Y., Ip, Y. T. and Jiang, J. (2010) 'Hippo signaling regulates Drosophila intestine stem cell proliferation through multiple pathways', *Proceedings of the National Academy of Sciences* 107(49): 21064-21069.

Rizki, T. M. and Rizki, R. M. (1992) 'Lamellocyte differentiation in Drosophila larvae parasitized by Leptopilina', *Developmental & Comparative Immunology* 16(2–3): 103-110.

Sinenko, S. A., Shim, J. and Banerjee, U. (2012) *Oxidative stress in the haematopoietic niche regulates the cellular immune response in Drosophila*.

Wu, S., Liu, Y., Zheng, Y., Dong, J. and Pan, D. (2008) 'The TEAD/TEF Family Protein Scalloped Mediates Transcriptional Output of the Hippo Growth-Regulatory Pathway', *Developmental Cell* 14(3): 388-398.

Appendix

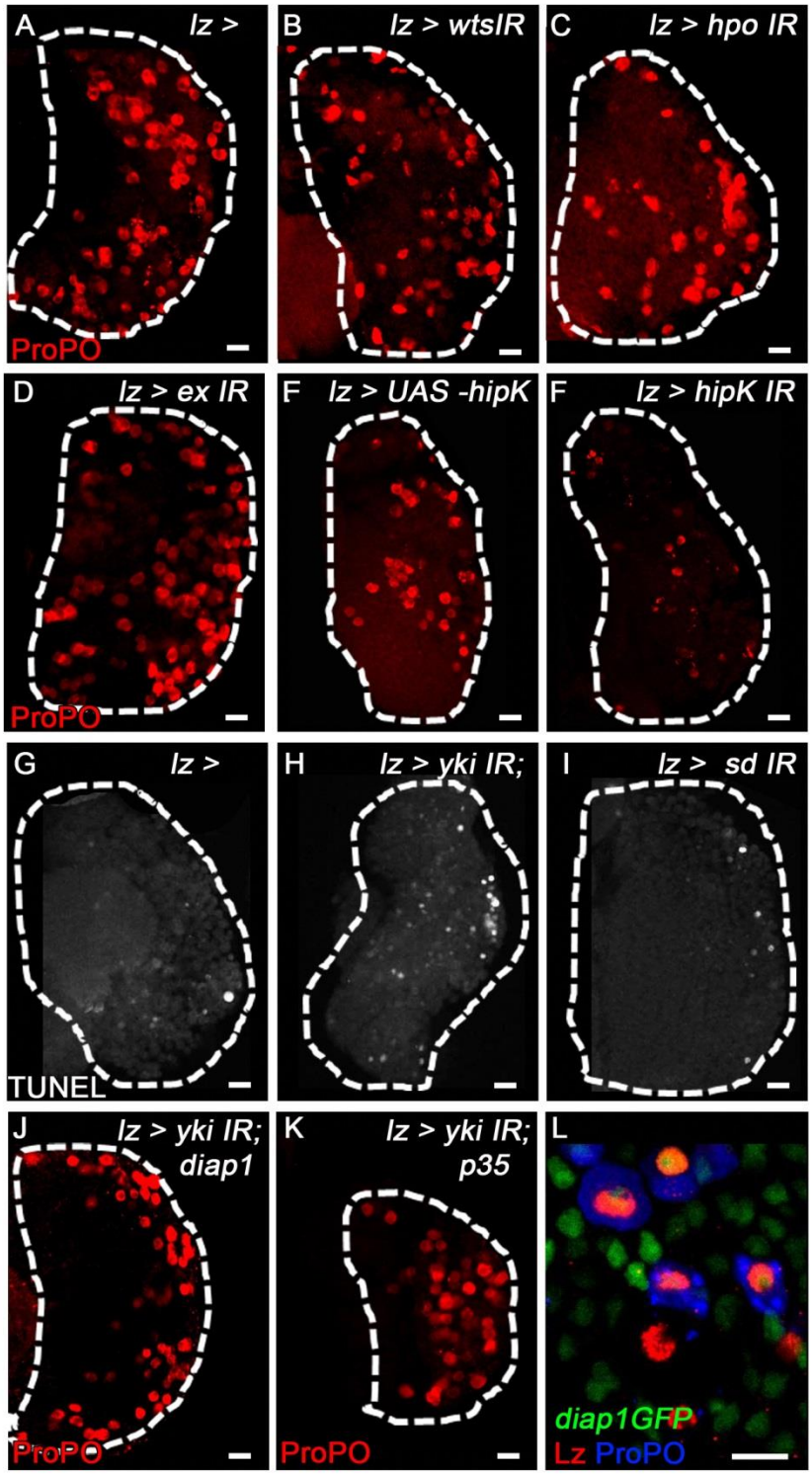

Figure A-1. Hippo pathway regulation of crystal cell differentiation and survival. (A-F) ProPO labels mature crystal cells (Red). (A) WT lymph gland. (B) Depletion of *warts* in crystal

cell progenitors does not affect crystal cell numbers. (C) Depletion of *hippo* in crystal cell progentiors does not affect crystal cell numbers. (D) Depletion of *expanded* in crystal cell progenitors does not affect crystal cell numbers. (E) Over-expression of *hipK* in crystal cell progenitors does not affect crystal cell numbers. (F) Depletion of *hipK* in crystal cell progenitors leads to complete loss of mature crystal cells. (G-I) TUNEL labels apoptotic cells (White) (G) WT (H) *yki* and (I) *sd* depletion in crystal cell progenitors increases apoptosis, that is rescued by over-expression of (J) *diap1* and (K) *p35.* (F) *diap1*(green) is expressed in the Cortical Zone of 3rd instar lymph glands, and is specifically co-expressed with Lz (Red) and ProPO (Blue). Scale bar 10 µm.

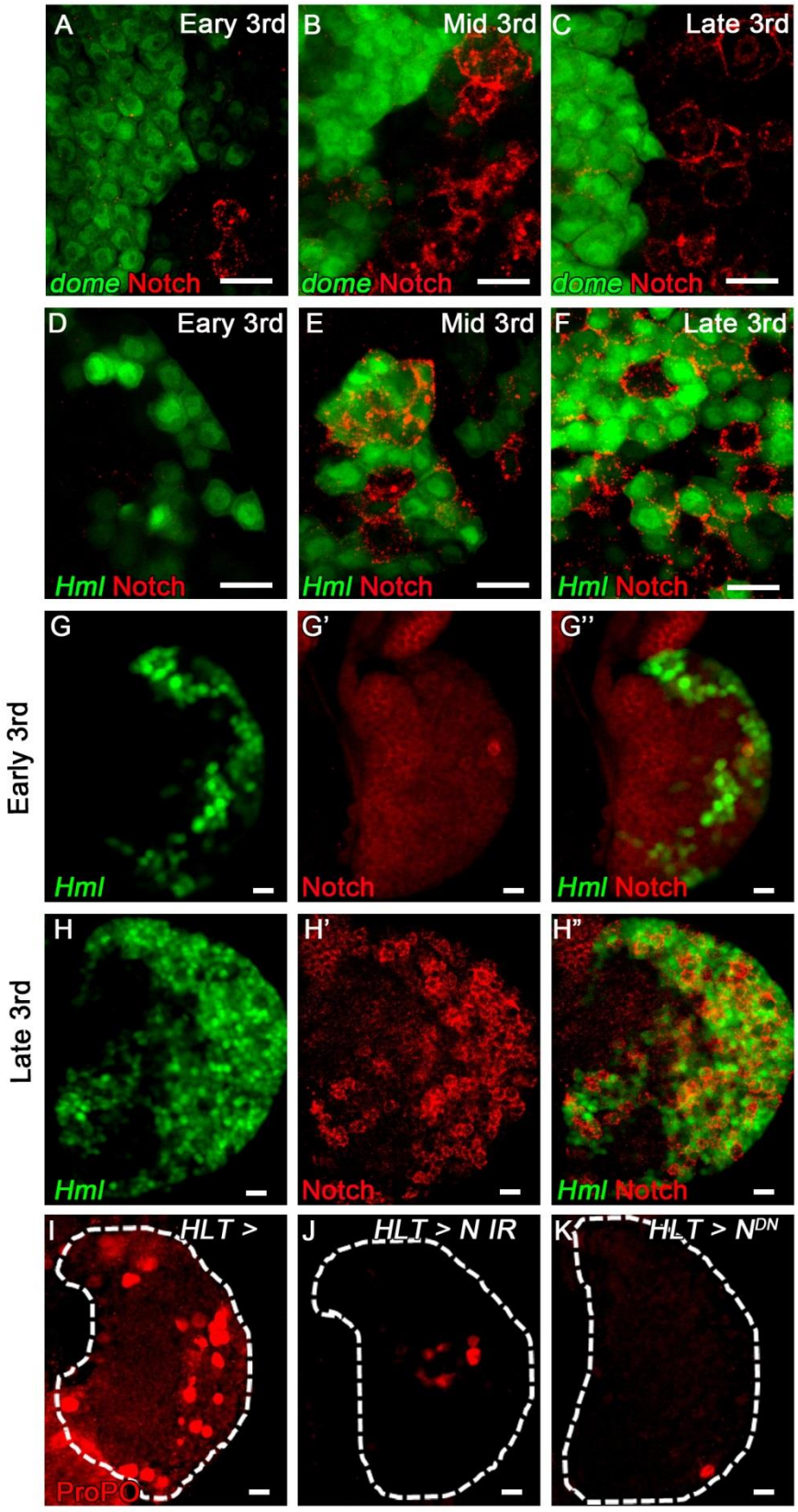
A
Eary 3rd
dome Notch
B
Mid 3rd
dome Notch
C
Late 3rd
dome Notch
D
Eary 3rd
Hml Notch
E
Mid 3rd
Hml Notch
F
Late 3rd
Hml Notch
Early 3rd
G
Hml
G'
Notch
G''
Hml Notch
Late 3rd
H
Hml
H'
Notch
H"
Hml Notch
I
HLT >
ProPO
J
HLT > N IR
K
HLT > N^DN

Figure A-2. Notch expression and function in the lymph gland. (A) In the early 3rd instar lymph gland, Notch (Red) expression is only observed in a few hemocytes, but not in prohemocytes (*dome*, Green). (B) By the mid 3rd instar lymph gland, Notch (Red) is more strongly expressed, but is still absent in prohemocytes (*dome*, Green). (C) In the late 3rd instar larva, Notch (Red) expression remains high, but is not observed in prohemocytes (*dome*, Green). (D) Notch (Red) is not expressed at high levels in the early 3rd instar lymph gland. (E) By the mid 3rd instar lymph gland Notch (Red) is highly expressed in differentiating hemocytes (*Hml*, Green). (F) In the late 3rd instar lymph gland, Notch (Red) is highly expressed throughout differentiating hemocytes (*Hml*, Green). (G-G") The developing Cortical Zone in the early 3rd instar lymph gland is populated by (G) *Hml* (Green) expressing cells. (G') Notch (Red) is expressed at low levels throughout the organ, but high levelsof Notch expression is restricted to rare cells of the Cortical Zone. (G") Merge channels. (H-H") In the late 3rd instar lymph gland (H) differentiating hemocytes (*Hml*, Green) populate a large Cortical Zone. (H') Notch (Red) is expressed throughout the lymph gland, but is only highly expressed in the Cortical Zone. (H") Merge channels. (I-K) ProPO (Red) labels mature crystal cells. (I) WT lymph gland. (J) Clonal depletion of *Notch* in the lymph gland with *HLT* largely blocks crystal cell differentiation. (K) Clonal over-expression of $Notch^{DN}$ strongly inhibits crystal cell differentiation. Scale bar 10 μm.

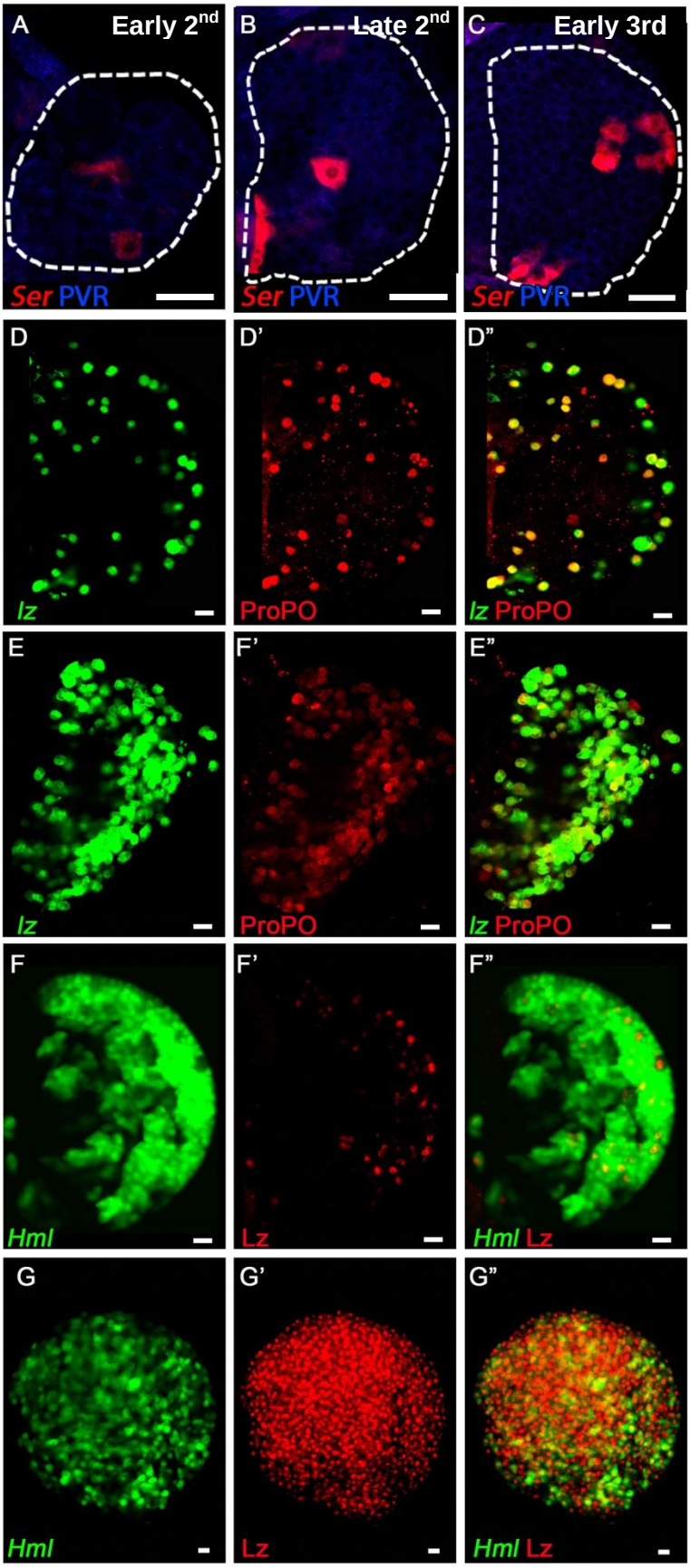
A
Early 2nd
Ser PVR
B
Late 2nd
Ser PVR
C
Early 3rd
Ser PVR
D
lz
D'
ProPO
D"
lz ProPO
E
lz
F'
ProPO
E"
lz ProPO
F
Hml
F'
Lz
F"
Hml Lz
G
Hml
G'
Lz
G"
Hml Lz

Figure A-3. Aberrant Serrate expression or Notch activation induces massive crystal cell differentiation in the lymph gland. (A) *Serrate* expression is first observed in the early 2nd instar lymph gland. (B) *Serrate* expressing Lineage Specifying Cells are first observed in the late 2nd instar lymph gland. (C) By the early 3rd instar lymph gland, multiple Lineage Specifying Cells are observed at the periphery of the organ. (D-D") Crystal cell progenitors (*lozenge,* Green) and mature crystal cells (ProPO, Red) in the WT lymph gland. (E-E") Over-expression of *Serrate* in crystal cell progenitors (*lz,* Green) induces massive differentiation of crystal cells (Red) in the lymph gland. (F-F") Differentiating hemocytes (*Hml,* Green) and crystal cell progenitors (Lz, Red) in the 3rd instar WT lymph gland. (G-G") Over-expression of N^{Act} in differentiating hemocytes (*Hml,* Green) induces complete differentiation of the lymph gland into crystal cell progenitors (Lz, Red). Co-expression of *Hml* and Lz indicates plasticity of *Hml+* cells to become crystal cells if given active Notch signal. Scale bar 10 µm.

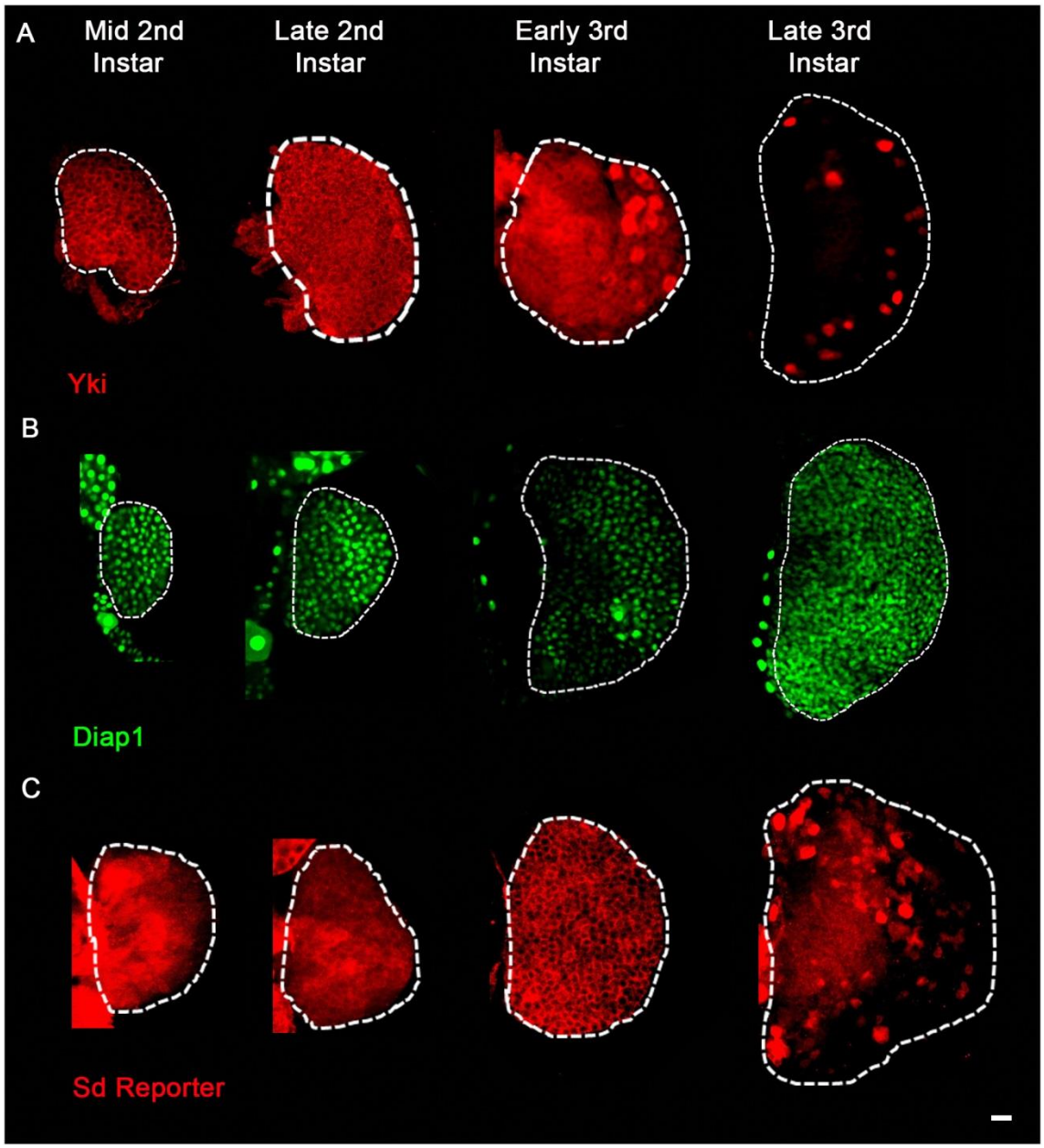

Figure A-4. Expression of Yorkie and Yorkie/Scalloped reporters in the lymph gland. (A) Staged expression of Yki (Red) in the lymph gland. Yki expression is first observed in the mid 2nd instar lymph gland in prohemocytes and is maintained throughout the organ until the 3rd instar, where it becomes restricted to scattered cells in the Cortical Zone. (B) Diap1 is also

actively expressed throughout the 2nd instar lymph gland. In the 3rd instar, Diap1 is mostly strongly expressed in the Cortical Zone, but is still maintained to a lesser degree in the Medullary Zone. (C) A reporter for Sd/Yki activity is strongly expressed in prohemocytes of the 2nd instar lymph gland and is expressed throughout the lymph gland until early 3rd instar. By the late 3rd instar, Sd/Yki activity is diminished in the Medullary Zone and is only expressed in scattered cells of the Cortical Zone. Scale bar 10 μm.

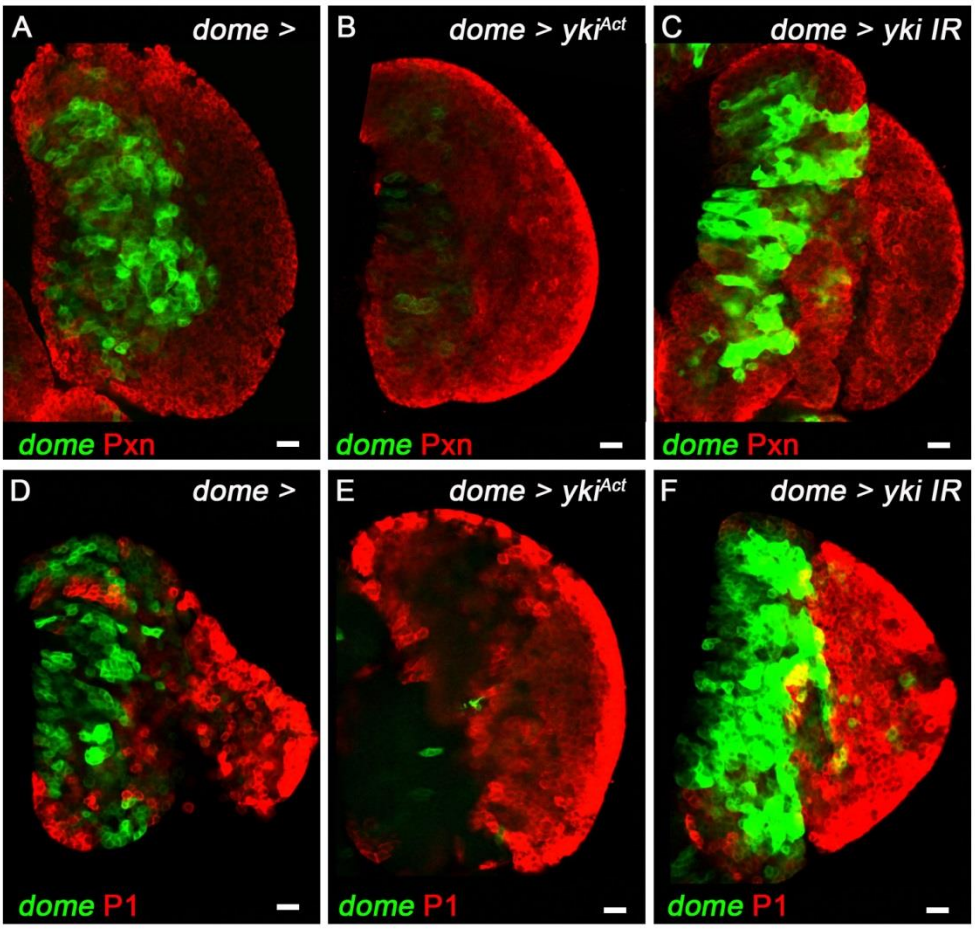

Figure A-5. Yorkie affects expression of *domeless* in prohemocytes, and has an inhibitory role in differentiation and maturation of plasmatocytes. (A) Prohemocytes labeled with *dome* (Green) and differentiating hemocytes labeled with Pxn (Red) in the WT 3rd instar lymph gland. (B) Over-expression of *yki*^{Act} in prohemocytes (*dome*, Green) does not have a significant effect on differentiation (Pxn, Red), but *dome* expression is significantly reduced. (C) Depletion of *yki* in prohemocytes (*dome*, Green) induces increased differentiation of hemocytes (Pxn, Red), and increases expression of *dome.* (D) Prohemocytes labeled with *dome* (Green) and mature plasmatocytes labeled with P1 (Red) in the WT 3rd instar lymph gland. (E) Over-expression of

yki^{Act} in prohemocytes (*dome*, Green) does not have a significant effect on maturation of plasmatocytes (P1, Red). Depletion of *yki* in prohemocytes (*dome*, Green) increases plasmatocyte maturation (P1, Red). Scale bar 10 μm.

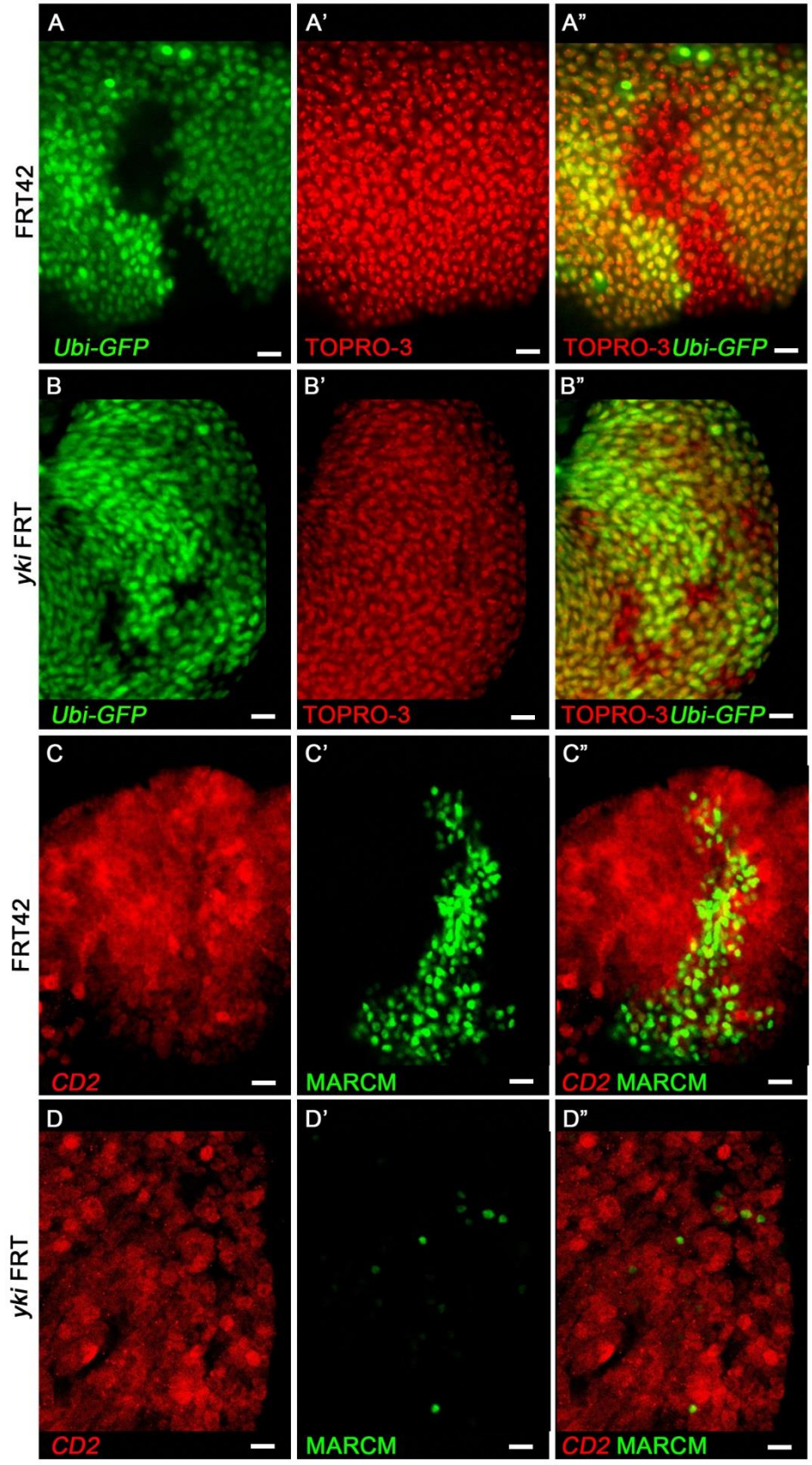
A
A'
A"
FRT42
Ubi-GFP
TOPRO-3
TOPRO-3 Ubi-GFP
B
B'
B"
yki FRT
Ubi-GFP
TOPRO-3
TOPRO-3 Ubi-GFP
C
C'
C"
FRT42
CD2
MARCM
CD2 MARCM
D
D'
D"
yki FRT
CD2
MARCM
CD2 MARCM

Figure A-6. Yorkie clones in the lymph gland do not properly grow. (A-A") Control clones in the 3rd instar lymph gland. (A) Absence of GFP labels the clone, while enhanced GFP (Bright Green) is observed in the twin-clone. (A') Topro-3 (Red) labels nuclei of cells. (A") Merge channels. (B-B") *yki* clones in the 3rd instar lymph gland. (B) Absence of GFP labels clones, which are significantly smaller than their twin-clones (Bright Green). (B') Topro-3 labels nuclei of cells. (B") Merge channels. (C-C") Control MARCM clones in the 3rd instar lymph gland. (C) GFP (Green) labels clone, and (C') CD2 (Red) labels unaffected cells. (C") Merge channels. (D-D") *yki* clones in the 3rd instar lymph gland. (D) GFP (Green) labels clone, and (D') CD2 (Red) labels unaffected cells. (D") Merge channels. Scale bar 10 μm.

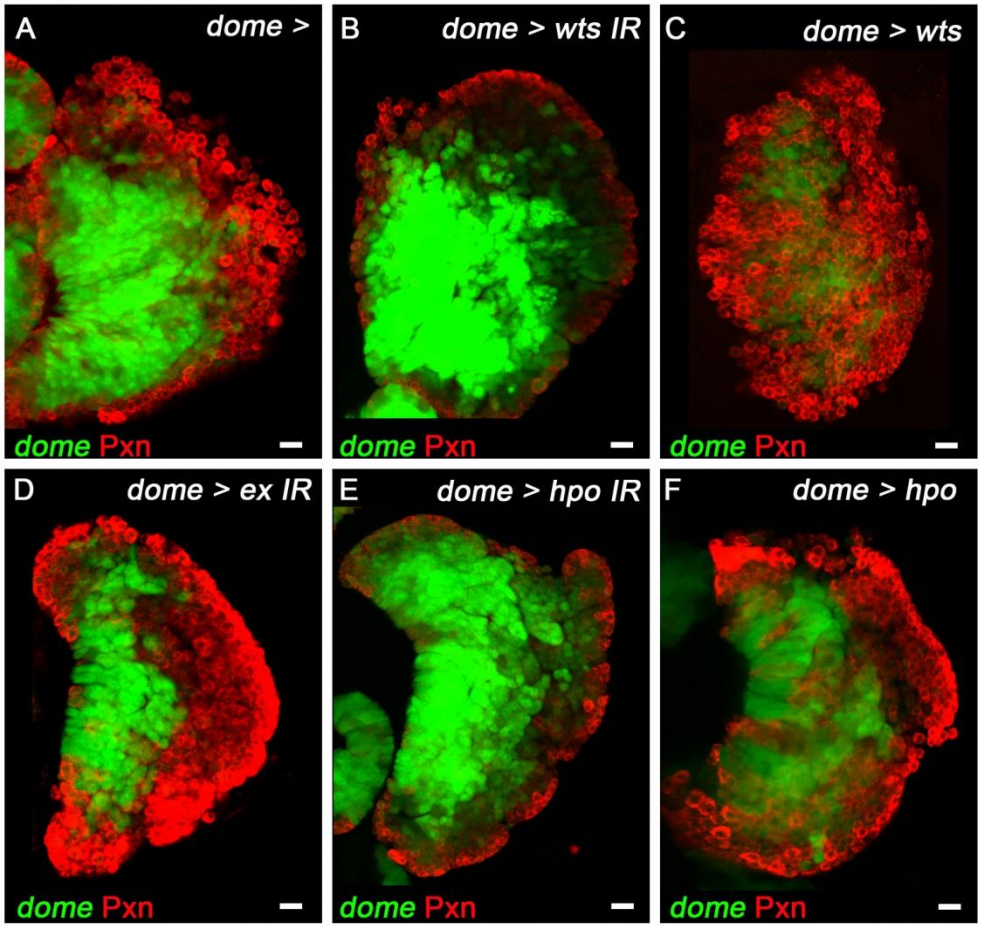

Figure A-7. Manipulation of Hippo Pathway components in prohemocytes variably affects differentiation. (A) Prohemocytes (*dome*, Green) and differentiating hemocytes (Pxn, Red) in the 3rd instar lymph gland. (B) Depletion of *warts*, the kinase responsible for direct inhibition of Yki, expands prohemocytes (*dome*, Green) at the expense of differentiating hemocytes (Pxn, Red). (C) Over-expression of *warts* in prohemocytes (*dome*, Green) induces increased differentiation of hemocytes (Pxn, Red). (D) Depletion of *expanded*, an upstream component in the Hippo Pathway, in prohemocytes has no effect on differentiation (Pxn, Red). (E) Depletion of *hippo*, an inhibitor of Yorkie, in prohemocytes (*dome*, Green) expands prohemocytes at the

expense of differentiating hemocytes (Pxn, Red). (F) Over-expression of *hippo* in prohemocytes induces an expansion of differentiation. (Pxn, Red). Scale bar 10 μm.

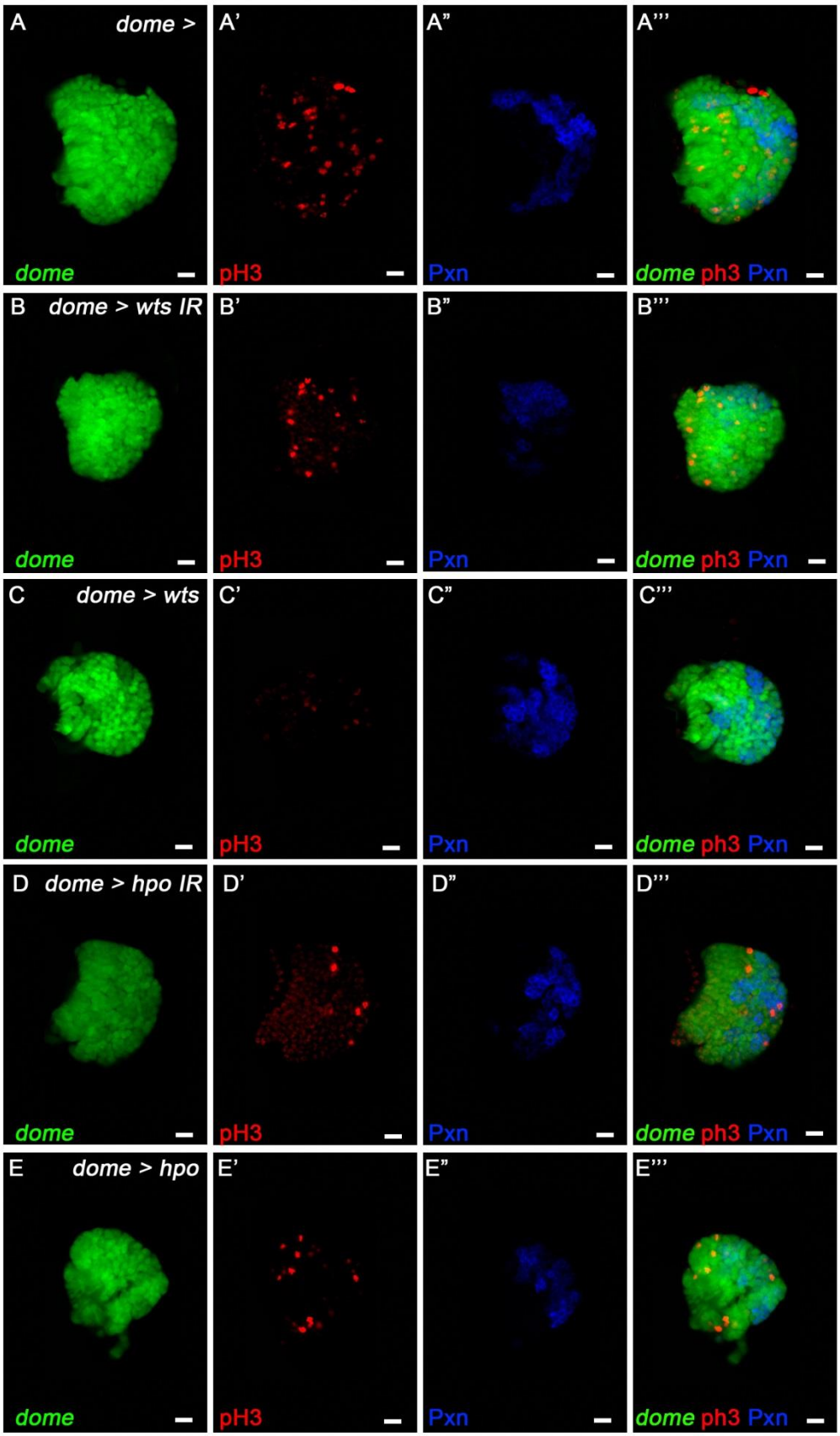
A dome >
A'
A"
A'''
dome
pH3
Pxn
dome ph3 Pxn
B dome > wts IR
B'
B"
B'''
dome
pH3
Pxn
dome ph3 Pxn
C dome > wts
C'
C"
C'''
dome
pH3
Pxn
dome ph3 Pxn
D dome > hpo IR
D'
D"
D'''
dome
pH3
Pxn
dome ph3 Pxn
E dome > hpo
E'
E"
E'''
dome
pH3
Pxn
dome ph3 Pxn

Figure A-8. Warts and Hippo regulate proliferation and differentiation in the 2nd instar lymph gland. (A-A’’’) Prohemocytes (*dome,* Green) populate the majority of the 2nd instar lymph gland. (A”) Differentiating hemocytes are labeled with Pxn (Blue) and (A’) proliferative cells are labeled with pH3 (Red). (A’’’) Merge channels. (B-B’’’) Depletion of *warts* in prohemocytes (*dome,* Green). (B’) Proliferation (pH3, Red) is not affected, but a decrease in (B”) differentiation (Pxn, Blue) is observed at this stage. (B’’’) Merge Channels. (C-C’’’) Over-expression of *warts* in prohemocytes (*dome,* Green) inhibits (C’) proliferation (pH3, Red), and (C”) increases differentiation (Pxn, Blue). (C’’’) Merge channels. (D-D’’’) Depletion of *hippo* in prohemocytes (*dome,* Green). (D’) Proliferation (pH3, Red) is reduced, but no significant changes to (D”) differentiation (Pxn, Blue) are observed. (D’’’) Merge Channels. (E-E’’’) Over-expression of *hpo* in prohemocytes (*dome,* Green). (E’) Proliferation is not affected (pH3, Red), but (E”) differentiation (Pxn, Blue) is delayed. Scale bar 10 μm.

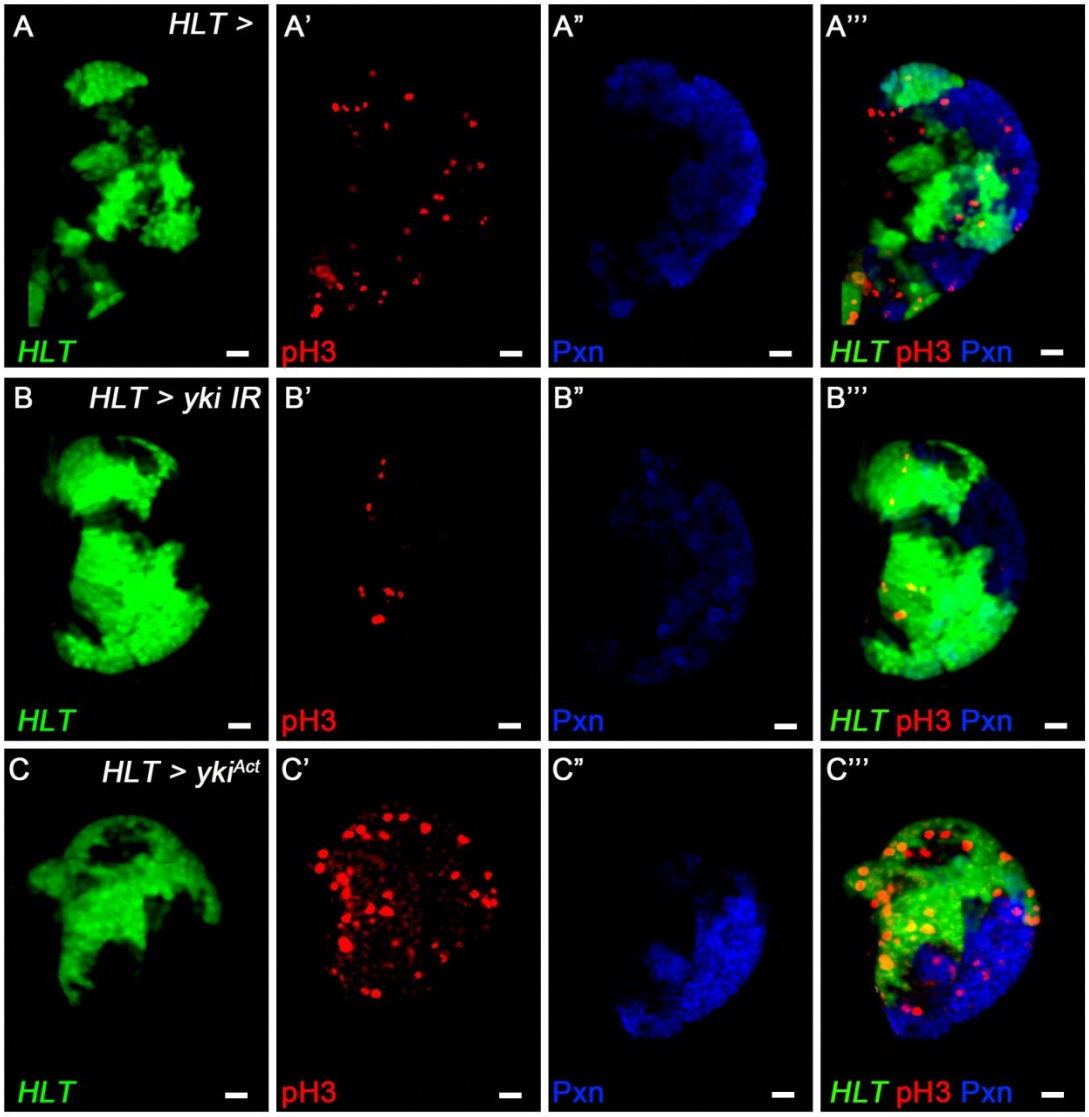

Figure A-9. Yorkie promotes proliferation in the early 3rd instar lymph gland. (A-A'") *HLT* (Green) is expressed in a clonal manner. (A') Proliferation (ph3, Red) is largely restricted to the (A") Cortical Zone (Pxn, Blue) of the early 3rd instar WT lymph gland. (A''') Merge channels. (B-B") Clonal (*HLT*, Green) depletion of *yki* in the early 3rd instar lymph inhibits (B') proliferation (pH3, Red), but has no effect on (B") differentiation. (B''') Merge Channels. (C-

C’’’) Clonal (*HLT,* Green) over-expression of *yki*^{Act} promotes massive (C’) proliferation (pH3, Red), and inhibits (C”) differentiation (Pxn, Blue). (C’’’) Merge channels, note lack of Pxn (Blue) in GFP^{+} (Green) cells. Scale bar 10 µm.

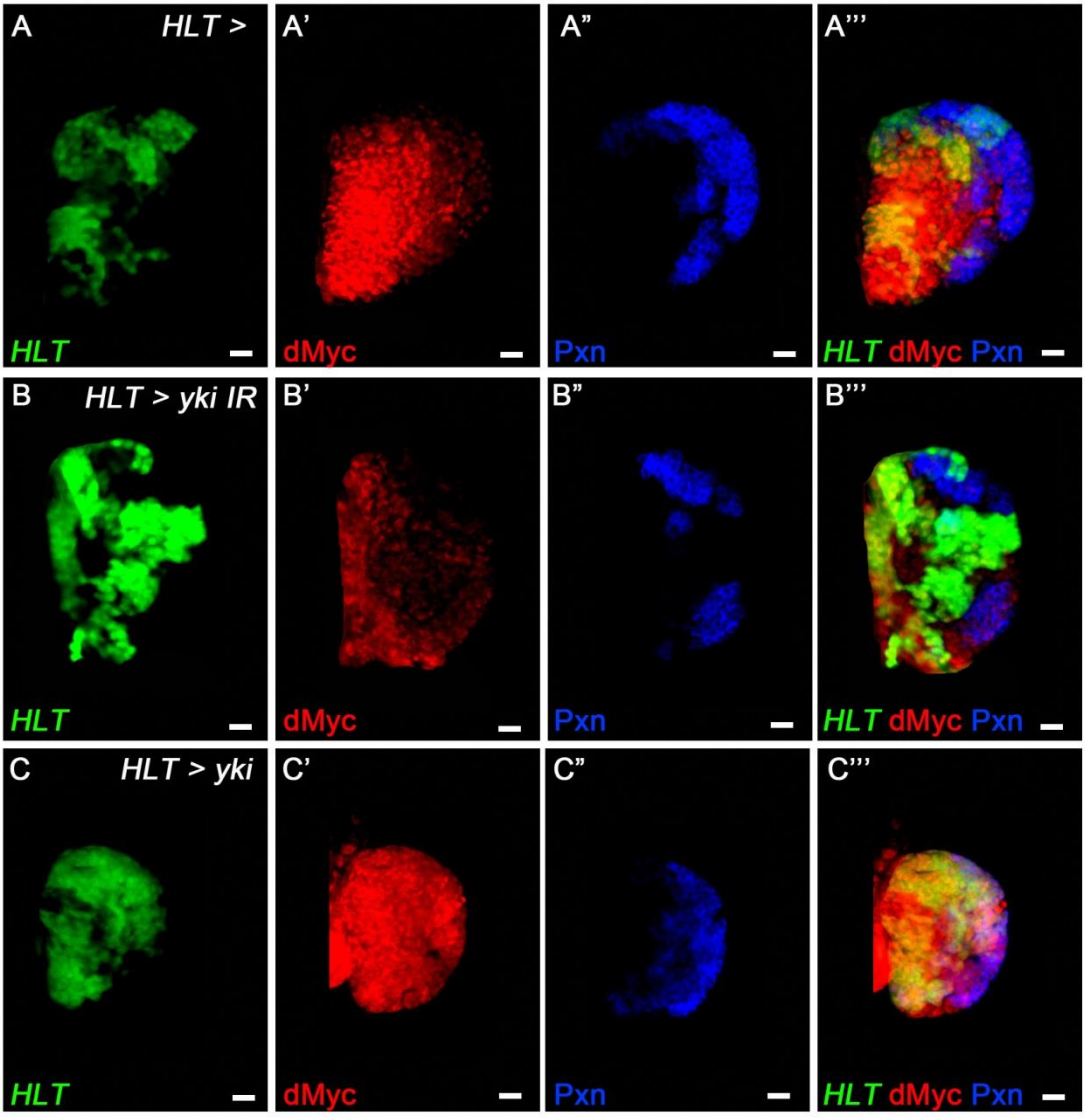

Figure A-10. Yorkie promotes expression of dMyc in the developing lymph gland. (A-A''') *HLT* (Green) is clonally expressed in the early 3rd instar lymph gland. (A') dMyc (Red) is highly expressed in the Medullary Zone, but is only expressed at low levels in (A") differentiating hemocytes (Pxn, Blue). (A''') Merge channels. (B-B''') Clonal (*HLT*, Green)

depletion of *yki* in the early 3rd instar lymph gland greatly reduces (B') dMyc (Red) expression. (B") Differentiation is not affected. (B''') Merge channels. (C-C''') Clonal (*HLT*, green) over-expression of yki^{Act} expands expression of (C') dMyc (Red). (C")Differentiating hemocytes (Pxn, Blue) are still excluded from clones. (C''') Merge channels. Scale bar 10 µm.

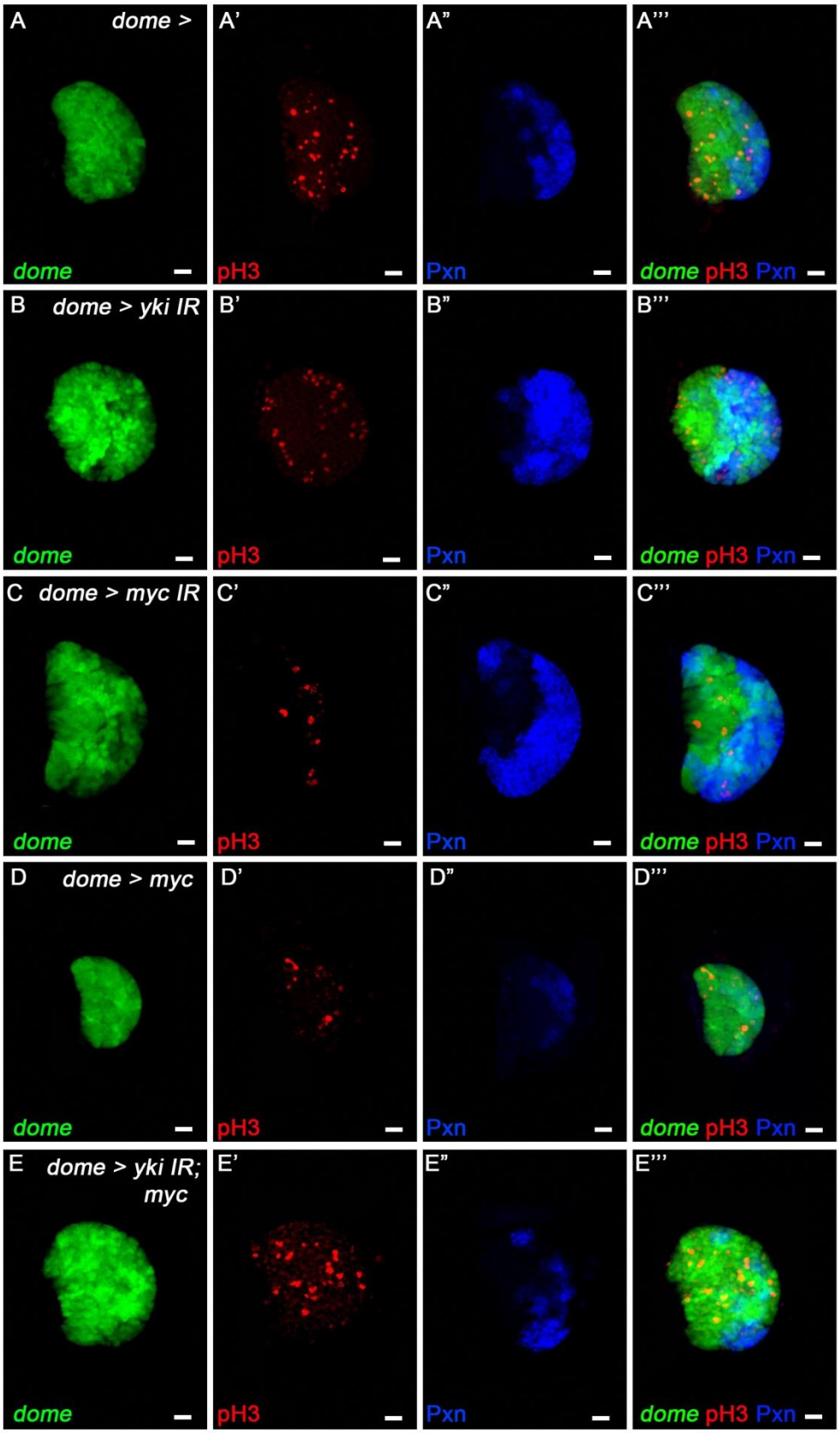
A dome >
A'
A"
A"'
dome
pH3
Pxn
dome pH3 Pxn
B dome > yki IR
B'
B"
B"'
dome
pH3
Pxn
dome pH3 Pxn
C dome > myc IR
C'
C"
C"'
dome
pH3
Pxn
dome pH3 Pxn
D dome > myc
D'
D"
D"'
dome
pH3
Pxn
dome pH3 Pxn
E dome > yki IR; myc
E'
E"
E"'
dome
pH3
Pxn
dome pH3 Pxn

Figure A-11. Myc functions downstream of Yorkie in prohemocytes of 2nd instar lymph gland and inhibits differentiation. (A-A’’’) Prohemocytes (*dome,* Green) populate the majority of the 2nd instar lymph gland. (A’) proliferative cells are labeled with pH3 (Red), and (A”) Differentiating hemocytes are labeled with Pxn (Blue). (A’’’) Merge channels. (B-B’’’) Depletion of *yki* in prohemocytes (*dome,* Green) does not alter numbers of (B’) proliferating hemocytes, but increases (B”) differentiation (Pxn, Blue). (B’’’) Merge channels. (C-C’’’) Depletion of *myc* in prohemocytes (*dome*, Green) decreases (C’) proliferation (pH3, Red), and promotes (C”) differentiation (Pxn, Blue). (C’’’) Merge channels. (D- D’’’) Over-expression of *myc* in prohemocytes (*dome,* Green) perturbs growth and decreases number of (D’) proliferative cells in the 2nd instar lymph gland. (D”) Differentiation (Pxn, Blue) is inhibited by over-expression of *myc.* (D-D’’’) Merge channels. (E-E’’’) Over-expression of *myc* with co-depletion of *yki* does not alter (E’) proliferation compared to WT, but does rescue (E”) increased differentiation (Pxn, Blue) phenotype induced by loss of *yki.* (E’’’) Merge channels. Scale bar 10 μm.

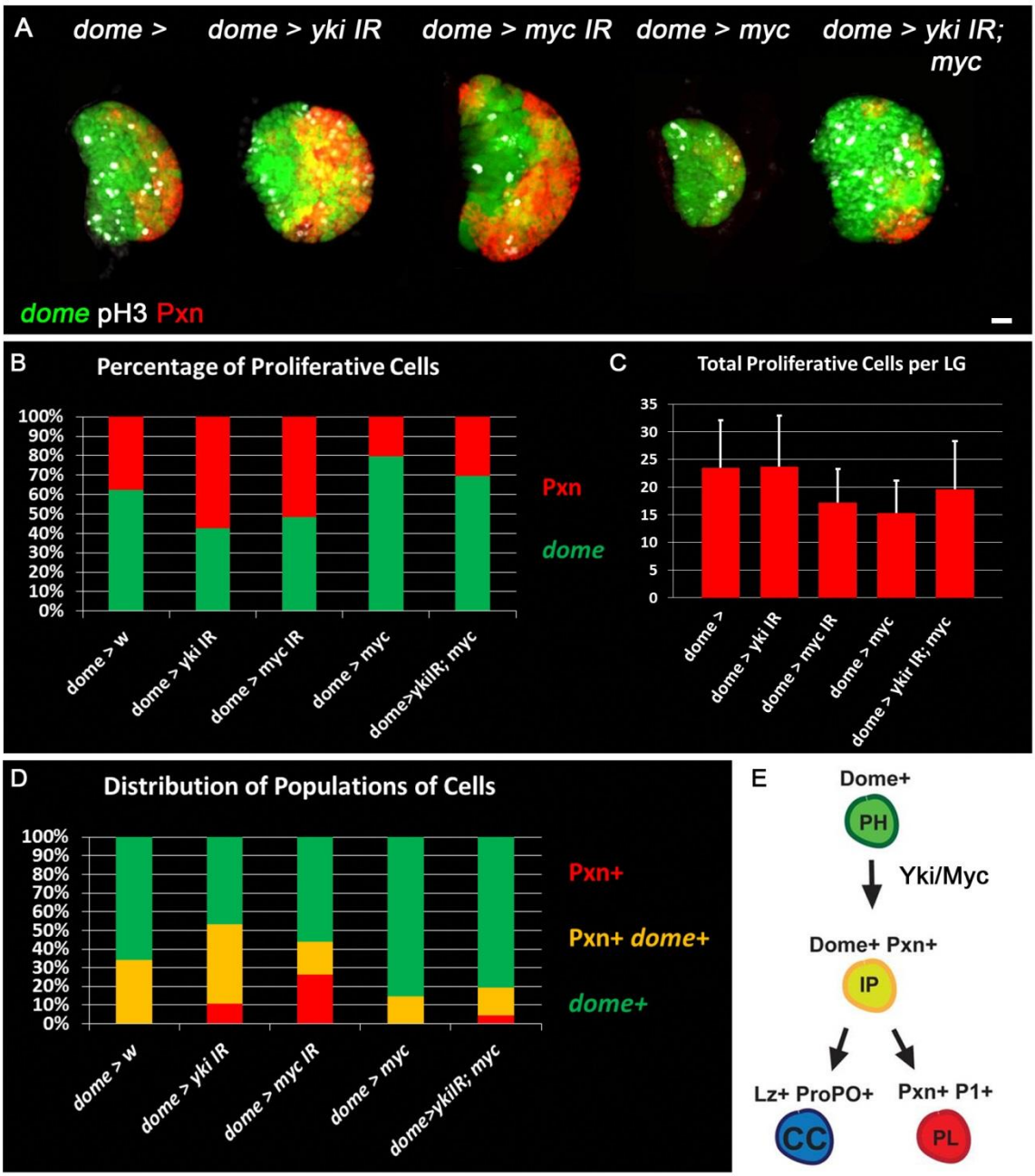

Figure A-12. Yorkie regulates dMyc to properly control differentiation. (A) Comparison of

Comparison of *yki* and *myc* manipulation in prohemocytes (*dome*, Green) of 2nd instar lymph

glands. Significant expansion of differentiating hemocytes (Pxn, Red) is observed upon both *yki* and *myc* depletion, while over-expression of *myc* inhibits differentiation and rescues differentiation induced by depletion of *yki.* (B) The majority (60%) of proliferative cells in the 2nd instar lymph gland are observed in prohemocytes. Depletion of *yki* and *myc* shifts proliferation into the differentiating hemocyte population. Over-expression of *myc* shifts proliferation to the prohemocyte population, even after *yki* depletion. (C) Total numbers of proliferative cells remain fairly constant upon *yki* depletion, but are reduced upon depletion or over-expression of *myc.* (D) *dome* is expressed in all cells of the WT 2nd instar lymph gland, including differentiating hemocytes. Depletion of *yki* expands this population of *dome*$^{+}$ Pxn^{+} hemocytes and induces loss of *dome* in a small percentage of differentiated cells. Depletion of *myc* expands the number of completely differentiated *dome*$^{-}$ hemocytes. Over-expression of *myc* expands the population of prohemocytes at the expense of differentiating hemocytes, and restores levels of differentiation to WT upon loss of, and restores levels of differentiation to WT upon loss of *yki.* (E) Schematic representation of differentiation in the lymph gland. Yki and dMyc mediate *dome*$^{+}$ prohemocytes giving rise to *dome*$^{+}$ Pxn^{+} intermediate progenitors which then fully differentiate into mature crystal cells or plasmatocytes. Scale bar 10 μm.

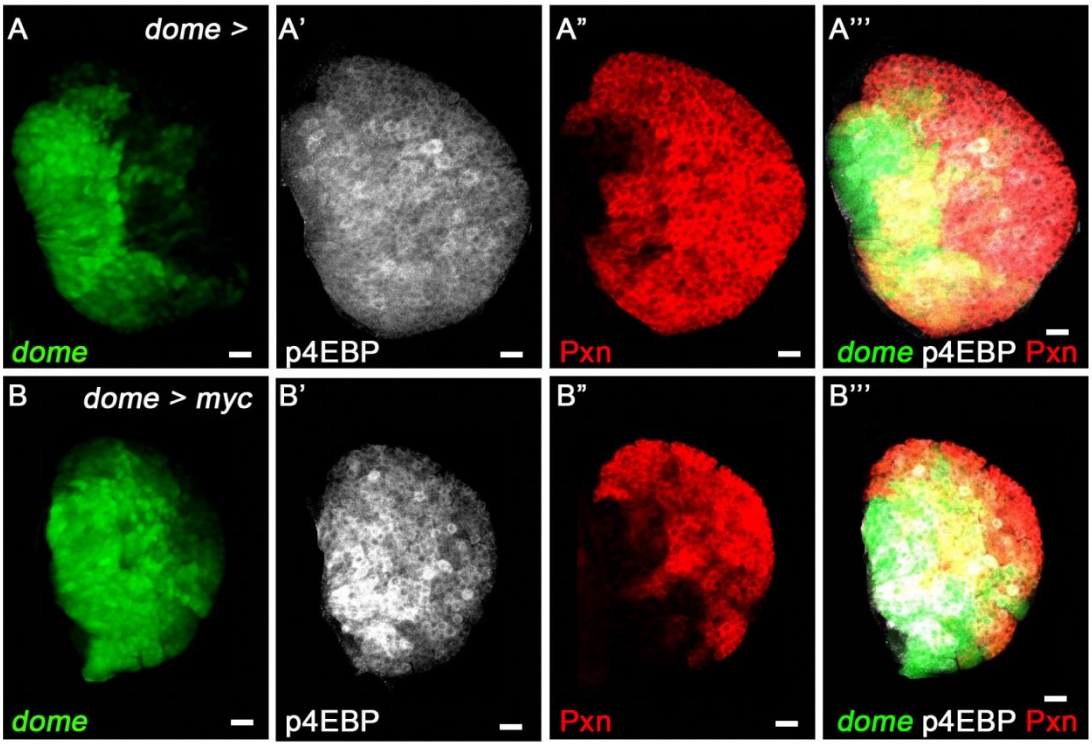

Figure A-13. dMyc activates TOR signaling. (A-A’’’) *dome* (Green) labels prohemocytes in the mid 3rd instar lymph gland when TOR signaling, identified by (A’) p4EBP (White), is most active. (A”) Differentiating hemocytes are labeled by Pxn (Red). (A’’’) Merge channels idenitifies *dome*$^{+}$ Pxn^{+} intermediate progenitors. (B-B’’’) Over-expression of *myc* in prohemocytes (*dome*, Green) expands (B) *dome*$^{+}$ population and TOR signaling (B’) (4EBP, White), while decreasing numbers of differentiating hemocytes (Pxn, Red). (B’’’) Merge channels. Scale bar 10 µm.

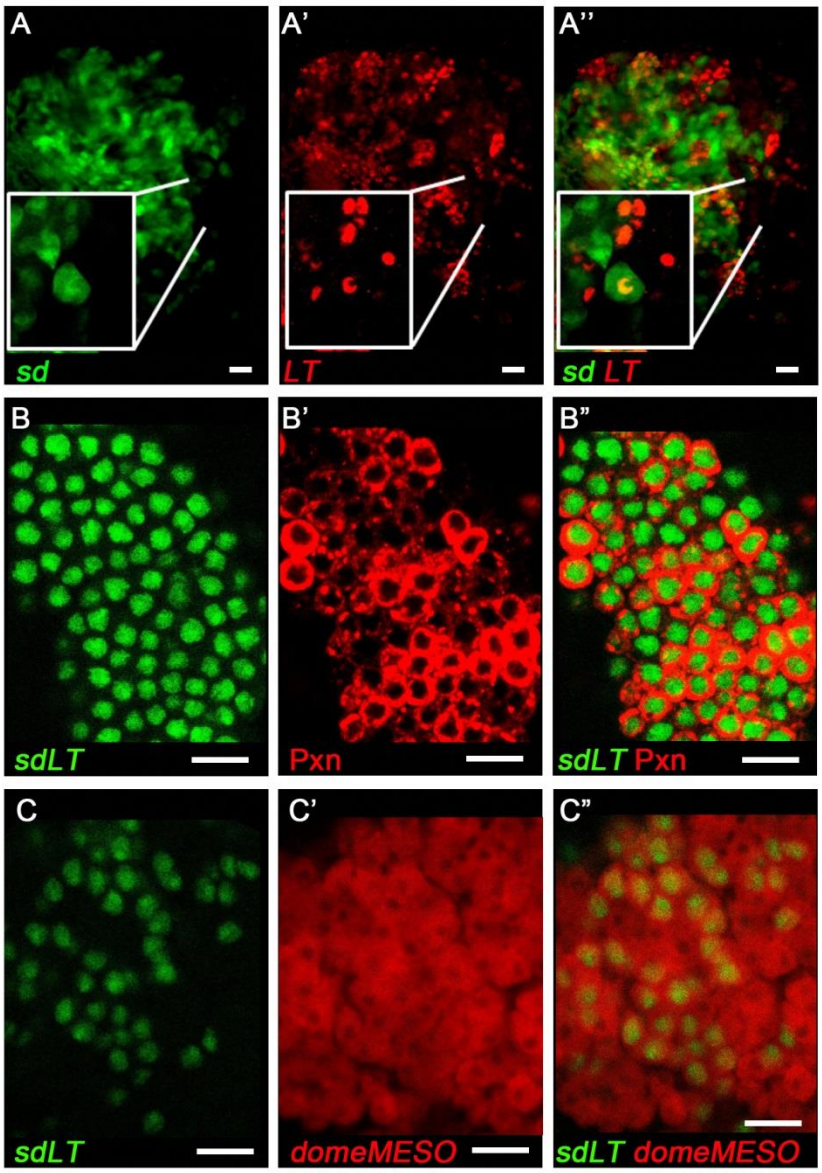

Figure A-14. Lineage tracing of *scalloped* expressing cells in the lymph gland. (A-A") *sd* (Green) expression in the 3rd instar lymph gland. (A') Lineage traced cells (Red) are observed throughout the lymph gland. (A") Merge channels identifies numerous lineage traced cells not actively expressing *sd.* (B-B") *sd* lineage traced cells (Green) are observed expressing (B') Pxn (Red). (B") Merge channels. (C-C") *sd* lineage traced cells (Green) are observed expressing (C') *domMESO* (Red). (C") Merge channels. Scale bar 10 µm.

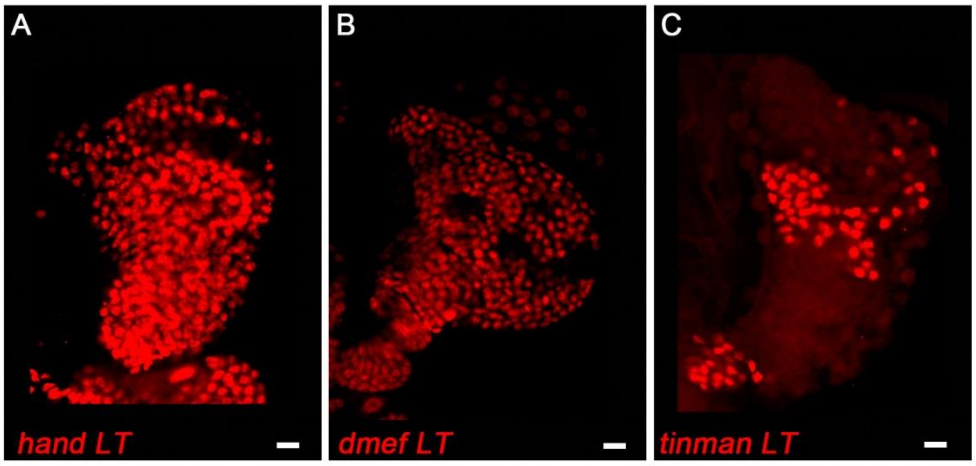

Figure A-15. Lineage tracing analysis reveals that lymph gland hemocytes are derived from a common Dorsal Vessel progenitor. (A) Lineage traced cells from *Hand* expressing cells populate the entire lymph gland. *Hand* is expressed in early prohemocytes of the lymph gland. (B) *dmef2* which is expressed in the cardiogenic mesoderm of the embryo and later in the Dorsal Vessel gives rise to lymph gland hemocytes. (C) *tinman* which is expressed in the dorsal mesoderm of the embryo and also strictly expressed in the Dorsal Vessel of the larva gives rise to lymph gland hemocytes. Scale bar 10 μm.

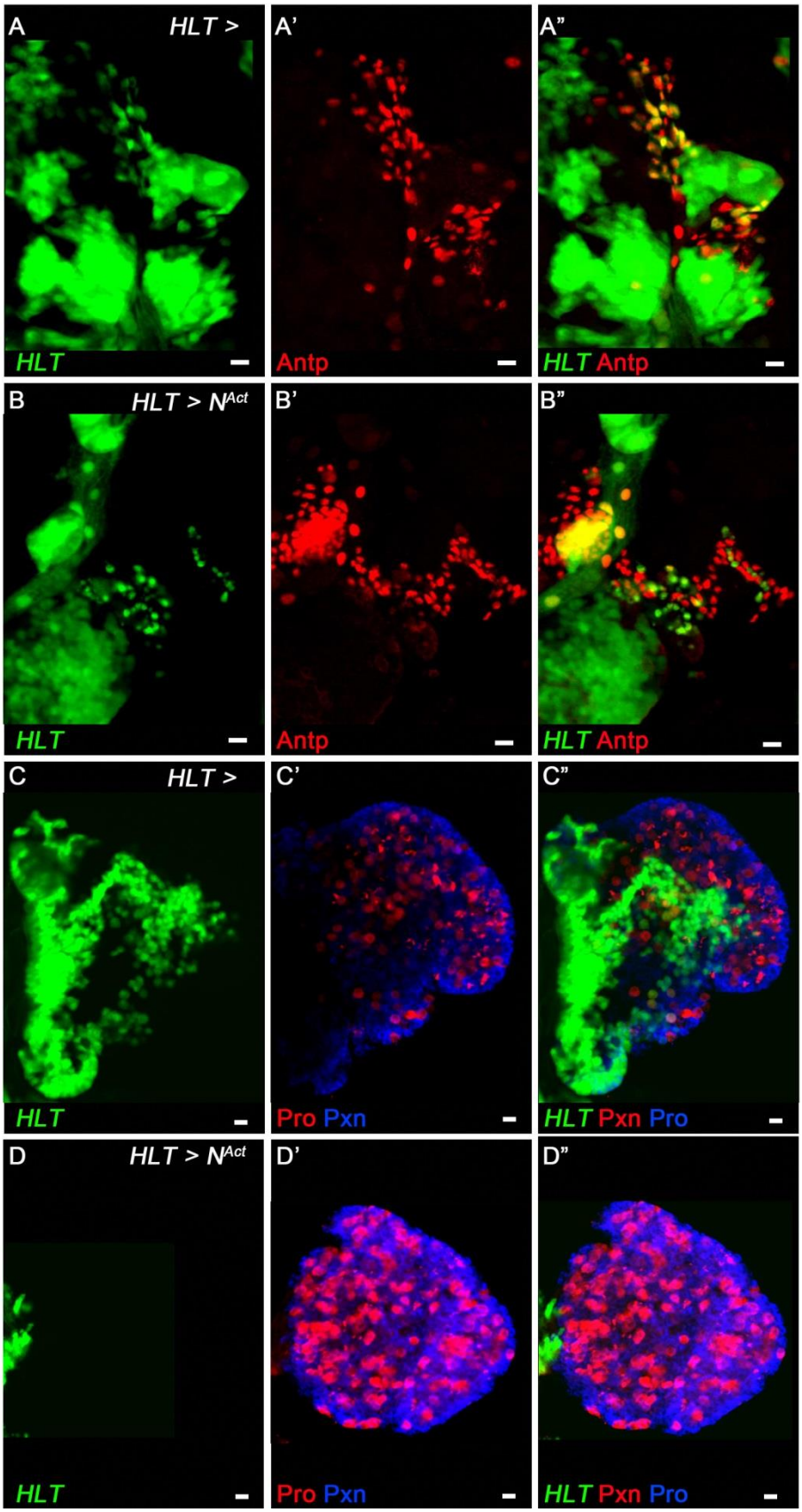
A
HLT >
HLT
A'
Antp
A"
HLT Antp
B
HLT > N^Act
HLT
B'
Antp
B"
HLT Antp
C
HLT >
HLT
C'
Pro Pxn
C"
HLT Pxn Pro
D
HLT > N^Act
HLT
D'
Pro Pxn
D"
HLT Pxn Pro

Figure A-16. Activation of Notch in lymph gland inhibits growth and PSC formation while non-autonomously inducing differentiation. (A-A") *HLT* (Green) is clonally expressed in the lymph gland, including (A') the PSC (Antp, Red). (A") Co-expression of Antp and *HLT* in the WT lymph gland. (B-B") Clonal (*HLT*) over-expression of $Notch^{Act}$ in the lymph gland inhibits (B') Antp expression. (B") Antp is not observed in *HLT* cells over-expressing $Notch^{Act}$. (C-C") *HLT* (Green) expression in the 3rd instar lymph gland. (C') Differentiating hemocytes (Pxn, Blue) and crystal cells (ProPO, Red) are observed in the Cortical Zone of the WT 3rd instar lymph gland. (C") Merge channels. (D-D") Clonal (*HLT*, Green) over-expression of $Notch^{Act}$ induces complete (D') differentiation of the lymph gland (Pxn , Blue and ProPO, Red) while (D") *HLT* expression is not observed in the lymph gland, perhaps due to early requirements for Notch in hemangioblast development. Scale bar 10 μm.

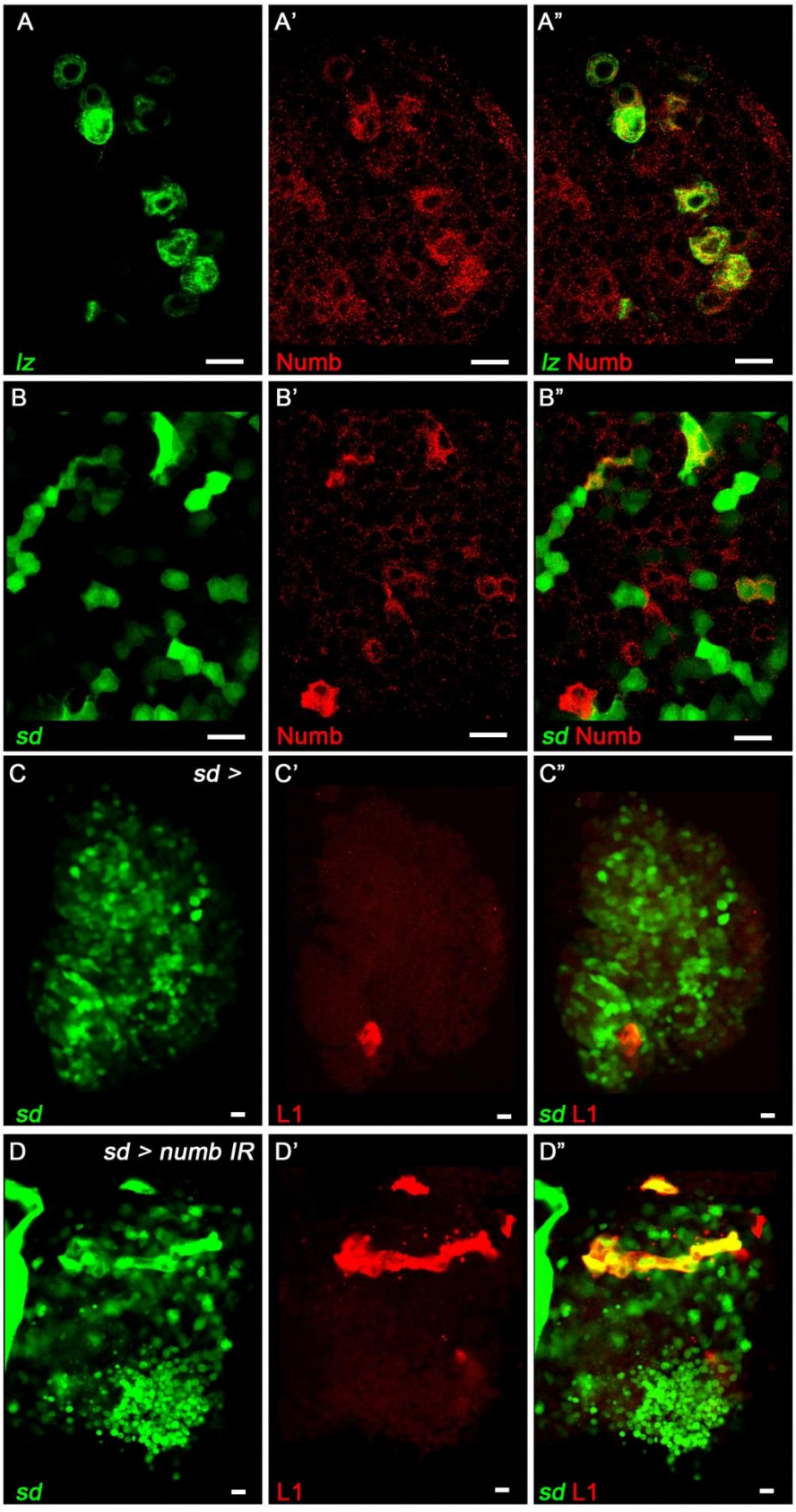
A
A'
A"
lz
Numb
lz Numb
B
B'
B"
sd
Numb
sd Numb
C
sd >
C'
C"
sd
L1
sd L1
D
sd > numb IR
D'
D"
sd
L1
sd L1

Figure A-17. Expression and function of Numb in the lymph gland. (A-A") *lz* (Green) labels crystal cell progenitors in the 3rd instar lymph gland. (A') Numb (Red) is expressed in scattered cells of the lymph gland, and is (A") co-expressed with *lz*. (B-B") *sd* (Green) expression in the 3rd instar lymph gland. (B') Numb (Red) is expressed in scattered cells of the lymph gland, and is (B") co-expressed with *sd* in some cells while also appearing adjacent to *sd* expressing cells. (C-C") *sd* (Green) expression in the WT lymph gland. (C') L1 (Red) labels lamellocytes and is rarely observed in WT lymph glands. (C") Merge channels. (D-D") Depletion of *numb* in *sd* (Green) expressing cells (D') induces differentiation of lamellocytes (L1, Red). (D") Merge channels. Scale bar 10 µm.

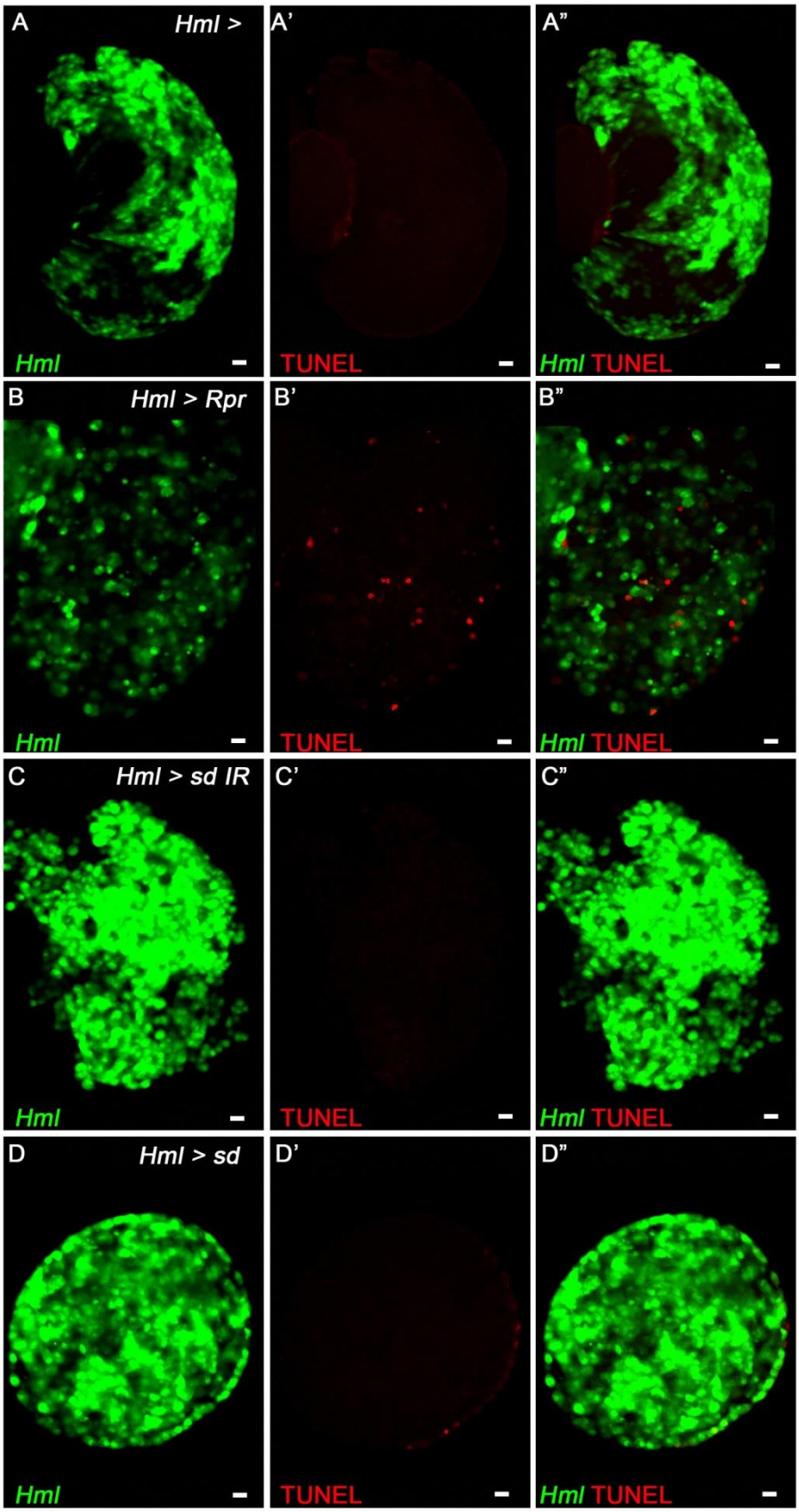
A
Hml >
Hml
A'
TUNEL
A"
Hml TUNEL
B
Hml > Rpr
Hml
B'
TUNEL
B"
Hml TUNEL
C
Hml > sd IR
Hml
C'
TUNEL
C"
Hml TUNEL
D
Hml > sd
Hml
D'
TUNEL
D"
Hml TUNEL

Figure A-18. Differentiation phenotypes induced by depletion or over-expression of *scalloped* is not caused by apoptosis. (A-A") Differentiating hemocytes (*Hml*, Green) in the WT 3rd instar lymph gland (A') are not apoptotic (Tunel, Red). (A") Merge channels. (B-B") Over-expression of *Rpr* in differentiating hemocytes (*Hml*, Green) induces (B') apoptosis (Tunel, Red). (B") Merge channels. (C-C") Depletion of *sd* in differentiating hemocytes (*Hml*, Green) does not induce (C') apoptosis (Tunel, Red). (C") Merge channels. (D-D") Over-expression of *sd* in differentiating hemocytes (*Hml*, Green) does not induce (D') apoptosis (Tunel, Red). (D") Merge channels. Scale bar 10 μm.