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FGFR signaling and the tumor suppressors TSC and PTEN regulate growth and differentiation of Drosophila blood progenitors

Author

Dragojlovic, Michelle Lindsay

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FGFR signaling and the tumor suppressors TSC and PTEN regulate growth and differentiation of *Drosophila* blood progenitors

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Molecular Biology

by

Michelle Lindsay Dragojlovic

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ABSTRACT OF THE DISSERTATION

FGFR signaling and the tumor suppressors TSC and PTEN regulate growth and differentiation of *Drosophila* blood progenitors

by

Michelle Lindsay Dragojlovic

Doctor of Philosophy in Molecular Biology Institute

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Professor Julian Martinez-Agosto, Chair

The tight regulation of hematopoietic progenitors is required to continuously supply blood cells during the lifespan of an organism and to make cells rapidly available in response to stress conditions, such as injury or infection. In *Drosophila*, a large pool of multipotent blood progenitors are maintained in the larval hematopoietic organ, the lymph gland (LG), by a complex network of signaling pathways that are mediated by niche-, progenitor-, or differentiated hemocyte-derived signals. Despite extensive studies in the LG during recent years, several important questions remain unanswered. In particular, the nature of the signals required to regulate the stereotypical proliferation and differentiation of LG blood progenitors during larval development remain unidentified.

In this dissertation we identify the Target of Rapamycin (TOR) and Fibroblast Growth Factor Receptor (FGFR) signaling pathways as critical regulators of growth and differentiation

of the *Drosophila* LG. In particular, we demonstrate that the tumor suppressors TSC and PTEN control blood progenitor proliferation through a common TOR- and 4EBP-dependent pathway. *Tsc* or *Pten* deficiency in progenitors increases TOR signaling and causes LG overgrowth by increasing the number of actively dividing cells during a critical window of growth. These phenotypes are associated with increased reactive oxygen species (ROS) levels in the LG, and scavenging ROS in progenitors is sufficient to rescue overgrowth. Differences between *Tsc* and *Pten* function become apparent at later stages. While loss of *Tsc* specifically expands the number of intermediate progenitors and limits terminal differentiation, absence of PTEN induces the myeloproliferative expansion of terminally differentiated blood lineages. This increased malignancy is associated with non-autonomous TOR activation within peripheral differentiating hemocytes, culminating in their premature release into circulation.

Since reduced TOR signaling in progenitors also induces increased differentiation, our findings demonstrate that although TOR signaling is sufficient, it is not required for inducing differentiation in the LG during development. In contrast, we identified the *Drosophila* FGFR, Heartless (Htl), and its two ligands, Pyramus and Thisbe, to be both required and sufficient for inducing blood progenitor differentiation in the LG. This Htl-mediated differentiation response of progenitors is dependent on two transcriptional regulators, the ETS protein, Pointed, and the Friend-of-GATA protein, U-Shaped, as well as TOR signaling, which is required specifically downstream of Thisbe-mediated Htl activation. Finally, we identify the *Drosophila* heparan sulfate proteoglycan (HSPG), Trol, as a critical negative regulator of FGF signaling in the LG, and suggest that sequestration of this potent differentiation signal by the extracellular matrix is a unique mechanism employed in blood progenitor maintenance in the *Drosophila* LG.

The dissertation of Michelle Lindsay Dragojlovic is approved.

Volker Hartenstein

Hanna Mikkola

Frank Laski

Shuo Lin

Julian Martinez-Agosto, Committee Chair

University of California, Los Angeles

2013

To my husband, Timothy

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LIST OF ABBREVIATIONS

4EBP:	translation initiation factor 4E-binding protein
AEH:	after egg hatching
ALK:	Anaplastic lymphoma kinase
Antp:	Antennapedia
Arm:	Armadillo
Bnl:	Branchless
Btl:	Breathless
CC:	crystal cell
Csw:	Corkscrew
CZ:	cortical zone
Dilp:	*Drosophila* insulin-like peptide
Dof:	Downstream-of-FGFR
Dome:	Domeless
EGFR:	Epidermal Growth Factor Receptor
eL2:	early-second larval instar
eL3:	early-third larval instar
FGFR:	Fibroblast Growth Factor Receptor
FOG:	Friend-of-GATA
Gbb:	Glass bottom boat
GFP:	Green fluorescent protein
Hml:	Hemolectin
Htl:	Heartless

JAK/STAT: Janus Tyrosine Kinase/ Signal Transducers and Activators of Transcription

Jeb: Jelly-Belly

L2: second larval instar

L3: third larval instar

LG: lymph gland

lL2: late-second larval instar

lL3: late-third larval instar

LM: lamellocyte

MAPK: Mitogen activated protein kinase

MARCM: mosaic analysis with a repressible cell marker

mL3: mid-third larval instar

MMP1: Matrix Metalloproteinase 1

MZ: medullary zone

p4EBP: phosphorylated-4EBP

pAKT: phosphorylated-AKT

PH: prohemocyte

phH3: phospho-histone H3

PI3K: Phosphatidylinositol-3-kinase

PL: plasmatocyte

Pnt: Pointed

PPO: Prophenoloxidase

PSC: Posterior Signaling Center

PTEN: Phosphatase and tensin homolog

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

Pxn: Peroxidasin

Pyr: Pyramus

Rheb: ras homolog enriched in brain

ROS: reactive oxygen species

RTK: receptor tyrosine kinase

S6K: S6 kinase

Shg: Shotgun [*Drosophila* E-Cadherin (dE-Cad)]

Spi: Spitz

Ths: Thisbe

TORC1: Target of rapamycin complex 1

TSC: Tuberous sclerosis complex

UAS: upstream activation system

Upd: Unpaired

Ush: U-Shaped

VHL: von Hippel-Lindau

Wg: Wingless

wL3: wandering-third instar

WT: wild-type

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dedicated to him. I am also grateful to my mom and dad for always supporting me and reminding me to keep a healthy perspective in my life and work.

Chapter 2 is a reprint of the article: "Multifaceted roles of PTEN and TSC orchestrate growth and differentiation of Drosophila blood progenitors" by Michelle Dragojlovic-Munther and Julian A. Martinez-Agosto, *Development* 139, 3752-3763. The authors retained the right to reprint this article. The final publication of this reprint is available at www.dev-biologists.org.

Chapter 3 is a version of the following article, which is in preparation for resubmission to *Developmental Biology*: "Extracellular matrix-modulated FGF signaling in *Drosophila* blood progenitors regulates their differentiation via a ras/ETS/FOG pathway and TORC1 function."

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VITA

2003-2006	B.S. Molecular Biology and Biochemistry University of California, Irvine Cumulative GPA: 4.00
2012-2013	Dissertation Year Fellowship (UCLA)
2009-2012	Cellular and Molecular Biology (CMB) Training Grant (UCLA)
Winter 2010	Teaching Assistant, Developmental Biology (MCDB 138) Department of Molecular, Cell and Developmental Biology University of California, Los Angeles
2009	Paul D. Boyer Teaching Award (MBI, UCLA)
Winter 2009	Teaching Assistant, Developmental Biology (MCDB 138) Department of Molecular, Cell and Developmental Biology University of California, Los Angeles
2007-2008	Chancellor's Prize (UCLA)
2003-2006	University of California, Regents Scholar (UCI)
2006	Honors in Biological Sciences (UCI)
2003-2006	Campuswide Honors Program (UCI)

PUBLICATIONS

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Chapter 1:

Introduction

The tight regulation of hematopoiesis and hematopoietic progenitors is required to continuously replenish blood cells during the lifespan of an organism and to make cells rapidly available in response to stress conditions, such as injury or infection. Blood development in *Drosophila* demonstrates several interesting parallels with vertebrate hematopoiesis. Although the variety of *Drosophila* blood cell types and their functions are more limited than in vertebrates, the conservation in *Drosophila* of several key mechanisms employed during vertebrate hematopoiesis makes the fruit fly a valuable genetic tool for studying hematopoiesis during development and disease. As in vertebrate systems, hematopoiesis in *Drosophila* occurs in spatially and temporally distinct waves and requires a population of multipotent progenitors, which differentiate into all mature blood lineages of the fly. The relative genetic simplicity of *Drosophila*, coupled with the rapid development of scientific technologies, including genome-wide expression analyses, RNAi-mediated gene silencing and a nearly complete collection of mutants, have established *Drosophila* as a valuable model organism with which to study development. In the following sections we review the origins, development and cellular makeup of *Drosophila* hematopoiesis and highlight key findings in the field which have identified complex signaling networks involved in blood homeostasis and blood progenitor maintenance in *Drosophila*. These and future studies of hematopoiesis in *Drosophila* will continue to enhance our understanding of fundamental mechanisms of hematopoiesis during development and disease.

Part I. *Drosophila* hematopoiesis: origins, development and hemocyte lineages

A. *Drosophila* hematopoiesis generates hemocytes of the myeloid lineage

Hematopoietic lineages in *Drosophila* are not as diverse as in vertebrates. Most obviously, erythroid and lymphoid blood lineages, which are responsible for oxygen delivery and

adaptive immunity in vertebrates, are altogether absent in *Drosophila*. Rather, the three mature hemocyte lineages in *Drosophila* all resemble the more ancient myeloid hematopoietic lineage, functioning in development and immune processes. The vast majority of mature hemocytes in *Drosophila* (90-95%) are plasmatocytes, which are most akin to the vertebrate monocyte/macrophage lineage (Evans et al., 2003; Lanot et al., 2001; Tepass et al., 1994). As phagocytic cells, plasmatocytes engulf and degrade apoptotic cells and cellular debris during development, particularly important during metamorphosis when histolysis and remodeling of larval tissues occurs (Evans et al., 2003), as well as functioning in wound repair (Babcock et al., 2008). Plasmatocytes also secrete various extracellular matrix (ECM) proteins, including Peroxidasin, Collagen and Laminin, which are deposited in basement membranes (Fessler et al., 1994; Kiger et al., 2001; Murray et al., 1995; Wood and Jacinto, 2007; Yasothornsrikul et al., 1997). Finally, plasmatocytes also function in the innate immunity of the fly via the phagocytic removal of invading pathogens, secretion of antimicrobial peptides, and signaling to the fat body for promotion of antimicrobial peptide synthesis (Agaisse et al., 2003; Dimarcq et al., 1997; Roos et al., 1998; Samakovlis C et al., 1990). In addition to their vital activities during embryonic and larval development, plasmatocytes are the only hemocyte cell type identified in the adult (Lanot et al., 2001).

Crystal cells are platelet-like, nonphagocytic hemocytes that are crucially involved in the innate immune response via their participation in melanization, an insect-specific immune response involved in clotting, wound healing, and encapsulation of foreign invaders (De Gregorio et al., 2002; Rämet et al., 2002). To this end, large quantities of components of the melanization enzymatic cascade are stored within paracrystalline inclusions in the crystal cell cytoplasm (Rizki and Rizki, 1985; Söderhäll and Cerenius, 1998). Crystal cells represent a small

fraction of *Drosophila* embryonic and larval hemocytes (~5%) and are not observed in the adult (Krzemien et al., 2010a; Lanot et al., 2001). Finally, lamellocytes are a specialized cell type that are also non-phagocytic and encapsulate and neutralize foreign objects too large to be engulfed by plasmatocytes (Nappi, 1975; Rizki and Rizki, 1992). While lamellocytes are rarely present in a healthy larva, large numbers of lamellocytes rapidly differentiate in response to invasion of parasitic wasp eggs (Krzemien et al., 2010b; Lanot et al., 2001; Sorrentino et al., 2002). Together with the other hemocyte types, lamellocytes adhere to and surround the parasitoid egg, forming a multilayered melanotic capsule that generates localized cytotoxic compounds to kill the parasite (Nappi, 1975; Nappi and Vass, 1998; Nappi et al., 2000; Rizki and Rizki, 1992). In contrast, bacterial challenge does not induce a lamellocyte response (Ratcliffe and Rowley, 1979).

Mature blood lineages in *Drosophila* are derived from undifferentiated prohemocytes in both the embryo and larva. Of the various blood lineages, prohemocytes are the smallest in size, and generally have little cytoplasmic volume (Evans et al., 2003). Heterogeneity within *Drosophila* prohemocytes has been postulated, both between embryonic and larval prohemocyte populations (Bataillé et al., 2005; Jung et al., 2005) and within the pool of larval prohemocytes (Jung et al., 2005; Krzemien et al., 2010b; Minakhina and Steward, 2010). However, a paucity of specific markers for distinct hemocyte progenitor types has prevented their direct functional classification in *Drosophila* (see below).

B. Two waves of hematopoiesis in *Drosophila* supply hemocytes for the embryo, larva and adult and demonstrate fundamental parallels with vertebrate hematopoiesis

A first wave of hematopoiesis in Drosophila occurs in the head mesoderm

Two spatially and temporally distinct waves of hematopoiesis have been described in *Drosophila*, which originate from distinct mesodermal populations in the embryo. The first wave

of hematopoiesis occurs in the embryonic head procephalic mesoderm (Tepass et al., 1994), whose anlage has been mapped to 70-80% egg length of the cellular blastoderm (Holz et al., 2003). Homo- and hetero-topic transplantation experiments revealed that embryonic hemocytes are already determined at the blastoderm stage, in contrast to all other mesodermal cells (Holz et al., 2003), but embryonic prohemocytes can first be identified at embryonic stage 5 via expression of the GATA transcription factor, Serpent (Srp) (Lebestky et al., 2000; Rehorn et al., 1996; Tepass et al., 1994). Embryonic hemocytes emerge
as undifferentiated prohemocytes that undergo four rapid cell divisions before their differentiation into plasmatocytes and crystal cells (Lebestky et al., 2000; Tepass et al., 1994). Plasmatocytes migrate along invariant and developmentally regulated pathways to populate the embryo, while crystal cells remain localized around the proventriculus (Lebestky et al., 2000; Tepass et al., 1994). By the end of embryogenesis all embryonic prohemocytes have differentiated (Tepass et al., 1994). While the role of embryonic crystal cells remains unclear, the pool of primitive plasmatocytes generated in the embryo have various functions during embryonic development, including the removal of accumulating apoptotic cells and the production of ECM that supports the development of basement membranes surrounding all tissues (Fessler and Fessler, 1989; Hartenstein, 2006; Tepass et al., 1994; Tepass and Hartenstein, 1994).

Embryonic plasmatocytes are extremely long-lived; they persist during the larval stages and even a subset of embryonic plasmatocytes are detected in the adult (Holz et al., 2003). In the late embryo, plasmatocytes do not proliferate, even upon experimental stimulation of their phagocytic function (Tepass et al., 1994). Later, during larval development, embryonic-derived plasmatocytes contribute <400 circulating hemocytes in the first larval instar, which increases to

>6000 hemocytes by wandering third instar via extensive proliferation, implying cell cycle re-entry in the larva (Makhijani et al., 2011). A percentage of these plasmatocytes do not circulate, but form a sessile population, arranged in a segmental pattern just beneath the larval cuticle, which is actively involved in the immune response via lamellocyte differentiation (Márkus et al., 2009). More recently, these plasmatocytes were also found to colonize 'epidermal muscular pockets' which localize between the larval epidermis and muscular layers and are thus anatomically secluded from the hemocoel (Makhijani et al., 2011). This hematopoietic location has been described as a microenvironment, which is dependent on peripheral neurons, and which supports hemocyte proliferation and survival (Makhijani et al., 2011).

A second wave of Drosophila hematopoiesis occurs in the larval lymph gland

A second wave of hematopoiesis occurs in the larval lymph gland (LG), a hematopoietic organ derived during midembryogenesis from the lateral mesoderm of the trunk, in a domain termed the cardiogenic mesoderm (Mandal et al., 2004; Rugendorff et al., 1994). The cardiogenic mesoderm also gives rise to vascular cardioblasts and excretory pericardial nephrocytes (Mandal et al., 2004). Clonal analysis suggested the presence of a bipotential 'hemangioblast' in the cardiogenic mesoderm that can give rise to both hemocyte progenitors and cardioblasts (Mandal et al., 2004). Specification of the cardiogenic mesoderm requires the sequential activity of the GATA protein Pannier (Pnr), the homeobox protein Tin, the transforming growth factor-β (TGF-β) protein Dpp, the Fibroblast Growth Factor Receptor (FGFR) Heartless (Htl), and Wnt/Wingless (Wg) signaling (Beiman et al., 1996; Frasch, 1995; Klinedinst and Bodmer, 2003; Mandal et al., 2004; Wu et al., 1995). LG prohemocytes fail to develop in embryos with mutations in *pnr*, *tin*, *dpp*, *htl* or *wg* (Mandal et al., 2004). In contrast, active Notch (N) signaling in the cardiogenic mesoderm, mediated by the N ligand Delta (Dl),

restricts cardiogenic mesoderm fate (Grigorian et al., 2011a; Mandal et al., 2004). At embryonic stage 12, LG prohemocytes, cardioblasts and pericardial nephrocytes are specified from cardiogenic mesoderm and begin to differentiate. Tin and Pnr expression become restricted to cardioblasts, while Odd-Skipped (Odd) expression is activated in lateral cells that give rise to LG prohemocytes (from thoracic cardiogenic clusters) and nephrocytes (from abdominal cardiogenic clusters) (Mandal et al., 2004). Finally, the GATA factor Serpent (Srp) is activated in LG prohemocytes and plays a central role in LG specification (Mandal et al., 2004). In addition to the role of N signaling in restricting numbers of the pluripotent progenitor pool in the cardiogenic mesoderm, N signaling also promotes LG prohemocyte fate specification by antagonizing *tin* and *pnr* expression and promoting *odd* and *srp* expression (Mandal et al., 2004). This latter role of N is also mediated by Dl, whose expression becomes restricted to cardioblasts, such that high N signaling is activated in adjacent blood progenitors and nephrocytes (Grigorian et al., 2011a). Activated MAPK signaling via both FGFR and Epidermal Growth Factor Receptor (EGFR) contributes to maintenance of high Dl expression required for proper specification of cardiogenic mesoderm-derived lineages (Grigorian et al., 2011a).

Eight to ten pairs of blood progenitors arise from the three thoracic (T1-T3) cardiogenic segmented clusters (Grigorian et al., 2011a), and by embryonic stages 13-16, the T1-T3 paired cell clusters of the LG primordium coalesce, forming the early LG (Mandal et al., 2007). Thus, in the late embryo, the LG consists of a single pair of lobes containing approximately twenty prohemocytes each, which flank the anterior part of the closely related aorta (dorsal blood vessel), while the excretory pericardial nephrocytes are positioned posterior to the LG (Mandal et al., 2004). By the second larval instar (L2), two or three pairs of posterior LG lobes have formed, and the primary lobes have increased significantly in size via extensive cell proliferation (see

below) (Jung et al., 2005). Differentiation and continued proliferation proceed in the growing LG during the third larval instar (L3) (see below). At the onset of metamorphosis, extensive differentiation of LG prohemocytes occurs and the LG tissue breaks down, releasing plasmatocytes and crystal cells into circulation, in anticipation of their various roles during metamorphosis (Evans et al., 2003; Grigorian et al., 2011b; Robertson, 1936). LG-derived hemocytes persist in the adult hemocoel, in addition to a subset of embryo-derived hemocytes (Holz et al., 2003). No hematopoietic tissue has been described in the adult, and it remains unclear whether any hemocyte progenitors, LG-derived or not, are present in the adult fly.

Mammalian embryonic hematopoiesis involves multiple spatially and temporally distinct phases

Fetal hematopoiesis in vertebrates is a complex process, requiring the generation of differentiated blood cells that are immediately required for development and growth of the embryo, while also establishing a growing pool of pluripotent HSCs in a process that involves multiple anatomical sites (the yolk sac, the aorta-gonad-mesonephros , the placenta and the fetal liver) (Mikkola and Orkin, 2006). A primitive and transient wave of hematopoiesis occurs in both mouse and humans in the extra-embryonic yolk sac, which provides a limited number of primitive nucleated erythroid cells for oxygen delivery to the embryo (Orkin and Zon, 2002; Palis et al., 1999), as well as macrophages (Bertrand et al., 2005) and primitive megakaryocytes (Tober et al., 2007). Subsequently, a pool of multipotential myeloerythroid progenitors also develop in the yolk sac (Palis et al., 1999) which then seed the fetal liver and give rise to mature blood cells in the embryo (Mikkola and Orkin, 2006).

Definitive hematopoiesis sustains blood development in the fetus and adult by supplying a population of pluripotent hematopoietic stem cells (HSCs), which have the ability to self-renew

and differentiate into all blood cell lineages (Orkin and Zon, 2002; Weissman, 2000). HSCs that start definitive hematopoiesis in mammals are first identified in the aorta-gonad-mesonephros (AGM) (Cumano et al., 1996; Godin and Cumano, 2002; Medvinsky and Dzierzak, 1996; Müller et al., 1994). HSCs are also formed in umbilical and vitelline arteries, which connect the dorsal aorta to the placenta and yolk sac (de Bruijn et al., 2000), and, although the extra-embryonic yolk sac has also been suggested as a source of HSCs, it remains unclear whether the yolk sac is a true source of definitive HSCs/progenitors (Mikkola and Orkin, 2006). Finally, definitive HSCs also emerge de novo in the placental vasculature (Rhodes et al., 2008), in addition to a large pool of HSCs that are thought to temporarily colonize the placenta via circulation (Mikkola and Orkin, 2006; Rhodes et al., 2008). Following their emergence from hemogenic sites, HSCs colonize the fetal liver, which functions in HSC expansion and differentiation until later fetal life when hematopoiesis in the bone marrow is established (Martinez-Agosto et al., 2007; Mikkola and Orkin, 2006).

Hematopoietic origins in Drosophila demonstrate fundamental parallels with mammalian systems

The origins of hematopoiesis and generation of hematopoietic progenitors in *Drosophila*, although much simpler than observed in mouse or human, reveal significant parallels which further validate the use of *Drosophila* as a genetic model to study mechanisms of blood development and homeostasis. First, the transient and limited generation of primitive differentiated hemocytes during the first wave of hematopoiesis in *Drosophila,* which serve the immediate needs of the embryo (see above), is comparable to primitive hematopoiesis in the mammalian yolk sac. One major distinction of *Drosophila* ‘primitive’ hematopoiesis is that it is intra-embryonic in the head mesoderm, while the extra-embryonic yolk sac in mammals serves

this role in mammals. Additionally, the second wave of hematopoiesis in the *Drosophila* LG has been compared to vertebrate definitive hematopoiesis, as it generates the blood supply that persists in the adult fly. The cardiogenic mesoderm, from which LG progenitors are derived, has been likened to the vertebrate AGM, as both tissues give rise to endothelial cells and nephrocytes in addition to blood (Hartenstein, 2006; Mandal et al., 2004; Martinez-Agosto et al., 2007; Medvinsky and Dzierzak, 1996). Remarkable conservation of the molecular mechanisms and signaling/transcriptional components that operate in the *Drosophila* cardiogenic mesoderm and vertebrate AGM has been identified. Induction of the AGM region results from the convergence of several signaling pathways, which include Wnt, bone morphogenetic protein (BMP) and FGF signaling (Marshall et al., 2000; Nishikawa et al., 2001; Orelio and Dzierzak, 2003), bearing striking similarity to the convergence of Wg, Dpp (a TGF-β/BMP molecule) and FGF signaling required for cardiogenic mesoderm specification in *Drosophila* (Beiman et al., 1996; Frasch, 1995; Manaka, 2004; Wu et al., 1995). Further, GATA factors, which play a critical role in the specification of blood cell lineages derived from vertebrate progenitors (Fossett and Schulz, 2001), are crucial in the specification of blood (via Srp) versus vascular (via Pnr) progenitor lineages in *Drosophila* (Mandal et al., 2004). Finally, Notch signaling too plays a critical role in regulating the genetic switch between vascular cells and blood progenitors in both *Drosophila* and vertebrates (Burns et al., 2005; Hadland et al., 2004; Kumano et al., 2003; Mandal et al., 2004; Pajcini et al., 2011; Robert-Moreno et al., 2005). While the LG is retained throughout larval development as the site for 'definitive' hematopoiesis, the mammalian AGM only temporarily generates HSCs, which eventually seed the placenta and fetal liver (Mikkola and Orkin, 2006). Unlike mammalian definitive hematopoiesis, which supplies pluripotent HSCs that persist in the adult, LG-derived hematopoietic progenitors differentiate at the end of larval

development and have not been identified in the adult. However, the hemocytes provided by the larval LG are presumed to be sufficient to meet the needs of the adult fly.

C. Development, growth & organization of the *Drosophila* lymph gland – a model of hematopoiesis

Structure and cellular composition of the larval lymph gland

While the LG had long been classified as a hematopoietic organ in *Drosophila* (Lanot et al., 2001; Shrestha and Gateff, 1982; Sorrentino et al., 2002), its utility as a genetic and developmental model for hematopoiesis was not appreciated until recent studies outlined the structural features and cellular events regulating LG hematopoiesis (Jung et al., 2005; Krzemien et al., 2007; Mandal et al., 2007). The primary lobes of the third instar LG consist of three general 'zones' of distinct cell populations. Differentiated plasmatocytes and crystal cells localize to the peripheral cortical zone (CZ, **Fig. 1-1**), and are loosely arranged (Jung et al., 2005). Hemocytes that are compactly arranged in the medullary zone (MZ, **Fig. 1-1**) do not express differentiation markers, and can also be discerned by their specific expression of several cellular markers, including high expression of the *Drosophila* E-Cadherin, Shotgun (Shg), as well as the Janus Tyrosine Kinase/ Signal Transducers and Activators of Transcription (JAK/STAT) receptor homolog, *domeless* (*dome*) (Jung et al., 2005). At later third instar stages the MZ population is largely quiescent, while cell proliferation is limited to CZ cells (Jung et al., 2005). Lineage-tracing analysis using the MZ marker *dome* to initiate permanent clonal expression demonstrated that mature hemocytes in the CZ arise from $dome^{+}$ MZ cells (Jung et al., 2005). Based on their quiescent and undifferentiated state as well as their ability to give rise to mature CZ hemocytes, the MZ population has been classified as a pool of prohemocytes (Jung et al., 2005). Finally, a small group of cells at the posterior tip of both primary lobes is

designated the Posterior Signaling Center (PSC, **Fig. 1-1**) (Lebestky et al., 2003). The PSC was first described as a population of cells that express the Notch ligand, Serrate (Ser) (Lebestky et al., 2003), and was later found to function as a hematopoietic niche that is required for maintenance of MZ prohemocytes (Krzemien et al., 2007; Mandal et al., 2007).

Zonation of the LG into a distinct MZ and CZ is not observed before the third larval instar. Differentiation of prohemocytes in the LG does not begin until second instar and invariantly occurs at the LG periphery (**Fig. 1-2**) (Jung et al., 2005). Progenitor differentiation is progressive during second instar, as markers of terminal differentiation are not observed until the early third instar stage (Jung et al., 2005). The controlled expansion of the number of differentiating hemocytes during the second and third larval instar results in formation of a mature CZ by late third instar stages (**Fig. 1-2**). While the PSC is specified in the embryo (Mandal et al., 2007), expression of Ser and other PSC-specific signal molecules is not observed until second instar (Jung et al., 2005; Mandal et al., 2007).

An extensive branching network of ECM can be observed throughout the primary LG lobes at late third instar stages, containing Collagen, Laminin and Perlecan (Fessler and Fessler, 1989; Fessler et al., 1994; Grigorian et al., 2011b; Jung et al., 2005; Krzemien et al., 2010b). Small populations of hemocytes cluster within ECM-bound pockets or chambers (Jung et al., 2005). A denser ECM reticulum is present in the MZ than in the CZ of the third instar LG; much of the lamellae have disappeared in the CZ and larger clusters of CZ hemocytes share a common ECM-bound chamber (Grigorian et al., 2011b). The PSC forms a morphologically discrete structure that is also surrounded by ECM (Krzemien et al., 2010b). During metamorphosis, the dispersing hemocytes leave behind the LG-associated ECM that they deposited, unlike in other larval tissues, where cell dispersal is preceded by digestion of the ECM (Grigorian et al., 2011b).

The role of the ECM and of such described ECM chambers during larval hematopoiesis and also during metamorphosis remains largely uncharacterized.

Heterogeneity in the lymph gland hemocyte progenitor population

The LG hemocyte progenitor population likely consists of a heterogeneous pool of progenitors, both with regard to different developmental stages and among the progenitors of a single larval stage. Two independent groups have demonstrated that inducing marked mitotic clones in the embryo generates LG clones of great variation in size and cell type by the third instar, suggesting that even the earliest LG embryonic prohemocytes are heterogeneous in their proliferative and self-renewal potential (Krzemien et al., 2010b; Minakhina and Steward, 2010). Large clones, which span the MZ and also give rise to CZ plasmatocytes and crystal cells, have been classified as 'persistent' clones, whereas 'transient' clones mostly contain CZ cells with few MZ cells included (Minakhina and Steward, 2010). From such clonal analyses, Minakhina and Steward suggested that pluripotent, self-renewing HSCs are present in the embryo and early larva and give rise to 'persistent' clones, while transient pluripotent progenitor cells undergo only a limited number of divisions before their differentiation and give rise to 'transient' clones. The presence of HSCs in second or third instar LGs was also assessed based on the following criteria: slow-cycling (retain BrdU), expression of specific markers common to other *Drosophila* stem cells, distinct nucleolar size, or expression of markers of asymmetric divisions. From these criteria, no evidence for a population of HSCs in contact with the PSC was identified in the LG (Krzemien et al., 2010b). Intriguingly, a small group of undifferentiated hemocytes are observed in the second instar, which do not express the prohemocyte marker, *dome* (Jung et al., 2005). This population, which localizes closest to the dorsal vessel in the most medial LG region, has been proposed to function as pre-prohemocytes, which give rise to $dome^{+}$ prohemocytes (Jung et

al., 2005). Whether this cell population has greater proliferative or self-renewal potential than *dome*$^{+}$ prohemocytes remains to be determined.

Among the multipotent prohemocytes of the MZ, the question also remains whether prohemocytes are homogeneous or restricted in their differentiation potential (Krzemien et al., 2010b). By analyzing mitotic LG clones generated during different developmental time-points, Krzemien et al. suggested that by the second instar, LG progenitors are already fated to give rise to either plasmatocytes or crystal cells; at earlier stages, prohemocytes give rise to both cell lineages (Krzemien et al., 2010b). However, it remains possible that a population of multipotent progenitors persists after the second instar, which may not be detected via clonal analysis due to low abundance or acquired quiescence.

Finally, in addition to undifferentiated MZ prohemocytes, the existence of intermediate hemocyte progenitors in the LG has also been suggested (**Fig. 1-1**). Sinenko et al. first described a genetic background that does not affect terminal plasmatocyte differentiation, but does increase a population of hemocytes that are 'double-positive' for the prohemocyte marker, *dome*, and the early differentiation marker, Peroxidasin (Pxn) (Sinenko et al., 2009). These 'double-positive' hemocytes were described as a population of progenitors undergoing a transition in their fate. Krzemien et al. also identified a small population of hemocytes that does not express MZ or terminal differentiation markers and is mitotically active (Krzemien et al., 2010b). The authors classified this population as a pool of intermediate progenitors, which undergo a terminal division before their complete differentiation.

In summary, heterogeneity between early and late LG prohemocytes has been suggested, as determined by their proliferative properties and self-renewal potential. At later stages, the MZ population has been proposed to consist of a mixed population of plasmatocyte- or crystal cell-

fated progenitors, as well as peripheral intermediate progenitors transitioning in their fate. Unfortunately, the paucity of specific markers that distinguish any of these postulated progenitor cell types has limited their true identification and functional characterization in the LG.

A highly regulated proliferation program controls LG growth during development

Extensive growth of the LG occurs during larval development, such that approximately 20 progenitors in the late embryo give rise to approximately 5000 hemocytes per primary lobe by the third larval instar (Krzemien et al., 2010b; Mandal et al., 2004). Following the highly programmed four mitotic divisions of embryonic LG prohemocytes during embryogenesis (Evans et al., 2003), LG prohemocytes proliferate extensively during early larval development, expanding their numbers, via unidentified genetic and external signals (Jung et al., 2005). A high LG mitotic index during the second and early third larval stages substantially increases the population of LG prohemocytes, but drops significantly by mid third instar through late third instar stages, at a time when increased differentiation expands the growing CZ (Krzemien et al., 2010b). Starting at mid third instar, a significant number of mitoses start to occur outside of the MZ, which sometimes correlate with terminally differentiated plasmatocytes, but never with crystal cells (Jung et al., 2005; Krzemien et al., 2010b). It has been proposed that a pool of intermediate progenitors, not yet terminally differentiated, is mitotically active at mid third instar and later stages, contributing to CZ expansion, although positive markers for such cells have not been identified (Krzemien et al., 2010b). The pool of MZ progenitors becomes largely quiescent by late third instar stages (Jung et al., 2005; Krzemien et al., 2010b).

Candidate regulators of prohemocyte proliferation in the lymph gland

To date, the signaling mechanisms responsible for transitioning LG hemocyte progenitors from a period of extensive proliferation during the second and early third larval instar stages to

their stereotypical quiescence after mid third instar are poorly understood. While a number of genes have been suggested to regulate progenitor proliferation/quiescence, direct functional analyses for these candidate genes in the progenitor population are still required to characterize their role in regulating progenitor proliferation.

An attractive candidate effector of progenitor proliferation/quiescence is adenosine, which can activate a mitogenic signal in an adenosine receptor (AdoR)/G protein/ adenylate cyclase/protein kinase A (PKA)-dependent manner (Dolezelova et al., 2007). While changes in extracellular adenosine levels have been associated with prohemocyte proliferation during the second instar (Mondal et al., 2011), an autonomous effect of adenosine on progenitor proliferation during development has not been carefully examined. Further, downregulation of *adoR* in the MZ prohemocyte population does not appear to affect LG size (Mondal et al., 2011), suggesting that adenosine may not be the mitogenic signal required for progenitor proliferation during early LG development. The single *Drosophila* Friend-of-GATA transcriptional regulator, U-shaped (Ush), has also been implicated in regulating proliferation in the LG, as *ush* mutant LGs are hypertrophic (Gao et al., 2009; Sorrentino et al., 2007). However, a progenitor-specific role for *ush* in LG progenitors has never been examined, and the mitotic index of *ush* mutant LGs was not different from controls during either second instar (Sorrentino et al., 2007) or late third instar stages (Gao et al., 2009), making it difficult to assess the developmental role of Ush in regulating LG growth/proliferation. Likewise, Notch (N) signaling has also been suggested to play a role in progenitor proliferation, as *N* mutant clones are always small (Lebestky et al., 2003), but a direct role for N in regulating progenitor proliferation has not been demonstrated.

Components of the enzymatic cascade involved in SUMOylation post-translational protein modifications were also recently identified as important regulators of progenitor cell

quiescence in the LG, including the E1 SUMO-activating enzymes, Aos1 and Uba2, the E2 SUMO-conjugating enzyme, Ubc9, and the E3 SUMO ligase, PIAS (Kalamarz et al., 2012). Functional loss of these genes induces LG hyperplasia and formation of hematopoietic tumors that detach from the LG (Kalamarz et al., 2012). Importantly, the E2 enzyme Ubc9 is specifically required in MZ progenitors to prevent these hematopoietic malignancies (Kalamarz et al., 2012). The functional role of Ubc9 in LG hemocytes was linked to cell cycle regulation via the cyclin/cyclin-dependent kinase (CDK) inhibitor, Dacapo(Dap)/p21, whose expression in the LG is highest in MZ progenitors (Kalamarz et al., 2012; Stanyon et al., 2004). Dap/p21 blocks the G1/S transition of the cell cycle via binding to cyclin E/CDK2 complexes (Lane et al., 1996). Dap expression is downregulated in *Ubc9* mutant LGs, and overexpression of *dap/p21* in transitional progenitors is sufficient to rescue the hyperplasia and altered differentiation of *Ubc9* mutant LGs (Kalamarz et al., 2012). Although the mechanism by which Ubc9 regulates Dap protein in the LG is unclear, sumoylation-regulated cell cycle control represents a novel mechanism of blood progenitor quiescence in the LG. P21- regulated cell cycle control may also play a similar role in vertebrates, in that $p21^{CIPI/WAF1}$ promotes HSC quiescence (Cheng et al., 2000), while *p21* null aged mice develop tumors (Martín-Caballero et al., 2001).

In addition to p21, several additional cell cycle regulatory genes have also been linked to hemocyte proliferation in the LG via their association with zinc finger protein RP8 (Zfrp8), the *Drosophila* ortholog of the highly conserved vertebrate protein, programmed cell death 2 (PDCD2), whose molecular function is largely uncharacterized (Minakhina et al., 2007; Tan et al., 2012). In *Drosophila*, *zfrp8* mutant LGs present with a mild increase in cellular proliferation/mitotic index, but the steady increase in growth rate throughout development culminates in LGs, which, only slightly larger than controls during late embryogenesis, become

10-50 times larger than controls by the third instar (Minakhina et al., 2007). In screening for potential interacting genes of Zfrp8, mutations in several cell cycle regulatory genes were found to dominantly enhance the *zfrp8/+* heterozygous mutant phenotype and contribute to increased LG growth (Minakhina et al., 2007; Tan et al., 2012). These include: *cdc27,* a subunit of the anaphase-promoting complex (APC) that regulates cyclin turnover and the spindle checkpoint (Deák et al., 2003; Minakhina et al., 2007); *lethal (1) discs degenerate 4* [*l(1)dd4*], encoding the gamma-ring protein Dgrip91, a component of the centrosome involved in γ-Tubulin anchoring (Minakhina et al., 2007); *mutagen-sensitive 304* (*mus304*), involved in the mitotic cycle, DNA repair and DNA damage checkpoint (Bi et al., 2005; Boyd et al., 1981; Brodsky et al., 2000; Tan et al., 2012); and *no poles* (*nopo*), a cell cycle regulator that encodes an E3 ubiquitin ligase (Merkle et al., 2009; Tan et al., 2012). *Zfrp8-/-* mutant clonal analysis in the LG demonstrated no change in clone size to controls, which the authors interpreted to mean that Zfrp8 is only required in a rare pool of HSCs, which don't survive in the absence of Zfrp8 function (Minakhina and Steward, 2010). Further, *cdc27* mutant LGs do not have a proliferative phenotype in the absence of single-copy loss of *Zfrp8* (Tan et al., 2012). Therefore, although a functional role for Zfrp8 and these associated cell regulatory genes has been suggested in hemocyte proliferation, further analyses are required to clearly elucidate their function autonomously in prohemocytes during LG development.

Origin and specification of the Posterior Signaling Center

The PSC is specified early in embryonic development by the homeotic gene, Antennapedia (Antp), and is lineage segregated from the rest of the LG cells (Mandal et al., 2007). In fact, a mutually exclusive functional relationship exists between Antp and the homeodomain cofactor, Homothorax (Hth), such that Hth, which is involved in specification of

the LG primordium, must be downregulated in PSC cells for Antp-dependent PSC specification (Mandal et al., 2007). Within the LG primordium at embryonic stages 11-12, Antp expression is limited to cells in the third thoracic segment (T3) of the cardiogenic mesoderm, while LG hemocyte progenitors develop from T1 and T2 segments (Mandal et al., 2007). By stages 13-16, the T1-T3 paired cell clusters of the LG primordium coalesce, and Antp expression is detected in 5-6 cells at the posterior edge of the embryonic LG (Mandal et al., 2007). Antp expression is then maintained in the PSC throughout larval LG development (Mandal et al., 2007). The *Drosophila* ortholog of mammalian early B-cell factor (EBF), Collier (Col), is also an important component of the PSC (Crozatier et al., 2004). Col is initially detected throughout the LG primordium but by embryonic stage 16 its expression is refined to cells of the PSC (Crozatier et al., 2004). Although an $Antp^{+}$ PSC is initially specified in *col* mutant embryos, the cells of the PSC are entirely absent by late larval stages (Crozatier et al., 2004; Mandal et al., 2007). Additionally, Col expression is not detected in *Antp* mutant embryos, placing Antp genetically upstream of Col in PSC cell specification in the embryo.

Functional characterization of the PSC as a hematopoietic niche

Although the PSC was initially identified as a signaling center with instructive functions (Crozatier et al., 2004; Lebestky et al., 2003), its functional characterization as a hematopoietic niche came from elegant studies examining the effects of absence or increase of PSC cells on LG hemocyte progenitors (Krzemien et al., 2007; Mandal et al., 2007). In the absence of a PSC (in *col* mutant LGs), the population of undifferentiated MZ prohemocytes is almost entirely lost (Krzemien et al., 2007; Mandal et al., 2007). Premature loss of the reservoir of MZ prohemocytes in the LG also prevents *col* mutant larvae from mounting a lamellocyte response upon wasp infestation (Crozatier et al., 2004), suggesting that the LG acts as a primary immune

defense against parasitization by maintaining multipotent progenitors that can rapidly differentiate into lamellocytes (Krzemien et al., 2010b; Lanot et al., 2001; Sorrentino et al., 2002). Interestingly, loss of prohemocytes in the absence of a PSC is evident by mid-third instar, but not earlier during second or early-third instar stages (Krzemien et al., 2007). This finding suggests that the PSC is strictly required during late stages of hematopoiesis, whereas independent mechanisms operate to maintain the proliferating prohemocytes of earlier larval stages. Converse to PSC loss, increasing PSC size via *Antp* overexpression increases the size of the MZ at the expense of the CZ at late third instar stages (Mandal et al., 2007).

The role of the PSC in cellular immunity

Recent studies have identified an additional role for the PSC, independent of its function as a hematopoietic niche that maintains prohemocytes, as a sensor of oxidative stress and regulator of the cellular immune response in *Drosophila* (Sinenko et al., 2012). In contrast to the relatively high levels of ROS detected in MZ prohemocytes (see below) (Owusu-Ansah and Banerjee, 2009), the cells of the PSC maintain very low levels of ROS expression (Sinenko et al., 2012). Inducing oxidative stress in the PSC by PSC-specific mitochondrial dysfunction or pathogenic infection increases ROS levels in the PSC and causes a robust increase in circulating lamellocyte numbers (Sinenko et al., 2012). The ROS-dependence of this lamellocyte response is demonstrated via the phenotypic rescue that occurs upon the following genetic manipulations in the PSC: overexpression of ROS scavenger genes, superoxide dismutase-2 (SOD2) or catalase; overexpression of the Forkhead box O (FoxO) transcription factor, a positive regulator of antioxidant enzyme gene expression; and inactivation of Akt1, a negative regulator of FoxO (Sinenko et al., 2012). Oxidative stress induces secretion of the Epidermal Growth Factor Receptor (EGFR) ligand Spitz (Spi) from the PSC, which signals to EGFR in hemocytes to

induce differentiation of lamellocytes in circulation (Sinenko et al., 2012). These results highlight a novel role of the PSC in mediating the cellular immune response, through its immunocompetence as a sensor of oxidative stress and concomitant secretion of the Spi cytokine.

PART II. Mechanisms of hemocyte progenitor regulation by niche-, progenitor- and differentiated hemocyte-derived signals in the lymph gland

A unique obstacle faced by LG hematopoiesis is the need to maintain large numbers of blood progenitors by a few 'niche' cells, which are many cell diameters away from the majority of MZ prohemocytes. This contrasts stem cell niche paradigms identified in other *Drosophila* stem cells types, where stem cells more often maintain intimate contact with the niche that supports their maintenance (Losick et al., 2011). In this section we outline a number of mechanisms that have been elucidated in the *Drosophila* LG to overcome this challenge of prohemocyte maintenance, which include niche-, progenitor- and differentiated hemocyte-generated maintenance signals.

A. Convergence of multiple signaling pathways regulates size & function of the hematopoietic niche in progenitor maintenance

PSC-derived hedgehog signaling is required for prohemocyte maintenance

Since its functional characterization as a hematopoietic niche, extensive efforts have been made to identify signaling networks that operate in the PSC for hemocyte progenitor maintenance. The first and best characterized signal identified in the PSC that non-autonomously maintains hemocyte progenitors is the growth factor Hedghehog (Hh) (Mandal et al., 2007), which has created a paradigm for niche-dependent blood progenitor maintenance in the *Drosophila* LG. Hh is expressed in the second and third instar LG PSCs, whereas downstream

signaling components, Patched (the Hh receptor) and activated Cubitus interruptus (Ci, a downstream pathway effector), are detected in MZ prohemocytes (Mandal et al., 2007). Blocking Hh signaling in the LG via a *hh* mutation or impairing Ci activity in the MZ induces the premature loss of MZ progenitors while not affecting PSC cell number (Mandal et al., 2007), demonstrating that while Hh is not required for specification of the PSC, non-autonomous Hh signaling from the PSC is functionally required for MZ maintenance at late LG stages. Delivery of the PSC-derived Hh signal to distal MZ progenitors was hypothesized to involve the long fillapodial cellular extensions that are characteristic of PSC cells (Mandal et al., 2007). These cellular extensions represent one mechanism whereby a small group of posterior niche cells can interact with MZ progenitors not directly adjacent to them.

The remarkable similarity of phenotype in *hh* mutant LGs with *col* mutant or *Antp* hypomorphic LGs, which lack a PSC, highlights the critical role of Hh in niche-mediated progenitor maintenance in the LG. The transcriptional network that operates in the LG to allow selective expression of Hh in cells of the PSC is thus important for PSC function and progenitor maintenance. The *Drosophila* GATA factor Serpent (Srp) was recently identified as a direct transcriptional activator of *hh* expression in the LG (Tokusumi et al., 2010). Although Srp is expressed ubiquitously in all LG cells (Jung et al., 2005), negative transcriptional regulation of *hh* expression in non-PSC LG hemocytes by two factors, Suppressor of Hairless [Su(H)] and U-shaped (Ush), facilitates the localized expression of *hh* to the PSC. Su(H) is a transcriptional regulator that activates Notch (N) target genes (Weinmaster, 2000), but in the absence of N signaling, Su(H) can function as a repressor of gene expression (Koelzer and Klein, 2003, 2006). In a N-independent manner Su(H) was found to negatively regulate *hh* transcription in MZ progenitors (Tokusumi et al., 2010). Likewise, the single *Drosophila* Friend-of-GATA (FOG)

protein, Ush, which is expressed in most LG cells but absent in the PSC (Gao et al., 2009), negatively regulates *hh* expression both within MZ progenitors and CZ differentiated hemocytes, thus refining Srp-mediated *hh* transcription to the small population of PSC cells in the LG (Tokusumi et al., 2010). More recently, several components of the BAP chromatin-remodeling complex were shown to be required for *hh* expression in PSC cells, while not affecting $Antp^{+}$ PSC cell number (Tokusumi et al., 2012). One component of the BAP complex, Osa, a DNA binding protein, was demonstrated to functionally interact with Srp in the regulation of PSC *hh* expression, possibly suggesting a role for the BAP complex in facilitating access of Srp to the PSC-specific *hh* enhancer (Tokusumi et al., 2012).

Pvf1 emanating from the PSC induces a CZ-derived signal for MZ progenitor maintenance

The *Drosophila* platelet-derived growth factor/vascular endothelial growth factor (PDGF/VEGF) receptor homolog, PVR, has three ligands, Pvf1-3. Pvf1 is co-expressed with Hh in PSC cells and Pvf1 protein has been proposed to be transported via transcytosis from the PSC to the CZ, where it activates PVR (Mondal et al., 2011). Pvf1-mediated activation of PVR in differentiated hemocytes activates a signaling pathway that functions in MZ progenitor maintenance, in parallel to the PSC-derived Hh signal (see below) (Mondal et al., 2011).

The functional role of Ser in the PSC remains unclear

The N ligand Ser was the first ligand molecule whose expression was identified in the PSC (Lebestky et al., 2003). N and Ser activity in the LG are required for expression of Lozenge (Lz), an acute myeloid leukemia 1(AML1)/RUNX1 factor homolog that is required for crystal cell lineage differentiation (Duvic et al., 2002; Lebestky et al., 2003). In addition to Ser expression in the PSC, however, scattered hemocytes in the LG are also Ser^{+}, and are often found adjacent to Lz^{+} crystal cell progenitors, suggestive of an inductive relationship (Lebestky

et al., 2003). Surprisingly, crystal cell differentiation is not affected in *col* mutant LGs, which lack a $Ser^{+}/Antp^{+}$ PSC but still retain Ser^{+} expression in scattered LG hemocytes (Crozatier et al., 2004), suggesting that Ser expression in the PSC is not exclusively required for crystal cell lineage specification. In fact, blocking Ser activity in the PSC has been suggested to decrease MZ size and actually increase crystal cell, but not plasmatocyte, differentiation (Krzemien et al., 2007). Further analyses are required to tease out the functional role of Ser expression in the PSC and whether it is involved in MZ prohemocyte maintenance/differentiation in a N-dependent or independent manner.

Convergence of Wg and BMP signaling in the PSC regulates PSC cell number

Components of the *Drosophila* Wingless (Wg)/Wnt/β-catenin signaling pathway are expressed in PSC cells, including the Wg ligand, its receptor, Frizzled 2 (DFz2), and intracellular pathway components, β-catenin/Armadillo (Arm) and Disheveled (Dsh) (Sinenko et al., 2009). Localized Wg signaling in the PSC autonomously regulates its size, such that reduced pathway activation impairs, while Wg overexpression expands, PSC cell number (Sinenko et al., 2009). While a decrease in MZ size accompanies the decreased PSC size upon Wg pathway disruption (Sinenko et al., 2009), the effect on MZ size of Wg-induced increases in PSC size has not been examined. One candidate effector of PSC size downstream of Wg signaling is Myc, a well characterized regulator of cell proliferation. Forcibly overexpressing *myc* in the PSC dramatically increases PSC number several-fold, and reducing *myc* expression upon Wg activation in the PSC restores PSC numbers to WT (Pennetier et al., 2012). Myc expression in the PSC is inhibited by Decapentaplegic/bone morphogenetic protein (Dpp/BMP) signaling, a member of the transforming growth factor (TGF)-β family, which negatively regulates PSC size (Pennetier et al., 2012). Localized BMP signaling in the PSC is mediated by the membrane-

bound heaparan-sulfate proteoglycan (HSPG) Dally-like (Dlp), which is highly expressed in the PSC and is functionally required for active BMP signaling in the PSC (Pennetier et al., 2012). Similar to *Antp* overexpression in the PSC, the increase in PSC cell number induced by inhibiting BMP signaling or increasing *myc* expression in the PSC induces an expansion of prohemocytes at the expense of terminally differentiated lineages (Pennetier et al., 2012). Although Wg is epistatic to Dpp signaling in the PSC, the molecular pathways by which Wg and Dpp control *myc* expression in the PSC are unknown (Pennetier et al., 2012). These studies demonstrated striking parallels with vertebrate systems, wherein osteoblasts, a major component of the mammalian bone marrow niche, are reduced in number upon decreasing Wnt signaling via β-catenin inactivation (Nemeth et al., 2009), but increase in number upon conditional knockout of the Type 1A BMP receptor (Zhang et al., 2003).

Insulin Receptor/Target of Rapamycin signaling and nutritional status regulate PSC cell number

The interconnected insulin/insulin growth factor (IGF, IIS) and Target of Rapamycin (TOR) signaling pathways function as a major nutrient-sensing system, which integrate nutrition status to tissue growth in *Drosophila* (Mirth and Shingleton, 2012; Tennessen and Thummel, 2011). Recent studies have highlighted a major role for these pathways in autonomously regulating PSC size in *Drosophila* (Benmimoun et al., 2012; Tokusumi et al., 2012). A recent screen, assessing the function of 820 different genes in PSC cell production and differentiation, examined 33 different gain- or loss-of-function genetic conditions of positive and negative regulators of IIS/TOR signaling, and harmoniously demonstrated that activation of IIS or TOR signaling expands the number of $Hh^{+}/Antp^{+}$ PSC cells, while diminishing IIS or TOR signaling restricts PSC cell number (Tokusumi et al., 2012). Positive pathway regulators/components

examined included insulin receptor (InR), insulin receptor substrate (chico), Akt, phosphatidyl inositol-3 kinase (PI3K), Rheb, Raptor, Rictor and S6 kinase (S6k), whereas negative pathway components examined included phosphatase and tensin homolog (PTEN), Tuberous Sclerosis Complex 1 and 2 (TSC1/2), FoxO and the translational initiation factor 4E-binding protein (4EBP) (Tokusumi et al., 2012). In addition, conditions of starvation also inhibit PSC cell number (Tokusumi et al., 2012). The phenotypic consequences of IIS/TOR pathway-induced changes to PSC size on maintenance of MZ progenitors were not examined.

A second study, also examining several IIS/TOR genetic components in the PSC, highlighted a similar effect of IIS/TOR signaling on PSC size (Benmimoun et al., 2012). This latter study suggested that increased PSC size, induced by hyperactive IIS/TOR signaling in the PSC, only subtly reduced terminal differentiation into plasmatocytes and crystal cells despite a greater than two-fold increase in PSC cell number (Benmimoun et al., 2012). This finding was in contrast to previous reports of *Antp* overexpression in the PSC, which increase PSC cell number and strongly impairs terminal differentiation in the LG (Mandal et al., 2007). Further studies are required to more clearly elucidate the effects of TOR-mediated PSC expansion on the MZ prohemocyte population, and should include the use of MZ markers as well as earlier markers of differentiation that demarcate differentiating hemocytes. Since *hh* expression is intact in the expanded $Antp^{+}$ PSC populations upon IIS/TOR activation (Benmimoun et al., 2012; Tokusumi et al., 2012), it will be interesting to identify if conditions of hyperactive IIS/TOR signaling that increase PSC size but only subtly affect progenitors are mediated by a mechanism that does not involve changes in *hh* activity, perhaps identifying a new criterion for PSC functional integrity.

What defines a functional PSC cell?

Since its functional characterization as a hematopoietic niche (Krzemien et al., 2007; Mandal et al., 2007), the limited number of recent studies of the LG PSC have demonstrated that not all 'PSC' cells function as a niche. While several genetic conditions have been described which increase PSC cell number (Benmimoun et al., 2012; Pennetier et al., 2012; Sinenko et al., 2009; Tokusumi et al., 2012), only the increases in PSC size induced by increasing *myc* expression or inhibiting BMP signaling have demonstrated an associated expansion of the MZ progenitor population (Pennetier et al., 2012), similar to *Antp* overexpression (Mandal et al., 2007). A late expansion in PSC cell number occurs upon *col* downregulation, yet these PSC cells fail to increase prohemocyte maintenance/MZ size or decrease differentiation. This lack of an effect is attributed to decreased *hh* expression per PSC cell (Pennetier et al., 2012). Further, Su(H) mutant LGs, which expand *hh* expression to MZ cells, are prematurely differentiated (Tokusumi et al., 2010), and ectopically expressing Hh in MZ cells does not expand blood progenitors (Tokusumi et al., 2010). Thus, in contrast to the effects of increasing total PSC size by *Antp* overexpression, which decreases hemocyte differentiation (Mandal et al., 2007), increasing the number of $Antp^+$ PSC cells via *col* downregulation fails to decrease terminal differentiation due to downregulation of *hh* expression levels that are compensated by the increased PSC cell number. In contrast, the expanded $Antp^+$ PSC populations upon IIS/TOR activation do maintain *hh* expression, but only cause a subtle decrease in terminal differentiation in the LG (Benmimoun et al., 2012; Tokusumi et al., 2012), suggesting that IIS/TOR signaling in the PSC may regulate another aspect of PSC function required for proper MZ progenitor maintenance. Finally, ectopic expression of *hh* in the MZ is not sufficient to prevent progenitor differentiation (Tokusumi et al., 2010), suggesting a defect in its secretion or signaling outside of the context of the PSC.

In addition to a requirement for *hh* expression in PSC cells, the presence of numerous filopodia that extend among MZ progenitors has been suggested to be required for PSC cells to relay maintenance signals to hemocyte progenitors of the MZ (Krzemien et al., 2007; Mandal et al., 2007). A number of genes have been identified that affect the generation of filopodial extensions (Tokusumi et al., 2012; Tokusumi et al., 2010). However, the genetic conditions examined also affect $Antp^{+}$ PSC number, *hh* expression, or both, preventing the ability to isolate the role of filopodial extensions in PSC-dependent progenitor maintenance.

B. Autonomous regulation of MZ hemocyte progenitor maintenance/differentiation

JAK/STAT signaling in the MZ

Mixed results have been reported regarding the role of the JAK/STAT signaling pathway in the LG. Hematopoietic progenitors in the MZ are identified by their specific expression of the JAK/STAT receptor, Dome (Jung et al., 2005), and active JAK/STAT signaling is observed in these cells via expression of the *dome-MESO lacZ* transgene, which monitors active pathway signaling (Krzemien et al., 2007). LGs mutant for *stat92E*, the transcriptional regulator activated downstream of JAK/STAT signaling in *Drosophila*, lose the MZ due to premature differentiation (Krzemien et al., 2007). Because this *stat92E* mutant phenotype phenocopies *col* mutant LGs, which lack a PSC, it was inferred that the PSC non-autonomously maintains JAK/STAT signaling in the MZ, which is required for progenitor maintenance (Krzemien et al., 2007). Of the three JAK/STAT cytokine lignads, Unpaired (Upd) 1-3, Upd3 and low levels of Upd 2 are expressed in the LG (Makki et al., 2010). Upd2 has no role in LG hematopoiesis, but Upd3, which is expressed in the PSC and MZ, was found to specifically function in the MZ, and not the PSC, for maintenance of JAK/STAT signaling in the LG (Makki et al., 2010), and has no role in the PSC for progenitor maintenance (Mandal et al., 2007). A role for Upd3 in the MZ was

identified during the immune response, wherein wasp infestation induces reduced levels of *upd3* transcripts in the LG, which in turn decreases JAK/STAT signaling levels and thus transcription of the target gene, *dome* (Makki et al., 2010). JAK/STAT signaling is further decreased in the MZ upon wasp infestation by a mechanism involving a short, non-signaling, cytokine *dome*-cognate receptor, Latran, which is expressed in the MZ, heteromers with Dome, and negatively regulates Dome-mediated JAK/STAT signaling in a dose-dependent manner (Makki et al., 2010). The decreased *dome* transcription induced by wasp infestation increases Latran/Dome ratios in the MZ, to further inhibit JAK/STAT signaling, which is required for the massive lamellocyte differentiation induced by wasp infestation (Makki et al., 2010). Although regulated JAK/STAT signaling in the MZ is required as part of the LG immune response (Makki et al., 2010), recent studies have suggested that canonical JAK/STAT signaling is not actually required for progenitor maintenance, as was previously reported (Krzemien et al., 2010a; Krzemien et al., 2007): first, no functional role for STAT92E was identified in autonomously regulating progenitor maintenance/differentiation (Minakhina et al., 2011), and second, loss-of-function of *dome* or the single JAK kinase, *Hopscotch*, does not alter progenitor differentiation (Mondal et al., 2011). Rather, a non-autonomous effect of STAT92E on progenitor maintenance has been proposed from the CZ, and is likely responsible for the precocious differentiation induced in *stat92E* mutant LGs (see below) (Mondal et al., 2011).

Wingless signaling autonomously maintains hemocyte progenitors in the lymph gland

The first autonomous signal identified in LG hemocyte progenitors, shown to both promote and be required for proper progenitor maintenance, was the canonical Wingless (Wg)/Wnt signaling pathway (Sinenko et al., 2009). Wg expression is initiated in LG hemocyte progenitors as early as the first instar stage, and is maintained in progenitors throughout larval

development (Sinenko et al., 2009). In contrast, Wg expression is turned off prior to the differentiation of peripheral progenitors in the second larval instar, and is thus largely absent in the peripheral CZ, except in crystal cell progenitors, until a late re-initiation of Wg expression in CZ hemocytes at the late third instar stage (Sinenko et al., 2009). Disruption of Wg signaling in hemocyte progenitors causes a reduction of Shotgun expression, which leads to the mislocalization of *dome* expression to more cortical LG regions (Sinenko et al., 2009). Although differentiation is not initially affected during the second instar and the number of terminally differentiated plasmatocytes are not increased in the LG by the third instar, the proportion of *dome*$^{+}$/Pxn^{+} hemocytes is increased upon disruption of Wg signaling (Sinenko et al., 2009). Conversely, activation of Wg signaling in hemocyte progenitors impairs their differentiation, blocking proper CZ formation (Sinenko et al., 2009). Together, these data suggest that canonical Wg signaling autonomously maintains LG hemocyte progenitors by impairing their transition to a more differentiated intermediate progenitor cell type. The absence of an effect on terminal differentiation upon disruption of Wg signaling demonstrates that the terminal differentiation of hemocyte progenitors requires, in addition to the loss of a maintenance signal, additional unidentified signals that direct progenitor differentiation into mature CZ hemocytes.

Interestingly, the PVR ligand, Pvf2, is also expressed in MZ progenitors, and like Wg, regulates Shotgun expression, such that *pvf2* downregulation in progenitors also causes the inappropriate mislocalization of *dome*$^{+}$ progenitors to cortical LG regions, while not altering progenitor differentiation (Mondal et al., 2011). Further analyses are required to examine a potential functional relationship between Pvf2 and Wg signaling in maintaining Shotgun expression and thus MZ integrity.

Autonomous ROS levels prime Drosophila MZ prohemocytes for differentiation

While excessive levels of reactive oxygen species (ROS) are damaging to cells, physiologically moderate ROS levels can function as signaling molecules that promote, for example, cell proliferation and survival (Trachootham et al., 2008). Mouse common myeloid progenitors (CMPs) maintain ROS levels 100X higher than HSCs, although the functional role of high ROS levels in CMPs remains uncharacterized (Tothova et al., 2007). In *Drosophila*, a significant upregulation of ROS levels occurs at the third larval instar stage, following a period of mitotically active prohemocytes limited to the early larval instars (Owusu-Ansah and Banerjee, 2009). ROS levels are then decreased upon progenitor differentiation into CZ hemocytes (Owusu-Ansah and Banerjee, 2009). These developmentally regulated, relatively high levels of ROS in MZ prohemocytes of the third instar sensitizes them for differentiation; increasing ROS in the LG via disruption of complex 1 proteins of the mitochondrial electron transport chain induces precocious differentiation into all mature hemocyte types (Owusu-Ansah and Banerjee, 2009). Increased ROS levels activates Jun kinase (JNK) signaling, which mediates the differentiation response of hemocyte progenitors via two major effectors: activation of FoxO (which mediates plasmatocyte and crystal cell differentiation), and downregulation of Polycomb proteins (which increases lamellocyte number) (Owusu-Ansah and Banerjee, 2009). Developmentally regulated ROS levels thus serve a dual role in MZ hemocyte progenitors, both in oxidative stress sensing and in the developmental regulation of progenitor differentiation.

Although inducing oxidative stress in LG progenitors via mitochondrial dysfunction was not reported to affect their proliferation, a link between ROS and proliferation of circulating hemocyte precursors, both normal and malignant, has been demonstrated in *Drosophila* larvae (Sinenko et al., 2010). Expression of the human AML1-ETO oncogenic fusion protein in *Drosophila* circulating hemocytes and hemocyte precursors induces the aberrant/malignant

expansion of a ROS^+ precursor hemocyte population in circulation, demonstrating a leukemic phenotype that is associated with a switch in cell fate from differentiation to self-renewal (Sinenko et al., 2010). The hyperproliferation of AML1-ETO-induced hemocyte precursors was suppressed by scavenging ROS via expression of *superoxide dismutase 2* (*SOD2*) or *foxo*, a direct positive regulator of *SOD2* expression (Sinenko et al., 2010). In addition to this role for ROS in a malignant pool of ROS^+ blood precursors, genetic disruption of mitochondrial function in circulating hemocytes also induces significant proliferation (Sinenko et al., 2010). From these studies it is evident that the role of ROS and ROS-interacting genes (i.e., FoxO) in *Drosophila* hematopoiesis is context-dependent, functioning both in differentiation (Owusu-Ansah and Banerjee, 2009) and proliferation (Sinenko et al., 2010).

The roles of nutrition, insulin and TOR signaling on hemocyte progenitor maintenance

Recent analyses of the effects of metabolic stress on LG hemocyte progenitors have demonstrated that MZ prohemocytes directly respond to both systemic and nutritional signals (Benmimoun et al., 2012; Shim et al., 2012). Extensive differentiation occurs in the LG at the expense of MZ prohemocytes in response to starvation, and the molecular mechanisms responsible for this hematopoietic response were tied to both systemic insulin and nutritional amino acid signaling (Benmimoun et al., 2012; Shim et al., 2012). Ablation of the neuroendocrine insulin-producing cells (IPCs) in the brain or mutation of the *Drosophila* insulin-like peptide 2, Dilp2, which is produced and secreted from IPCs, increases LG differentiation similar to starvation (Shim et al., 2012). *Drosophila* LG hemocyte progenitors may directly respond to this systemic insulin signal, as they express high levels of insulin receptor (Inr), and downregulation of *inr* in prohemocytes increases their differentiation (Benmimoun et al., 2012; Shim et al., 2012). Suppression of progenitor differentiation is observed upon increasing

neuronal *dilp2* expression or secretion, which led Shim et al. to conclude that maintenance of MZ hemocyte progenitors requires direct sensing, via Inr, of IPC-derived Dilp2 (Shim et al., 2012). It is well established that the fat body can indirectly control insulin secretion from brain IPCs upon sensing amino acid levels via the amino acid transporter protein Slimfast (Slif) (Colombani et al., 2003; Géminard et al., 2009). Disruption of Slif function in the fat body increases LG progenitor differentiation (Benmimoun et al., 2012; Shim et al., 2012), likely via its effect on Dilp2 secretion. While Shim et al. suggested that Slif also functions directly in hemocyte progenitors to sense amino acids and regulate progenitor maintenance, Benmimoun et al. did not observe any change to LG progenitor maintenance upon progenitor-specific Slif disruption (Benmimoun et al., 2012; Shim et al., 2012). Despite this discrepancy, these studies demonstrate a response of *Drosophila* MZ prohemocytes to nutrient restriction/metabolic stresses. Shim et al. have suggested that the effects of dietary restriction on LG progenitors are mediated via Wg signaling; Wg expression is decreased in the LG upon both *inr* and *slif* downregulation in progenitors, and forced overexpression of *wg* in progenitors is sufficient to maintain progenitors upon starvation (Shim et al., 2012).

Conflicting findings have been reported for the role of the nutrient-responsive Target of Rapamycin (TOR) growth signaling pathway in regulating LG progenitors. Shim et al. report increased differentiation upon reduced TOR signaling in LG progenitors, but reduced differentiation upon TOR activation, suggesting that Dilp-Inr-TOR signaling functions in progenitor maintenance (Shim et al., 2012). In contrast, Benmimoun et al. report that both activation and inhibition of TOR signaling in hemocyte progenitors increases their differentiation (Benmimoun et al., 2012). While both groups demonstrate that the LG response to nutrient restriction and reduced TOR activation includes precocious progenitor differentiation, the role of

TOR signaling during normal LG development remains unclear. Previous studies in the LG have highlighted a dual role for signaling pathways in regulating both normal developmental processes and stress responses (Mukherjee et al., 2011; Owusu-Ansah and Banerjee, 2009). Further examination of the role(s) of TOR signaling in MZ prohemocytes will likely uncover novel insights into the dual use of a nutrient-responsive signal by the *Drosophila* myeloid system during normal development and in response to stress signals.

Bag of marbles and microRNA 7 regulate hemocyte progenitor maintenance in the LG

Bag of marbles (Bam) is a *Drosophila* germline stem cell differentiation factor that can function in selective mRNA translation control (Kim et al., 2010; Li et al., 2009; McKearin and Ohlstein, 1995; McKearin and Spradling, 1990; Shen et al., 2009), which was found to be specifically expressed and required in the population of MZ LG prohemocytes for their maintenance; absence of *bam* in prohemocytes increases differentiation into all three hemocyte lineages at the expense of progenitors, while forcibly expressing *bam* in prohemocytes impairs their differentiation (Tokusumi et al., 2011). While microRNA-7 (mir-7) had previously been identified as a repressor of *bam* expression during male germline differentiation (Pek et al., 2009), Tokusumi et al. found identical loss- and gain-of-function phenotypes for *mir-7* and *bam* in the LG and suggested that cooperativity of these two genes functions in progenitor maintenance. Finally, Yan, an ETS-domain transcriptional repressor (Lai and Rubin, 1992), was identified to have opposite effects on progenitor differentiation upon loss- and gain-of-function analyses, compared to Bam and mir-7 (Tokusumi et al., 2011). Since Yan is expressed in scattered cells in the LG that do not overlap with the MZ marker TepIV or the plasmatocyte marker, P1, Tokusumi et al. suggested that Yan functions in a pool of intermediate progenitors. However, it remains to be determined whether Yan expression overlaps with crystal cell

markers, as a subset of Yan+ cells localize to the LG periphery (Tokusumi et al., 2011), where intermediate progenitors have never been identified. Whether Yan labels intermediate progenitors or another differentiated hemocyte cell type, the number of Yan+ hemocytes increases in *bam* or *mir-7* mutant LGs, concomitant with an increase in all differentiated hemocyte lineages (Tokusumi et al., 2011). While the authors suggested from these data that Bam and mir-7 cooperate to negatively regulate Yan mRNA translation, the increase in the number of Yan+ hemocytes upon *bam* or *mir-7* genetic manipulation is likely reflective of the overall increase in differentiation status within the LG, and does not directly demonstrate a relationship between Bam and mir-7 with Yan function. Although Tokusumi et al. highlight three potential regulators of progenitor maintenance (two positive and one negative), further investigation is required to identify the functional and epistatic relationships between Bam, mir-7 and Yan in LG progenitor maintenance.

C. Regulation of MZ prohemocyte maintenance/differentiation by CZ hemocytes

PVR- and STAT92E-mediated Adgf-A expression is required in the CZ for MZ prohemocyte maintenance

A recent elegant analysis by Mondal et al. has deciphered a signaling network that is dependent on differentiating CZ hemocytes for maintenance of MZ prohemocytes (Mondal et al., 2011). Expression of Pvf1, one of three ligands for the *Drosophila* platelet-derived growth factor/vascular endothelial growth factor (PDGF/VEGF) receptor homolog, PVR, in the PSC, initiates a signaling cascade that commences with transcytosis of Pvf1 from the PSC to CZ hemocytes, where it activates PVR. Activated PVR in differentiating hemocytes induces the activation of STAT92E, specifically in differentiating intermediate progenitors and not terminally differentiated hemocytes, which results in expression of the secreted factor, adensoine

deaminase growth factor A (AdgfA). This Pvf1-PVR-STAT92E-Adgf-A signaling cascade is required for MZ prohemocyte maintenance, as prohemocytes are lost due to differentiation by the third larval instar upon disruption of PSC-derived Pvf1 or CZ-derived PVR, STAT92E or Adgf-A signaling. Prior to this late effect on progenitor maintenance, disruption of Pvf1, PVR or Adgf-A initially increases progenitor proliferation during the second instar stage, suggesting that this signal cascade is first required to maintain progenitor quiescence during second instar, which in turn prevents premature differentiation during the third instar.

The primary function of Adgf-A is to inactivate extracellular adenosine by deamination (Dolezal et al., 2003; Maier et al., 2001). Extracellular adenosine signals through the *Drosophila* adenosine receptor (AdoR) to transmit a G protein/adenylate cyclase/cAMP-dependent protein kinase A (PKA) signal into the cell (Dolezelova et al., 2007). Decreasing adenosine signaling autonomously in MZ progenitors, for example by downregulation of *adoR* or disruption of G protein, adenylate cyclase, or PKA function, concomitantly increases MZ size/maintenance. Interestingly, PKA is known to antagonize Hh signaling by promoting the proteolysis of active Cubitus interuptus (Ci), the downstream effector of Hh signaling, to its repressor form (Chen et al., 1998). Therefore, active PKA signaling in MZ progenitors may reduce the effectiveness of the PSC-derived Hh maintenance signal. In fact, Mondal et al. report that decreasing *adoR* expression or inhibiting PKA function in hemocyte progenitors increases active Ci levels, demonstrating a previously undescribed function for AdoR-dependent PKA activation in modulating Ci activity. These results collectively describe a model where a PSC-derived signal functions in CZ differentiating hemocytes to maintain MZ progenitors in an Adgf-A-dependent manner, by limiting the amount of extracellular adenosine/AdoR-induced PKA activation that would interfere with the PSC-derived Hh maintenance signal in progenitors.

This study highlights a novel mechanism for progenitor maintenance in the LG that is dependent on differentiating hemocytes, and may be particularly important in other systems for which a large number of progenitors must be maintained that are not all in direct contact with the niche. It remains unclear, however, what signal operates in the LG to initiate progenitor differentiation, both in wild-type and in conditions of reduced maintenance caused by inhibition of PVR-STAT92E-Adgf-A and Ado-adenylate cyclase-PKA signaling. Despite previous studies that have established a correlation between negative regulators of cell proliferation and their effects on progenitor differentiation in the LG (Kalamarz et al., 2012), establishing the link between loss of quiescence and premature differentiation requires further investigation.

Part III. Signaling mechanisms affecting LG hemocyte differentiation and number

In addition to the identified signaling pathways required for hemocyte progenitor maintenance in the PSC, progenitor cells, or differentiated hemocytes, several additional genes have been demonstrated to function in LG homeostasis. In this section, we discuss additional effectors that operate in the LG to maintain homeostasis of the different hemocyte populations.

U-shaped regulates LG homeostasis and differentiation

U-shaped (Ush) is the single *Drosophila* Friend-of-GATA (FOG) Zinc-finger domain transcriptional cofactor. Ush is expressed throughout the MZ and in a subset of differentiated plasmatocytes and crystal cells of the CZ, but is absent in PSC cells (Gao et al., 2009). While Ush expression was not initially detected in second instar LGs (Sorrentino et al., 2007), a later analysis did identify Ush protein expression in the LG by this stage (Gao et al., 2009). *Ush trans* heterozygous mutant LGs, carrying one null and one hypomorphic *ush* allele, are hyperplastic (Gao et al., 2009; Sorrentino et al., 2007) and present with a reduction in plasmatocyte and crystal cell lineages but a large increase in lamellocyte number in the LG (Gao et al., 2009).

Mutant larvae also demonstrate an increase in circulating hemocyte numbers, including circulating lamellocytes, although this is likely due to the autonomous loss of *ush* function in circulating larval hemocytes, where Ush is also expressed (Sorrentino et al., 2007). Intriguingly, *ush* heterozygous LGs present with elevated plasmatocyte and crystal cell numbers, but no change in lamellocytes (Gao et al., 2009). Further, *ush* overexpression in differentiated hemocytes of the CZ limits plasmatocyte differentiation (Gao et al., 2009). These latter findings prompted Gao et al. to suggest that Ush normally functions as a maintenance signal for LG prohemocytes, despite the decrease in plasmatocyte and crystal cell lineages observed in *ush trans* heterozygous mutants. Further, analysis of the *ush* gene hematopoietic *cis*-regulatory module identified a putative STAT binding site, and functional analyses suggested that STAT can directly regulate *ush* expression. Since previous studies suggested that the PSC maintains MZ hemocyte progenitors by activating JAK/STAT signaling in the MZ (Krzemien et al., 2007), Gao et al. proposed a model whereby Ush, activated by STAT in progenitors, is a downstream effector of the PSC that functions in prohemocyte maintenance. Further analyses later clarified that the PSC does not regulate JAK/STAT signaling in the MZ (Makki et al., 2010; Mandal et al., 2007), and that STAT does not autonomously regulate MZ prohemocyte differentiation (Minakhina et al., 2011; Mondal et al., 2011). Further analyses, including prohemocyte-specific loss- and gain-of function analyses of Ush, are required to tease apart the role of this transcriptional regulator in prohemocyte maintenance/differentiation.

At least five GATA factors are present in *Drosophila*, but only Serpent (Srp) and Pannier (Pnr) have been suggested to function in *Drosophila* hematopoiesis (Mandal et al., 2004; Minakhina et al., 2011; Rehorn et al., 1996). Ush has been shown to bind to both Srp and Pnr GATA factors (Haenlin et al., 1997; Waltzer et al., 2002), yet it remains unclear how Ush and

these GATA factors function together in hematopoiesis. In the embryo, Ush interacts with Srp in direct competition with Lz to anatagonize crystal cell lineage commitment (Waltzer et al., 2003). A genetic interaction between Ush and Srp has also been suggested in the regulation of circulating lamellocyte numbers (Sorrentino et al., 2007), but further analyses are required to define the functional relationship between these factors in circulating hemocytes. As both GATA and FOG factors are critical regulators of vertebrate hematopoiesis, teasing apart the roles of Ush in LG hemocyte populations and how Ush intereacts with Srp and/or Pnr to this end may provide insight into conserved or novel GATA-FOG functions in hematopoiesis.

Terminal plasmatocyte differentiation in the lymph gland may require Pannier and STAT

In addition to the findings of Mondal et al., the role of STAT92E in the CZ was also proposed as part of a mechanism by which STAT92E positively regulates expression of the GATA factor Pnr, and that both factors are autonomously required for terminal plasmatocyte differentiation of committed Pxn^{+} hemocytes (Minakhina et al., 2011). The authors found that both *stat92e-/-* and *pnr-/-* mutant clones in the LG started to differentiate into Pxn^{+} hemocytes or could form terminal crystal cells, but didn't differentiate into terminal plasmatocytes (Minakhina et al., 2011). The authors also suggest that STAT92E inhibits differentiation of adjacent hemocytes non-autonomously, as ectopic differentiation of hemocytes occurred adjacent to medial *stat92e-/-* clones which localize close to the MZ. These conclusions are puzzling, as downregulation of *stat92E* and *pnr* in CZ hemocytes led to opposite phenotypes: an increase versus a decrease, respectively, in plasmatocyte differentiation in the LG (Minakhina et al., 2011). Further, larger *stat92e-/-* LG clones that were generated by Mondal et al., demonstrated terminal plasmatocyte differentiation in peripheral mutant cells (Mondal et al., 2011). In light of these discrepancies, it remains unclear whether STAT92E is required autonomously in peripheral

differentiating hemocytes for their terminal plasmatocyte differentiation. However, the work of Minakhina et al. supports the findings of Mondal et al. that STAT92E in peripheral CZ hemocytes non-autonomously blocks differentiation of adjacent MZ prohemocytes, contributing to their maintenance (Minakhina et al., 2011; Mondal et al., 2011).

Crystal cell differentiation in the lymph gland requires N signaling, endocytic trafficking, and Sima

A role for canonical Notch (N) signaling in crystal cell fate specification is well established, and is known to require the N ligand Serrate (Ser) (Duvic et al., 2002; Lebestky et al., 2003). In addition to the PSC, Ser expression is often found adjacent to Lz^{+} crystal cell progenitors, suggestive of an inductive relationship in crystal cell specification (Lebestky et al., 2003). Continued N signaling is also required in crystal cells for their expansion and maintenance, demonstrated by the 'bursting' phenotype induced upon the late disruption of *N* expression in already-specified crystal cell progenitors (Mukherjee et al., 2011). However, the Ser^{+} cell does not remain in close proximity to terminally differentiated crystal cells, and further, the late removal of *Ser* expression in the LG does not affect crystal cell maintenance (Mukherjee et al., 2011). Mukherjee et al. thus propose a model in which ligand-independent N stabilization occurs after crystal cell specification in a manner dependent on the *Drosophila* hypoxia inducible factor-α (HIF-α) homolog, Sima. Sima is stably expressed in crystal cells, even under normoxic conditions (Mukherjee et al., 2011). Importantly, disruption of *sima* expression in the LG reduces crystal numbers and also suppresses the increased crystal cell differentiation that occurs upon N activation in the LG (Mukherjee et al., 2011). Conversely, *sima* overexpression increases crystal cell number and also increases endogenous N expression in crystal cells. Colocalization of Sima and internalized N is evident in Hrs-positive early endocytic vesicles, and expression of

Rab5, which enhances the turnover of endocytic full length N, suppresses the Sima-induced crystal cell phenotype (Mukherjee et al., 2011). These experiments highlight the role of endocytic vesicles in the N/Sima interaction, and suggest that non-canonical N activation after crystal cell specification relies on Sima-dependent stabilization of full length N receptor in the enodcytic pathway, even in the absence of ligand signaling. Importantly, hypoxia, which stabilizes Sima from degradation, increases crystal cell numbers (Mukherjee et al., 2011), demonstrating that the same Sima/N signaling pathway is required for both normal development and in response to hypoxic stress.

A second study has also suggested a role for endocytic trafficking in LG homeostasis. Recent reports have demonstrated that Asrij, a conserved endocytic protein that localizes to a subset of endocytic vesicles in the LG, regulates the number of Lz^{+} crystal cell progenitors in the LG (Kulkarni et al., 2011). This observed phenotype is likely the result of N intracellular domain accumulation in sorting endosomes, which is suggestive of aberrant endocytic trafficking and which can induce increased N activity in the LG (Kulkarni et al., 2011). Kulkarni et al. also demonstrate an increase in plasmatocyte differentiation and hyperproliferation of LG secondary lobes in *asrij* mutants, and suggest an important role for cellular trafficking in regulation of LG homeostasis. Cell-type specific genetic and functional analyses of Asrij and all other endocytic pathway components are required, however, to tease apart the roles of endocytic trafficking in modulating the various signaling pathways known to function within distinct hemocyte lineages of the LG.

Myeloid Leukemia Factor regulates Drosophila lymph gland hematopoiesis

The vertebrate myeloid leukemia factor 1 (*MLF1*) gene encodes a conserved protein that has been associated with acute myeloid leukemia (AML) (Yoneda-Kato et al., 1996), although its

physiological role in hematopoiesis remains largely unknown. In *Drosophila*, the single MLF homolog was shown to have multiple roles in hematopoiesis (Bras et al., 2012). In embryonic hemocytes and circulating larval hemocytes, MLF was shown to regulate crystal cell number via protecting Lz, a transcription factor required for crystal cell differentiation, from protein degradation. A second role for MLF in LG homeostasis was characterized; *mlf* is expressed at low levels throughout LG hemocytes, and *mlf* mutant LGs are hypertrophied and prematurely differentiate into both plasmatocyte and crystal cell lineages at the expense of prohemocytes (Bras et al., 2012). These results suggest a potential role for MLF in regulating prohemocyte maintenance in the LG, although further analyses are required to identify the functional role for MLF in different LG hemocyte populations to this end.

Part IV. Conclusions

The conservation of several fundamental mechanisms contributing to mammalian hematopoiesis has been demonstrated in the *Drosophila* LG during recent years, including the multilineage potential of *Drosophila* hemocyte progenitors and the strict dependence of their maintenance on a hematopoietic niche. Further, the ability to perform countless genetic analyses, including cell-type specific, with direct *in vivo* imaging of interacting cell populations, highlights the utility of the *Drosophila* LG as a powerful genetic model for studying conserved mechanisms of hematopoiesis during development and disease. In addition, the demonstrated hematopoietic response to multiple stresses in the LG, including nutrient restriction, hypoxia, infection and oxidative stress, highlights the utility of this genetic model to tease apart the dual use of genetic pathways involved in both normal hematopoiesis and in response to stress.

Since its functional characterization as a hematopoietic niche, extensive analyses have been performed during recent years in the PSC of the *Drosophila* LG to identify the mechanisms

of niche-dependent blood progenitor maintenance in this system. The required maintenance of *Drosophila* progenitors faces obstacles not seen in other *Drosophila* stem cell types, which are more often in intimate contact with the niche responsible for their maintenance (Losick et al., 2011). In the LG, a large pool of progenitors must be maintained, despite the vast majority not being in direct contact with the niche. Several mechanisms have been proposed to operate in the LG to combat this apparent problem. These include, the extension of long fillapodial cellular processes from PSC cells, which emanate from the PSC and could deliver maintenance signals to distant progenitors, use of endocytic vesicles to transport signaling molecules to distant hemocytes, and the operation of a feedback signal, emanating from differentiated hemocytes, for the maintenance of adjacent progenitors.

Despite extensive studies in the LG during recent years, many important questions remain unanswered. In particular, the nature of the signal required to trigger differentiation of hemocyte progenitors during the second instar remains unidentified. Further, genetic dissection of the interaction between such positive effectors of differentiation and known maintenance signals in the LG, including Wg and Hh signaling, is required to understand how the tightly regulated expansion of differentiating hemocytes during the larval stages contributes to the formation of a mature CZ by late larval development, while also maintaining the population of undifferentiated MZ prohemocytes. Additionally, while a number of genes have been implicated in hemocyte proliferation in the LG, the signals required to regulate cell proliferation of progenitors and in turn LG growth and final tissue size, remain unidentified. The LG thus represents a relatively new system that can be used to tease apart mechanisms of blood progenitor cell maintenance and differentiation decisions and will likely continue to enhance our understanding of mammalian hematopoiesis during both development and disease.

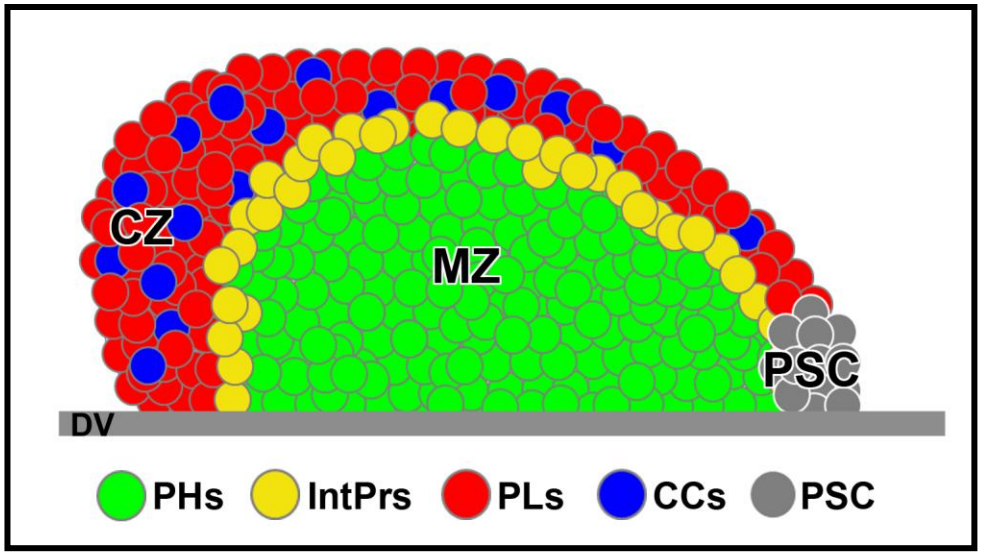

Figure 1-1. The late third instar lymph gland is divided into three distinct cellular domains

Schematic diagram of a late third instar lymph gland primary lobe. The pool of undifferentiated prohemocytes (PHs, green) are compactly arranged in the medullary zone (MZ) and give rise to differentiated plasmatocytes (PLs, red) and crystal cells (CCs, blue), which localize to the peripheral cortical zone (CZ). The posterior signaling center (PSC, gray) is a small group of cells at the posterior tip of each lymph gland primary lobe and functions as a hematopoietic niche by supplying pro-maintenance signals to adjacent MZ PHs. DV= dorsal vessel.

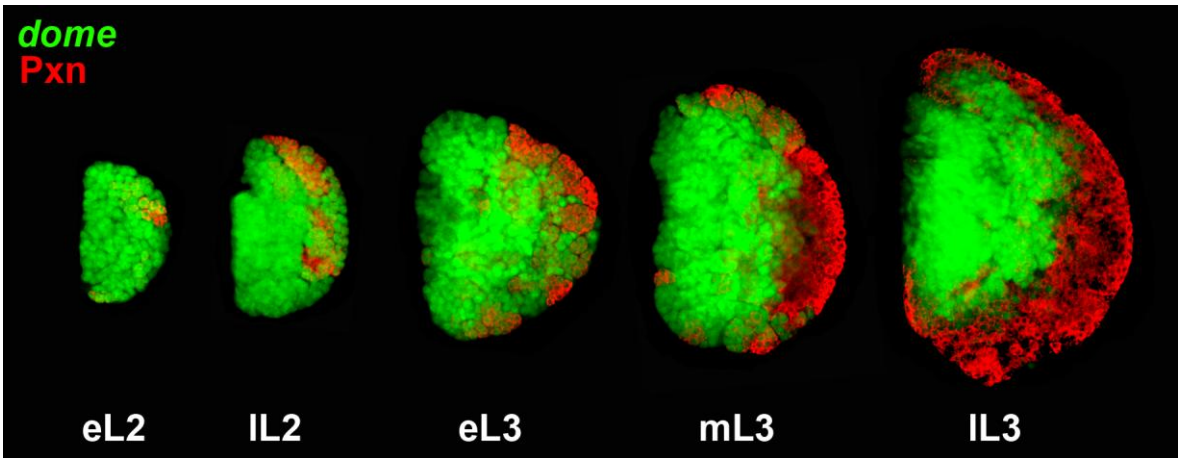

Figure 1-2. Lymph gland development during the second and third larval instars stages

Progression of wild-type lymph gland (*dome-gal4, UAS-2xEGFP*) growth at early second (eL2), late second (lL2), early third (eL3), mid-third (mL3) and late third (lL3) instar stages. Differentiation invariable begins during eL2 at the periphery of the lymph gland, and the controlled expansion of the number of differentiating hemocytes leads to the development of a mature cortical zone by lL3. Hemocyte progenitors of the medullary zone are shown in green (*dome*$^{+}$), and differentiating hemocytes are labeled with Pxn expression in red. Figure adapted from Dragojlovic-Munther and Martinez-Agosto, Development, 2012.

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Chapter 2:

Multifaceted roles of PTEN and TSC orchestrate growth and differentiation of *Drosophila* blood progenitors

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Multifaceted roles of PTEN and TSC orchestrate growth and differentiation of *Drosophila* blood progenitors

Michelle Dragojlovic-Munther[1] and Julian A. Martinez-Agosto[2,*]

SUMMARY

The innate plasticity of hematopoietic progenitors is tightly regulated to supply blood cells during normal hematopoiesis and in response to stress or infection. We demonstrate that in the *Drosophila* lymph gland (LG) the tumor suppressors TSC and PTEN control blood progenitor proliferation through a common TOR- and 4EBP-dependent pathway. *Tsc2* or *Pten* deficiency in progenitors increases TOR signaling and causes LG overgrowth by increasing the number of actively dividing cells that accumulate high levels of phosphorylated (p) 4EBP during a critical window of growth. These phenotypes are associated with increased reactive oxygen species (ROS) levels in the LG, and scavenging ROS in progenitors is sufficient to rescue overgrowth. Blood progenitor number is also sensitive to starvation and hypoxia in a TOR-dependent manner. Differences between *Tsc1/2* and *Pten* function become apparent at later stages. Loss of *Tsc1/2* autonomously increases p4EBP and decreases pAKT levels, expands the number of intermediate progenitors and limits terminal differentiation, except for a late induction of lamellocytes. By contrast, absence of PTEN increases p4EBP and pAKT levels and induces myeloproliferative expansion of plasmatocytes and crystal cells. This increased malignancy is associated with non-autonomous increases in p4EBP levels within peripheral differentiating hemocytes, culminating in their premature release into circulation and demonstrating potential non-autonomous effects of *Pten* dysfunction on malignancy. This study highlights mechanistic differences between TSC and PTEN on TOR function and demonstrates the multifaceted roles of a nutrient-sensing pathway in orchestrating proliferation and differentiation of myeloid-specific blood progenitors through regulation of ROS levels and the resulting myeloproliferative disorder when dysregulated.

KEY WORDS: *Drosophila*, Lymph gland, Myeloid, TSC, PTEN, TOR

INTRODUCTION

The innate plasticity of hematopoietic progenitors is tightly regulated to continuously replenish blood cells under steady-state conditions and to supply cells that are immediately available in response to stress. Similar to vertebrates, hematopoiesis in *Drosophila* requires a population of multipotent progenitor cells (Mandal et al., 2007). These progenitors give rise exclusively to myeloid-like lineages through mechanisms that are conserved at the cellular and molecular levels (Evans and Banerjee, 2000; Evans et al., 2003; Martinez-Agosto et al., 2007).

Mature myeloid cell types derived from *Drosophila* progenitors include: macrophage-like plasmatocytes (PLs); crystal cells (CCs); and a specialized cell type, the lamellocyte, which is not normally present in the larva but can be induced to differentiate in response to specific immune challenges (Lanot et al., 2001; Sorrentino et al., 2002). One wave of hematopoiesis in *Drosophila* occurs in the larval lymph gland (LG) (Lebestky et al., 2000). In the LG, maturing hemocytes populate the peripheral cortical zone (CZ; Fig. 1A), and originate from a pool of multipotent progenitors that are compactly arranged in the medullary zone (MZ; Fig. 1A) and are genetically distinguishable by expression of the JAK/STAT receptor *domeless* (*dome*) (Jung et al., 2005). Additionally, a small population of intermediate progenitors is localized at the MZ/CZ boundary (Fig. 1A) (Sinenko et al., 2009; Krzemien et al., 2010).

[1]Molecular Biology Institute, University of California, Los Angeles, CA 90095, USA. [2]Department of Human Genetics, David Geffen School of Medicine, University of California, Los Angeles, CA 90095, USA.

*Author for correspondence (julianmartinez@mednet.ucla.edu)

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The posterior signaling center (PSC; Fig. 1A), a small group of cells at the posterior LG tip, functions as a hematopoietic niche by supplying pro-maintenance signals to progenitors (Krzemien et al., 2007; Mandal et al., 2007).

Proliferative and pro-differentiation cellular responses in *Drosophila* blood progenitors are mobilized in response to specific stimuli, such as increased reactive oxygen species (ROS) levels or parasitic wasp infection (Lanot et al., 2001; Sorrentino et al., 2002; Owusu-Ansah and Banerjee, 2009; Krzemien et al., 2010). This plasticity of the LG to adapt to stress scenarios and regulate its growth is reminiscent of the Target of rapamycin (TOR) signaling network. TOR is a conserved serine/threonine kinase that forms part of a multiprotein complex (TORC1) that coordinates cell growth in response to environmental signals such as energy status, nutrient availability and cellular stressors (Yang and Guan, 2007; Wang and Proud, 2009; Russell et al., 2011). Its growth-promoting function is largely effected by regulating protein translation downstream of two major effectors: S6 kinase (S6K) and the translational initiation factor 4E-binding protein (4E-BP; Fig. 1B) (Fingar et al., 2002; Holz et al., 2005; Ma and Blenis, 2009).

Regulation of TORC1 signaling through upstream mediators occurs largely at the level of the pathway inhibitor Tuberous sclerosis complex 1 and 2 (TSC1/2; Fig. 1B). TSC1/2 blocks TORC1 activation by inhibiting RHEB, the direct activator of TORC1 (Garami et al., 2003; Inoki et al., 2003; Tee et al., 2003). Perhaps the best-characterized modulator of TORC1 signaling is AKT, a kinase that is activated downstream of growth factor signaling in a phosphatidylinositol 3-kinase (PI3K)-dependent manner (Fig. 1B). Activated AKT phosphorylates TSC2 on multiple residues, which relieves RHEB inhibition and activates TORC1 (Inoki et al., 2002; Potter et al., 2002; Miron et al., 2003). PI3K-dependent activation of AKT is inhibited by the Phosphatase

and tensin homolog (PTEN; Fig. 1B) as well as by a negative-feedback loop downstream of TORC1 signaling (Harrington et al., 2004; Kockel et al., 2010), adding additional layers of regulation to AKT-mediated TORC1 activation.

In this study, we demonstrate the common effects of *Tsc2* and *Pten* loss on the regulation of early blood progenitor proliferation in a TORC1-, 4EBP- and ROS-dependent manner. We highlight differences in the expansion of intermediate progenitors and differentiated hemocytes that are unique to TSC and PTEN, as well as the roles of stress, feedback inhibition and AKT activity in TOR-dependent LG regulation.

MATERIALS AND METHODS

Drosophila stocks

All stocks were obtained from stock centers (VDRC, NIG, Bloomington) except: *UAS-Tsc*$^{1+2}$ [A. Gould (Tapon et al., 2001)]; *UAS-Pten*^{wt} and *Pten*^{C494} (A. Wodarz, Georg-August-Univ, Gottingen, Germany); *Tsc2*^{129} (T. Ip, University of Massachusetts, Medical Center, Worcester, MA, USA); *UAS-GTPx1* (U. Banerjee, University of California, Los Angeles, CA, USA); and *UAS-Akt*^{myr} [E. Hafen (Stocker et al., 2002)]; *w*^{1118} was used for controls. Gal4 lines used: *dome-gal4, UAS-2xEGFP*; and *dome-gal4; P{tubP-gal80[ts]}20* (U. Banerjee). *dome-gal4* crosses were performed at 29°C, except *dome>Tsc*$^{1+2}$, *dome>Pten*^{wt} and *dome>Tor*^{DN} crosses which were grown at 18°C.

Larval staging experiments

Staging of *dome>Tsc2*RNAi and *dome>Pten*RNAi was performed at 29°C, and the following time points after egg hatching (AEH) were used for larval stages: 30 hours AEH (early second instar, eL2); 36 hours AEH (late second instar, lL2); 42 and 48 hours AEH (early third instar, eL3); 54 hours AEH (mid third instar, mL3); 65 hours AEH (late third instar, lL3); and 72 hours AEH (wandering third instar, wL3).

Clonal analysis

FLP-out clones in the LG were generated using *HHLT* (*hand-gal4, hml-gal4, UAS-2xEGFP, UAS-FLP, A5C-FRT-STOP-FRT-gal4*) as described (Evans et al., 2009). For lineage tracing of circulating hemocytes, H-TRACE (*hand-gal4, UAS-2xEGFP, UAS-FLP, A5C-FRT-STOP-FRT-gal4*) was used. Genotypes for MARCM FRT clones are as follows: *hs-flp FRT82B Tsc1*^{29} *FRT82B Tub-mCD8-GFP* and *hs-flp FRT40A Pten*^{2L117} *FRT40A Tub-nGFP*. Clones were induced by a 2-hour heat shock at 37°C within 2 hours AEH.

Immunohistochemistry, BrdU labeling and ROS detection

Immunohistochemistry was performed as previously described (Jung et al., 2005). Antibodies used: mouse anti-PXN (1:400; L. Fessler, University of California, Los Angeles, CA, USA); rabbit anti-PPO (1:400; M. Kanost, Kansas State University, Manhattan, KS, USA); mouse anti-P1 and anti-L1 (both 1:20; I. Ando, Hungarian Academy of Sciences, Szeged, Hungary); rat anti-SHG (1:400; V. Hartenstein, University of California, Los Angeles, CA, USA); rabbit anti-p4EBP and anti-pAKT (1:300 and 1:40, respectively; Cell Signaling); mouse anti-LZ and anti-TSC2 (both 1:100; DSHB); rabbit anti-RHEB (1:200; Thermo Scientific); rabbit anti-PTEN (1:400; A. Wodarz); mouse anti-Histone (pan) (1:100; Millipore); and anti-phH3 (1:300; Millipore and AbCam). BrdU labeling was performed as previously described with a 45-minute BrdU pulse (Mondal et al., 2011). ROS detection was performed as previously described (Owusu-Ansah and Banerjee, 2009) using dihydroethidium.

Rapamycin experiments

For early treatment, crosses were set up on 15-20 µM rapamycin food plates, and larvae were grown at 25°C until wL3. For later treatment, larvae were reared on normal food until eL3 and transferred to rapamycin food plates until wL3.

Circulating hemocyte analysis

Circulating hemocytes from a single larva were isolated in 20 µl PBS and evenly dispersed on a 14 multi-well glass slide (Thermo Scientific), incubated for 30 minutes, and then fixed and immunostained as above. Relative quantification of circulating hemocytes is reported as the number of hemocytes per 0.25 mm^2 section of the glass well.

Quantitative real-time PCR (Q-PCR)

Isolation of total RNA and reverse transcription were performed according to standard methods. Q-PCR was performed using an ABI 7900HT Q-PCR machine and SYBR Green (Qiagen). Relative quantification of transcript levels was calculated using the comparative C_t method and normalized against *rp49* (*RpL32* – FlyBase). *Tsc2* and *rp49* primers were used as described (Amcheslavsky et al., 2011). *Pten* primers were: forward, 5′-GCCACAGAAAATGCAAAGCCA-3′; reverse, 5′-GCCGGAAAC-TGGTATTGATGGT-3′.

Distribution of hemocyte and progenitor cell populations

Methodology to determine ratios of *dome*$^+$/PXN prohemocyte, *dome*$^+$/PXN$^+$ intermediate progenitor and *dome* /PXN$^+$ differentiated hemocyte populations was adapted from Shim et al. (Shim et al., 2012). Two-way ANOVA analysis was used to evaluate significance between hemocyte population distributions among genotypes.

Mitotic index and CC ratios

Mitotic index and ratio of CCs in the LG were measured as the total number of phH3$^+$ or LZ$^+$/PPO$^+$ cells divided by the total number of cells. The method for estimating total cell numbers in the LG was adapted from Krzemien et al. (Krzemien et al., 2010). The distribution of mitoses at mL3 was assessed by quantifying the number of phH3$^+$ cells in each population for all confocal sections as a ratio to total phH3$^+$ hemocytes. Statistical significance was evaluated with Student's *t*-test (mitotic index and CC ratios) and two-way ANOVA analysis (distribution of mitoses).

Starvation and hypoxic stress

Acute starvation was induced by transferring (fed) eL3 larvae (48 hours AEH at 29°C) to agar plates containing PBS/1% sucrose for 24 hours; larvae were dissected at 72 hours AEH. For hypoxia experiments, larvae were reared in normoxia until lL2 (36 hours AEH at 29°C) and then transferred to a 5% O_2 hypoxia chamber until wL3.

RESULTS

TSC and PTEN regulate TORC1 signaling in *Drosophila* blood progenitors

A search for cell type-specific markers in the LG identified higher expression of RHEB and TSC2 in MZ progenitors (prohemocytes) than in CZ hemocytes at wL3 (Fig. 1C-D′). Similar expression was observed for the upstream regulator of TORC1 activity, PTEN (Fig. 1E,E′), as well as for phosphorylated (p) AKT (AKT1 – FlyBase) (Fig. 1F,F′), indicating that active PI3K-AKT signaling occurs in prohemocytes. Expression of p4EBP (THOR – FlyBase), a marker of active TOR kinase activity (Miron et al., 2003), was also higher in MZ prohemocytes (Fig. 1G,G′). We confirmed the central role for TSC in regulating TORC1 signaling during normal *Drosophila* hematopoiesis by examining p4EBP expression upon *Tsc1/2* loss-of-function (LOF). *Tsc1* and *Tsc2* (*gigas* – FlyBase) homozygous mutants die at early larval stages (Ito and Rubin, 1999). Interestingly, heterozygosity for mutations in *Tsc1* or *Tsc2* specifically increases p4EBP levels in *dome* progenitors at wL3 (supplementary material Fig. S1A-C′), whereas heterozygosity for a *Pten* mutation increases p4EBP throughout the LG (supplementary material Fig. S1D,D′). A similar effect occurs upon progenitor-specific downregulation of *Tsc2* or *Pten* in the LG (supplementary material Fig. S1E-F′).

We next examined the phenotypic consequence of TORC1 hyperactivation, induced by downregulation of *Tsc1/2* or *Pten* function, on hemocyte progenitors. Growth of the LG occurs via extensive cell proliferation throughout larval development, from an initial group of ~30 progenitors per lobe in the late embryo to a

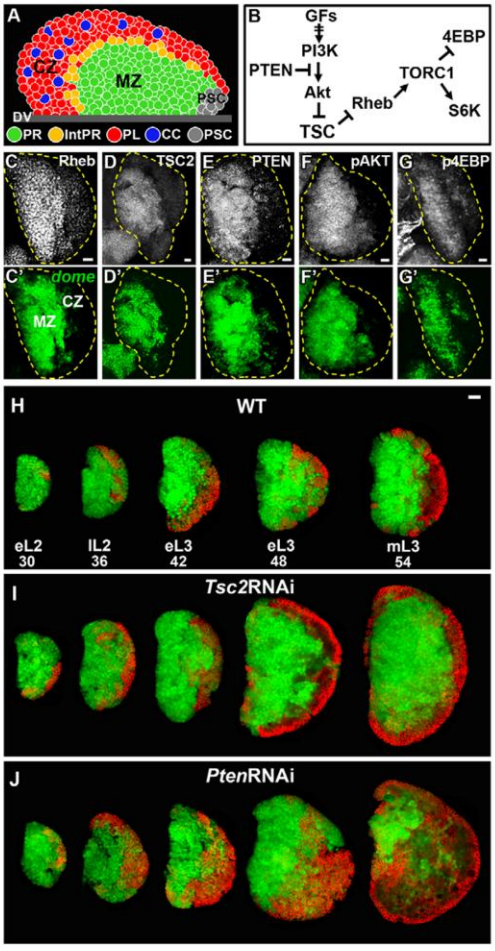

Fig. 1. TORC1 signaling in *Drosophila* hemocyte progenitors regulates early LG growth. (**A**) Diagram of a wL3 lymph gland (LG) primary lobe, highlighting undifferentiated progenitors (PRs) in the medullary zone (MZ), plasmatocytes (PLs) and crystal cells (CCs) in the cortical zone (CZ), intermediate progenitors (IntPrs) and the posterior signaling center (PSC). DV, dorsal vessel. (**B**) TOR signaling to its targets 4EBP and S6K is inhibited by TSC and PTEN. GFs, growth factors. (**C-G′**) TORC1 pathway components are expressed in MZ progenitors. All panels show wild-type (WT; *dome-gal4, UAS-2xEGFP*) wL3 LGs (outlined by dashed line). Expression of RHEB (C,C′), TSC2 (D,D′), PTEN (E,E′), pAKT (F,F′) and p4EBP (G,G′) is elevated in prohemocytes (green). (**H-J**) *dome-gal4, UAS-2xEGFP* (H) is used to express *UAS-Tsc2*RNAi (I) or *UAS-Pten*RNAi (J) in prohemocytes (green). The early differentiation marker PXN is in red. (H) Progression of WT growth from eL2 to mL3 instar stages. (I,J) *Tsc2* (I) and *Pten* (J) downregulation increases LG growth rate throughout development. *Pten* LOF increases differentiation by 36 hours AEH and throughout later stages. Scale bars: 20 μm.

mature LG consisting of ~5000 hemocytes (Krzemien et al., 2010). Onset of differentiation occurs during the second larval instar stage (L2), invariably at the periphery of the tissue (Fig. 1H). Early differentiating hemocytes express both the prohemocyte marker *dome* and the early differentiation marker Peroxidasin (PXN) at low levels (*dome* /PXN), and represent a population of intermediate progenitors. By contrast, fully differentiated hemocytes lose *dome*, upregulate PXN expression (*dome*⁻/PXN), and proceed to express a marker of terminal differentiation: P1 (PLs), Prophenoloxidase (PPO; CCs) or L1 (lamellocytes). *Tsc2* or *Pten* downregulation in prohemocytes increases the rate of tissue growth during L2, eL3 and mL3 instar stages (Fig. 1I,J). In *Tsc2*-deficient LGs, differentiation onset and progression remain intact (compare Fig. 1H with 1I), and, accordingly, the proportion of *dome* /PXN⁻ prohemocytes by mL3 (64%) is the same as in wild type (WT; 65%) (supplementary material Fig. S1G). By contrast, *Pten* deficiency expands differentiating hemocytes from lL2 to mL3 (Fig. 1J and supplementary material Fig. S1G), suggesting a specific role for PTEN in prohemocytes in regulating their transition to differentiation during development.

TORC1 activity correlates with early proliferating progenitors

The accelerated rate of tissue growth that we observe upon *Tsc2* and *Pten* LOF suggested that they might regulate LG proliferation. We tested this hypothesis by comparing the populations of phospho-histone H3 (phH3) cells in WT versus *Tsc2*- or *Pten*-deficient LGs, and observed an increased number of mitotically dividing cells upon *Tsc2* or *Pten* disruption throughout all stages (compare Fig. 2A,D,G with 2B,E,H and 2C,F,I). The percentage of phH3 cells in the developing LG (mitotic index) is highest during L2 and eL3 (Fig. 2J) (Krzemien et al., 2010). Importantly, we found that the mitotic index of *Tsc2*- or *Pten*-deficient LGs is significantly increased during this critical window of LG growth (Fig. 2J), accounting for the increased rate of tissue growth observed (Fig. 1H-J). Interestingly, high levels of cytoplasmic p4EBP (p4EBP^{high}) are expressed in a small population of scattered cells (Fig. 2A′,D′) in WT LGs during L2 and eL3, the majority of which colocalize with phH3 and represent mitotically dividing hemocytes (Fig. 2A″,D″). Downregulation of either *Tsc2* or *Pten* in progenitors increases the population of phH3 /p4EBP^{high} cells at these early stages (Fig. 2B″,E″ and 2C″,F″), suggesting that TORC1 activity promotes the early proliferation and expansion of progenitor pools. Expression of high p4EBP levels in phH3 cells during early larval development is specific for M phase, as overlap of p4EBP with the S-phase marker BrdU is not observed in any background (supplementary material Fig. S1H-M).

By mL3 the mitotic index drops significantly in WT, but more dramatically in *Tsc2*- or *Pten*-deficient backgrounds (Fig. 2J), emphasizing their role during the phases of highest proliferation in early LG development. Moreover, a higher proportion of mitoses in *Pten*-deficient LGs at mL3 occur within PXN hemocytes as compared with WT or *Tsc2*-deficient LGs (supplementary material Fig. S1N), consistent with the increased differentiation observed at mL3 (Fig. 1J and supplementary material Fig. S1G). In addition, the population of p4EBP^{high} cells at mL3 in *Tsc2*- and *Pten*-deficient LGs is much larger than that of phH3 cells, although some colocalization still occurs (Fig. 2H″,I″).

Dynamic p4EBP expression downstream of *Pten* and *Tsc2* deficiencies at later third instar stages

The significant increase in p4EBP levels in *Tsc2*- or *Pten*-deficient LGs by mL3 (Fig. 2H″,I″) occurs during a stage when increased differentiation in the LG expands the early CZ. In both *Tsc2*- and *Pten*-deficient backgrounds at mL3, a pronounced accumulation of p4EBP occurs in differentiating cells that express low levels of

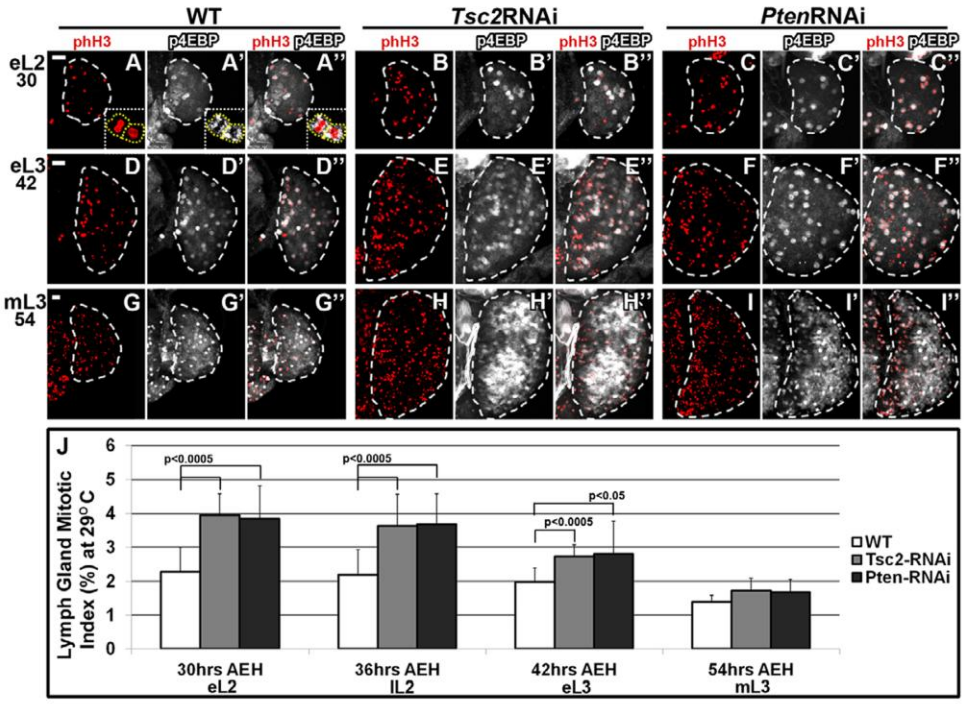

Fig. 2. TORC1 activity in early proliferating prohemocytes. (**A-A″,D-D″,G-G″**) p4EBP expression (white) in WT (*dome>*) overlaps with mitotically dividing cells (phH3, red). Inset (A-A″) demonstrates cytoplasmic p4EBP accumulation. (**B-C″,E-F″,H-I″**) Downregulation of *Tsc2* (*dome>Tsc2*RNAi) or *Pten* (*dome>Pten*RNAi) increases the number of proliferating cells and p4EBP^{high} cells, which colocalize at early stages (B″,E″,C″,F″) but not at mL3 (H″,I″). (**J**) Quantification of LG mitotic index. *Tsc2* or *Pten* downregulation significantly increases LG mitotic index at early developmental stages. Data are mean ± s.d., *n*=12. Scale bar: 20 μm (for each row).

PXN along the early MZ/CZ boundary (Fig. 3A-F‴), marking a transition in high TORC1 activation from proliferating hemocytes to differentiating hemocytes at mL3. A subset of these p4EBP^{high} cells express low levels of P1 (supplementary material Fig. S2A-C‴, arrows) but not the CC progenitor marker Lozenge (LZ ; supplementary material Fig. S2D-F‴), demonstrating that high p4EBP expression is specific for differentiating *dome* /PXN or P1^{low} PLs, and not for fully differentiated (*dome*$^{-}$) PLs at the LG periphery or CC progenitors. Lamellocytes are not observed at this stage.

At lL3 (Fig. 3G-I‴), the p4EPB patterns become markedly distinct between *Tsc2*- and *Pten*-deficient LGs (compare Fig. 3H″ with 3I″). High p4EBP expression extends medially in *Tsc2*-deficient LGs, overlapping largely with *dome* /PXN cells, and is absent in the LG periphery (Fig. 3H-H‴). By contrast, high p4EBP expression extends peripherally in differentiating hemocytes in *Pten*-deficient LGs, including within buds of tissue that pinch off from the CZ, and is expressed at lower levels in MZ prohemocytes (Fig. 3I-I‴). By wL3, p4EBP^{high} cells are observed in *dome* hemocytes in both backgrounds, but continue to extend peripherally upon progenitor-specific *Pten* downregulation (supplementary material Fig. S1E-F′). Collectively, these data demonstrate non-autonomous activation of TORC1 activity upon *Pten*, but not *Tsc2*, downregulation in progenitors at late stages.

We further confirmed our findings from *Tsc1/2* and *Pten* deficiencies by performing mutant clonal analysis. Consistent with our findings from when *Tsc2* is downregulated in progenitors, high levels of p4EBP are autonomously upregulated in *Tsc1*$^{-/-}$ clones (Fig. 4B-B″). Although low levels of PXN are expressed in *Tsc1*$^{-/-}$ cells (Fig. 4B‴, gray), fully differentiated PXN^{high} cells are found only at the very periphery of the LG (Fig. 4B‴, white), which are p4EBP negative. By contrast, accumulation of p4EBP in *Pten*$^{-/-}$ cells depends on clone location. Medially localized *Pten*$^{-/-}$ cells are p4EBP^{low} and undifferentiated, similar to *Pten* downregulation in prohemocytes (Fig. 4D-D‴, yellow arrows; compare with Fig. 3I-I‴). Scattered p4EBP^{high} expression is observed within a subset of more peripheral *Pten*$^{-/-}$ cells, as well as non-autonomously within WT PXN^{high} cells (Fig. 4D-D‴, white arrows). Interestingly, the localization of large *Pten*$^{-/-}$ clones in the most peripheral LG regions was more likely associated with autonomous p4EBP upregulation (Fig. 4E,E′), differentiation, and 'pinching off' of LG tissue (Fig. 4F,F′), suggesting that the absence of *Pten* function might selectively affect differentiating hemocyte progenitors as opposed to the undifferentiated prohemocyte population. Furthermore, these findings substantiate the differences observed upon progenitor-specific downregulation of *Pten* and *Tsc*.

Tsc2 and *Pten* deficiencies expand distinct cell populations at wandering third instar

In addition to their divergent p4EBP patterns at late stages, striking differences are observed between *Tsc2*- and *Pten*-deficient LGs in the expansion of distinct cell lineages by wL3. Whereas WT LGs

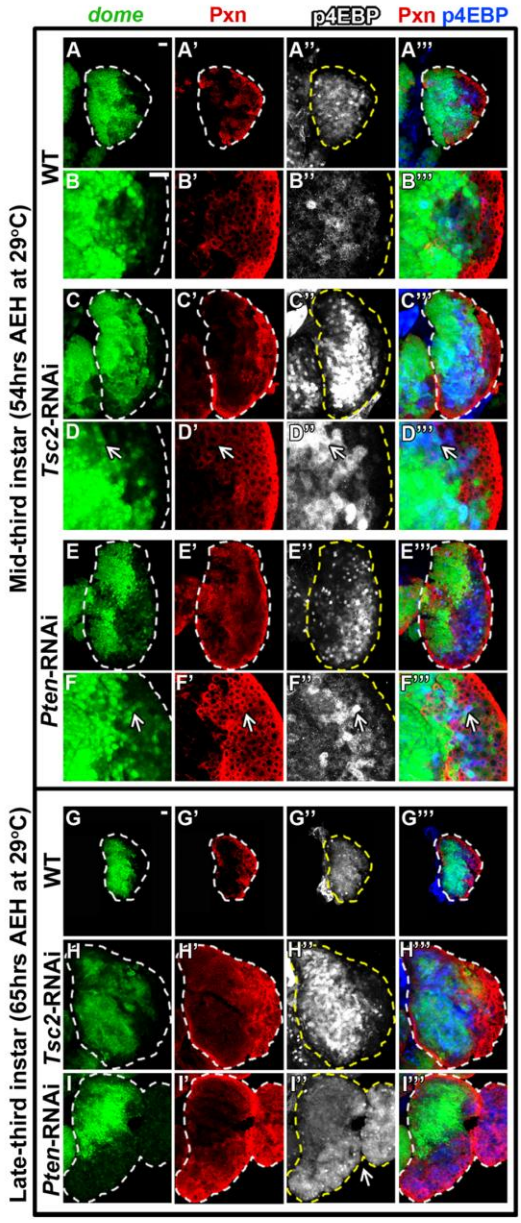

Fig. 3. Dynamic p4EBP expression at mid and late third instar stages. p4EBP expression is white in column 3 and blue in column 4. (**A-B‴,G-G‴**) In WT (*dome-gal4, UAS-2xEGFP*), p4EBP is expressed at low levels and in scattered p4EBP^{high} cells. (**C-F‴,H-I‴**) Downregulation of *Tsc2* (*dome>Tsc2*RNAi) or *Pten* (*dome>Pten*RNAi) in progenitors expands a population of p4EBP^{high} cells along the MZ/CZ boundary at mL3 (C-F‴). Arrows in D-D‴ and F-F‴ point to PXN^{+}/p4EBP^{high} cells. By lL3, high p4EBP expression expands throughout medial regions upon *Tsc2* downregulation (H-H‴), but is highest upon *Pten* downregulation in differentiating cells at the LG periphery (I-I‴), including within buds of CZ tissue (arrow, I′). Scale bars: 20 μm; in A for A-A‴,C-C‴,E-E‴; in B for B-B‴,D-D‴,F-F‴; in G for G-I‴.

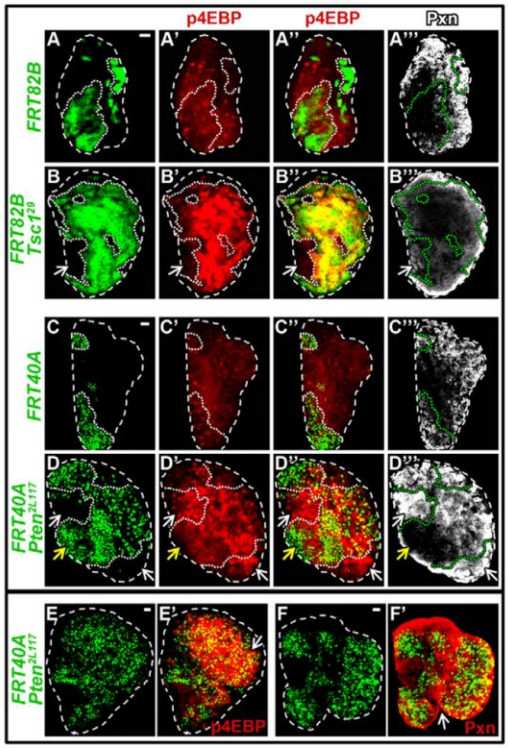

Fig. 4. Mutant clonal analysis of *Tsc1* and *Pten* in the LG. All panels show lL3. Clones are outlined by a white or green dotted line. (**A-B‴**) *Tsc1* MARCM clones. (A-A‴) WT MARCM clones (*hs-flp FRT82B Tub-mCD8-GFP*). (B-B‴) *Tsc1^{29}* clones (*hs-flp FRT82B Tsc1^{29} FRT82B Tub-mCD8-GFP*) express elevated p4EBP (red) in *Tsc1$^{-/-}$* clones (green) relative to surrounding WT tissue (arrows). High PXN expression (white) is observed only at the tissue periphery, whereas *Tsc1$^{-/-}$* cells express low PXN levels (gray, B‴). (**C-F′**) *Pten* MARCM clones. (C-C‴) WT MARCM clones (*hs-flp FRT40A Tub-nGFP*). (D-F′) *Pten^{2L117}* clones (*hs-flp FRT40A Pten^{2L117} FRT40A Tub-nGFP*) express high p4EBP (red) within scattered *Pten$^{-/-}$* cells as well as in surrounding WT cells, which are also PXN^{+} (white arrows in D-D‴). Medial *Pten$^{-/-}$* cells are PXN negative and express low p4EBP levels (yellow arrows in D-D‴). *Pten$^{-/-}$* clones located in peripheral LG regions are associated with high p4EBP (red) levels (arrow, E,E′) and pinching off of PXN^{+} (red) tissue (arrow, F,F′). Scale bars: 20 μm.

have a small population of intermediate progenitors at the MZ/CZ boundary (5±2% of the LG; Fig. 5A-A″,P), as identified by co-expression of *dome* and PXN (Fig. 5A-A″, insets), *Tsc2* downregulation in the MZ induces severe LG overgrowth and dramatically expands the proportion of *dome*$^{+}$/PXN^{+} intermediate progenitors (51±10%; P<0.0001; Fig. 5E-E″,P). This proportion of *dome*$^{+}$/PXN^{+} cells at wL3 is much higher than that observed at mL3 (supplementary material Fig. S1G), demonstrating a late accumulation of intermediate progenitors upon *Tsc2* downregulation. By contrast, the population of fully differentiated hemocytes (*dome*$^{-}$/PXN^{+}) decreases relative to WT (23±4% versus 40±3%, respectively; P<0.0001; Fig. 5P). Terminally differentiated PLs and CCs are found only in a thin cortical layer around the LG

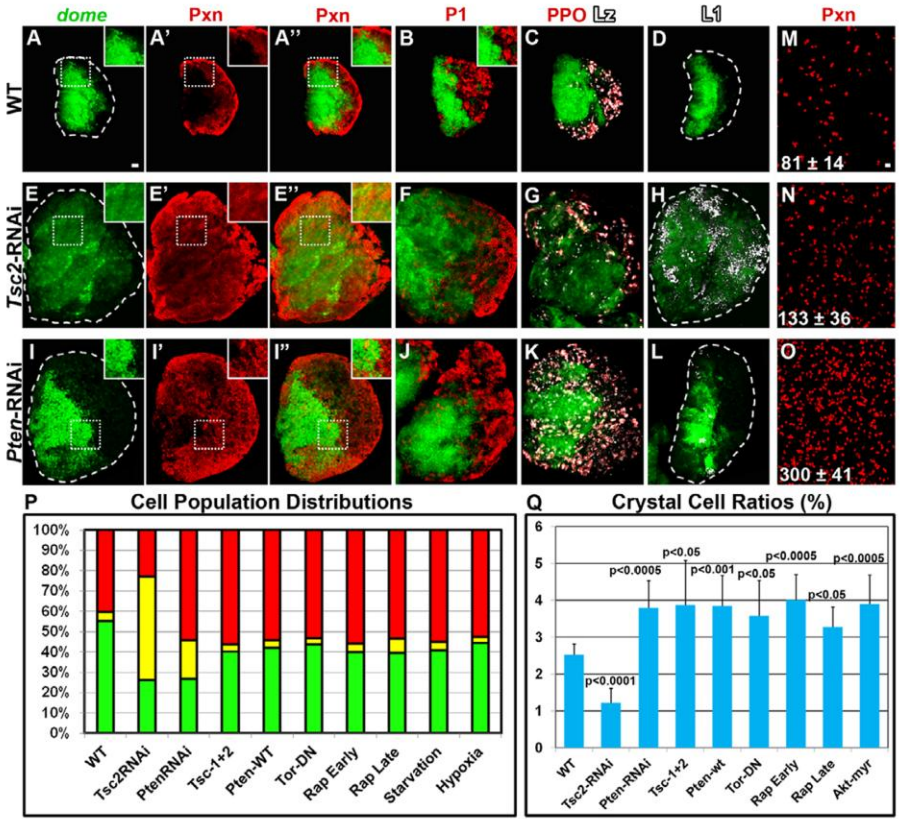

Fig. 5. Effects of *Tsc2* and *Pten* deficiencies on differentiation. All panels show wL3. (**A-D**) In WT (*dome-gal4, UAS-2xEGFP*), PXN (red) is expressed in differentiated hemocytes as well as in a small number of intermediate progenitors (*dome*$^+$/PXN$^+$) at the MZ/CZ boundary (insets, A-A″). (**B**) P1 (red) labels terminally differentiated PLs but not intermediate progenitors (inset). (**C**) PPO (red) marks fully differentiated CCs. LZ (white) marks both fully differentiated CCs (LZ$^+$/PPO$^+$) and CC progenitors (LZ$^+$/*dome*$^+$). (**D**) L1 marks lamellocytes, which are not present in WT. (**E-H**) *Tsc2* downregulation (*dome>Tsc2*RNAi) increases LG size and expands the population of intermediate progenitors (E-E″), decreases PLs (F) and CCs (G), and increases lamellocyte number (H). (**I-L**) *Pten* downregulation (*dome>Pten*RNAi) increases LG size and numbers of intermediate progenitors (I′,I″), PLs (J) and CCs (K) and of rare lamellocytes (L). (**M-O**) *Pten* downregulation (*dome>Pten*RNAi) (O) increases the relative number of circulating PXN$^+$ hemocytes compared with WT (P<0.0001; M) or *Tsc2* downregulation (P<0.0001; N). Data are mean ± s.d., n=8. (**P**) Distribution of *dome*$^+$/PXN$^-$ prohemocytes (green), *dome*$^+$/PXN$^+$ intermediate progenitors (yellow) and *dome*$^-$/PXN$^+$ differentiated hemocytes (red). Data are mean (n=12), and two-way ANOVA revealed significant changes (P<0.0001) in the distribution of hemocyte populations in all conditions compared with WT. (**Q**) Ratio of CCs to total LG cells (percentage). Data are mean ± s.d., n=10. Scale bars: 20 μm, except for 1.2× magnification for H,K.

periphery (Fig. 5F,G), and the ratio of CCs to total LG cells is significantly reduced compared with WT (Fig. 5Q). By contrast, lamellocytes, which are not normally present in a healthy larva (Fig. 5D), are dispersed throughout the LG (Fig. 5H). LG-specific clonal downregulation of *Tsc2* also induces overgrowth (supplementary material Fig. S3D-F), expands the population of PXN hemocytes (supplementary material Fig. S3D′,D″) and induces lamellocytes (supplementary material Fig. S3F), demonstrating the LG-specific effects of *Tsc2* deficiency.

By contrast, *Pten* deficiency expands the populations of *dome* /PXN intermediate progenitors (19±9%, compared with 5±2% in WT; P<0.0005; Fig. 5P), *dome*$^-$/PXN cells (54±8%, compared with 40±3% in WT; P<0.0005; Fig, 5I-I″,P) and fully differentiated PLs (Fig. 5J) at the cost of the MZ. Unlike *Tsc2* downregulation, the ratio of CCs to total LG cells is significantly increased in the LG upon progenitor-specific *Pten* downregulation (Fig. 5K,Q), and lamellocytes are rarely observed (Fig. 5L). Increased lamellocyte differentiation upon *Tsc1/2* versus *Pten* LOF was also demonstrated by LG-specific clonal downregulation (supplementary material Fig. S3F,I) and mutant clonal analysis (supplementary material Fig. S3M-P). *Pten* downregulation also increases the number of circulating hemocytes compared with WT or *Tsc2* deficiency (compare Fig. 5O with 5M,N), suggesting premature dissemination of LG hemocytes upon *Pten* disruption, which do not normally enter circulation until the onset of metamorphosis when the LG disintegrates (Grigorian et al., 2011). Lineage-tracing analysis demonstrated the LG origin of the increased circulating hemocytes upon LG-specific *Pten* downregulation (supplementary material Fig. S3J-L). These phenotypic differences in *Tsc2*- and *Pten*-deficient LGs are not the

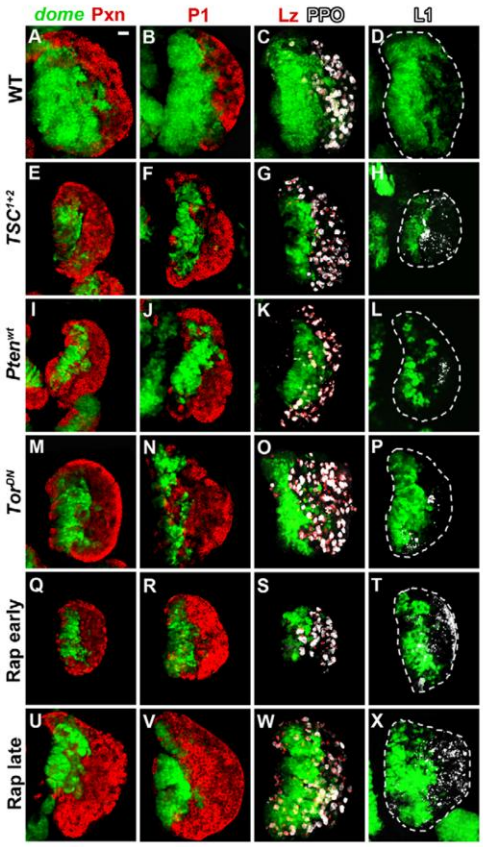

Fig. 6. Inhibition of TORC1 signaling reduces progenitor pools. All panels show wL3. *dome-Gal4, UAS-2xEGFP* (A-D) is used to express *UAS-Tsc*$^{1+2}$ (E-H), *UAS-Pten*^{wt} (I-L) and *UAS-Tor*^{DN} (M-P) in prohemocytes. (**A-D**) WT. The populations of PXN^{+} hemocytes (A), PLs (P1, B), CCs (LZ^{+}/PPO^{+}, C) and lamellocytes (L1, D) are marked. (**E-T**) Overexpression of *Tsc*$^{1+2}$ (E-H), *Pten*^{wt} (I-L) and *Tor*^{DN} (M-P) or rapamycin treatment throughout larval development (Q-T) all decrease overall LG size (compare with A-D) and increase differentiation. (**U-X**) Rapamycin treatment only at late stages (eL3-wL3) does not affect LG size, but does increase differentiated hemocytes. Scale bar: 20 μm.

result of non-autonomous changes in PSC size (data not shown) or differences in knockdown efficiencies for their respective RNAi constructs (71% and 76% knockdown for *Tsc2*RNAi and *Pten*RNAi, respectively; supplementary material Fig. S3Q).

The premature dissemination of *Pten*-deficient LGs (Fig. 3I‴, Fig. 4F′ and supplementary material Fig. S3L) and scattering of *Pten* mutant cells within the LG (Fig. 4D) suggested that changes in cell adhesion might be associated with the greater malignancy of *Pten* versus *Tsc1/2* deficiency. High levels of Shotgun (SHG, DE-cadherin) expression are retained in *Tsc1*$^{-/-}$ clones, which express low levels of PXN (gray) and are not fully differentiated (PXN^{high}, white) (supplementary material Fig. S4B-B‴). Similarly, medially localized *Pten*$^{-/-}$ cells, which are undifferentiated, retain high SHG levels (supplementary material Fig. S4D-D‴, arrows). By contrast, scattered *Pten*$^{-/-}$ cells are PXN^{high} and downregulate SHG expression (supplementary material Fig. S4D-D‴). Although the differentiation status of these scattered *Pten*$^{-/-}$ cells may account for the downregulation of SHG, we cannot rule out the possibility that decreased adhesion has a causative role in the increased differentiation and dissemination of *Pten*-deficient LGs.

Inhibition of TORC1 signaling reduces progenitor pools and impairs early, but not late, LG growth

We next examined the phenotypic consequence of *Tsc1/2* and *Pten* overexpression on prohemocytes. Inhibition of TORC1 signaling by overexpression of *Tsc*$^{1+2}$ and *Pten*^{wt} in progenitors reduces overall LG size at wL3 and significantly increases the proportion of PXN^{-} hemocytes (Fig. 6A,E,I and Fig. 5P). Terminally differentiated hemocyte lineages are also expanded (Fig. 6F-H,J-L) and the ratio of CCs to total LG cells is significantly increased (Fig. 5Q). LG-specific clonal expression of *Tsc*$^{1+2}$ and *Pten*^{wt} also decreases total LG size and increases differentiation throughout the tissue (supplementary material Fig. S5A-C). At L2, LGs in which prohemocytes overexpress *Tsc*$^{1+2}$ or *Pten*^{wt} are already markedly smaller than controls (supplementary material Fig. S5D-F), which can be attributed to reduced progenitor proliferation at this stage (supplementary material Fig. S5G).

The decreased LG size and increased differentiation that we observe upon *Tsc*$^{1+2}$ or *Pten*^{wt} overexpression is phenocopied by overexpression of a dominant-negative *Tor* allele in progenitors and through systemic treatment with the TORC1 inhibitor rapamycin (Fig. 6M-T). Both conditions increase the proportion of *dome*$^{-}$/PXN^{-} hemocytes in the LG as well as the ratio of CCs to total LG cells (Fig. 5P,Q). Delaying treatment with rapamycin until later stages is sufficient to increase hemocyte differentiation as well (Fig. 6U-X and Fig. 5P,Q), but does not significantly affect further LG growth. A similar effect occurs upon conditionally overexpressing *Tsc*$^{1+2}$ in prohemocytes only after larvae reach later stages (supplementary material Fig. S5H-I″). These results highlight the early role of TORC1 signaling in regulating prohemocyte proliferation and a later role in regulating differentiation.

Tsc2 and *Pten* phenotypes are sensitive to stress and depend on TORC1, 4EBP and pAKT

That a nutrient-sensing signaling pathway regulates progenitor number during normal *Drosophila* hematopoiesis raises the intriguing possibility that nutritional/environmental stress signals might utilize the same developmental hematopoietic signaling pathway to regulate progenitor adaptations to stress. The response of blood progenitors to stress conditions is evident upon rearing WT larvae under acute starvation and hypoxia, both of which increase the size of the CZ at the expense of MZ progenitors (Fig. 7A-A″ and Fig. 5P) and phenocopy conditions of reduced TORC1 signaling induced by overexpression of *Tsc*$^{1+2}$ and *Pten*^{wt} (Fig. 5P). Furthermore, the LG overgrowth characteristic of *Tsc2*- and *Pten*-deficient LGs is attenuated by these stressors (Fig. 7B-B″,C-C″), suggesting that the effects of starvation and hypoxia on hemocyte progenitors might be mediated by reducing TORC1 signaling.

We confirmed the TORC1 dependence of *Tsc2* or *Pten* deficiency-induced LG overgrowth by treating larvae with rapamycin throughout development, which was sufficient to rescue the phenotype in both backgrounds (Fig. 7B‴,C‴). The tight correlation of p4EBP expression with mitotically dividing cells during early LG development suggested that 4EBP might

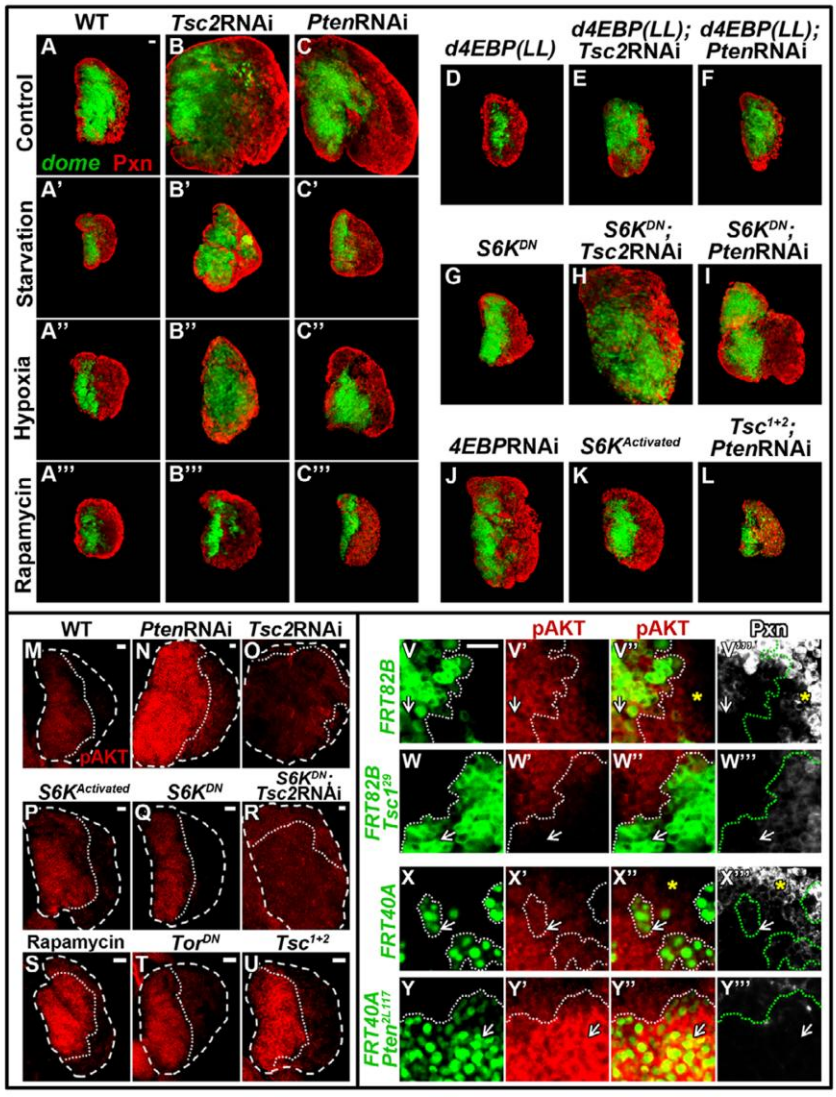

Fig. 7. Dependence of *Tsc2* and *Pten* phenotypes on TORC1 and 4EBP but not S6K. All panels show wL3. In A-U, *dome-gal4, UAS-2xEGFP* is used to drive expression of the specified genetic constructs (green channel is omitted in M-U, but *dome*$^+$ progenitors are outlined by dotted lines). PXN is in red in A-L and pAKT is in red in M-Y‴. (**A-A‴**) WT LGs (*dome>*). Starvation (A′), hypoxia (A″) or rapamycin treatment (A‴) all increase PXN$^+$ hemocytes (red) at the expense of MZ prohemocytes (green) compared with controls (fed/normoxic, A). (**B-C‴**) Overgrowth associated with *Tsc2* and *Pten* downregulation (B,C) is attenuated in conditions of starvation (B′,C′), hypoxia (B″,C″) and rapamycin treatment (B‴,C‴). (**D-F**) Overexpression of *d4EBP(LL)* decreases LG size (D) and is sufficient to attenuate LG overgrowth in *Tsc2*-deficient (E) or *Pten*-deficient (F) LGs (compare with B,C). (**G-I**) Reducing S6K function (*dome>S6K^{DN}*) does not affect LG size and differentiation (G), and does not rescue LG overgrowth in *Tsc2*-deficient (H) or *Pten*-deficient (I) LGs. (**J**,**K**) Downregulation of *4EBP* (J) increases LG size to a greater extent than activation of S6K (K). (**L**) Overexpression of *Tsc^{1+2}* upon *Pten* downregulation rescues LG overgrowth but does not rescue the increased differentiation observed upon *Pten* downregulation alone (compare with C). (**M**) WT LG. Expression of pAKT is highest medially, within *dome*$^+$ progenitors. (**N**,**O**) *Pten* downregulation increases pAKT levels in progenitors (N), whereas *Tsc2* deficiency reduces pAKT expression (O). (**P**,**Q**) Activating (*dome>S6K^{Activated}*, P) or reducing (*dome>S6K^{DN}*, Q) S6K function does not affect pAKT expression. (**R**) Downregulation of *S6K* activity upon *Tsc2* downregulation does not increase pAKT levels compared with conditions of *Tsc2* downregulation alone (compare with O). (**S-U**) Reducing TORC1 signaling via systemic rapamycin treatment (S) or overexpression of *Tor^{DN}* (T) or *Tsc^{1+2}* (U) in prohemocytes increases pAKT expression. (**V-Y‴**) MARCM clones for WT [*hs-flp FRT82B Tub-mCD8-GFP* (V-V‴) and *hs-flp FRT40A Tub-nGFP* (X-X‴)], *Tsc1^{29}* (*hs-flp FRT82B Tsc1^{29} FRT82B Tub-mCD8-GFP*, W-W‴) and *Pten^{2L117}* (*hs-flp FRT40A Pten^{2L117} FRT40A Tub-nGFP*, Y-Y‴)]. In WT, pAKT expression is highest in PXN-negative cells (arrows, V-V‴ and X-X‴) and gradually decreases in PXN$^+$ cells (asterisk, V″,V‴ and X″,X‴). *Tsc1$^{-/-}$* clones demonstrate reduced pAKT expression in progenitors (PXN-negative cells; arrows, W-W‴). *Pten$^{-/-}$* clones in medial LG regions demonstrate autonomous increases in pAKT expression (PXN-negative cells; arrows, Y-Y‴). Scale bars: 20 μm; A for A-L; in V for V-Y‴.

itself be regulating the proliferation of blood progenitors. Phosphorylation of 4EBP by active TORC1 relieves translation initiation factor 4E (eIF-4E) from inhibitory 4EBP binding and promotes growth (Gingras et al., 2001). Expression of a mutant form of *Drosophila* 4EBP [*d4EBP(LL)*] that binds more tightly to eIF-4E decreases LG size and increases differentiation, similar to rapamycin treatment (compare Fig. 7D with 7A,A‴). Overexpression of *d4EBP(LL)* upon *Tsc2* or *Pten* downregulation in progenitors is sufficient to block LG overgrowth (Fig. 7E,F), confirming a role for 4EBP downstream of TSC and PTEN in hemocyte progenitor proliferation. Unexpectedly, overexpression of $S6k^{DN}$ in prohemocytes is not sufficient to significantly alter LG size and differentiation (Fig. 7G), or to rescue overgrowth of *Tsc2*- and *Pten*-deficient LGs (Fig. 7H,I). The more prominent role for 4EBP as compared with S6K in the LG is also demonstrated in that downregulation of *4EBP* in prohemocytes increases LG size compared with controls (compare Fig. 7J with 7A), whereas overexpression of

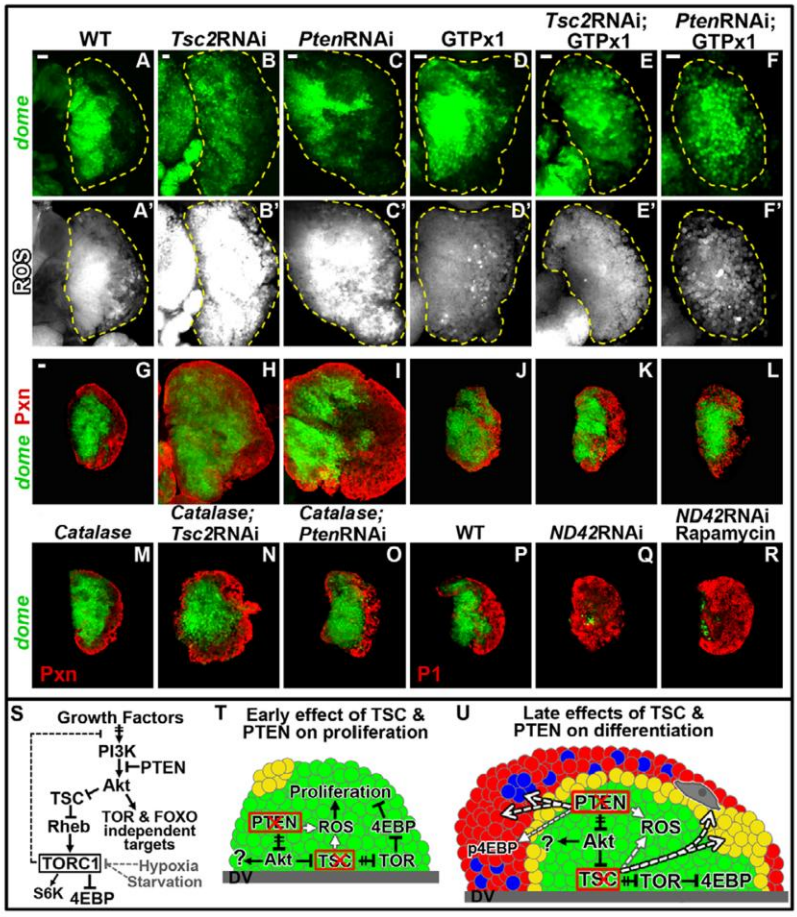

Fig. 8. ROS-dependent effects of TSC and PTEN in prohemocytes. All panels show wL3. *dome-gal4, UAS-2xEGFP* (green) drives expression of each genetic construct in prohemocytes. ROS levels are in white (A′-F′). PXN (G-O) or P1 (P-R) are in red. (**A**,**A′**) WT. ROS levels are highest in *dome*$^{+}$ prohemocytes. (**B-C′**) Downregulation of *Tsc2* (B,B′) or *Pten* (C,C′) increases ROS levels. (**D**,**D′**) Overexpression of the ROS scavenger GTPx1 reduces ROS levels. (**E-F′**) GTPx1 expression reduces ROS levels in *Tsc2*-deficient (E,E′) and *Pten*-deficient (F,F′) LGs. (**G-O**) Overexpression of the ROS scavengers GTPx1 (J) or catalase (M) upon *Tsc2* or *Pten* downregulation rescues their overgrowth (compare K,L and N,O with H,I). (**P**) WT LG. (**Q**) Downregulation of *ND42* increases PL differentiation and reduces the progenitor population (green) while LG size is unaffected. (**R**) Systemic rapamycin treatment of ND42-deficient LGs does not reverse the increase in differentiation. (**S**) TORC1 signaling to 4EBP (and S6K) is inhibited by TSC and PTEN. The presence of a TORC1-dependent negative-feedback loop in the LG regulates AKT activity upon TORC1 activation (crosshatched line). *Pten* disruption hyperactivates AKT, which can activate TORC1 signaling as well as TOR- and FOXO-independent effectors. Conditions of hypoxia and starvation are able to reduce TORC1 signaling downstream of PTEN and TSC. (**T**) Disruption of *Tsc2* and *Pten* in hemocyte progenitors (green) increase proliferation in a TOR-, 4EBP- and ROS-dependent manner at early stages. (**U**) At late LG stages, *Tsc1/2* and *Pten* deficiencies affect distinct hemocyte lineages. *Tsc1/2* disruption autonomously increases p4EBP and ROS levels and expands intermediate progenitors (yellow) and lamellocytes (gray). *Pten* disruption autonomously increases AKT activation and ROS levels, non-autonomously induces p4EBP expression and expands fully differentiated PLs (red) and CCs (blue). White arrows with gray outlines represent increases in specified marker expression; white arrows with black outlines represent increases in specified cell lineages. DV, dorsal vessel. Scale bars: 20 μm; in G for G-R.

$S6k^{Activated}$ does not (Fig. 7K). Epistasis of *Pten* and *Tsc1/2* was also examined, and, similar to rapamycin treatment, $Tsc^{1\ 2}$ overexpression is sufficient to block overgrowth of *Pten*-deficient LGs, although substantial differentiation still occurs (Fig. 7L). These data suggest that, although a common TOR-mediated signaling network regulates progenitor proliferation downstream of TSC and PTEN, TSC/TOR-independent effectors might contribute to the increased differentiation and myeloproliferative effects observed upon *Pten* deficiency.

One candidate effector molecule that may be differentially regulated upon *Tsc2* and *Pten* downregulation is AKT. Whereas *Pten* loss leads to constitutive activation of AKT, hyperactive TORC1 signaling is known to trigger a negative-feedback loop that attenuates PI3K-AKT signaling (Harrington et al., 2004; Kockel et al., 2010). *Pten* downregulation in prohemocytes increases pAKT levels in the LG (Fig. 7N), whereas pAKT expression is nearly absent in *Tsc2*-deficient LGs (Fig. 7O). Although this feedback inhibition downstream of TORC1 signaling could be S6K mediated (Harrington et al., 2004), we found no evidence of S6K-dependent feedback in *Drosophila* prohemocytes (Fig. 7P-R). By contrast, reducing TORC1 signaling in prohemocytes via rapamycin treatment (Fig. 7S), Tor^{DN} overexpression (Fig. 7T), or $Tsc^{1\ 2}$ overexpression (Fig. 7U) did increase pAKT levels. Our data thus describe a TORC1-dependent, S6K-independent negative feedback that attenuates signaling to AKT in the LG.

Disparate pAKT expression among *Tsc2*- and *Pten*-deficient LGs raises the possibility that AKT might be a functionally relevant target downstream of PTEN but not TSC signaling, which might account for some of the observed phenotypic differences in these backgrounds. We confirmed differential regulation of pAKT upon *Tsc1/2* and *Pten* LOF through MARCM clonal analysis (Fig. 7V-Y^{m}), which demonstrates cell-autonomous increases in pAKT expression in medial $Pten^{-/-}$ cells, but absence of pAKT in $Tsc1^{-/-}$ cells. Although constitutive activation of AKT in prohemocytes does not induce hypertrophy, unlike in *Pten*-deficient LGs, we often observed an increase in PXN cells, with occasional fragments of differentiated tissue 'pinching' off from the LG, similar to the effects of *Pten* loss (compare supplementary material Fig. S6C with S6B). Additionally, the ratio of CCs to total LG cells is significantly increased upon AKT activation (Fig. 5Q), whereas lamellocyte numbers are not altered (data not shown), further demonstrating the similarity of AKT-dependent phenotypes to *Pten* but not *Tsc1/2* loss. Perhaps the best-described targets of AKT are the Forkhead box O (FoxO) family of transcription factors, which are inhibited by activated AKT (Castellino and Durden, 2007). Downregulation of *foxo* in prohemocytes does not phenocopy *Pten* silencing or AKT activation (compare supplementary material Fig. S6D with S6B,C), and overexpression of *foxo* is not sufficient to rescue the increased differentiation or overgrowth of *Pten*-deficient LGs (supplementary material Fig. S6E,F). Although a common TORC1- and 4EBP-dependent signaling pathway mediates the early proliferative effects downstream of *Tsc2* and *Pten* deficiencies, the additive effects of activating both AKT and TORC1 signaling upon *Pten* disruption may account for the increased differentiation and myeloproliferation observed.

ROS dependence of *Tsc2* and *Pten* phenotypes

Analysis of MZ markers that are affected upon progenitor-specific *Tsc2* or *Pten* disruption identified significant changes in ROS in these backgrounds. WT prohemocytes maintain elevated ROS levels at wL3 compared with differentiated hemocytes (Fig. 8A,A′) (Owusu-Ansah and Banerjee, 2009), and downregulation of *Tsc2* or *Pten* in progenitors increases ROS levels (Fig. 8B-C′). These levels are reduced upon overexpression of the ROS scavenger protein glutathione peroxidase-like 1 (GTPx1) in prohemocytes (Fig. 8E-F′). Scavenging ROS in *Pten*- and *Tsc2*-deficient LGs with GTPx1 (Fig. 8K,L) or catalase (Fig. 8N,O) is sufficient to rescue their overgrowth.

Downregulation of complex I proteins of the mitochondrial electron transport chain in prohemocytes, such as ND75 and ND42, has been previously shown to trigger precocious differentiation into mature hemocyte lineages (Owusu-Ansah and Banerjee, 2009). We examined whether elevated ROS is sufficient to induce the phenotypes associated with *Tsc2* and *Pten* deficiencies. Although downregulation of *ND42* in prohemocytes increases differentiation, LG size is unaffected (Fig. 8P,Q). Further, treating *ND42*-deficient larvae with rapamycin to suppress TORC1 signaling did not rescue the increased differentiation (Fig. 8R). These data demonstrate that the proliferative effects observed upon *Tsc2* and *Pten* disruption are ROS dependent, but differ in their phenotypic consequences and mechanistic basis from other conditions that may increase ROS in the LG (Owusu-Ansah and Banerjee, 2009).

DISCUSSION

TORC1 signaling regulates hemocyte progenitor proliferation

Our data demonstrate a crucial role of TORC1 signaling in regulating the proliferation of *Drosophila* hemocyte progenitors at early developmental stages, when rapid growth of the LG occurs. The specific accumulation of p4EBP in phH3 cells at early stages highlights the central function of TORC1 signaling in actively dividing hemocyte progenitors. These data complement recent findings in *Drosophila* ovarian germline stem cells (GSCs) in which p4EBP levels were specifically elevated in M phase (LaFever et al., 2010). However, in contrast to *Drosophila* GSCs, in which 4EBP does not mediate *Tor*-associated phenotypes (LaFever et al., 2010), our data demonstrate a central role for 4EBP in regulating hemocyte progenitor proliferation downstream of *Tsc* and *Pten* disruption (Fig. 8S,T). The sufficiency of scavenging ROS in *Pten*- and *Tsc2*-deficient LGs to rescue associated LG overgrowth also highlights the role of ROS in mediating hemocyte proliferation upon TORC1 hyperactivation (Fig. 8T). A limited number of studies in vertebrate hematopoietic stem cells (HSCs) deficient for *Tsc1* and *Pten* consistently demonstrate their initial expansion due to aberrant cell cycling, but eventual exhaustion caused by reduced self-renewal capacity (Yilmaz et al., 2006; Zhang et al., 2006; Chen et al., 2008). Our findings suggest that 4EBP-mediated TORC1 activity might be particularly relevant in hematopoietic progenitors, warranting its further investigation in vertebrates.

Differential effects of *Tsc1/2* and *Pten* function on blood progenitors

Our findings demonstrate phenotypic differences upon *Tsc1/2* and *Pten* manipulation in blood progenitors. Mutant *Tsc1* clones autonomously express high levels of p4EBP and are associated with low PXN expression. Progenitor-specific downregulation of *Tsc2* does not alter differentiation onset and progression, but by late L3 stages the population of $p4EBP^{high}$ *dome* /PXN intermediate progenitors dramatically expands, and retention of this pool of progenitors restricts the populations of fully differentiated PLs and CCs while still allowing large numbers of lamellocytes to differentiate (Fig. 8U). By contrast, *Pten* silencing promotes an

early increase in differentiation that proceeds to expand terminally differentiated PLs and CCs and also leads to premature release of hemocytes from the LG (Fig. 8U). Interestingly, the increased malignancy induced by *Pten* disruption in progenitors is associated with non-autonomous increases in p4EBP levels within peripheral differentiating hemocytes and 'buds' of CZ tissue (Fig. 8U, Fig. 3I″, Fig. 4D″ and supplementary material Fig. S1F). Further studies are needed to clarify whether non-autonomous TORC1 activation induced by *Pten* disruption is common to other cell types and might contribute to the greater malignancy potential associated with *PTEN* mutations as compared with *TSC* mutations in human disease (Manning et al., 2005).

In contrast to the non-autonomous effects of *Pten* LOF on p4EBP accumulation, *Pten* loss autonomously increases AKT activation. Further, the phenotypic similarities between AKT activation and *Pten* loss in progenitors suggests that the additive effects of activating both AKT and TORC1 signaling in *Pten*-deficient hemocytes might contribute to the more dramatic effects on terminal differentiation and premature release of LG hemocytes into circulation. Previous studies in vertebrates have shown that *Pten* loss (Yilmaz et al., 2006; Zhang et al., 2006) and AKT activation (Kharas et al., 2010) in HSCs as well as in myeloid progenitors (Yu et al., 2010) induces myeloproliferative disorder (MPD) and leukemia. Although reduced expression of *TSC* has been reported in a subset of acute leukemia patients (Xu et al., 2009), *Tsc1* loss in HSCs is not sufficient to induce leukemia in mice (Chen et al., 2008). Attenuation of AKT signaling upon *Tsc1/2* disruption might account for the decreased malignant potential of *Tsc1/2*-deficient LGs compared with *Pten* loss, similar to previous reports (Harrington et al., 2004; Manning et al., 2005).

Unexpectedly, both activation and suppression of TORC1 signaling increases the proportion of PXN cells in the LG at the expense of *dome* progenitors. The increased AKT activation that we observe upon conditions of TORC1 pathway suppression, induced via relief of a TOR-dependent negative-feedback loop, may account for the expansion of differentiated hemocytes and CCs observed, although we cannot rule out the possibility that other pathways might contribute to this effect, such as changes in Wingless levels (Shim et al., 2012).

TORC1-dependent effects of stress and elevated ROS in progenitors

Consistent with the nutrient-sensing function of TOR signaling (Wang and Proud, 2009) and our findings concerning its central role in regulating *Drosophila* hemocyte progenitor proliferation and differentiation, we observed increased differentiation under starvation and low oxygen conditions, which phenocopied reduced TORC1 signaling in the LG. Furthermore, starvation specifically decreased WT LG size (Fig. 7A′), and rearing *Tsc2*-deficient LGs under conditions of starvation rescues their overgrowth, demonstrating the TOR-dependent effects of nutrient availability on proliferation, similar to findings for neural progenitors in *Drosophila* (Sousa-Nunes et al., 2011). An additional metabolic stressor that has been shown to affect LG progenitor differentiation is elevated ROS (Owusu-Ansah and Banerjee, 2009). Manipulation of mitochondrial electron transport chain proteins increases ROS levels in the LG and causes precocious progenitor differentiation, but does not affect tissue size. These effects are not TOR dependent, consistent with their distinct phenotype compared with *Tsc2* and *Pten* disruption. The lack of change in LG size upon rapamycin treatment of *ND42*RNAi larvae is likely to be due to compensatory proliferation of differentiated hemocytes, independent of TOR, which may result from the early loss of progenitors in this background (Owusu-Ansah and Banerjee, 2009). The ROS-dependent LG overgrowth observed upon TORC1 hyperactivation demonstrates mechanistic differences in ROS signaling between conditions of mitochondrial dysfunction and of increased TOR signaling in blood progenitors. Conditional *Tsc1* deletion in mouse HSCs increases ROS, which induces a loss of quiescence and increased cycling that is rescued by decreasing ROS levels (Chen et al., 2008). This and several other studies consistently demonstrated a link between HSC proliferation/quiescence and intracellular oxidation status (Ito et al., 2006; Arai and Suda, 2007; Jang and Sharkis, 2007; Tothova et al., 2007), similar to our findings.

Our results highlight differences in the expansion of intermediate progenitors, differentiated hemocytes and LG-derived circulating hemocytes that are unique to TSC and PTEN, resulting from differential effects on feedback inhibition, AKT activity and autonomous versus non-autonomous TOR activation. We also demonstrate the common effects of *Tsc2* and *Pten* loss on regulating early blood progenitor proliferation in a TORC1-, 4EBP- and ROS-dependent manner and the effects of starvation and hypoxia on TOR-dependent LG growth. This study demonstrates the multifaceted roles of a nutrient-sensing pathway in orchestrating the growth and differentiation of myeloid-specific blood progenitors through regulation of ROS levels and the resulting MPD when dysregulated, highlighting potential non-autonomous effects of *Pten* dysfunction on malignancy.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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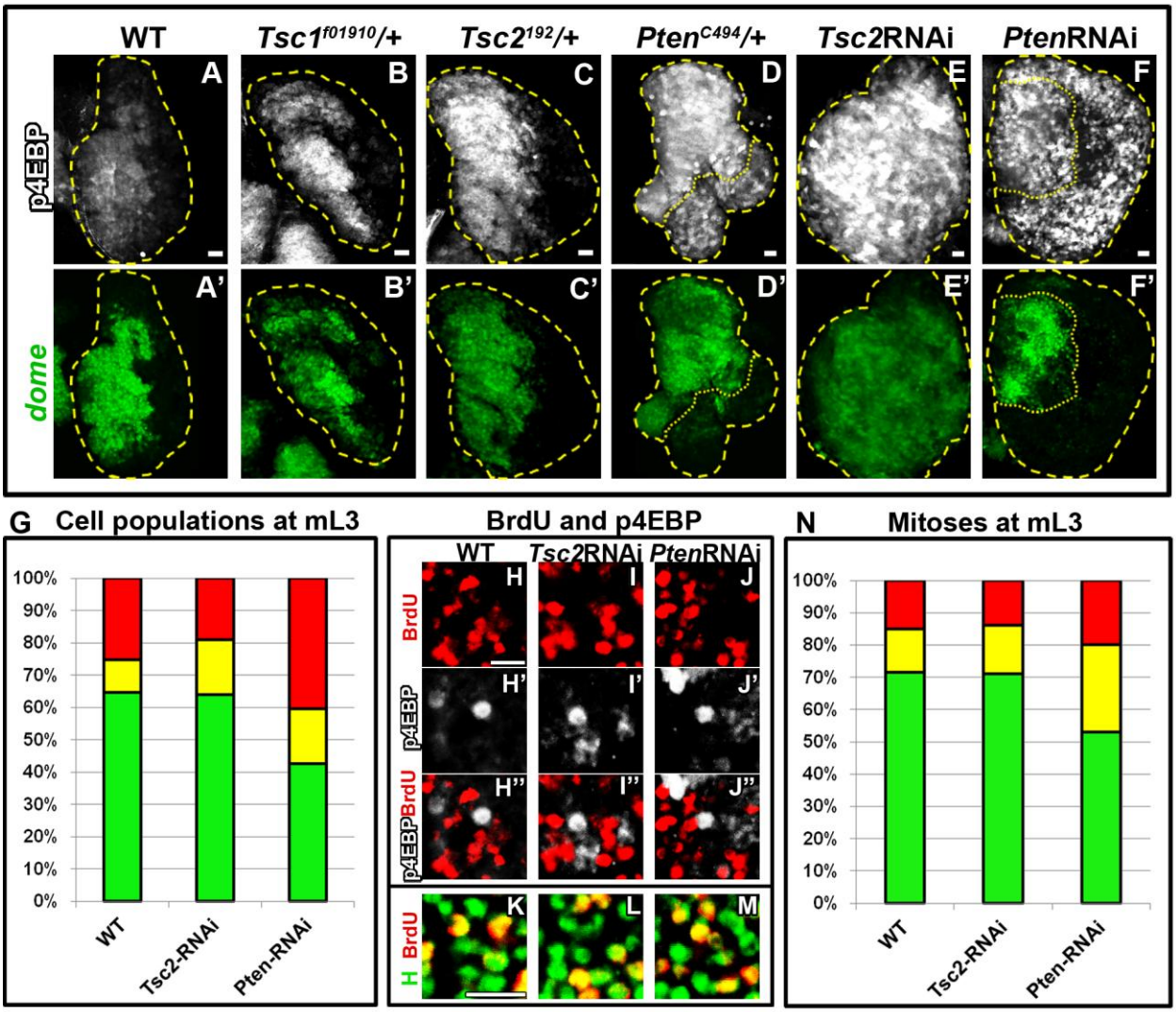

Figure 2-9. Supplementary Figure S1. Expression of p4EBP, cell population distributions and mitoses upon disruption of *Tsc* and *Pten* function

(**A-F'**) Increase in p4EBP expression upon TORC1 pathway manipulation at wL3. Single copy loss of *Tsc1* [(*Tsc1*^{f01910}/+) (**B-B'**)] or *Tsc2* [(*Tsc2*^{192}/+) (**C-C'**)] increases p4EBP expression (white) in *dome*$^{+}$ hemocyte progenitors (green), compared to WT (**A-A'**). Single copy loss of *Pten* [(*Pten*^{C494}/+) (**D-D'**)] increases p4EBP expression both within *dome*$^{+}$ and *dome*-negative hemocytes. Downregulation of *Tsc2* (*dome*>*Tsc2*RNAi, **E-E'**) in hemocyte progenitors autonomously increases p4EBP expression in *dome*^{low} hemocytes throughout the LG.

Downregulation of *Pten* (*dome>Pten*RNAi, **F-F'**) increases p4EBP autonomously within *dome*$^{+}$ hemocytes and non-cell autonomously in *dome*-negative hemocytes.

(**G**) Hemocyte and progenitor cell distributions at mL3 among the populations of *dome*$^{+}$/Pxn^{-} prohemocytes (PH, green), *dome*$^{+}$/Pxn^{+} intermediate progenitors (IP, yellow), and *dome*$^{-}$/Pxn^{+} differentiated hemocytes (DH, red). WT LGs are composed of 65 ± 5% PH, 10 ± 3% IP, and 25 ± 5% DH. *Tsc2* downregulation in progenitors does not affect prohemocyte population size at mL3: 64 ± 5% of the LG represents PH, whereas 17 ± 3% of cells are IP and 19 ± 6% are DH. *Pten* deficiency increases differentiation at the cost of prohemocytes: 43 ± 8% of the LG represents PH, whereas 17 ± 4% are IP and 40 ± 9% are DH.

Data are represented as mean ± s.d. (n=10). Two-way ANOVA statistics showed significant changes (p<0.0001) in the distribution of hemocyte populations in *Tsc2* or *Pten* deficient LGs compared to WT at mL3.

(**H-M**) Bromodeoxyuridine (BrdU, red) incorporation in WT (*dome>gal4*) (**H-H"** and **K**), *dome>Tsc2*RNAi (**I-I"** and **L**) and *dome>Pten*RNAi (**J-J"** and **M**) LGs at eL2. BrdU, a marker of cells in S-phase, does not colocalize with p4EBP^{high} cells in all backgrounds (**H-J"**). Histone (H, green), a nuclear marker, overlaps with BrdU (red), indicating nuclear localization of BrdU (**K-M**).

(**N**) Distribution of mitoses in mL3 LGs among the populations of *dome*$^{+}$/Pxn^{-} prohemocytes (PH, green), *dome*$^{+}$/Pxn^{+} intermediate progenitors (IP, yellow), and *dome*$^{-}$/Pxn^{+} differentiated hemocytes (DH, red). In WT, 71.5 ± 1.8% of mitoses occur in PH, whereas 13.5 ± 3.4% occur in IP and 15 ± 2.5% in DH. *Tsc2* downregulation does not affect the proportion of mitoses that occur in PH: 71 ± 6.6% of mitoses occur within PH, whereas 15 ± 5.3% occur in IP and 13.9 ±

5.3% of mitoses occur in DH. In contrast, *Pten* downregulation decreases the proportion of mitoses in PH to 53.1 ± 5.8%, whereas 27.1 ± 5.3% of mitoses occur in IP and 19.8 ± 7% in DH. Data are represented as mean ± standard deviation (n=10). Two-way ANOVA statistics comparing the distribution of mitoses at mL3 in *Tsc2* and *Pten* deficient LGs compared to WT showed no difference for *Tsc2*RNAi LGs ($p>0.05$), but a significant change upon *Pten* deficiency ($p<0.0001$).

Scale bars=20μm in **A-F**. Scale bars=10μm in **H-M**.

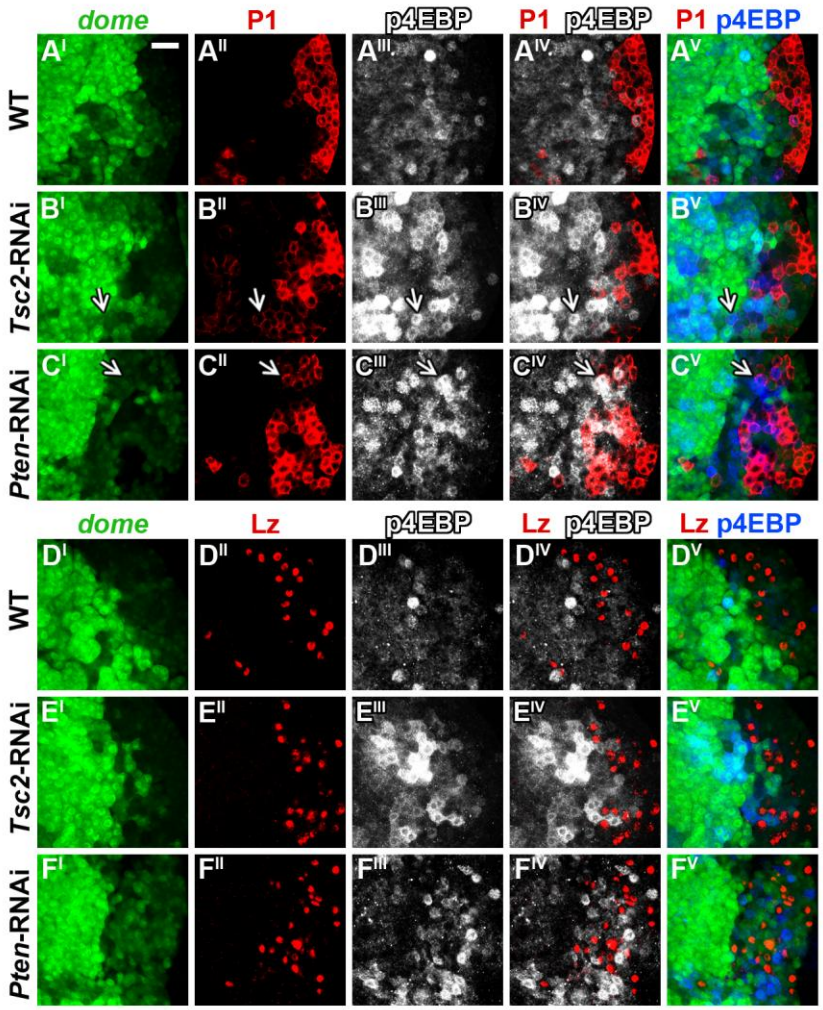

Figure 2-10. Supplementary Figure S2. Distribution of p4EBP^{high} cells in *Tsc2* and *Pten* deficient LGs at mL3

All panels represent mL3 LGs. p4EBP is shown in white in columns III and IV and blue in V. P1 (**A^{I}-C^{V}**) labels differentiated PLs, and Lz (**D^{I}-F^{V}**) labels CCs and their progenitors. L1^{+} lamellocytes were not observed at this stage in any of the genetic backgrounds. (**A^{I-V}**and **D^{I-V}**) WT. p4EBP is expressed throughout the primary lobe at low levels with some scattered p4EBP^{high} cells. A small population of P1^{+} (**A^{I-V}**) and Lz^{+} (**D^{I-V}**) hemocytes is present.

(**B**$^{\mathbf{I\text{-}V}}$ and **E**$^{\mathbf{I\text{-}V}}$**)** Downregulation of *Tsc2* (*dome>Tsc2*RNAi) expands the population of p4EBP^{high} cells. Rare p4EBP^{high} colocalize with P1^{low} (**B**$^{\mathbf{I}}$**-B**$^{\mathbf{V}}$) hemocytes (arrows), but not with P1^{high} hemocytes or with Lz^{+} cells (**E**$^{\mathbf{I\text{-}V}}$).

(**C**$^{\mathbf{I\text{-}V}}$ and **F**$^{\mathbf{I\text{-}V}}$) Downregulation of *Pten* (*dome>Pten*RNAi) expands the population of p4EBP^{high} cells. A subset of P1^{+} hemocytes (arrows, **C**$^{\mathbf{I\text{-}V}}$) are p4EBP^{high}, including some P1^{high} cells. Lz^{+} cells are often observed adjacent to p4EBP^{high} cells but they do not overlap (**F**$^{\mathbf{I\text{-}V}}$).

Scale bar =20μm.

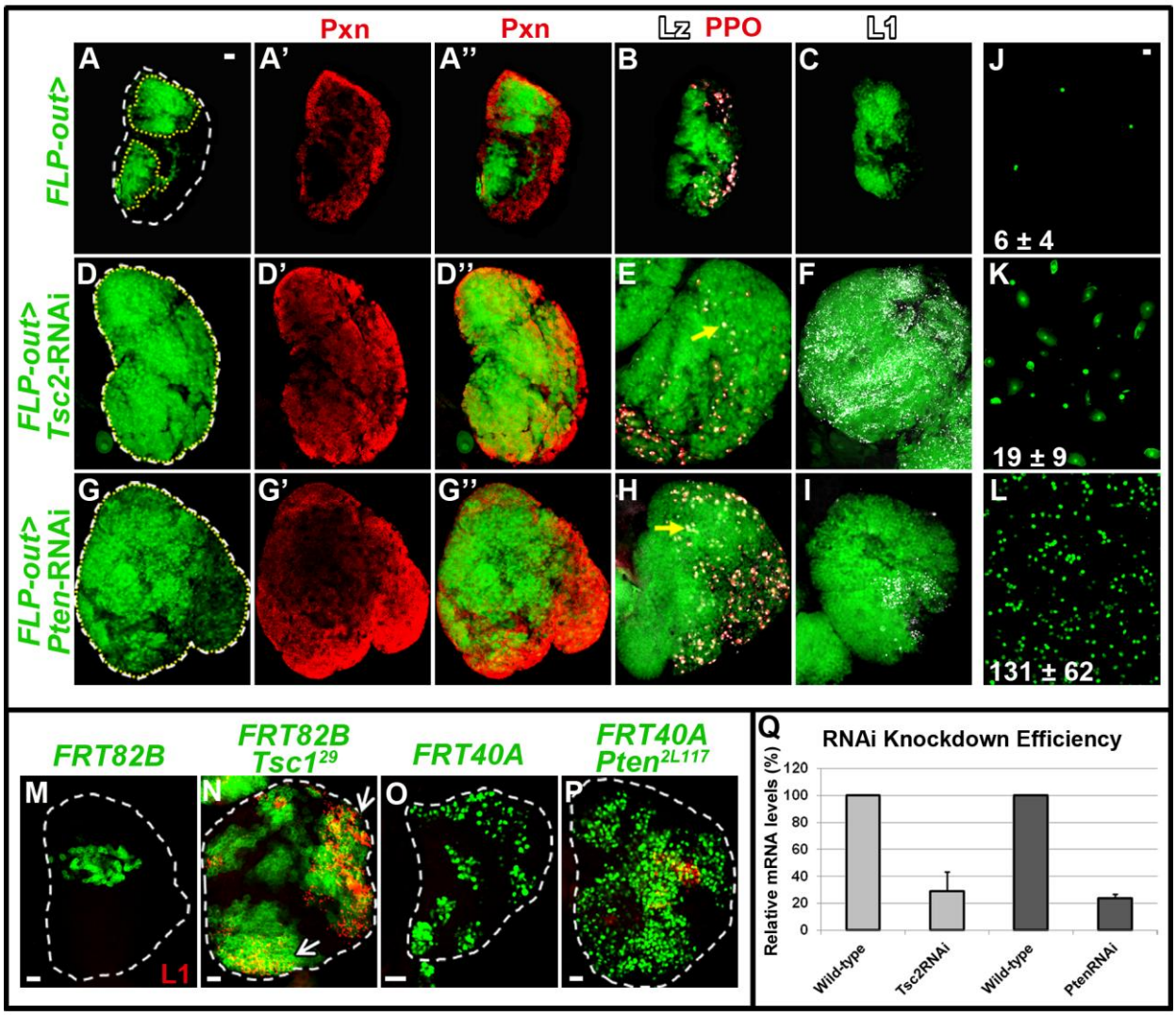

Figure 2-11. Supplementary Figure S3. Clonal analysis and knockdown efficiencies of *Tsc2*RNAi and *Pten*RNAi

Clones are demarcated in yellow in panels **A, D,** and **G**.

(**A-C**) WT LGs (*FLP-out>*): clonal expression of GFP (green) and normal expression of differentiation markers Pxn (red, **A'-A"**), PPO (red, **B**), Lz (white, **B**), and L1 (white, **C**, not normally present in WT).

(**D-F**) Clonal expression of *Tsc2*RNAi (*FLP-out>Tsc2*RNAi) increases LG size and GFP-marked clones encompass the entire LG lobe (compare to **A-C**). Pxn (**D'-D"**) and L1 expression

(**F**) expands throughout the LG. A small number of Lz^{+}/PPO^{-} CC progenitors (arrow) are also seen in medial regions of the LG (**E**), unlike in WT.

(**G-I**) Clonal expression of *Pten*RNAi (*FLP-out>Tsc2*RNAi) increases LG size and increases differentiation (**G'-H**) with few lamellocytes observed (**I**).

(**J-L**) Hemocyte bleeds from LG-specific lineage-traced larvae. GFP marks hemocytes in circulation that are derived from the lymph gland. Very few LG-derived, GFP^{+} hemocytes are observed in circulation in WT (**J**). Downregulation of *Tsc2* in the LG induces the release of LG-derived hemocytes, particularly lamellocytes, into circulation (**K**). Downregulation of *Pten* in the LG increases the relative number of LG-derived hemocytes, but not lamellocytes, released into circulation (**L**) compared to WT ($p<0.001$; **J**) or *Tsc2* downregulation ($p<0.001$; **K**). Data are indicated as mean ± s.d., n=10.

(**M-P)** All panels represent wL3. MARCM clones for WT [(*hs-flp FRT82B Tub-mCD8-GFP*, **M**) and (*hs-flp FRT40A Tub-nGFP*, **O**)], $Tsc1^{29}$ (*hs-flp FRT82B* $Tsc1^{29}$ *FRT82B Tub-mCD8-GFP*, **N**) and $Pten^{2L117}$ (*hs-flp FRT40A* $Pten^{2L117}$ *FRT40A Tub-nGFP*, **P**)]. In WT, lamellocytes (red) are not observed (**M** and **O**). $Tsc^{-/-}$ clones autonomously induce lamellocyte differentiation (arrows, **N**). $Pten^{-/-}$ clones induce a small number of lamellocytes (**P**).

(**Q**) Knock-down efficiency of *Tsc2*RNAi and *Pten*RNAi constructs. RT-quantitative PCR was performed to assess the relative levels of *Tsc2* or *Pten* at wL3, following ubiquitous expression of their respective RNAi constructs with *daughterless-Gal4*. Data represent the mean of three replicates ± s.d. *Tsc2* mRNA transcripts were detected at 29.02% of WT, and *Pten* mRNA transcripts were detected at 23.88% of WT.

Scale bars =20μm, except for X1.2 magnification for panel **I**.

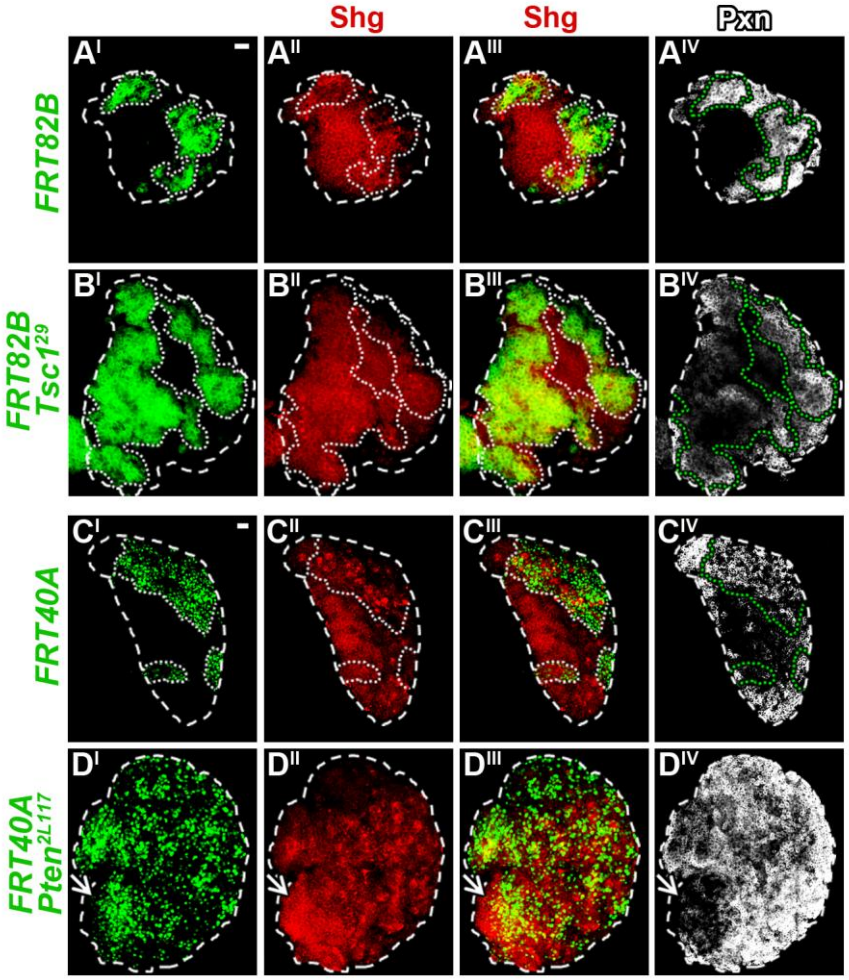

Figure 2-12. Supplementary Figure S4. Shotgun expression in *Tsc* and *Pten* LOF backgrounds.

All panels represent wL3. Clones are outlined by a white or green dotted line. (**A^{I}-B^{IV}**) *Tsc* MARCM clones. (**A^{I-IV}**) WT MARCM clones (*hs-flp FRT82B Tub-mCD8-GFP*). (**B^{I-IV}**) *Tsc1^{29}* clones (*hs-flp FRT82B Tsc1^{29} FRT82B Tub-mCD8-GFP*) maintain Shotgun (Shg, dE-Cadherin) expression (red) in *Tsc$^{-/-}$* clones (green). High Pxn expression (white) is observed only at the tissue periphery, while *Tsc$^{-/-}$* cells express low Pxn levels (grey, **B^{IV}**).

(**C**$^{\mathbf{I}}$-**D**$^{\mathbf{IV}}$) *Pten* MARCM clones. (**C**$^{\mathbf{I\text{-}IV}}$) WT MARCM clones (*hs-flp FRT40A Tub-nGFP*) express highest Shg expression in medial, Pxn-negative tissue. Cells in the periphery are differentiated and express reduced Shg levels, except for non-specific expression of Shg in scattered cells. (**D**$^{\mathbf{I\text{-}IV}}$) *Pten*^{2L117} clones (*hs-flp FRT40A Pten*^{2L117} *FRT40A Tub-nGFP*) that are medially localized (arrow) are Pxn-negative and express high Shg levels. Scattered *Pten*$^{-/-}$ cells in the periphery are Pxn-high (white) and express reduced levels of Shg, except for some scattered cells.
Scale bars=20μm.

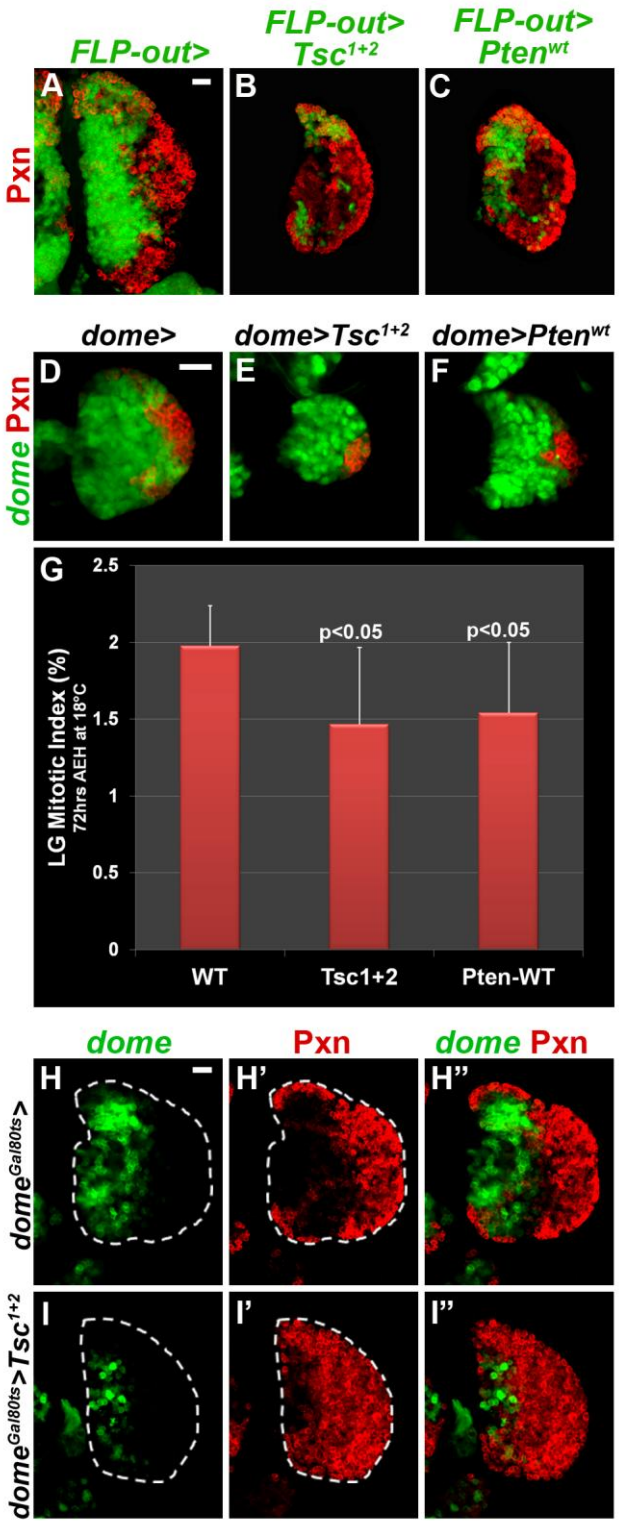

Figure 2-13. Supplementary Figure S5. Inhibition of TORC1 signaling in prohemocytes impairs early LG growth

Figure 2-13. Supplementary Figure S5. Inhibition of TORC1 signaling in prohemocytes impairs early LG growth

(**A-C**) FLP-out clones were generated specifically in the LG for WT (*FLP-out>*; **A**), Tsc^{1+2} (*FLP-out>* Tsc^{1+2}; **B**) and $Pten^{wt}$ (*FLP-out>* $Pten^{wt}$; **C**). Clonal overexpression of Tsc^{1+2}(**B**) or $Pten^{wt}$ (**C**) reduces overall LG size at wL3 and increases the population of Pxn^{+} hemocytes (red). (**D-F**) Overexpression of Tsc^{1+2} (**E**) and $Pten^{wt}$ (**F**) in prohemocytes decreases overall LG size at lL2, compared to WT (*dome>*, **D**), but the onset of differentiation of a small number of Pxn^{+} (red) hemocytes at the LG periphery occurs normally. Staging of *dome>*Tsc^{1+2} and *dome>*$Pten^{wt}$ was performed at 18ºC and lL2 larvae were dissected at 72hrs AEH.

(**G**) Quantification of mitotic index at lL2. Overexpression of Tsc^{1+2} (1.47 ± 0.5%, $p<0.0001$) or $Pten^{wt}$ (1.54 ± 0.46, $p<0.0001$), in prohemocytes decreases mitotic index, compared to WT (1.98 ± 0.26%). Data are indicated as mean ± s.d., n=10.

(**H-I"**) Delaying expression of Tsc^{1+2} in prohemocytes until eL3 does not alter LG size (compare **I"** to **H"**) but increases the number of Pxn^{+} differentiated hemocytes (red). Late expression of *UAS-*Tsc^{1+2} in progenitors was induced using *dome-gal4; P{tubP-gal80[ts]}20* and shifting larvae to the restrictive temperature (29ºC) at eL3.

Scale bars=20μm and apply to corresponding rows.

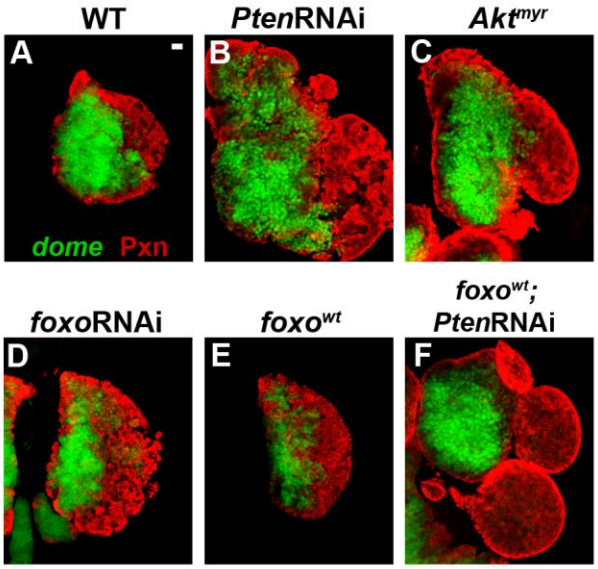

Figure 2-14. Supplementary Figure S6. FoxO-independent role of Akt in mediating *Pten* LOF phenotypes

All panels represent wL3 LGs. In all panels, *dome-gal4, UAS-2xEGFP* (green) drives expression of genetic constructs listed. Pxn expression is shown in red. Scale bar = 20µm.

(**A)** WT LG.

(**B**) *Pten* downregulation in prohemocytes increases LG size and expands the population of Pxn^{+} hemocytes.

(**C**) Overexpression of activated *Akt* (Akt^{myr}) increases the population of Pxn^{+} hemocytes which sometimes 'bud' at the LG periphery.

(**D**) Downregulation of *foxo* in prohemocytes does not phenocopy Akt^{myr} overexpression (**C**) or *Pten* downregulation (**B**) in the LG.

(**E**) Overexpression of *foxo* increases Pxn^{+} hemocytes at the expense of prohemocytes.

(**F**) Overexpression of *foxo* upon *Pten* downregulation does not rescue the accumulation of differentiated hemocytes associated with *Pten* deficiency (**B**).

Chapter 3:

Extracellular matrix-modulated FGF signaling in *Drosophila* blood progenitors regulates their differentiation via a ras/ETS/FOG pathway and TORC1 function

Abstract

Maintenance of hematopoietic progenitors ensures a continuous supply of blood cells during the lifespan of an organism. Thus, understanding the molecular basis for progenitor maintenance is a continued focus of investigation. A large pool of undifferentiated blood progenitors are maintained in the *Drosophila* hematopoietic organ, the larval lymph gland (LG), by a complex network of signaling pathways that are mediated by niche-, progenitor-, or differentiated hemocyte-derived signals. In this study we examined the function of the *Drosophila* Fibroblast Growth Factor Receptor (FGFR), Heartless (Htl), a critical regulator of early LG progenitor specification in the late embryo, during larval LG hematopoiesis. Activation of Htl signaling in hemocyte progenitors by its two ligands, Pyramus (Pyr) and Thisbe (Ths), is both required and sufficient to induce progenitor differentiation and formation of the plasmatocyte-rich LG cortical zone. We identify two transcriptional regulators that function downstream of Htl signaling in LG progenitors, the ETS protein, Pointed, and the Friend-of-GATA (FOG) protein, U-Shaped, which are required for this Htl-induced differentiation response. Furthermore, cross-talk of FGFR and Target of Rapamycin (TOR) signaling in hemocyte progenitors is required for lamellocyte differentiation downstream of Ths-mediated Htl activation. Finally, we identify the *Drosophila* heparan sulfate proteoglycan (HSPG), Trol, as a critical negative regulator of FGF signaling in the LG, demonstrating that sequestration of differentiation signals by the extracellular matrix is a unique mechanism employed in blood progenitor maintenance that is of potential relevance to many other stem cell niches.

Introduction

The maintenance of hematopoietic stem cells (HSCs) is a crucial process for the normal production of blood cells, but in addition, understanding its molecular basis could enhance the therapeutic benefits of this cell population. Studies of hematopoiesis and the regulation of hematopoietic progenitors have identified a variety of molecular mechanisms that regulate progenitor/stem cell maintenance (Arai and Suda, 2007; Seita and Weissman, 2010; Teitell and Mikkola, 2006). As in vertebrate systems, *Drosophila* hematopoiesis requires a population of multipotent progenitor cells that give rise to all differentiated hemocyte lineages (Mandal et al., 2007). Previous studies of *Drosophila* larval hematopoiesis have uncovered a complex network of signaling pathways that cooperate to regulate hemocyte progenitor maintenance. These include niche-derived Hedgehog (Hh) signaling (Krzemien et al., 2007; Mandal et al., 2007), an adenosine deaminase growth factor A (Adgf-A)- mediated signal emanating from differentiated hemocytes (Mondal et al., 2011), and Wingless (Wg) signaling, which autonomously regulates progenitor cell maintenance (Sinenko et al., 2009). In contrast, the signal(s) required for differentiation of *Drosophila* hemocyte progenitors during larval development remain largely unknown.

The major wave of hematopoiesis in *Drosophila* occurs in the larval lymph gland (LG) (Lebestky et al., 2000; Tepass et al., 1994). Studies of the LG have allowed genetic dissection of signaling networks that operate in a niche-, progenitor- or differentiated hemocyte-dependent manner to maintain blood homeostasis, owing to the ability to perform cell-type specific genetic manipulation with direct *in vivo* imaging of its effects on these distinct cell populations. In the LG, multipotent blood progenitors termed prohemocytes express high levels of dE-Cadherin and are compactly arranged in a medial region of both primary lobes termed the Medullary Zone

(MZ; **Fig. 1A**) (Jung et al., 2005). These prohemocytes give rise to all mature, myeloid-like hemocyte lineages (Jung et al., 2005): macrophage-like plasmatocytes (PLs), platelet-like crystal cells (CCs), and lamellocytes (LMs), which are not normally present in a healthy larva but are induced to differentiate upon parasitic wasp infection (Lanot et al., 2001; Sorrentino et al., 2002). Differentiated hemocytes that arise from LG prohemocytes populate the peripheral cortical zone (CZ; **Fig. 1A**) (Jung et al., 2005) and do not enter circulation until the onset of metamorphosis (Grigorian et al., 2011b). Finally, the posterior signaling center (PSC; **Fig. 1A**), a small group of cells at the posterior tip of each primary lobe, functions as a hematopoietic niche by supplying pro-maintenance signals, such as Hh, to progenitors (Krzemien et al., 2007; Lebestky et al., 2003; Mandal et al., 2007). The strict dependence of *Drosophila* progenitor maintenance on the PSC (Krzemien et al., 2007; Mandal et al., 2007) highlights the unique role within the LG of a hematopoietic niche devoted to the maintenance of myeloid lineage-restricted progenitors.

Similar to the vertebrate aorta-gonadal mesonephros (AGM), wherein hematopoietic and vascular lineages are derived from a common progenitor cell called the hemangioblast (Ema et al., 2003; Medvinsky and Dzierzak, 1996), a common origin of *Drosophila* vascular cells and hematopoietic LG progenitors has been described (Mandal et al., 2004). Specifically, the *Drosophila* LG, vascular cardioblasts (heart and aorta), and excretory cells (pericardial nephrocytes) all arise from the cardiogenic mesoderm late in embryonic development. Clonal analysis demonstrated that a single cardiogenic mesoderm cell can give rise to both LG and vascular cells, providing evidence for a *Drosophila* hemangioblast, akin to the vertebrate AGM (Mandal et al., 2004). Amongst the signaling pathways required for development of all three lineages derived from the cardiogenic mesoderm is the FGFR homolog, Heartless (Htl) (Beiman et al., 1996; Frasch, 1995; Mandal et al., 2004). Loss-of-function mutations in *Htl* result in

absence of a heart/cardioblasts (Shishido et al., 1997), and cause LG progenitors to not develop (Mandal et al., 2004). In contrast, constitutive activation of FGFR in the cardiogenic mesoderm increases the number of blood progenitors, nephrocytes, and cardioblasts (Grigorian et al., 2011a).

Htl is one of two *Drosophila* FGFRs and is activated by two FGF-8-like ligands, Pyramus (Pyr) and Thisbe (Ths) (Gryzik and Müller, 2004; Stathopoulos et al., 2004). Htl FGFR signaling has been studied extensively in the context of mesoderm development, migration during gastrulation, and differentiation of mesodermal lineages (Beiman et al., 1996; Gisselbrecht et al., 1996; Klingseisen et al., 2009; McMahon et al., 2010). It has also been shown to play a major role in glial cell migration and differentiation in the eye (Franzdottir et al., 2009). The second *Drosophila* FGFR, Breathless (Btl), is activated by a single FGF ligand, Branchless (Bnl), and is critical for morphogenesis of the trachea during embryonic, larval and pupal developmental stages (reviewed in Cabernard et al., 2004). With only 3 FGF-FGFR combinations, *Drosophila* FGFR signaling is less complex than in vertebrates, wherein 22 FGF ligands and 4 FGFRs contribute to over 120 potential FGF-FGFR interactions (Zhang et al., 2006). Despite this apparent simplicity, recent studies have provided evidence for overlapping functions of Pyr and Ths, in which they act redundantly to provide robust signaling, as well as distinct functions due to their differential effects on Htl activation (Franzdottir et al., 2009; Kadam et al., 2009; Klingseisen et al., 2009).

Despite the functional requirement of Htl signaling during development of early LG progenitors in the late embryo, a role for Htl signaling in the larval LG is currently unknown. Here, we provide evidence that activation of Htl signaling by both its ligands, Ths and Pyr, is both required and sufficient for differentiation of hemocyte progenitors in the LG. We

demonstrate that these effects are Ras-mediated, and dependent on two downstream transcriptional effectors, Pointed (Pnt) and U-Shaped (Ush) as well as on cross-talk with the target of rapamycin (TOR) growth signaling pathway. Finally, we identify the *Drosophila* heparan sulfate proteoglycan (HSPG), Trol, as a crucial modulator of FGF signaling in the LG, demonstrating that sequestration of differentiation signals by the extracellular matrix (ECM) is a unique mechanism utilized to maintain blood progenitors, in addition to niche-, progenitor-, and differentiated hemocyte-generated maintenance signals.

Materials and Methods

Drosophila stocks and crosses

All RNAi lines used in the described experiments were obtained from the Vienna *Drosophila* RNAi Center (VDRC, Vienna, Austria). The following *Drosophila* stocks were used: *UAS-Htl^{DN}*, *UAS-[d4EBP(LL)]*, and *Ths-Gal4* (Bloomington); *UAS-Htl^{Act}* (A. Michelson); *UAS-Ths^{WT}* and *UAS-Pyr^{WT}* (A. Stathopoulos); *UAS-Ras^{DN}*, *UAS-Ras85B^{Act}*, *UAS-Rolled-MAPK^{Act}*, *UAS-PntP2^{Act}*, and *Trol-GFP* protein trap line ZCL1973X (V. Hartenstein); *UAS-Vein*, *UAS-secreted-Spitz*, *UAS-Pvf1*, and *UAS-Pvf2* (U. Banerjee); *UAS-Ush^{WT}* (N. Fossett); *UAS-secreted-Gurken* and *UAS-secreted-Keren* (A. Simcox); *UAS-Jeb* (J. Weiss); *UAS-Bnl* (M. Krasnow).

FLP-out clones in the LG were generated using *HHLT* (*hand-gal4, hml-gal4, UAS-2xEGFP, UAS-FLP; A5C-FRT-STOP-FRT-gal4*) as described (Evans et al., 2009). Hemocyte progenitor-specific gene expression was performed using *dome-gal4, UAS-2xEGFP* (S. Noselli). Expression of *UAS-Pyr^{WT}*, *UAS-Ush^{WT}* or *UAS-Pnt*RNAi genetic constructs in hemocyte progenitors was performed using *dome-gal4; P{tubP-gal80[ts]}20* (U. Banerjee) to permit larval viability; larvae were shifted from the permissive temperature (18°C) to the restrictive temperature (29ºC) at the onset of L2 stage.

Larval staging

Staging was performed at 25°C and the following time-points after egg hatching (AEH) were used for larval stages: 54 hrs AEH (L2), 72 hrs AEH (eL3), 116 hrs AEH (wL3).

Immunohistochemistry

Primary antibodies used: mouse anti-Pxn (1:300; J. Fessler), rabbit anti-ProPO (1:300; M. Kanost), mouse anti-P1 and anti-L1 (1:20; I. Ando), rabbit anti-p4EBP (1:300; Cell Signaling), and rat anti-Heartless (1:400, B. Shilo). LGs were dissected from staged larvae in 1xPBS and

then fixed for 20 minutes in 3.7% formaldehyde. LGs were blocked in 10% NGS with 0.4% TritonX-100 in 1xPBS (PBST) for 1hr at room temperature. Primary antibodies were diluted in PBST and incubated with LGs in 96-well MicroWell plates (Thermo Scientific) overnight at 4ºC in a humidified chamber. LGs were then washed 4 x 15 minutes in PBST with gentle shaking. Secondary antibodies (Jackson Immunoresearch) were prepared in 10% NGS in 0.4%PBST at a concentration of 1:400 and incubated with LGs overnight in a dark, humidified chamber at 4ºC. LGs were then washed 4 x 15 minutes in PBST with gentle shaking and 1 x 15 minutes in 1xPBS. LGs were mounted using Vectashield (Vector Laboratories). Samples were imaged using a Carl Zeiss LSM 310 Laser Scanning Confocal Microscope.

Rapamycin treatment

Crosses were set up on 15µM rapamycin food plates and larvae were grown at 25°C until wL3.

Quantification of Pxn^{+} hemocyte population distribution (% area) in the LG

Methodology to determine the proportion of Pxn^{+} hemocytes in the LG was performed as in Dragojlovic-Munther and Martinez-Agosto, 2012. Statistical significance of data was assessed with Student's *t*-test.

Results

The FGFR Heartless is expressed in LG hemocyte progenitors

Although Htl signaling is required to generate LG progenitors in the embryo, a role for FGFR signaling in the larval LG remains unknown. We reasoned that a role for Htl in hematopoiesis would extend to later stages of LG development, given its essential role in the embryo. We therefore first examined Htl expression in the LG at distinct larval stages. Undifferentiated hemocyte progenitors in the LG are identified by positive expression of the JAK/STAT receptor *domeless* (*dome*), and negative expression of the early differentiation marker, Peroxidasin (Pxn) (*dome*$^{+}$/Pxn^{-}). Early differentiating progenitors express both *dome* and Pxn at low levels (*dome*$^{+}$/Pxn^{+}) and represent a population of intermediate progenitors (Dragojlovic-Munther and Martinez-Agosto, 2012; Krzemien et al., 2010). During the second larval instar stage (L2) the LG is largely undifferentiated, but a small population of differentiating progenitors (*dome*$^{+}$/Pxn^{+}) begin to emerge at the LG periphery (**Fig. 1B**). Htl expression is detected throughout the entire L2 LG (**Fig. 1B'**), and overlaps completely with the progenitor marker, *dome* (**Fig. 1B"**). By early third instar (eL3), the number of Pxn^{+} hemocytes expands, forming an early CZ (**Fig. 1C**). Htl expression is still detected throughout the LG (**Fig. 1C'**) but is most strongly expressed in *dome*$^{+}$ progenitors (**Fig. 1C"**). By wandering third instar (wL3) a mature CZ is observed at the LG periphery (**Fig. 1D**). Htl expression is still detected throughout the population of *dome*$^{+}$ progenitors but is absent in a subset of *dome* $^{-}$/Pxn^{+} hemocytes at the periphery (white arrows, **Fig. 1D'-D"**).

Interestingly, expression of the Htl FGFR ligand, *Ths*, is distinct from, yet overlaps with, Htl expression in the LG. Unlike the homogenous expression of Htl at L2 and eL3, expression of *Ths* is more restricted in the LG, and is not found in all Htl^{+} hemocytes (**Fig. 1E-F'**).

Interestingly, *Ths*-negative hemocytes often correlate with Pxn expression in the LG periphery, suggesting that *Ths* expression may turn off upon hemocyte differentiation (**Fig. 1H-I'**). By wL3, *Ths* expression occurs strongest in medial (Htl^+) LG regions (**Fig. 1G-G'**), adjacent to but rarely overlapping with Pxn^+ differentiated hemocytes at the periphery (**Fig. 1J-J'**). In addition to peripheral Ths^-/Pxn^+ hemocytes (white arrows, **Fig. 1I-J'**), a few scattered Ths^- cells are also sometimes observed in medial LG regions, which are undifferentiated (yellow arrows, **Fig. 1I-J'**). This pattern of Htl and *Ths* expression in the LG suggested a potential role for Htl signaling in regulating LG progenitors.

Heartless signaling in LG progenitors regulates their differentiation

We next examined the functional role of Htl in prohemocytes by performing progenitor-specific loss-of-function (LOF) and gain-of-function (GOF) analyses in the LG. Wild-type (WT) LGs at wL3 are composed of 42±5% Pxn^+ hemocytes (**Fig. 2A** and **K**). Measuring the proportion of Pxn-labeled hemocytes in the LG is a good indicator of relative changes in CZ size and LG differentiation status (Dragojlovic-Munther and Martinez-Agosto, 2012; Krzemien et al., 2010; Shim et al., 2012). Specifically decreasing Htl signaling in prohemocytes by overexpression of a dominant-negative (DN) *Htl* allele significantly decreases the proportion of Pxn^+ differentiated hemocytes in the LG to 20±6% ($p<0.0001$, **Fig. 2B** and **K**). Terminally differentiated PLs and CCs, labeled with P1 and Prophenoloxidase (PPO), respectively, are restricted to a thin peripheral layer compared to WT (compare **Fig. 2B'** to **A'**), and LMs ($L1^+$) are rarely observed (**Fig. 2B"**).

Surprisingly, downregulating either of the FGF ligands, *Ths* or *Pyr*, in LG progenitors had an even stronger effect on hindering progenitor differentiation, such that LGs are composed of only 12±4% or 11±5% Pxn^+ hemocytes, respectively ($p<0.0001$, **Figs. 2C**, **D**, and **K**). PLs and

CCs are likewise restricted to a very thin peripheral LG layer (**Fig. 2C'** and **D'**). LMs are not observed upon *Ths* downregulation (**Fig. 2C"**), but are detected throughout the LG upon *Pyr* downregulation, intermixed among the progenitor population (**Fig. 2D"**).

In contrast to the decreased differentiation that occurs upon decreasing Htl signaling, progenitor-specific overexpression of a constitutively active *Htl* allele increases Pxn expression in the LG (81±8%) compared to WT (p<0.0001, **Fig. 2E** and **K**). PLs and CCs are also observed throughout the LG (**Fig. 2E'**). Interestingly, hemocytes expressing low levels of the progenitor marker *dome* are observed in medial LG regions (**Fig. 2E-E'**), but these $dome^{low}$ hemocytes largely co-express Pxn and the terminal differentiation markers, P1 and PPO, suggesting that they are mature hemocytes (**Fig. 2E-E'**). Finally, LMs are observed throughout the LG (**Fig. 2E"**). Forcibly overexpressing *Ths* and *Pyr* in prohemocytes is sufficient to induce almost complete differentiation of the LG, such that the proportion of Pxn^+ differentiated hemocytes in the LG increases to 89±7% and 88±6%, respectively (p<0.0001, **Fig. 2F**, **G** and **K**). Terminally differentiated PLs and CCs are detected throughout the LG (**Fig. 2F'** and **G'**), and large numbers of LMs are induced to differentiate upon *Ths*, but not *Pyr*, overexpression (**Fig. 2F"** and **G"**), demonstrating an inverse effect to *Ths* and *Pyr* downregulation (**Fig. 2C"** and **D"**). LG secondary lobes, which consist mostly of undifferentiated $dome^+$ progenitors in WT (**Fig. 2H**), are severely hypertrophied and differentiated upon both *Ths* and *Pyr* overexpression in $dome^+$ progenitors (**Fig. 2I-J**). Altogether these data demonstrate that Htl and its ligands, Ths and Pyr, are necessary and sufficient to promote hemocyte progenitor differentiation in the LG, with distinct opposing effects on LM differentiation.

Ras-MAPK, Pointed and U-shaped regulate LG progenitor differentiation

We next assessed potential downstream regulators of FGFR in the LG which might mediate the observed differentiation response of hemocyte progenitors. Similar to other receptor tyrosine kinases (RTKs), ligand-dependent activation of FGFRs results in the recruitment of adaptor proteins which can activate multiple signal transduction pathways (Eswarakumar et al., 2005; Turner and Grose, 2010). Perhaps the best characterized effector of activated RTK signaling is Ras, which activates the mitogen activated protein kinase (MAPK) growth signaling pathway (McKay and Morrison, 2007). We assessed the role of Ras signaling in the LG via progenitor-specific overexpression of a DN *Ras* allele. Similar to *Htl* LOF in prohemocytes (**Fig. 2B-B"**), Ras^{DN} overexpression reduces the proportion of Pxn^{+} differentiated hemocytes in the LG compared to WT (20±11%, $p<0.0001$, **Figs 2K** and **3B**). The populations of PLs and CCs are also limited to a thin peripheral layer (**Fig. 3B'**). As in *Htl* LOF, rare LMs are sometimes observed (**Fig. 3B"**). In contrast, overexpressing activated *Ras* or its downstream effector, *Rolled MAPK*, in LG prohemocytes expands the populations of Pxn^{+} hemocytes and terminally differentiated PLs and CCs throughout the LG (**Figs 2K**, **3C-C'** and **3D-D'**), at the expense of $dome^{+}$ progenitors. While LMs are not observed in the LG upon progenitor-specific Ras activation (**Fig. 3C"**), a small number of LMs differentiate upon Rolled MAPK activation (**Fig. 3D"**).

A transcription factor that can be activated downstream of both Btl-FGFR and Htl-FGFR signaling in *Drosophila* is the ETS-domain protein, Pointed (Pnt) (Cabernard and Affolter, 2005; Franzdottir et al., 2009). We hypothesized that Pnt could mediate the strong differentiation phenotypes upon manipulation of Htl-FGFR signaling in prohemocytes. The *Drosophila Pnt* gene encodes two protein isoforms, PntP1 and PntP2, arising from the use of two alternative promoters that are separated by 50kb of genomic sequence (Klambt, 1993; Scholz et al., 1993).

Whereas PntP1 is a constitutive transcriptional activator, phosphorylation of PntP2 at Thr151 by MAPK potently activates PntP2 transcriptional activity (Brunner et al., 1994; O'Neill et al., 1994). Consistent with a role for Pnt downstream of Htl signaling, downregulation of *Pnt* expression in *Drosophila* LG prohemocytes blocks their differentiation, such that the proportion of Pxn^+ hemocytes in the LG is only 2±1% (**Figs 2K** and **3E**). While few scattered PLs and CCs are observed at wL3 (**Fig. 3E'**), a significant induction of LMs occurs upon *Pnt* downregulation (**Fig. 3E"**), similar to *Pyr* downregulation (**Fig. 2D"**). Overexpression of activated PntP2 in prohemocytes increases the proportion of Pxn^+ LG hemocytes (80±10%, **Figs 2K** and **3F**) while not affecting LM numbers (**Fig. 3F"**).

We next examined the function of the transcriptional regulator U-shaped (Ush), the single *Drosophila* Friend-of-GATA (FOG) zinc finger protein, in LG prohemocytes. Similar to Htl, Ush function is required for mesoderm migration early in embryonic development (Fossett et al., 2000). Ush has previously been shown to be expressed within LG prohemocytes of the MZ as well as a subset of differentiated PLs and CCs (Gao et al., 2009), yet progenitor-specific manipulation of *Ush* expression has not been examined. Remarkably, *Ush* downregulation in LG prohemocytes potently inhibits LG CZ formation, as the proportion of Pxn^+ hemocytes in the LG is only 6±4% (**Figs 2K** and **3G**). Few PLs and rare CCs are observed (**Fig. 3G'**), whereas induction of LMs occurs throughout the LG (**Fig. 3G"**), phenocopying *Pyr* and *Pnt* downregulation. Conversely, overexpression of WT *Ush* in $dome^+$ progenitors increases the proportion of Pxn^+ differentiated hemocytes in the LG to 80±6% (**Figs 2K** and **3H**). The populations of PLs and CCs expand to more medial LG regions, (**Fig. 3H-H'**), whereas LMs are not observed (**Fig. 3H"**). These findings demonstrate a role for ras signaling and ETS and FOG transcription factor function in blood cell differentiation.

RTK signaling effects on differentiation are specific to Pyramus and Thisbe

Our findings suggest that canonical Ras-MAPK signaling through Pnt may mediate the effects of activated Htl signaling in the LG. Previous studies have demonstrated functional roles of other RTKs in circulating hemocytes (Munier et al., 2002; Zettervall et al., 2004) as well as in the LG, including Epidermal Growth Factor Receptor (EGFR) (Sinenko et al., 2012) and the *Drosophila* platelet derived growth factor/ vascular endothelial growth factor (PDGF/VEGF) receptor homolog, PVR (Mondal et al., 2011). In order to assess the specificity of Ths and Pyr in inducing progenitor differentiation, we monitored changes in differentiation upon forcibly overexpressing other RTK ligands in LG progenitors.

Progenitor-specific overexpression of the Btl-FGFR ligand, Bnl, does not alter LG differentiation (**Fig. 4B** and **K**), demonstrating specificity for Htl FGFR and its ligands in mediating LG progenitor differentiation. EGFR is expressed in the LG (JA Martinez-Agosto, unpublished results). However, forcibly overexpressing the EGFR ligands, *Vein*, *Spitz*, *Gurken*, or *Keren* in *dome*$^{+}$ prohemocytes does not affect LG differentiation (**Fig. 4C-F** and **K**), and neither does overexpression of the Anaplastic Lymphoma Kinase (ALK) ligand, *Jelly-Belly* (*Jeb*, **Fig. 4G** and **K**). Recent studies have demonstrated low PVR expression in MZ progenitors (Mondal et al., 2011). The PVR ligand, Pvf1, is expressed in PSC cells, whereas Pvf2 is expressed in PSC and most medial MZ cells (Mondal et al., 2011). While overexpression of *Pvf1* in LG progenitors does not induce a differentiation response (**Fig. 4H** and **K**), *Pvf2* overexpression is sufficient to induce almost complete differentiation of the LG (**Fig. 4I** and **K**). In contrast, downregulation of *Pvf2* in the MZ has no effect on progenitor differentiation (Mondal et al., 2011), suggesting that Pvf2 is not required to induce differentiation during normal LG development, but is sufficient to induce a differentiation response upon increased

ligand expression. Pvf2 signals independently from Htl in blood progenitors, as reducing Htl function in prohemocytes upon *Pvf2* overexpression does not impair the differentiation response observed upon *Pvf2* overexpression alone (**Fig. 4J**, compare to **I**). Our data thus identifies two independent positive effectors of differentiation in LG prohemocytes, one that is necessary and sufficient for normal hemocyte differentiation and the other able to promote differentiation upon conditions that cause its upregulation.

From these experiments, we conclude that the requirement of *Ths* and *Pyr* in LG progenitors for their differentiation is not a general response of activating RTK signaling, but is specific to the Htl-FGFR signaling pathway.

Cell-autonomous effects of Htl signaling in LG hemocytes

Downregulation of *Ths* or *Pyr* ligands within the *dome*$^{+}$ progenitor population leads to the same phenotype as LOF conditions of Htl and potential effectors, Ras and Pnt in progenitor cells (**Fig. 2K**). Further, having demonstrated the co-expression of the ligand *Ths* in Htl^{+} cells in the LG (**Fig. 1E'**, **F'** and **G'**), we hypothesized that cell-autonomous FGF signaling may operate in LG progenitors to regulate their differentiation. We investigated a potential role for cell autonomous FGF signaling by clonally manipulating expression of Htl, its ligands, and intracellular pathway components, specifically in the LG, using G-TRACE (Evans et al., 2009). Clonal overexpression of *Htl*^{DN} in the LG impairs differentiation cell autonomously within the clones, such that clonal cells minimally overlap with the early differentiation marker, Pxn (**Fig. 5B-B''**). Likewise, LG-specific clonal downregulation of *Ths* or *Pyr* strongly impairs differentiation autonomously within the mutant cells (green, Pxn^{-}), whereas peripheral WT cells differentiate (red, Pxn^{+}) (**Fig. 5C-D''**). Conversely, clonally overexpressing *Htl*^{Act}, *Ths*^{WT}, or

Pyr^{WT} in the LG autonomously induces differentiation, such that Pxn expression is strongly upregulated within mutant cells (yellow, **Fig. 5E-G''**).

Clonally impairing Ras activity in the LG autonomously blocks differentiation, while WT cells differentiate (**Fig. 5H-H''**). Conversely, clonal activation of Ras or Rolled MAPK in the LG strongly induces differentiation autonomously (yellow, **Fig. 5I-J''**). The crucial effect of Pnt activity in regulating differentiation of LG progenitors (**Fig. 3E-E''**) was confirmed and further demonstrated by the strong autonomous block in differentiation of *Pnt*RNAi clones, which compose almost the entire LG (**Fig. 5K-K''**). Similarly, LG-specific clonal downregulation of *Ush* blocks differentiation in clonal cells, whereas adjacent WT cells differentiate (**Fig. M-M''**). Finally, clonal activation of PntP2 (**Fig. 5L-L''**) or overexpression of *Ush*^{WT} (**Fig. N-N''**) autonomously induces differentiation.

Taken together, these findings confirm the strong autonomous effects of Htl FGFR and its potential downstream effectors Ras, Pnt and Ush in regulating LG hemocyte differentiation, and also suggest that Ths and Pyr may function either autonomously or within a very limited diffusion range to regulate progenitor differentiation.

Pnt and Ush function downstream of Htl in LG progenitors

We confirmed that Pnt functions downstream of Htl signaling in the LG to mediate effects on hemocyte differentiation by performing epistasis analysis. LG-specific clonal downregulation of *Pnt* upon overexpression of *Htl*^{Act} is sufficient to block the differentiation response of Htl activation alone (compare **Fig. 6B-C**) and to autonomously hinder clonal hemocyte differentiation, similar to *Pnt*RNAi expression alone (**Fig. 5K''**). Likewise, clonal downregulation of *Pnt* upon *Ths* or *Pyr* overexpression also effectively hinders differentiation of

clonal cells (**Fig. 6D-G**). These findings suggest that the differentiation response downstream of activated Htl signaling in the LG is dependent on Pnt activity.

We next assessed the importance of Pnt activation downstream of Htl signaling by examining the sufficiency of PntP2 activation to induce differentiation in conditions of reduced Htl signaling. Co-expression of $PntP2^{Act}$ with Htl^{DN}, *Ths*RNAi, or *Pyr*RNAi in $dome^{+}$ hemocyte progenitors is sufficient to significantly increase LG differentiation in these backgrounds (**Fig. 6I-N**). Importantly, the proportion of Pxn^{+} hemocytes in these backgrounds (72±11%, 71±10%, or 67±11%, respectively; **Fig. 6U**) is similar to progenitor-specific $PntP2^{Act}$ overexpression alone (80±10%; **Fig. 2K**), but dramatically differs from conditions of Htl^{DN}, *Ths*RNAi, or *Pyr*RNAi overexpression alone (20±6%, 12±4%, or 11±5%, respectively; **Fig. 2K**).

Given the similar phenotypes of *Ush* downregulation with conditions of reduced Htl signaling (compare **Fig. 3G-G''** and **5M-M''** with **Fig. 2B-D''** and **5B-D''**), we examined whether Ush also functions downstream of Htl signaling in the LG. Downregulating *Ush* upon overexpression of Htl^{Act} or Ths^{WT} in $dome^{+}$ hemocyte progenitors significantly reduces progenitor differentiation in the LG (**Fig. 6P** and **R**) compared to overexpression of Htl^{Act} or Ths^{WT} alone (**Fig. 6O-Q**). The proportion of Pxn^{+} hemocytes in these backgrounds is dramatically decreased (16±6% and 20±10%, respectively; **Fig. 6U**), compared to Htl^{Act} or Ths^{WT} overexpression alone (81±8% and 89±7%, respectively; **Fig. 2K**). Having observed that reducing either *Pnt* or *Ush* expression in the LG is sufficient to rescue differentiation phenotypes induced by activated Htl signaling, we next examined the epistatic relationship of Ush and Pnt in MZ prohemocytes. Interestingly, downregulation of *Ush* upon $PntP2^{Act}$ overexpression in $dome^{+}$ progenitors dramatically impairs progenitor differentiation in the LG (**Fig. S-T**), phenocopying *Ush*RNAi

alone (**Fig. 3G**), and reduces the proportion of Pxn^{+} hemocytes in the LG to only 7±5% (**Fig. 6U**).

Altogether, our epistasis analysis demonstrates that Pnt activation downstream of Htl signaling mediates the effects of Ths/Pyr-Htl signaling on hemocyte progenitor differentiation. Our data also describes Ush as a potent effector of differentiation, which either functions downstream of Pnt or is required for Pnt activity in mediating the Htl-induced differentiation response of hemocyte progenitors.

Activation of TOR signaling upon Htl activation

TOR complex 1 (TORC1) signaling in *Drosophila* LG progenitors regulates their early proliferation (during L2 and eL3) and later differentiation (Dragojlovic-Munther and Martinez-Agosto, 2012). Interestingly, activation of TORC1 through *Tsc1/2* disruption in LG progenitors induces significant LM differentiation (Dragojlovic-Munther and Martinez-Agosto, 2012), similar to activation of Htl or overexpression of *Ths* in progenitors (**Fig. 2E''** and **F''**). Given that LMs are not observed in the LG upon progenitor-specific Ras or Pnt activation (**Fig. 3C''** and **F''**), we reasoned that a different pathway might mediate the LM differentiation induced by Ths or Htl and examined the possibility that it could be TORC1-mediated. In WT, p4EBP, a marker of active TORC1 signaling, is expressed at low levels in the LG (**Fig. 7A-A''**, Dragojlovic-Munther and Martinez-Agosto, 2012). Overexpression of Htl^{Act} or Ths^{WT} in hemocyte progenitors increases p4EBP expression throughout the LG, suggesting active mTORC1 signaling upon FGF signaling (**Fig. 7B-C''**). We therefore examined the TORC1-dependence of Htl- and Ths-induced phenotypes by treating larvae with the TORC1 inhibitor, Rapamycin. Systemic Rapamycin treatment of WT larvae increases differentiation in the LG (compare **Fig. 7E** to **D**, Dragojlovic-Munther and Martinez-Agosto, 2012; Shim et al., 2012).

Interestingly, Rapamycin treatment partially restores LG zonation upon overexpression of Htl^{Act} in hemocyte progenitors (compare **Fig. 7H** to **G**), and moderately affects LG differentiation upon *Ths* overexpression (compare **Fig. 7K** to **J**). Unlike its limited effects on PL differentiation, Rapamycin treatment is sufficient to block the LM differentiation normally observed upon progenitor-specific Htl^{Act} or Ths^{WT} overexpression (compare **Fig. 7N** and **Q** to **M** and **P**).

Our previous studies identified 4EBP as a potent effector of TORC1-dependent phenotypes in *Drosophila* hemocyte progenitors (Dragojlovic-Munther and Martinez-Agosto, 2012). Phosphorylation of 4EBP by TORC1 promotes growth by relieving translation initiation factor 4E (IF-4E) from inhibitory 4EBP binding (Gingras et al., 2001). Similar to systemic Rapamycin treatment, progenitor-specific overexpression of a mutant form of *Drosophila* 4EBP [*d4EBP(LL)*] that binds more tightly to IF-4E increases differentiation (compare **Fig. 7F** to **D**). Overexpression of *d4EBP(LL)* upon Htl^{Act} or Ths^{WT} overexpression in progenitors blocks LM differentiation (**Fig. 7O** and **R**) and partially restores zonation of Htl^{Act} LGs (**Fig. 7I**), similar to Rapamycin treatment. Together, these experiments suggest that the dramatic expansion of LMs observed upon progenitor-specific Htl activation or *Ths* overexpression is mediated by active TORC1 signaling through 4EBP, rather than Ras-Pnt signaling, which is required for PL differentiation.

Regulation of FGF signaling in the LG by HSPG

Intriguingly, *Ths* expression in the LG is highest in Pxn^{-} hemocyte progenitors (**Fig. 1H-J'**), and forcibly overexpressing *Ths* in the progenitor population is sufficient to induce almost complete differentiation of the LG (**Fig. 2F** and **K**). We reasoned that FGF ligand signaling in LG progenitors must be tightly regulated, both to allow the differentiation of peripheral progenitors that forms the maturing CZ during development, and also to prevent the premature

differentiation of medial prohemocytes before the onset of metamorphosis, when MZ progenitors differentiate and enter circulation (Grigorian et al., 2011b). One candidate modulator of FGF signaling in the LG is the *Drosophila* Perlecan homolog, Trol, a member of the heparan sulfate proteoglycan (HSPG) family (Park et al., 2003). Perlecans are mainly distributed in the extracellular matrix (ECM) and exclusively have heparan sulfate (HS) chains (Perrimon and Bernfield, 2000). FGF signaling is among the most well characterized pathways regulated by HSPGs, which serve at least two context-dependent functions in regulating signaling through FGFR. First, HSPGs can serve as a co-factor that stabilizes the FGF/FGFR interaction. Secondly, binding of FGF ligands to highly sulfated HS sequences can sequester FGFs, regulate their movement and diffusibility in the ECM, and also protect them from proteolytic degradation (Dowd et al., 1999; Saksela et al., 1988).

Previous studies have demonstrated that a dense *Trol*-positive ECM reticulum surrounds progenitors in the MZ at later L3 stages (Grigorian et al., 2011b). We hypothesized that signaling of FGF ligands in the LG might be modulated spatially and/or temporally through Trol. Consistent with this hypothesis, *Trol* downregulation in LG progenitors increases differentiation in the LG (68±14% Pxn-labeled LG hemocytes, **Fig. 8B** and **I**), compared to WT (42±5%, **Fig.8A** and **I**) by wL3. We next examined whether this increased differentiation can be attributed to altered FGF signaling in the LG. Reducing expression of *Ths* or *Pyr* upon downregulation of *Trol* in progenitors is sufficient to significantly reduce the proportion of Pxn^+ cells in the LG (31±8% and 29±8%, respectively, **Fig. 8D**, **F** and **I**) compared to WT ($p<0.05$) or downregulation of *Trol* alone ($p<0.0001$). However, the proportion of Pxn^+ hemocytes in these backgrounds is also significantly higher than *Ths* or *Pyr* downregulation alone ($p<0.0001$, **Figs 2K** and **8C** and **E**), suggesting that increased availability of both Ths and Pyr is required for the

differentiation response observed upon *Trol* downregulation. Furthermore, overexpression of Htl^{DN} upon progenitor-specific *Trol* downregulation is sufficient to significantly decrease the proportion of Pxn^{+} hemocytes (25±8%, **Fig. 8H-I**) compared to WT or *Trol*RNAi alone ($p<0.0001$), and is not significantly different than overexpression of Htl^{DN} alone (20±6%, $p>0.05$, **Figs 2K** and **8G**). This finding further suggests that the differentiation observed upon *Trol* downregulation is FGF/FGFR-mediated.

We next examined Trol expression at earlier stages to determine its distribution at the onset of hemocyte differentiation in the developing LG. In contrast to wL3 LGs, wherein high Trol expression is detected in the ECM surrounding MZ progenitors (Pxn^{-}) but is absent in the peripheral CZ (Pxn^{+}, inset, **Fig. 8J**), high Trol levels extend to the LG periphery during L2 (**Fig. 8K**). We also observed large pockets of Trol-negative tissue in the LG periphery which contained groups of early differentiating hemocytes during this stage (inset, **Fig. 8K**). Interestingly, increased differentiation in the LG associated with progenitor-specific *Trol* downregulation is already observed by L2 and eL3 stages (**Fig. 8M** and **O**), compared to WT (**Fig. 8L** and **N**), culminating in the observed phenotype at wL3 (**Fig. 8B**). Altogether, our data suggests that Trol modulates Htl-FGFR signaling in the LG to prevent precocious prohemocyte differentiation. This likely occurs via sequestration of FGF ligands in the ECM until they are required to induce hemocyte differentiation during LG development .

Discussion

In this study we highlight a crucial role for Htl FGFR signaling in LG blood progenitors for maintaining the homeostatic balance of MZ progenitors and differentiated hemocytes of the CZ. Whereas activation of Htl signaling in blood progenitors dramatically increases differentiation at the expense of the MZ, reducing Htl signaling in progenitors impairs CZ formation. To date, the signals that induce progenitor differentiation and CZ formation during larval LG development remain largely unknown. Hyperactivation of TORC1 signaling by progenitor-specific downregulation of its inhibitors TSC and PTEN autonomously increases LG differentiation at late larval stages (Dragojlovic-Munther and Martinez-Agosto, 2012). However, reducing TORC1 signaling also increases differentiation in the LG at the expense of prohemocytes (Benmimoun et al., 2012; Dragojlovic-Munther and Martinez-Agosto, 2012; Shim et al., 2012). Furthermore, while *Pvf2* overexpression also autonomously increases progenitor differentiation (this study), downregulation of *Pvf2* in progenitors does not affect their differentiation (Mondal et al., 2011). These findings demonstrate that neither Pvf2 nor TORC1 are required for LG progenitor differentiation developmentally. Our analysis of Pyr/Ths-Htl signaling through Pnt uncovers a progenitor-specific signaling network that is not only sufficient, but is also required, for PL differentiation and CZ formation in the LG.

The mechanisms that operate to regulate the population of undifferentiated *dome*$^{+}$ hemocyte progenitors in the LG secondary lobes have not been characterized, but maintenance of this hemocyte population provides a reservoir of progenitors during larval development. Our functional analysis of Pyr and Ths in *dome*$^{+}$ progenitors demonstrated the sufficiency of these ligands to autonomously induce both differentiation and hypertrophy of LG secondary lobes.

Heartless signaling in the LG is mediated by Pnt and Ush

Distinct roles in different hemocyte populations have been identified for the single *Drosophila* FOG transcriptional regulator, Ush, during hematopoiesis. These include roles as a negative regulator of CC lineage specification during embryonic hematopoiesis (Fossett et al., 2001), as well as a regulator of the number of circulating larval hemocytes (Sorrentino et al., 2007) and an inhibitor of LM transformation of embryo-derived circulating PLs (Avet-Rochex et al., 2010). Ush is strongly expressed in the LG MZ, and it has previously been proposed to regulate prohemocyte maintenance (Gao et al., 2009). In this study we performed blood progenitor-specific *Ush* downregulation in the LG, which dramatically increases the size of the MZ at the expense of CZ PLs and CCs, but also induces LM differentiation. These data complement previous studies that have demonstrated a reduction of PLs but significant induction of LMs in LGs from *Ush* transheterozygous mutant larvae (Gao et al., 2009).

The similarity of phenotypes induced by *Ush* genetic manipulation in blood progenitors compared to that of *Htl*, *Pyr*, and *Ths* suggested a potential interaction of Ush with Htl signaling in blood homeostasis. Previous studies have demonstrated a genetic interaction between *Ush* and *Htl* in the context of early mesodermal cell migration, suggesting that these two genes function in a common genetic pathway (Fossett et al., 2000), although the mechanism remained unclear. Our data demonstrates that the GATA transcriptional co-factor, Ush, is required downstream of Htl signaling to induce differentiation of LG progenitors (**Fig. 8P**). A link between RTK-Ras activity and GATA transcriptional networks has also been demonstrated in vertebrates. Nearly half of non-small cell lung cancer (NSCLC) patients contain mutations in the RTK/Ras family, and recent studies demonstrated that GATA2 transcriptional activity is requisite for Ras-driven NSCLC cells (Kumar et al., 2012).

We also highlight similar effects of the *Drosophila* ETS protein Pnt and the FOG homolog Ush as transcriptional effectors regulating blood progenitor differentiation, and demonstrate that the differentiation of progenitors induced by activated Pnt requires Ush. Intriguingly, differentiation of the vertebrate myeloid megakaryocyte lineage requires cooperativity of ETS and FOG/GATA proteins for transcription of the megakaryocyte-restricted αIIb gene (Wang et al., 2002). Our study suggests that cooperativity between the *Drosophila* FOG and ETS proteins, Ush and Pnt, may also regulate the myeloid-restricted hemocyte differentiation that occurs in the LG (**Fig. 8P**), similar to vertebrates. Ush has been shown to bind to at least two *Drosophila* GATA factors, Serpent and Pannier (Haenlin et al., 1997; Waltzer et al., 2002), both of which have roles in blood development (Evans et al., 2003; Minakhina et al., 2011), yet it remains unclear how Ush and these GATA factors function together in hematopoiesis. Further studies are required to elucidate Ush/GATA transcriptional activity and its targets during *Drosophila* hematopoiesis, and the roles of Pnt in this process.

Distinct roles of Ras in LG hemocyte populations

Previous studies in *Drosophila* hematopoiesis have identified Ras as a potent effector of differentiated hemocyte proliferation, demonstrating dramatic increases in circulating hemocyte population numbers upon Ras activation (Asha et al., 2003; Evans et al., 2007; Zettervall et al., 2004). In this study we examined progenitor-specific Ras activation in the LG, allowing dissection of a distinct role for Ras activity in *Drosophila* larval hematopoiesis. Progenitor-specific Ras activation in the LG induces their precocious differentiation, expanding CZ size at the expense of the MZ (**Fig. 3**). In contrast to its proliferative effects on embryo-derived circulating hemocytes, Ras activation in LG progenitors does not affect LG size (**Fig. 3**). We have previously described TORC1 as a crucial regulator of early hemocyte progenitor

proliferation in the LG, dramatically increasing tissue size upon progenitor-specific TORC1 activation (Dragojlovic-Munther and Martinez-Agosto, 2012). Our data here suggests that this role of TORC1 in proliferating hemocyte progenitors is independent of Ras activity.

Unlike progenitor-specific Ras activation, clonal Ras activation in the LG autonomously induces hemocyte differentiation and also dramatically increases LG tissue size (**Fig. 5**). This increase in tissue size is likely due to the maintained expression of Ras^{Act} in differentiated clonal hemocytes. Our data thus describe a transition in Ras function in the LG, wherein Htl-induced Ras activation in hemocyte progenitors initially regulates their differentiation, after which, activation of Ras promotes proliferation of differentiated hemocytes, which may contribute to increasing CZ hemocyte numbers at late larval stages (Jung et al., 2005). Potential candidates for Ras activation in differentiated CZ hemocytes are EGFR, PVR, and/or ALK, which all induce differentiated hemocyte proliferation (Zettervall et al., 2004), but do not autonomously regulate progenitor differentiation in the MZ (**Fig. 4**).

Regulation of LM differentiation by Ths and Pyr

Previous studies in *Drosophila* hematopoiesis have identified a number of signals that contribute to LM differentiation, including PVR, ALK, Toll, and EGFR (Avet-Rochex et al., 2010; Sinenko et al., 2012; Zettervall et al., 2004), although these studies have been limited to manipulating gene expression in differentiated hemocytes (both circulating and in the LG) or within cells of the LG niche (PSC). Genetic alterations specifically in the LG prohemocyte population that induce LM differentiation remain poorly characterized. Progenitor-specific TORC1 activation through *Tsc2* downregulation is sufficient to induce significant LM differentiation throughout the LG (Dragojlovic-Munther and Martinez-Agosto, 2012). Here we identify that progenitor-specific overexpression of Ths^{WT} and Htl^{Act} in the LG also promotes

significant LM differentiation. Given that activation of Ras or Pnt in progenitors does not induce LM differentiation, we reasoned that Htl activation through Ths might branch to activate TORC1 signaling. Although FGFR signaling has never been shown to signal through TORC1 in *Drosophila*, recent studies have suggested synergy between Htl and TORC1 signaling during *Drosophila* post-embryonic gliogenesis (Avet-Rochex et al., 2012). Our study demonstrates that LM differentiation induced by Htl activation and *Ths* overexpression in blood progenitors is dependent on TORC1 and 4EBP (**Fig. 8P**), suggesting that the TORC1 axis may be particularly important in regulating progenitor-specific LM differentiation in the LG and confirming the ability of FGF signaling to activate TORC1.

Our study has also identified Pyr, Pnt and Ush as negative regulators of LM differentiation in LG blood progenitors, as their downregulation induces LM differentiation while inhibiting PL differentiation (**Fig. 8P**). This may be a useful mechanism for lineage restriction under circumstances in which progenitors primarily differentiate into LMs at the expense of other lineages, such as upon wasp infection (Krzemien et al., 2010; Rizki and Rizki, 1984). Further studies are required to elucidate the mechanism by which these pathway components impair LM differentiation, which likely involves repression of a signal that actively promotes LM differentiation.

Trol as a modulator of prohemocyte-autonomous Heartless signaling

Studies of FGFR signaling in *Drosophila* have largely demonstrated paracrine signaling of Bnl, Pyr and Ths to their respective receptors, which often occurs across different cell types. During embryonic mesoderm migration, ectoderm-derived Pyr and Ths signal to adjacent Htl-expressing mesoderm cells and regulate the collapse of the mesoderm tube onto the ectoderm as well as later monolayer formation after mesoderm spreading (McMahon et al., 2010). During

Drosophila embryonic tracheal development, expression of Bnl in small clusters of non-tracheal cells functions as a chemoattractant, activating Btl on tracheal cells (Sutherland et al., 1996). In the *Drosophila* eye imaginal disc neuronal-derived Ths regulates glial differentiation, and is preceded by either an autocrine or paracrine signal from glial-derived Pyr that regulates glial number and motility (Franzdottir et al., 2009). During *Drosophila* postembryonic brain gliogenesis, Pyr non-autonomously activates Htl on glia of the same subtype (i.e., perineural or cortex glia) to regulate their proliferation (Avet-Rochex et al., 2012). These all represent examples of non-autonomous FGFR signaling in *Drosophila* development.

In contrast, our data demonstrates a cell-autonomous block on hemocyte differentiation upon *Ths* or *Pyr* downregulation, phenocopying the cell-autonomous effects of disrupting Htl or its downstream effectors, Ras, Pnt and Ush, in hemocytes (**Fig. 5**). Furthermore, downregulation of *Pnt* in *Ths*- or *Pyr*- overexpressing hemocytes is sufficient to impair overall LG differentiation, suggesting that the ligand-expressing cells themselves require *Pnt* and thus respond to the Htl-induced differentiation signal (**Fig. 6**). Finally, we observed co-expression of *Ths* in Htl^{+} hemocytes. Together, these data suggest that in the LG, Htl/FGFR signaling may be activated cell autonomously by its ligands, representing a potential example of autocrine FGFR signaling that is rarely observed during *Drosophila* development. Given that both *Ths* and *Pyr* are required during normal LG development for progenitor differentiation and CZ formation, we hypothesize that autocrine signaling may allow for tightly regulated and localized differentiation, allowing potent FGF ligands to only induce differentiation autonomously.

Given this autonomous induction of differentiation by Ths and Pyr, it is surprising to observe *Ths* expression in many undifferentiated (Pxn^{-}) hemocyte progenitors from L2 through wL3. *Ths* expression extends peripherally, adjacent to differentiating hemocytes, both early in

development and just before pupation at wL3. Furthermore, the impaired CZ formation upon progenitor-specific *Ths* downregulation suggested to us that the most peripheral *dome*$^{+}$/*Ths*$^{+}$ cells may be actively signaling to induce differentiation in peripheral progenitors, which then turn off expression of *Ths* following differentiation. Such highly regulated Htl signaling would thus require a mechanism by which to sequester FGF ligands until a progenitor cell is prompted to start differentiating. We hypothesized that the *Drosophila* HSPG, Trol, could serve this function of modulating/sequestering available FGF ligands. In the LG, a dense *Trol*-positive ECM reticulum surrounds MZ progenitors, whereas in the CZ much of this ECM has disappeared. Reduced expression of *Trol* in the MZ increases prohemocyte differentiation at early and late LG stages, and this effect is rescued by impairing Htl signaling. Our findings are consistent with previous studies that have shown GOF phenotypes in *fgf* mutants with reduced affinity to HS (Harada et al., 2009; Makarenkova et al., 2009), and suggest that Trol contributes to maintain a reservoir of sequestered FGFs in the LG (**Fig. 8P**), which become available upon disruption of *Trol* expression.

Formation of the CZ in a WT LG occurs in a stereotypical manner, requiring tight regulation of differentiation both temporally and spatially, yet the signals that regulate this process remained unclear. Our study suggests that a highly regulated release of sequestered FGF ligand, perhaps via expression of heparanases, may be responsible for regulating progenitor differentiation and for patterning CZ formation. Further studies are required to investigate which signals are responsible for mediating such a stereotypical release of FGFs during LG development. Additionally, the continued presence of *Ths* in MZ prohemocytes by wL3 also suggests that FGF ligands may become available following the onset of metamorphosis, contributing to the rapid differentiation of LG progenitors that occurs before their release into

circulation upon pupation (Grigorian et al., 2011b). Consistent with this finding, the strong *Trol* expression observed in the MZ late in larval development is reduced at the onset of pupation, when metamorphosis begins (Grigorian et al., 2011b). Finally, a reservoir of immobilized FGF ligand, present in LG progenitors throughout development, may also be utilized to induce the rapid differentiation of hemocytes observed in response to larval infection or stress conditions (Krzemien et al., 2010; Lemaitre and Hoffmann, 2007; Shim et al., 2012).

Previous studies in the LG have revealed signaling networks that actively maintain progenitors, uncovering niche-, progenitor-, and differentiated hemocyte-derived maintenance signals that serve this function (Mandal et al., 2007; Mondal et al., 2011; Sinenko et al., 2009). Our study highlights the role of a Htl-Ras-Pnt signaling pathway that is both required and sufficient for progenitor differentiation and CZ formation. We uncover a novel mechanism of progenitor maintenance in the LG, wherein sequestration of a differentiation signal developmentally also supports progenitor maintenance and prevents their precocious differentiation. This mechanism of ligand sequestration may be relevant in vertebrate hematopoiesis, wherein FGFs have been mainly implicated in myeloid-lineage restricted progenitors and cell types (Allouche and Bikfalvi, 1995; Berardi et al., 1995; Moroni et al., 2002). Furthermore, signaling through ETS, FOG and the TOR pathway may be important in conditions of dysregulated FGF signaling, such as acute myeloid leukemias (AMLs, Bieker et al., 2003; Karajannis et al., 2006).

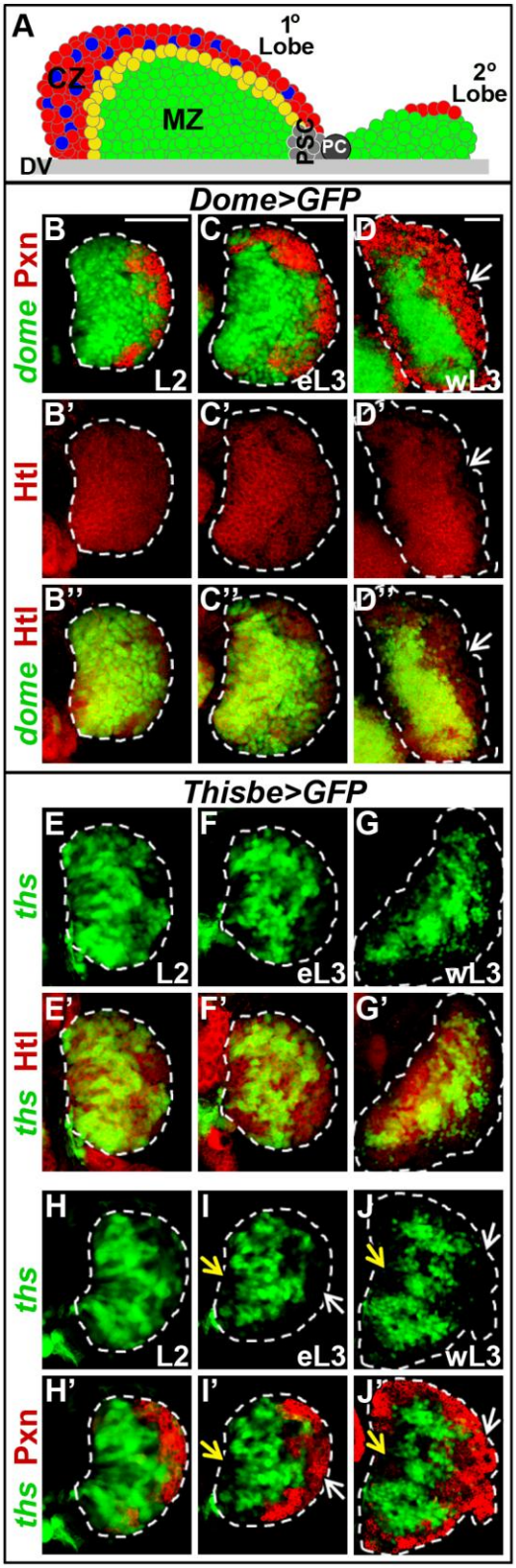

Figure 3-1. Htl/FGFR is expressed in the lymph gland

Figure 3-1. Htl/FGFR is expressed in the lymph gland

In panels **B-D''**, *dome-Gal4* drives *UAS-2xEGFP* (green), whereas in panels **E-J'** *ths-Gal4* drives *UAS-2xEGFP* (green). The early differentiation marker Pxn is shown in red in panels **B**, **C**, **D**, **H'**, **I'**, and **J'** and Htl FGFR is shown in red in panels **B'-B''**, **C'-C''**, **D'-D''**, **E'**, **F'** and **G'**.

(**A**) Schematic diagram of a wL3 LG primary and secondary lobe. Differentiated PLs (red) and CCs (blue) localize to the peripheral CZ. Prohemocytes (green) are compactly arranged in the MZ. PSC cells (grey) localize to the posterior tip of the primary lobe. Secondary lobes mostly consist of undifferentiated progenitors (green) with few differentiated hemocytes (red). Pericardial cells (PC) intercalate between the primary and secondary lobes. DV=dorsal vessel.

(**B-C''**) At the second larval instar stage (L2), few differentiating hemocytes (red, **B**) are observed in the LG, but by early third instar (eL3), an early CZ begins to form at the LG periphery (**C**). Htl expression (red, **B'-B''** and **C-C''**) is detected throughout the LG at these early stages.

(**D-D''**). By wandering third instar (wL3) a mature CZ has formed (**D**). Htl is expressed throughout the population of *dome*$^{+}$ progenitors in the MZ (**D'-D''**) but is absent in a subset of the most peripheral (*dome*$^{-}$) CZ hemocytes (white arrows).

(**E-G'**) At L2, *Ths* (green) expression is detected in most LG hemocytes, except for a few peripheral hemocytes (**E**). By L3, *Ths* expression becomes more restricted and is not detected at medial or peripheral LG borders (**F** and **G**). Overlap (yellow) of *Ths* (green) and Htl (red) expression is observed in LG hemocytes at all stages (**E-G'**).

(**H-J'**) Peripheral *Ths*$^{-}$ hemocytes often correlate with differentiating (**H-I'**) or differentiated (**J-J'**) Pxn^{+} hemocytes (red, white arrows). By L3 stages, *Ths*$^{-}$/Pxn^{-} hemocytes are also observed in medial regions (yellow arrows).

Scale bars=50μm. Scale bar in **B** corresponds to L2 stages (**B-B''**, **E-E'**, and **H-H'**). Scale bar in **C** corresponds to eL3 stages (**C-C''**, **F-F'**, and **I-I'**). Scale bar in **D** corresponds to wL3 stages (**D-D''**, **G-G'**, and **J-J'**).

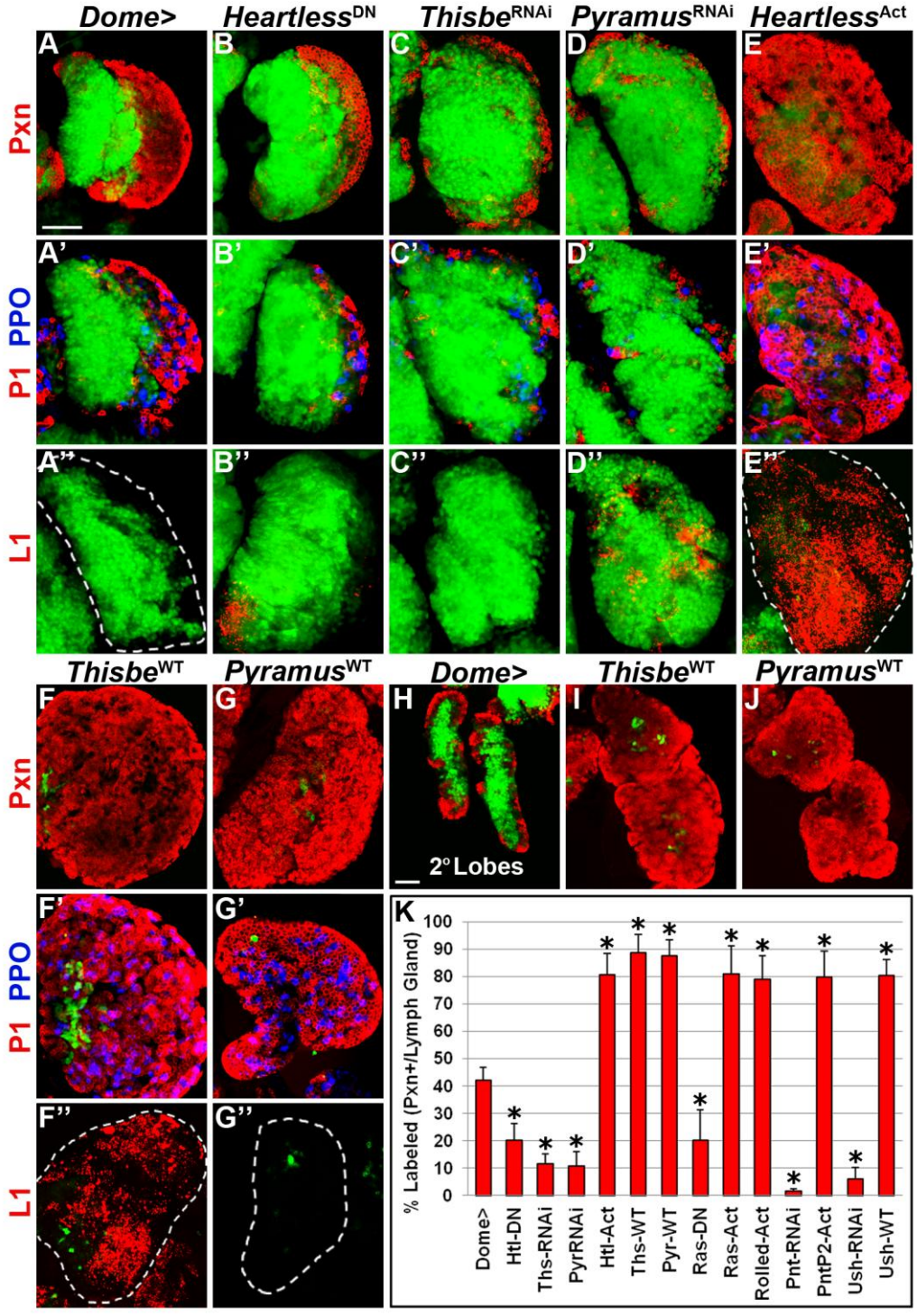

Figure 3-2. Htl/FGFR and its ligands regulate progenitor differentiation in the lymph gland

Figure 3-2. Htl/FGFR and its ligands regulate progenitor differentiation in the lymph gland

All panels represent wL3. In all panels, *dome-Gal4, UAS-2xEGFP* (WT, **A-A''**, **H**) is used to express *UAS-Htl*^{DN} (**B-B''**), *UAS-Ths*^{RNAi} (**C-C''**), *UAS-Pyr*^{RNAi} (**D-D''**), *UAS-Htl*^{Act} (**E-E''**), *UAS-Ths*^{WT} (**F-F''**, **I**), and *UAS-Pyr*^{WT} (**G-G''**, **J**), in prohemocytes (green). Differentiation markers are labeled to the left of corresponding rows.

(**A-A''**) WT LG primary lobe. Pxn (red, **A**) is expressed in differentiated hemocytes (*dome*$^{-}$) as well as in a small population of *dome*$^{+}$/Pxn^{+} intermediate progenitors at the MZ/CZ boundary. P1 (red, **A'**) and PPO (blue, **A'**) label terminally differentiated PLs and CCs, respectively. L1 (red, **A''**) marks LMs, not present in WT.

(**B-D''**) Overexpression of *Htl*^{DN} (**B**) or downregulation of *Ths* (**C**) or *Pyr* (**D**) in prohemocytes impairs CZ formation. PLs and CCs are restricted to a thin peripheral layer compared to WT (compare **B'**, **C'**, and **D'** to **A'**). Reduced *Htl* function in prohemocytes induces rare LMs in the LG (**B''**), but *Ths* downregulation does not (**C''**), while *Pyr* downregulation induces many LMs to differentiate in the LG (**D''**).

(**E-G''**) Overexpression of *Htl*^{Act}, *Ths*^{WT} or *Pyr*^{WT} is sufficient to induce almost the complete loss of *dome*$^{+}$/Pxn^{-} prohemocytes (**E**, **F**, and **G**) due to premature differentiation into PLs and CCs (**E'**, **F'**, and **G'**). While significant LM differentiation occurs upon *Htl*^{Act} (**E''**) or *Ths*^{WT} (**F''**) overexpression in prohemocytes, LMs are not observed upon *Pyr*^{WT} overexpression (**G''**).

(**H**) WT LG secondary lobes. LG secondary lobes consist mostly of undifferentiated *dome*$^{+}$/Pxn^{-} hemocytes, although a few differentiating Pxn^{+} hemocytes are observed at the periphery.

(**I-J**) Overexpression of *Ths*^{WT} (**I**) or *Pyr*^{WT} (**J**) in *dome*$^{+}$ hemocytes is sufficient to induce severe hypertrophy and differentiation of LG secondary lobes.

(**K**) Quantification of Pxn^{+} hemocyte population distribution (% area) in the LG. Data are represented as mean ± s.d., n=10. Asterisks denote statistically significant results compared to WT (*dome*>), measured by Student's *t*-test.

Scale bars=50µm. Scale bar in **A** corresponds to **A-G''** except for the following additional magnifications: X0.8 for **F** and **F'** and X0.7 for **E''** and **F''**. Scale bar in **H** corresponds to **H-J**.

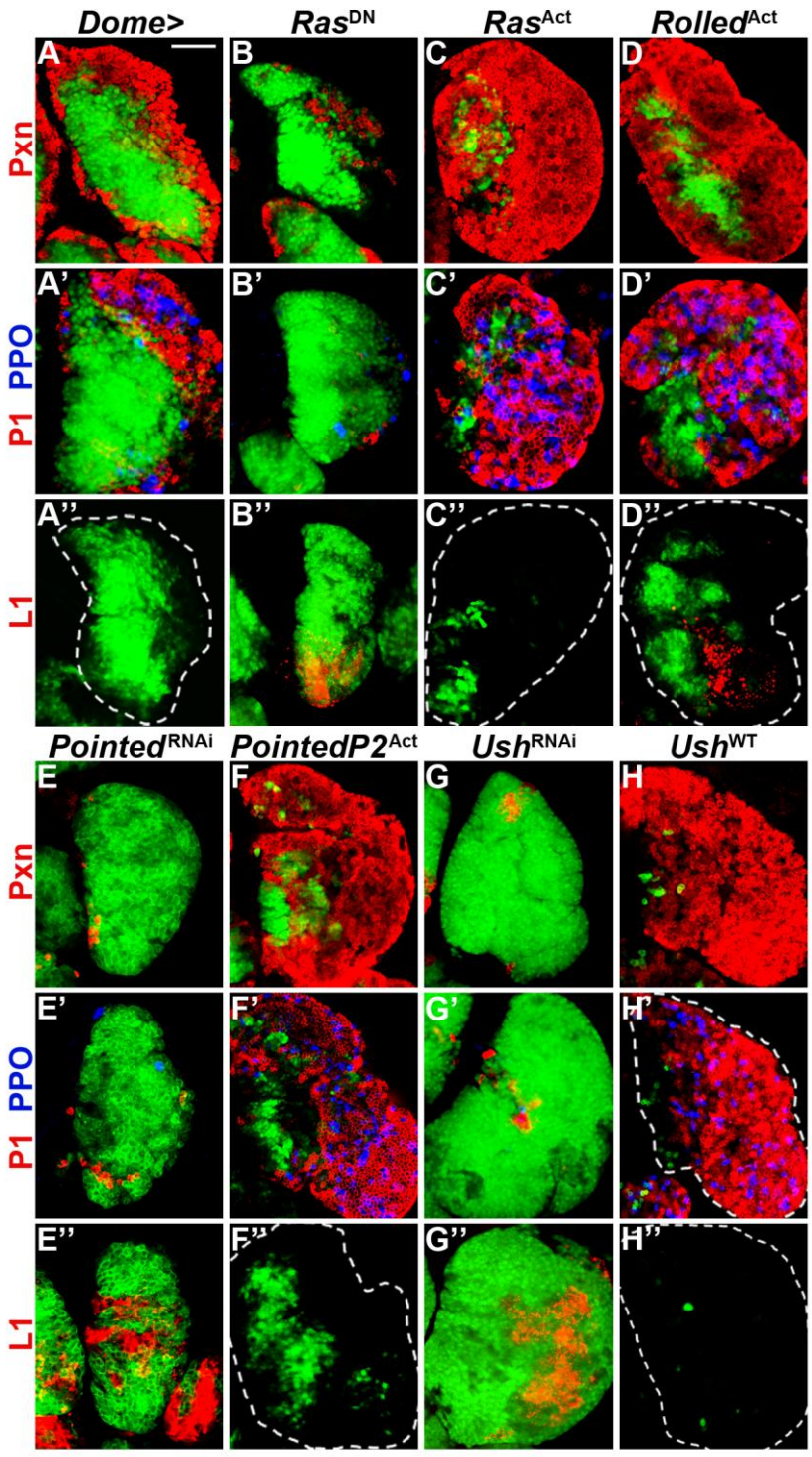

Figure 3-3. Ras-MAPK, Pointed and U-shaped regulate lymph gland progenitor differentiation

All panels represent wL3. In all panels, *dome-Gal4, UAS-2xEGFP* (WT, **A-A''**) is used to express *UAS-Ras*^{DN} (**B-B''**), *UAS-Ras*^{Act} (**C-C''**), *UAS-Rolled*^{Act} (**D-D''**), *UAS-Pnt*^{RNAi} (**E-E''**), *UAS-PntP2*^{Act} (**F-F''**), *UAS-Ush*^{RNAi} (**G-G''**) and *UAS-Ush*^{WT} (**H-H''**) in prohemocytes (green). Differentiation markers are indicated in **A-A''** for corresponding rows.

(**A-A''**) WT LG. The populations of Pxn^{+} hemocytes (**A**), PLs and CCs (**A'**) and LMs (**A"**) are labeled.

(**B-B''**) Overexpression of *Ras*^{DN} in prohemocytes reduces CZ size (**B**), limiting terminal PL and CC differentiation (**B'**) while allowing rare LMs to differentiate (**B''**).

(**C-D''**) Overexpression of *Ras*^{Act} or *RolledMAPK*^{Act} in prohemocytes increases CZ size at the expense of the MZ (**C** and **D**, respectively) and causes PL and CC differentiation in medial LG regions (**C'** and **D'**). LMs are not observed upon Ras activation (**C''**), but a small number of LMs differentiate upon Rolled activation (**D''**).

(**E-H''**) Downregulation of *Pnt* (**E**) and *Ush* (**G**) impair CZ formation and dramatically reduce PL or CC differentiation (**E'** and **G'**), while inducing significant LM differentiation throughout the LG (**E''** and **G''**). Overexpression of *PntP2*^{Act} (**F**) or *Ush*^{WT} (**H**) in prohemocytes increases CZ size at the expense of the MZ. While PLs and CCs are observed throughout the LG (**F'** and **H'**), LMs are not observed (**F"** and **H"**).

Scale bar=50μm corresponds to all panels except for the following additional magnifications: X0.9 for **F**, X0.8 for **D** and **H'**, and X0.7 for **F'**.

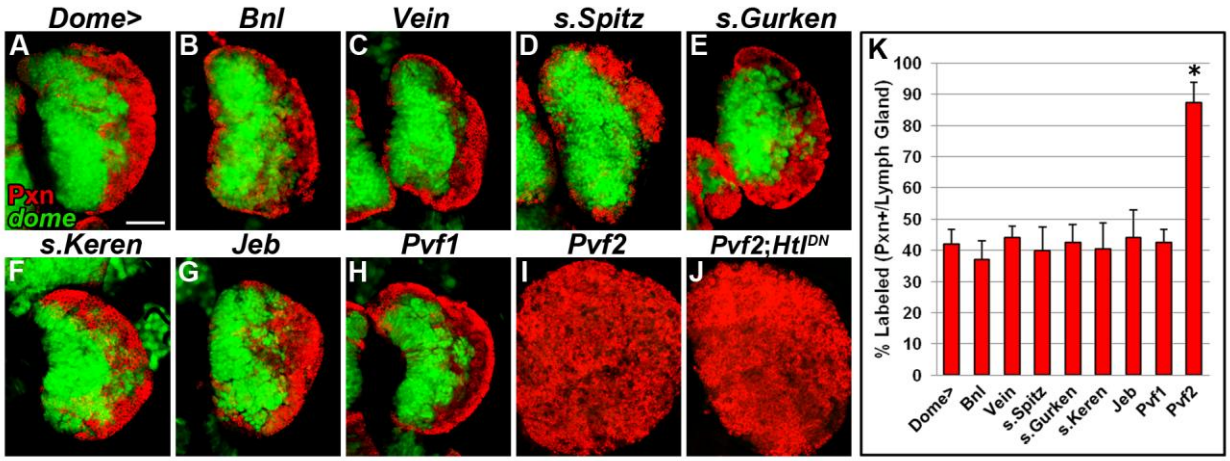

Figure 3-4. RTK signaling effects on differentiation are specific to Pyramus and Thisbe

All panels represent wL3. In all panels, *dome-Gal4, UAS-2xEGFP* is used to express the specified genetic constructs in prohemocytes (green). Pxn expression is shown in red.

(**A**) WT LG.

(**B-G**) Overexpression of the *Drosophila* FGFR ligand *Bnl* (**B**), the EGFR ligands, *Vein* (**C**), *secreted-Spitz* (*s.Spitz*, **D**), *secreted-Gurken* (*s.Gurken*, **E**), or *secreted-Keren* (*s.Keren*, **F**), or the ALK ligand *Jeb* (**G**) in prohemocytes does not affect size of the CZ.

(**H-I**) Overexpression of the PVR ligand, *Pvf1* (**H**), in prohemocytes does not affect LG differentiation, but overexpression of *Pvf2* (**I**) in prohemocytes induces their differentiation, dramatically increasing CZ size at the expense of MZ progenitors.

(**J**) Overexpression of Htl^{DN} upon *Pvf2* overexpression in prohemocytes does not impair the dramatic increase in differentiation observed upon *Pvf2* overexpression alone (**I**).

(**K**) Quantification of Pxn^{+} hemocyte population distribution (% area) in the LG. Data are represented as mean ± s.d., n=10. Asterisk denotes statistically significant result ($p<0.0001$) compared to WT (*dome>*), measured by Student's *t*-test.

Scale bar=50μm and corresponds to all panels except for the following additional magnifications: X0.5 for **I**, X0.8 for **D**, X0.9 for **J**, and X1.2 for **B**.

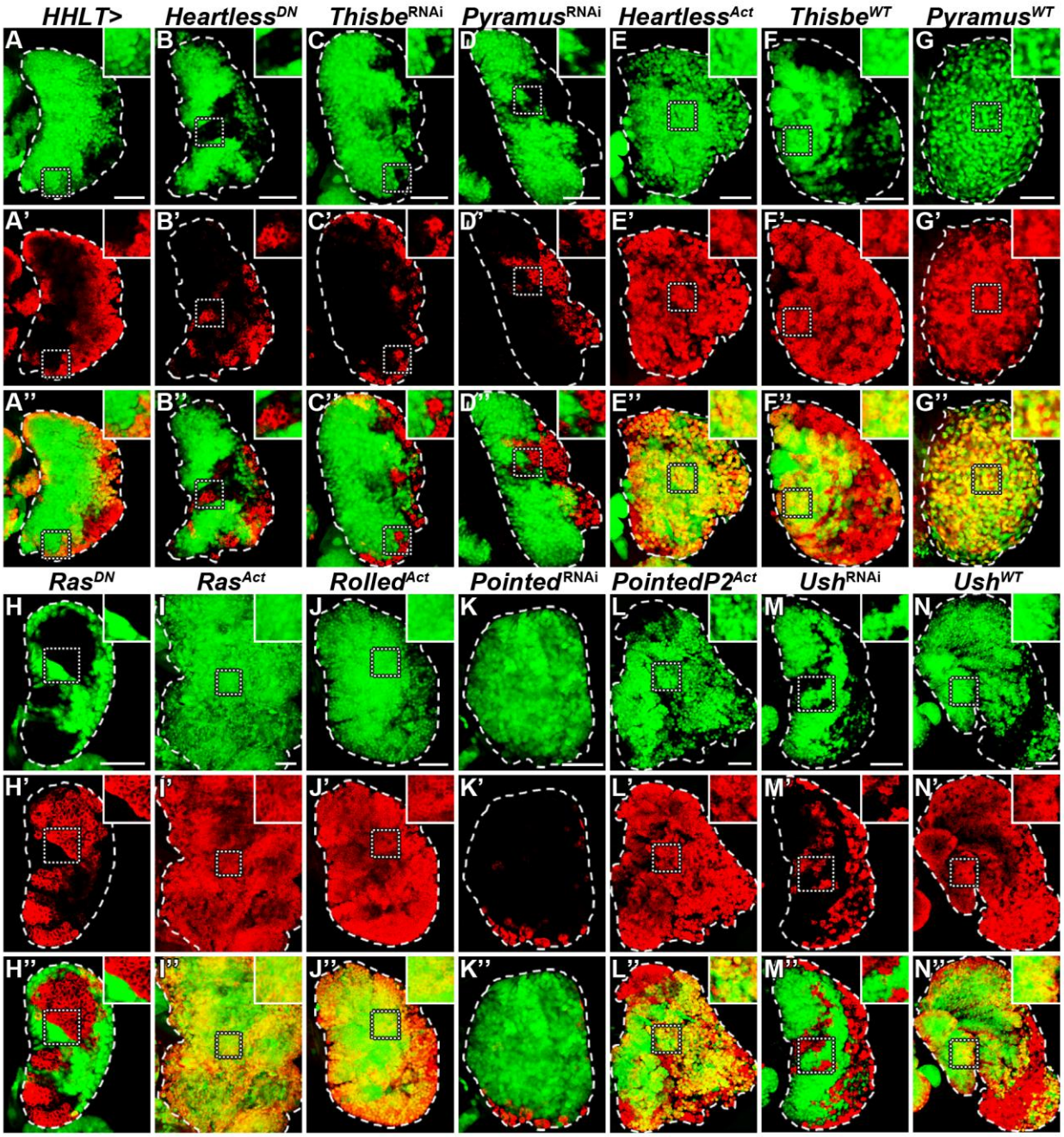

Figure 3-5. Cell-autonomous effects of Htl signaling on LG prohemocytes

All panels represent wL3. In all panels, G-TRACE (*hand-gal4, hml-gal4, UAS-2xEGFP, UAS-FLP; A5C-FRT-STOP-FRT-gal4*) is used to clonally express the specified genetic constructs,

specifically in the LG. Clonal cells are marked with GFP (green) and Pxn expression is shown in red.

(**A-A’’**) WT LG. WT clones are often localized to medial LG regions, and clonal cells (green) express Pxn (red) only in peripheral CZ regions (yellow).

(**B-D’’**) Clonally overexpressing Htl^{DN} (**B-B’’**) or downregulating *Ths* (**C-C’’**) or *Pyr* (**D-D’’**) in the LG restricts hemocyte differentiation to WT cells outside of the clone, demonstrating an autonomous block on differentiation.

(**E-G’’**) Clonally overexpressing Htl^{Act} (**E-E’’**), Ths^{WT} (**F-F’’**), or Pyr^{WT} (**G-G’’**) in LG hemocytes autonomously induces differentiation in clonal cells.

(**H-I’’**) Clonal overexpression of Ras^{DN} (**H-H’’**) autonomously impairs hemocyte differentiation, while clonal activation of Ras signaling (**I-I’’**) autonomously induces hemocyte differentiation and also dramatically increases tissue size.

(**J-J’’**) Clonal activation of Rolled MAPK autonomously induces hemocyte differentiation.

(**K-N’’**) Clonal downregulation of *Pnt* (**K-K’’**) or *Ush* (**M-M’’**) in the LG autonomously inhibits hemocyte differentiation, while clonally overexpressing $PntP2^{Act}$ (**L-L’’**) or Ush^{WT} (**N-N’’**) autonomously induces differentiation in clonal cells.

Scale bars=50μm.

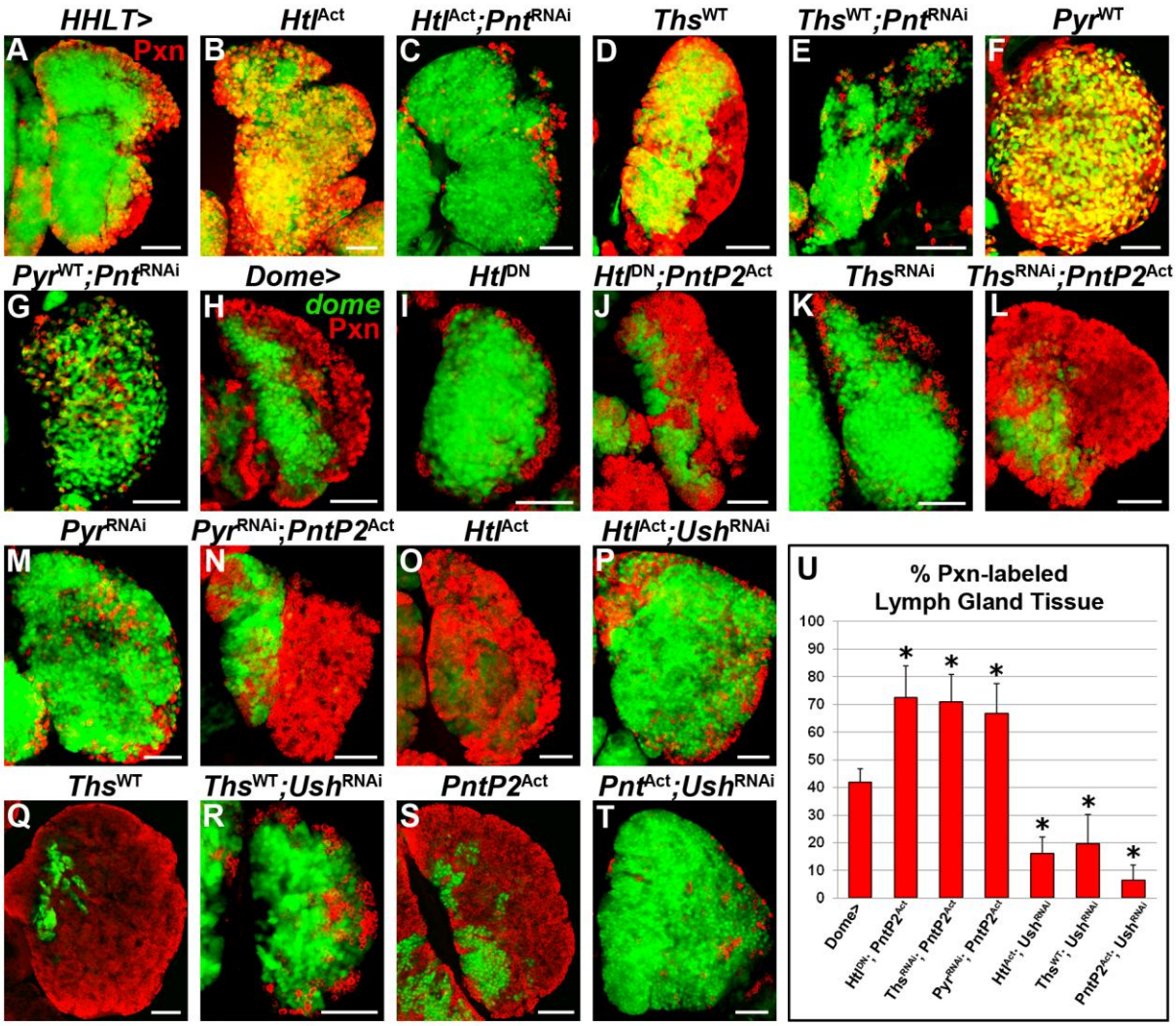

Figure 3-6. Pnt and Ush function downstream of Htl in LG progenitors

All panels represent wL3. In panels **A-G**, G-TRACE (*hand-gal4, hml-gal4, UAS-2xEGFP, UAS-FLP; A5C-FRT-STOP-FRT-gal4*) is used to clonally express the specified genetic constructs (green text) in LG hemocytes. In panels **H-T**, *dome-Gal4, UAS-2xEGFP* is used to express the specified genetic constructs (black text) in prohemocytes. GFP (green) labels clonal cells in **A-G** and prohemocytes in **H-T**. Pxn expression is shown in red.

(**A**) WT LG. WT clonal cells (green) express Pxn (red) only in peripheral CZ regions (yellow).

(**B-G**) Clonal overexpression of Htl^{Act} (**B**), Ths^{WT} (**D**), or Pyr^{WT} (**F**) in the LG autonomously induces hemocyte differentiation. Clonal downregulation of *Pnt* upon overexpression of Htl^{Act} (**C**), Ths^{WT} (**E**), or Pyr^{WT} (**G**) is sufficient to autonomously block hemocyte differentiation of clonal cells.

(**H**) WT LG. WT expression of *dome* (green) in the MZ and Pxn (red) in the CZ.

(**I-N**) Overexpression of Htl^{DN} (**I**) or downregulation of *Ths* (**K**) or *Pyr* (**M**) in prohemocytes impairs prohemocyte differentiation and CZ formation. Overexpression of $PntP2^{Act}$ upon Htl^{DN} (**J**), Ths^{RNAi} (**L**) or Pyr^{RNAi} (**N**) expression in prohemocytes is sufficient to induce their precocious differentiation.

(**O-T**) Progenitor-specific overexpression of Htl^{Act} (**O**), Ths^{WT} (**Q**), or $PntP2^{Act}$ (**S**) increases differentiation in the LG at the expense of prohemocytes. Downregulation of *Ush* upon overexpression of Htl^{Act} (**P**), Ths^{WT} (**R**), or $PntP2^{Act}$ (**T**) in prohemocytes impairs their differentiation, hindering CZ formation.

(**U**) Quantification of Pxn^{+} hemocyte population distribution (% area) in the LG. Data are represented as mean ± s.d., n=10. Asterisks denote statistically significant results compared to WT (*dome>*), measured by Student's *t*-test.

Scale bars=50µm.

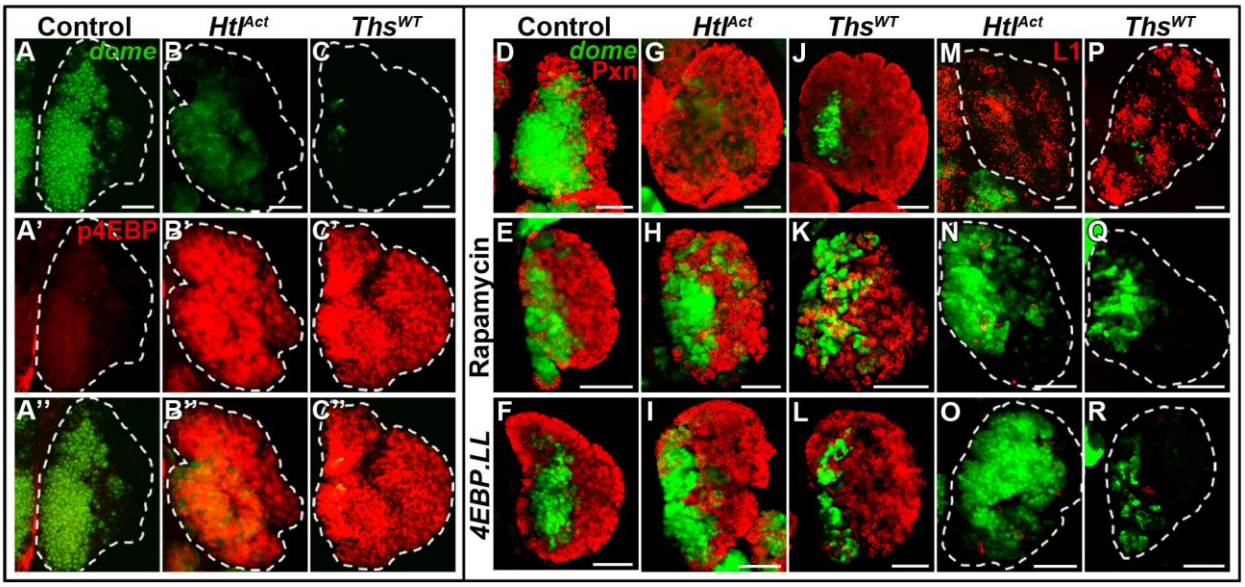

Figure 3-7. Activation of TOR signaling upon Htl activation

All panels represent wL3. In all panels, *dome-Gal4, UAS-2xEGFP* (WT, **A-A''** and **D**) is used to express the specified genetic constructs in prohemocytes (green). P4EBP is labeled in red in panels **A'-C''**, Pxn is labeled in red in panels **D-L**, and L1 is labeled in red in panels **M-R**.

(**A-A''**) WT LG. P4EBP is expressed at low levels in medial regions of the LG.

(**B-C''**) Overexpression of Htl^{Act} (**B-B''**) or Ths^{WT} (**C-C''**) in prohemocytes increases p4EBP levels throughout the LG.

(**D**) WT LG.

(**E**) Reducing TORC1 signaling via systemic Rapamycin treatment increases CZ size at the expense of the MZ.

(**F**) Overexpression of [*d4EBP(LL)*] in prohemocytes increases differentiation in the LG.

(**G**) Overexpression of Htl^{Act} in prohemocytes induces their differentiation.

(**H-I**) Rapamycin treatment (**H**) or overexpression of [*d4EBP(LL)*] (**I**) upon progenitor-specific Htl^{Act} overexpression partially restores zonation in the LG.

(**J**) Overexpression of *Ths*^{WT} in prohemocytes induces their differentiation.

(**K-L**) Rapamycin treatment (**K**) or [*d4EBP(LL)*] overexpression (**L**) upon progenitor-specific *Ths*^{WT} overexpression is not sufficient to block the increase in differentiation observed upon *Ths*^{WT} overexpression alone (**J**).

(**M-R**) Overexpression of *Htl*^{Act} (**M**) or *Ths*^{WT} (**P**) in prohemocytes induces LM differentiation throughout the LG, and systemic Rapamycin treatment (**N** and **Q**) or progenitor-specific overexpression of [*d4EBP(LL)*] (**O** and **R**) upon *Htl*^{Act} or *Ths*^{WT} overexpression, respectively, is sufficient to block this LM response.

Scale bars=50µm.

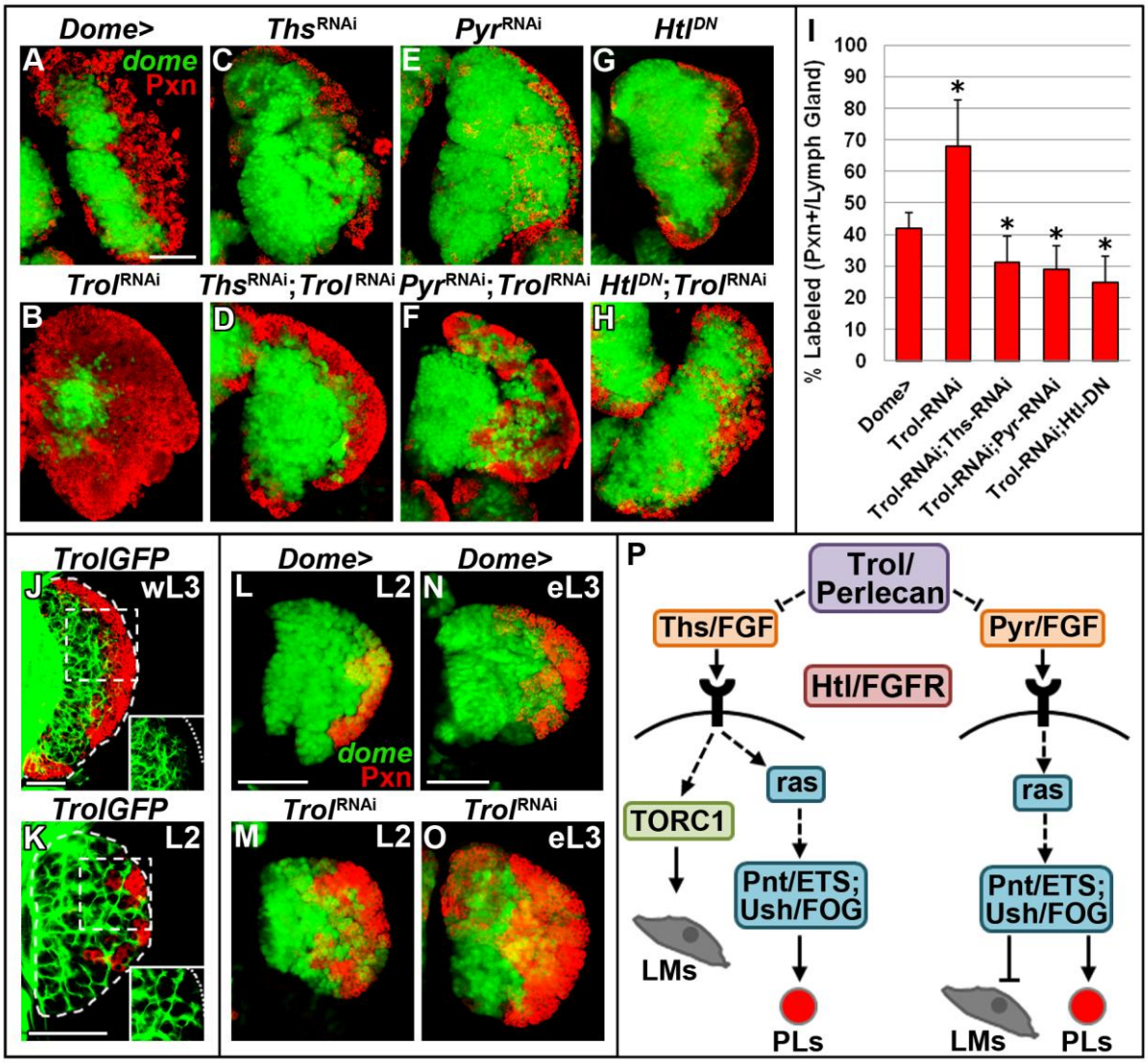

Figure 3-8. Regulation of FGF signaling in the LG by Trol

Panels **A-H** represent wL3. In panels **A-H** and **L-O**, *dome-Gal4, UAS-2xEGFP* (WT, **A**, **L** and **N**) is used to express the specified genetic constructs in prohemocytes (green). In panels **J-K**, the protein trap line ZCL1973X labels Trol with GFP expression. In all panels Pxn expression is shown in red.

(**A**) WT LG.

(**B**) Downregulating *Trol* expression in prohemocytes induces their differentiation into Pxn^{+} hemocytes, increasing the CZ at the expense of the MZ.

(**C-H**) Progenitor-specific downregulation of *Ths* (**C**) or *Pyr* (**E**) or overexpression of Htl^{DN} (**G**) reduces the differentiation of Pxn^{+} hemocytes, impairing CZ formation. Downregulation of *Ths* (**D**) or *Pyr* (**F**) or overexpression of Htl^{DN} (**H**) upon *Trol* downregulation in prohemocytes is sufficient to block the increased differentiation induced by *Trol* downregulation alone (**B**).

(**I**) Quantification of Pxn^{+} hemocyte population distribution (% area) in the LG. Data are represented as mean ± s.d., n=10. Asterisks denote statistically significant results compared to WT (*dome*>), measured by Student's *t*-test.

(**J**) At wL3, high levels of Trol are detected in the ECM amidst the population of Pxn^{-} hemocytes of the MZ, but Trol expression is not observed among Pxn^{+} hemocytes of the CZ (inset).

(**K**) During L2, high levels of Trol are observed in the ECM throughout the LG. Large pockets of Trol-negative tissue outline groups of early differentiating (Pxn^{+}) hemocytes.

(**L-O**) WT LGs at L2 (**L**) contain a small population of early differentiating hemocytes, which increases by eL3 (**N**) to begin forming the early CZ. Downregulation of *Trol* in prohemocytes expands the population of differentiating hemocytes during L2 and eL3 (**M** and **O**, respectively) compared to WT.

(**P**) Ths/Pyr-Htl signaling in *Drosophila* LG hemocyte progenitors. Ths- and Pyr-induced Htl activation in LG progenitors is modulated by Trol HSPG. Htl activation by its ligands induces PL differentiation in a Pnt- and Ush-dependent manner through Ras activation, affecting the ratio of MZ progenitors and CZ differentiated hemocytes in the LG. In contrast, LM differentiation in the LG is oppositely effected by Ths and Pyr: whereas Ths-specific Htl activation induces LM

differentiation in a TORC1-dependent manner, LM differentiation is inhibited by Pyr, Pnt and Ush.

Scale bars=50μm. Scale bar in **A** corresponds to panels **A-H**, except for the following additional magnifications: X0.75 for **E**, X0.8 for **B** and **H**, and X1.2 for **D**. Scale bar in **L** corresponds to **L-M**. Scale bar in **N** corresponds to **N-O**.

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Appendix

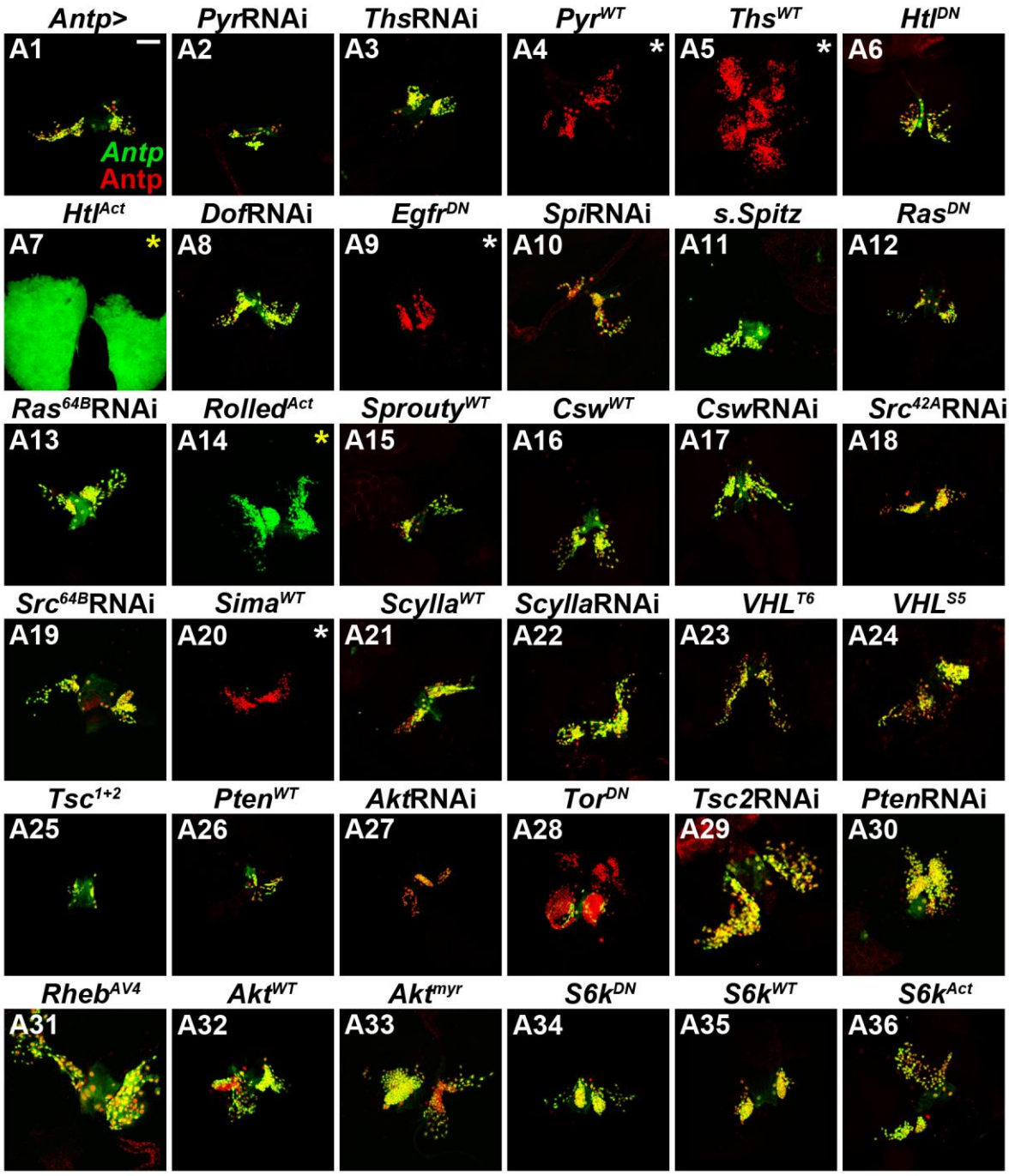


Figure A-1. Autonomous regulation of PSC cell number

All panels represent wL3 LGs. For all panels *Antp-gal4, UAS-GFP* labels PSC cells in green and is used to drive expression of the specified genetic constructs. Antp antibody (Ab) staining is shown in red. For panels **A4-A5**, **A9** and **A20** (white asterisks) a temperature-sensitive Gal80

repressor was used prior to the second instar stage in order to permit larval viability; $Antp^{+}$ cells are not labeled with GFP in these genetic backgrounds, but only with Antp Ab (red). For panels **A7** and **A14** (yellow asterisks) Antp Ab staining was not performed.

(**A1**) Wild-type (WT) PSC, demonstrating strong overlap of *Antp* and Antp Ab staining.

(**A2-A3**) Downregulation of the FGF ligand *Pyramus* (*Pyr*, **A2**), but not *Thisbe* (*Ths*, **A3**), in the PSC autonomously reduces PSC size.

(**A4-A5**) Overexpression of the FGF ligand *Ths* (**A5**), but not *Pyr* (**A4**), in the PSC robustly increases the number of $Antp^{+}$ PSC cells in the LG.

(**A6-A7**) Reducing Heartless (Htl) FGFR activity in the PSC by overexpression of Htl^{DN} does not change PSC size (**A6**), but Htl activation via overexpression of Htl^{Act} robustly increases PSC cell number (**A7**).

(**A8**) Downregulation of the FGFR-specific adaptor molecule, *Downstream of fgfr* (*Dof*), in the PSC does not alter PSC size.

(**A9**) Reducing Epidermal Growth Factor Receptor (EGFR) activity in the PSC by overexpression of $Egfr^{DN}$ does not alter PSC size.

(**A10-A11**) Downregulation (**A10**) or overexpression (**A11**) of the EGFR ligand, *Spitz* (*Spi*), in the PSC does not affect PSC cell number.

(**A12**) Reducing Ras activity in the PSC by overexpression of Ras^{DN} significantly reduces PSC size.

(**A13**) Downregulation of *Ras64B* in the PSC does not change PSC cell number.

(**A14**) Activation of Rolled MAPK in the PSC by overexpression of $Rolled^{Act}$ significantly increases PSC cell number.

(**A15**) Overexpression of *Sprouty*, an intracellular inhibitor of Ras signaling, in the PSC is sufficient to significantly reduce PSC size.

(**A16-A17**) Overexpression (**A16**) or downregulation (**A17**) of *Corkscrew* (*Csw*), a protein tyrosine phosphatase that functions as a positive signal transducer downstream of several receptor tyrosine kinases (RTKs), does not change PSC cell number.

(**A18-A19**) Downregulation of *Src42A* (**A18**) or *Src64B* (**A19**) in the PSC does not alter PSC cell number.

(**A20**) Overexpression of the *Drosophila* Hypoxia-Inducible Factor α (HIF-α) ortholog, *Sima*, in the PSC does not change PSC size.

(**A21-A22**) Overexpression of the *Drosophila* REDD1 homolog, *Scylla* (**A21**), a target of HIF/Sima, in the PSC does not change PSC size, but *Scylla* downregulation (**A22**) significantly increases PSC cell number.

(**A23-A24**) Overexpression of *von Hippel-Lindau*^{T6} (*VHL*^{T6}, **A23**) or *VHL*^{S5} (**A24**) in the PSC does not alter PSC cell number.

(**A25-A27**) Reducing Target of Rapamycin Complex 1 (TORC1) signaling in the PSC by overexpression of *Tsc*$^{1+2}$ (**A25**) or *Pten*^{WT} (**A26**) or downregulation of *Akt* (**A27**) significantly reduces PSC cell number.

(**A28**) Overexpression of *Tor*^{DN} in the PSC significantly increases the number of Antp^{+} cells (red) in the LG, while not autonomously affecting *Antp* expression (green).

(**A29-A31**) Hyperactivation of TORC1 signaling in the PSC by downregulation of *Tsc2* (**A29**) or *Pten* (**A30**) or overexpression of *Rheb*^{AV4} (**A31**) significantly increases the number of PSC cells in the LG.

(**A32-A33**) Overexpression of wild-type [Akt^{WT} (**A32**)] or activated [Akt^{myr} (**A33**)] Akt increases PSC size, expanding the number of $Antp^{+}$ cells (red) more than the number of $Antp^{+}$ cells (green).

(**A34-A36**) Overexpression of $S6k^{DN}$ (**A34**) or $S6k^{WT}$ (**A35**) in the PSC does not affect PSC size, but $S6k^{Act}$ overexpression increases PSC cell number (**A36**).

Scale bar = 50μm.

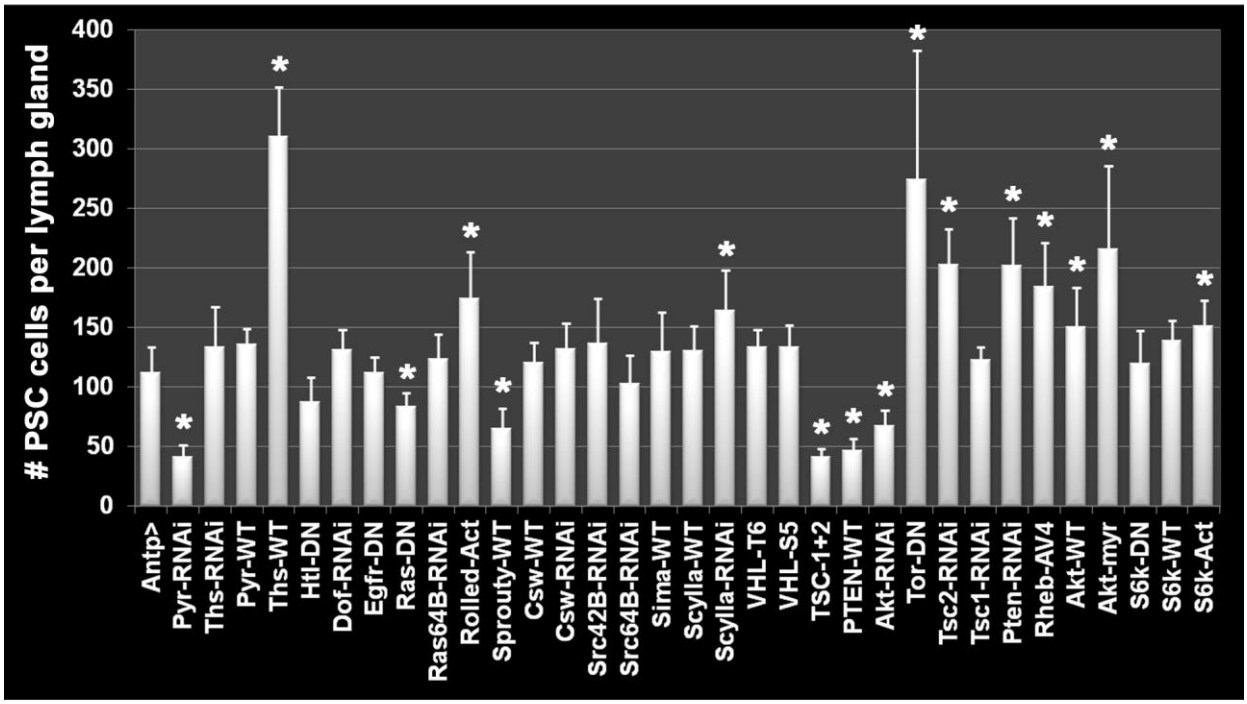


Figure A-2. Multiple genes autonomously change PSC cell number

Quantification of the number of PSC cells per lymph gland (both primary lobes) at wL3 upon expression of the specified genetic constructs in the PSC with *Antp-gal4*. Asterisks denote statistically significant results compared to controls (*Antp*>), measured by Student's *t*-test. Data are indicated as mean ± s.d., n=4-12.

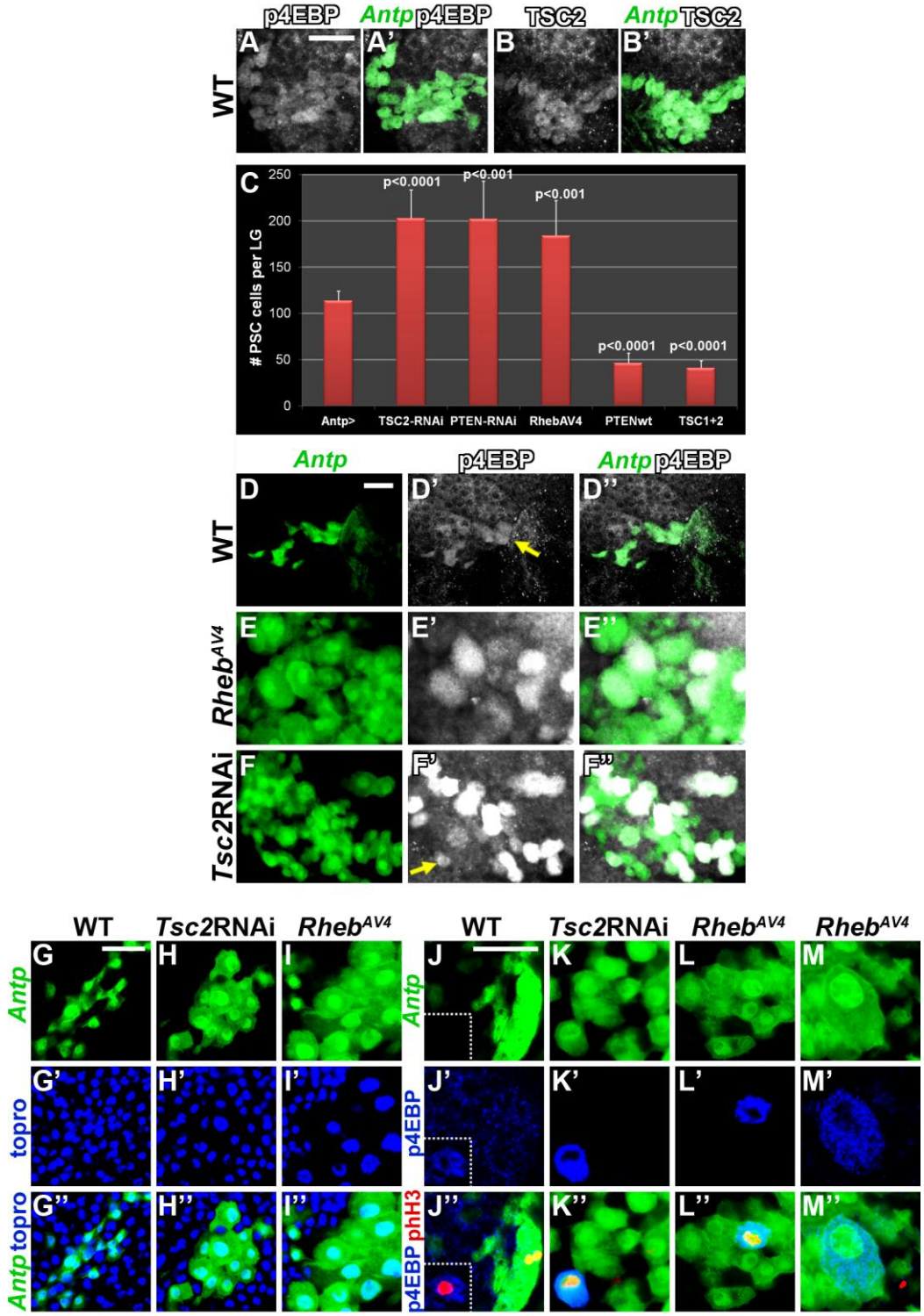


Figure A-3. Alterations to the PSC upon autonomous changes in TORC1 activity

Figure A-3. Alterations to the PSC upon autonomous changes in TORC1 activity

All panels represent wL3 LGs. For all panels, *Antp-gal4, UAS-GFP* labels PSC cells in green and is used to drive expression of the specified constructs.

(**A-B'**) WT PSC cells express phosphorylated-4EBP (p4EBP, white; **A-A'**) and TSC2 (white, **B-B'**).

(**C**) Quantification of the number of PSC cells per lymph gland (both primary lobes). Compared to WT (114 ± 10), *Tsc2* (203 ± 31) or *Pten* (202 ± 41) downregulation and *Rheb* overexpression (184 ± 38) significantly increases the number of PSC cells. In contrast, overexpression of Tsc^{1+2} (41± 8) or $Pten^{wt}$ (47± 11) significantly reduces the number of PSC cells. Data are indicated as mean ± s.d., n=8.

(**D-D"**) WT p4EBP (white) expression in the PSC (green). Arrow indicates nuclear localization of p4EBP in PSC cells.

(**E-F"**) Overexpression of *Rheb* (*Antp>*$Rheb^{AV4}$, **E-E"**) or *Tsc2* downregulation (*Antp>Tsc2*RNAi, **F-F"**) increases cell size autonomously in the PSC and induces $p4EBP^{high}$ expression in a subpopulation of cells.

(**G-I"**) The intensity of nuclear staining with TOPRO (blue) is not different between WT (**G-G"**) and *Tsc2*RNAi (**H-H"**) or $Rheb^{AV4}$ (**I-I"**) expressing PSC cells, suggesting that endoreplication does not occur in PSC cells upon TORC1 hyperactivation.

(**J-J"**) WT. Rare phospho-histone $H3^+$ ($phH3^+$, red) PSC cells are observed at wL3 (overlap between phH3 and *Antp* is yellow in **J"**) but do not express high levels of p4EBP (blue), unlike a $p4EBP^{high}/phH3^+$ prohemocyte from the same LG (insets).

(**K-M”**) A small number of p4EBP^{high} (blue) and phH3^{+} cells [red, but yellow from overlap with *Antp* (green)] are present upon *Tsc2* downregulation (**K-K”**) or *Rheb* overexpression (**L-L”**). Enlarged PSC cells that express elevated p4EBP do not divide (**M-M”**).

Scale bars = 20μm. Scale bar in **A** corresponds to **A-B’**. Scale bar in **D** corresponds to **D-F”**. Scale bar in **G** corresponds to **G-I”**. Scale bar in **J** corresponds to **J-M”**.

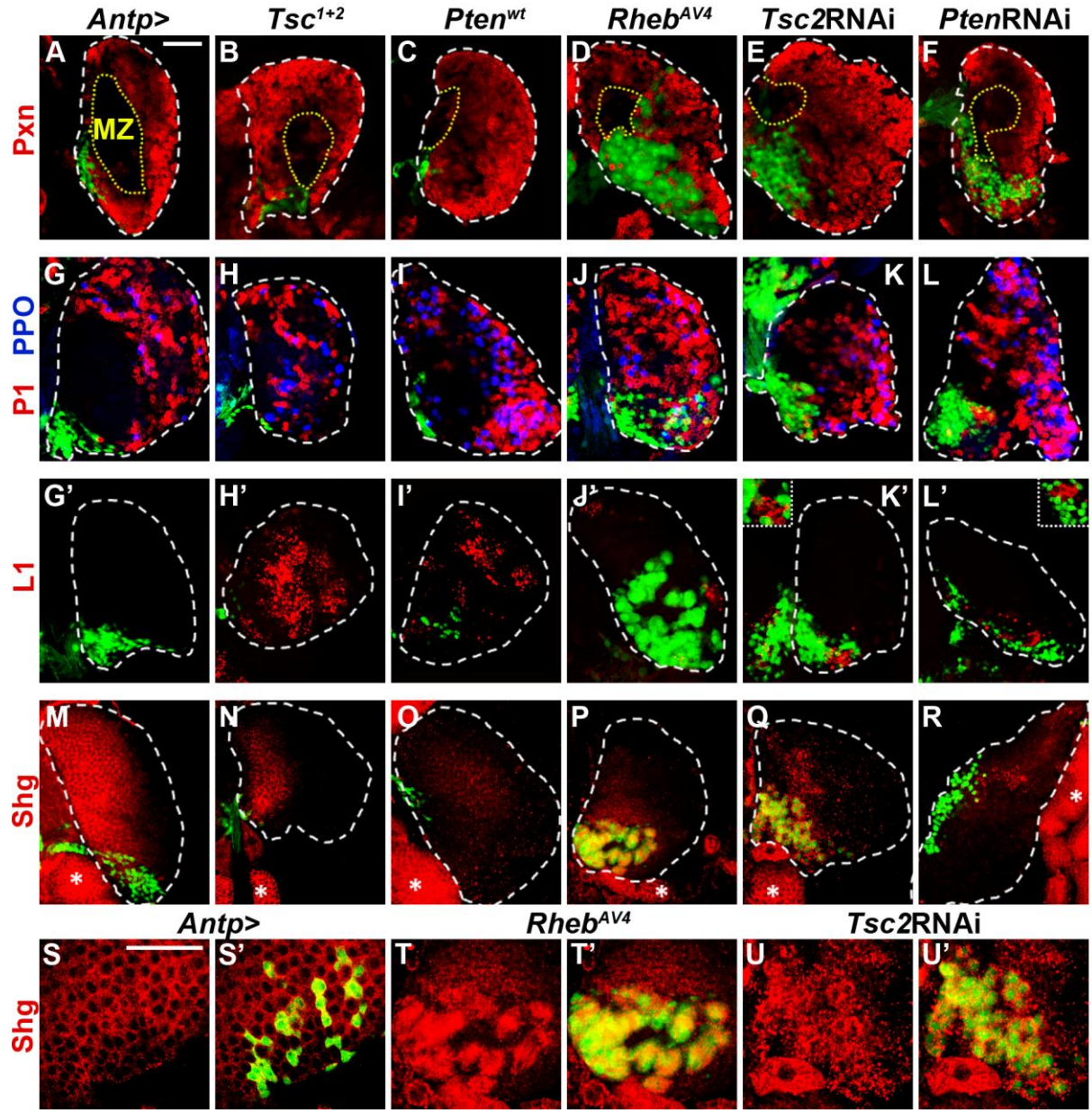


Figure A-4. Activating or reducing TORC1 activity in the PSC results in progenitor loss

All panels represent wL3 LGs. For all panels, *Antp-gal4, UAS-GFP* labels PSC cells in green and is used to drive expression of the specified constructs. Markers are specified to the left of corresponding rows.

(**A**) WT LG, showing the population of PSC cells (green) at the posterior tip of the LG, the MZ (dotted outline, yellow), and the early marker, Pxn (red), expressed in the CZ.

(**B-C**) Overexpression of Tsc^{1+2} (**B**) or $Pten^{wt}$ (**C**) in the PSC reduces the number of PSC cells, and increases the size of the CZ at the cost of the MZ. Overall LG size is not affected.

(**D-F**) Overexpression of *Rheb* (**D**) or downregulation of *Tsc2* (**E**) or *Pten* (**F**) in the PSC expands its size. In all cases, the CZ expands at the cost of the MZ.

(**G-G'**) WT LG, showing expression of markers of terminally differentiated hemocytes: P1 (plasmatocytes; red) and ProPO (crystal cells; blue) in (**G**) and absence of $L1^{+}$ lamellocytes (red) in (**G'**).

(**H-I'**) Reducing TORC1 signaling in the PSC via overexpression of Tsc^{1+2} (**H-H'**) or $Pten^{wt}$ (**I-I'**) subtly increases terminally differentiated plasmatocyte and crystal cell lineages (**H** and **I**) and also induces lamellocyte differentiation (**H'** and **I'**).

(**J-L'**) Hyperactivation of TORC1 signaling via overexpression of *Rheb* (**J-J'**) or downregulation of *Tsc2* (**K-K'**) or *Pten* (**L-L'**) increases differentiation of mature plasmatocyte and crystal cell lineages (**J, K,** and **L**). A small number of lamellocytes differentiate in close proximity to the PSC (**J'**, **K'** and **L'**).

(**M-U'**) Manipulation of TORC1 signaling in the PSC autonomously regulates Shotgun (Shg) expression and non-cell autonomously affects MZ size.

(**M** and **S-S'**) MZ progenitors are marked by high expression of the adhesion protein Shg (red, **M**), which is also expressed in PSC cells (**S-S'**). Asterisk in **M** demarcates high Shg expression in adjacent secondary lobe, similar to high Shg expression in the MZ.

(**N-O**) Inhibition of TORC1 signaling in the PSC via overexpression of Tsc^{1+2} (**N**) or $Pten^{wt}$ (**O**) reduces Shg expression in the LG (relative to secondary lobes, asterisks), demonstrating premature loss of MZ progenitors.

(**P-R** and **T-U'**) Hyperactivation of TORC1 signaling in the PSC via overexpression of *Rheb* (**P**) or downregulation of *Tsc2* (**Q**) or *Pten* (**R**) reduces Shg expression in the MZ (relative to secondary lobes, asterisks), demonstrating premature loss of MZ progenitors, although a cell autonomous increase in Shg expression in the PSC is observed specifically upon *Rheb* overexpression (**P** and **T-T'**) or *Tsc2* downregulation (**Q** and **U-U'**), but not *Pten* downregulation (**R**).

Scale bars = 50μm. Scale bar in **A** corresponds to **A-R**, except for the following additional confocal magnifications: X1.2 (**N-Q**) and X0.9 (**G** and **L**). Scale bar in **S** corresponds to **S-U'**.

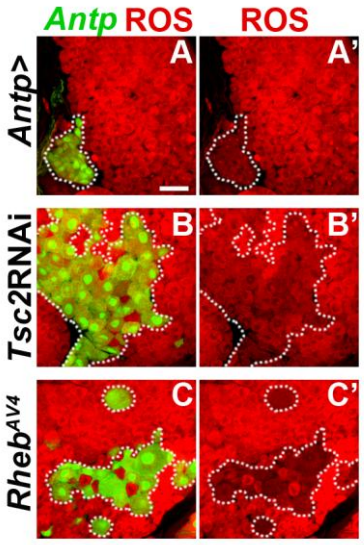


Figure A-5. Hyperactive TORC1 signaling in the PSC does not alter ROS levels

All panels represent wL3 LGs. In all panels, *Antp-Gal4, UAS-GFP* (WT, **A-A'**) is used to express the specified genetic constructs in PSC cells (green). Reactive oxygen species (ROS) are labeled with dihydroethidium dye, shown in red.

(**A-A'**) WT LG. ROS levels (red) are low in PSC cells (green) compared to adjacent MZ prohemocytes, which maintain relatively high ROS levels.

(**B-C'**) Hyperactive TORC1 signaling in the PSC induced by downregulation of *Tsc2* (**B-B'**) or overexpression of *Rheb* (**C-C'**) does not increase ROS levels in the PSC.

Scale bar = 20μm.

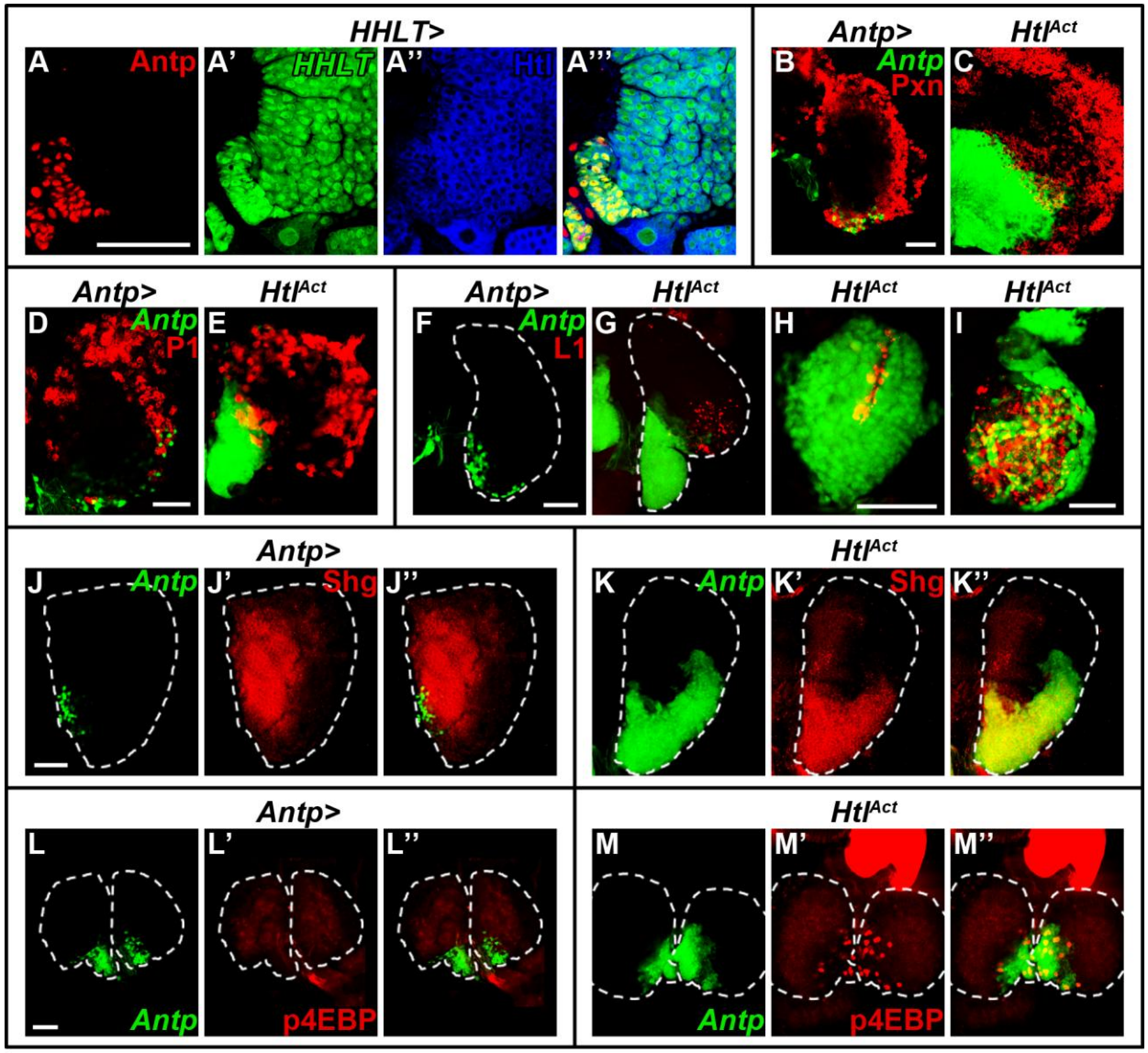


Figure A-6. Constitutive activation of Htl FGFR in the PSC disrupts PSC function

All panels represent wL3 LGs. In panels **A-A'''**, *HHLT* (*hand-gal4, hml-gal4, UAS-2xEGFP, UAS-FLP; A5CFRT-STOP-FRT-gal4*) is used to induce LG-specific clonal expression of GFP in hemocytes and in PSC cells. In panels **B-M"**, *Antp-Gal4, UAS-GFP* is used to express the specified genetic constructs in PSC cells (green).

(**A-A'''**) Expression of Htl (blue) overlaps with the PSC-specific marker, Antp (red), demonstrating that Htl FGFR is expressed in PSC cells of the LG (green).

(**B-C**) Overexpression of *Htl*^{Act} in PSC cells (**C**) dramatically increases PSC size in the LG, compared to WT (**B**), but does not affect expression of the early differentiation marker, Pxn (red).

(**D-E**) Overexpression of *Htl*^{Act} in PSC cells (**E**) does not affect terminal plasmatocyte differentiation in the LG, marked by P1 expression (red), compared to WT (**D**).

(**F**) WT LG. Lamellocytes, marked by L1 expression (red), are not normally observed in WT LGs.

(**G-I**) Overexpression of *Htl*^{Act} in PSC cells induces lamellocyte differentiation (red) in adjacent hemocytes (**G**) or even among and between the expanded population of PSC cells (**H-I**).

(**J-K"**) Whereas a WT LG demonstrates low expression of Shotgun (Shg, red) in PSC cells and high Shg expression in adjacent MZ prohemocytes (**J-J"**), overexpression of *Htl*^{Act} in PSC cells is sufficient to autonomously increase Shg levels in PSC cells, while decreasing Shg expression in adjacent hemocytes, (**K-K"**).

(**L-M"**) Whereas a WT LG demonstrates relatively low p4EBP levels in LG hemocytes and in the PSC (**L-L"**), overexpression of *Htl*^{Act} in PSC cells autonomously induces a population of p4EBP^{high} cells that are scattered within the expanded PSC population (**M-M"**).

Scale bars = 50μm. Scale bar in **B** corresponds to **B-C**. Scale bar in **D** corresponds to **D-E**. Scale bar in **F** corresponds to **F-G**. Scale bar in **J** corresponds to **J-K"**. Scale bar in **L** corresponds to **L-M"**.

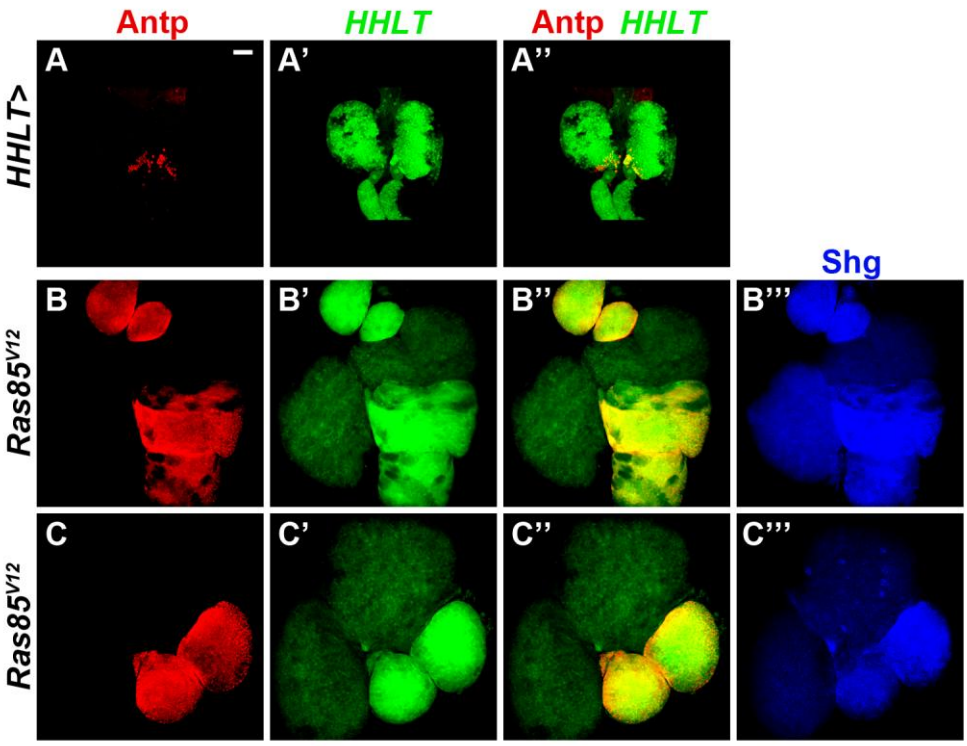


Figure A-7. Ras activation in the PSC autonomously increases PSC size

All panels represent wL3 LGs. In all panels, *HHLT* (*hand-gal4, hml-gal4, UAS-2xEGFP, UAS-FLP; A5CFRT-STOP-FRT-gal4*) is used to clonally induce Gal4 expression in the LG, such that clonal cells are labeled with GFP (green) and express the specified genetic constructs. The PSC is marked with Antp expression (red); Shotgun (Shg) expression is shown in blue.

(**A-A''**) WT LG, demonstrating a WT PSC (red) at the posterior tips of both primary lobes.

(**B-C'''**) Activating Ras in the LG by clonal overexpression of $Ras85^{V12}$ robustly increases PSC size (red) and also induces an autonomous increase in Shg expression (blue) within the expanded PSC population, relative to adjacent LG hemocytes.

Scale bar = 50µm.

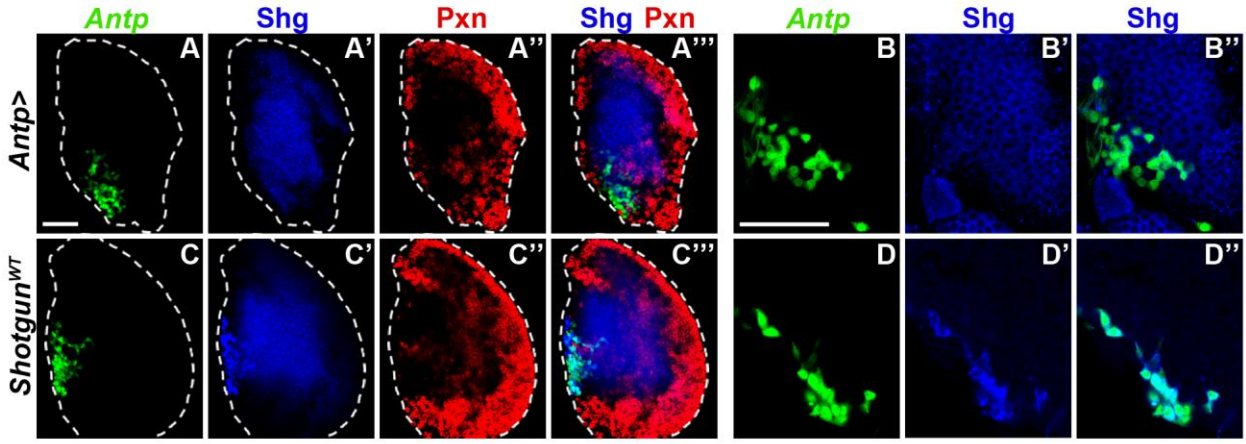


Figure A-8. Shotgun overexpression in the PSC does not affect PSC function

All panels represent wL3 LGs. In all panels, *Antp-Gal4, UAS-GFP* is used to express the specified genetic constructs in PSC cells (green). Pxn expression is shown in red and Shg is labeled in blue.

(**A-B"**) In WT LGs, Shg expression (blue) is higher in undifferentiated MZ prohemocytes, compared to the Pxn^{+} CZ (red, **A-A'''**). PSC cells (green) also express Shg (**B-B"**).

(**C-D"**) Overexpression of Shg^{WT} in the PSC autonomously increases Shg expression in the PSC (**D-D"**), but increased Shg expression does not impair PSC function, as the relative MZ/CZ ratio is similar to WT (compare **C-C'''** to **A-A'''**).

Scale bars = 50µm. Scale bar in **A** corresponds to **A-A'''** and **C-C'''**. Scale bare in **B** corresponds to **B-B"** and **D-D"**.

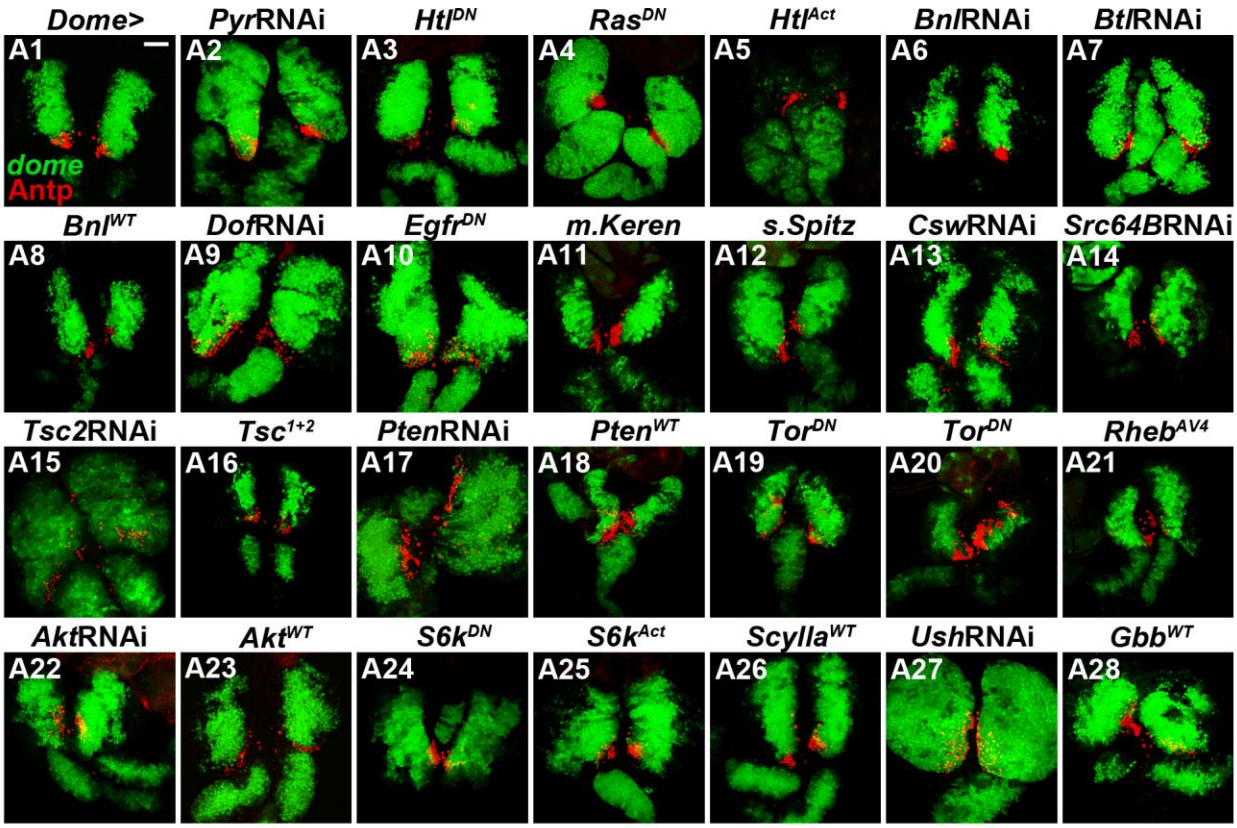


Figure A-9. Non-autonomous regulation of PSC size by hemocyte progenitors

All panels represent wL3 LGs. In all panels, *dome-Gal4, UAS-2xEGFP* (WT, **A1**) is used to express the specified genetic constructs in hemocyte progenitors (green). The PSC is labeled with Antp (red).

(**A1**) WT LG. The populations of MZ prohemocytes (green) and PSC cells (red) are marked.

(**A2-A4**) Reducing Htl FGFR-Ras signaling in hemocyte progenitors via downregulation of *Pyr* (**A2**) or overexpression of Htl^{DN} (**A3**) or Ras^{DN} (**A4**) does not affect PSC size.

(**A5**) Overexpression of Htl^{Act} in hemocyte progenitors does not affect PSC cell number. The primary lobes are largely *dome*-negative, reflective of premature prohemocyte differentiation, while enlarged secondary lobes maintain low *dome* expression.

(**A6-A7**) Reducing Breathless (Btl) FGFR signaling in prohemocytes by downregulation of the FGF ligand *Branchless* (*Bnl*, **A6**) or the FGFR *Btl* (**A7**) does not affect PSC cell number.

(**A8**) Overexpression of Bnl^{WT} in prohemocytes does not alter PSC size.

(**A9**) Downregulation of the FGFR-specific adaptor molecule, *Dof*, in progenitors does not alter PSC size.

(**A10**) Reducing EGFR activity in prohemocytes by overexpression of $Egfr^{DN}$ does not change PSC size.

(**A11-A12**) Overexpression of the EGFR ligands *Keren* (**A11**) or *Spitz* (**A12**) in prohemocyte does not affect PSC cell number.

(**A13-A14**) Downregulation of *Corkscrew* (*Csw*, **A13**) or *Src64B* (**A14**) in hemocyte progenitors does not change PSC cell number.

(**A15-A16**) Neither *Tsc2* downregulation (**A15**) nor overexpression of Tsc^{1+2} (**A16**) in prohemocytes significantly affects PSC cell number.

(**A17-A18**) Neither downregulation (**A17**) nor overexpression (**A18**) of *Pten* in prohemocytes significantly changes PSC size.

(**A19-A20**) Overexpression of Tor^{DN} in hemocyte progenitors is sufficient to induce ectopic $Antp^{+}$ expression in medial LG regions (**A19-A20**), and is also sufficient to significantly increase PSC cell number (**A20**).

(**A21**) Overexpression of $Rheb^{AV4}$ in prohemocytes does not affect PSC cell number.

(**A22-A23**) Neither downregulation (**A22**) nor overexpression (**A23**) of *Akt* in progenitors alters PSC size.

(**A24-A25**) Overexpression of $S6k^{DN}$ (**A24**) or $S6k^{Act}$ (**A25**) in hemocyte progenitors does not change PSC cell number.

(**A26**) Overexpression of *Scylla*^{WT} in prohemocytes does not affect PSC size.

(**A27**) Downregulation of the single *Drosophila* Friend-of-GATA gene, *U-shaped* (*Ush*), in prohemocytes does not change PSC cell number.

(**A28**) Overexpression of the transforming growth factor-β (TGF-β) gene, *Glass bottom boat* (*Gbb*^{WT}), in prohemocytes does not affect PSC size.

Scale bar = 50μm and applies to all panels except for an additional X0.6 magnification for **A15** and an additional X0.83 magnification for **A5**.

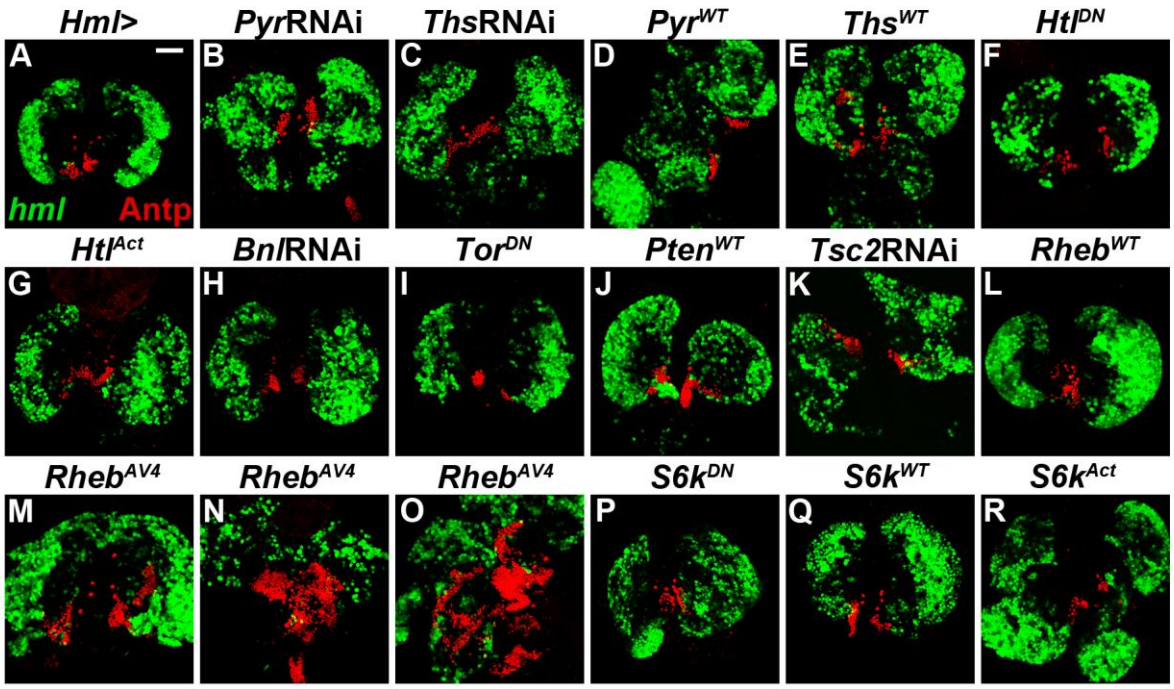


Figure A-10. Non-autonomous regulation of PSC size by differentiated hemocytes

All panels represent wL3 LGs. In all panels, *hml-Gal4, UAS-GFP* (WT, **A**) is used to express the specified genetic constructs in CZ hemocytes (green). The PSC is marked with Antp expression (red).

(**A**) WT LG. CZ hemocytes (green) and PSC cells (red) are labeled.

(**B-C**) Downregulation of the FGF ligands *Pyr* (**B**) or *Ths* (**C**) in CZ hemocytes does not alter PSC size.

(**D-E**) Overexpression of the FGF ligands Pyr^{WT} (**D**) or Ths^{WT} (**E**) in CZ hemocytes does not affect PSC cell number, but *Ths* overexpression does induce ectopic $Antp^{+}$ expression in medial LG regions.

(**F-G**) Reducing or activating Htl signaling in CZ hemocytes by overexpression of Htl^{DN} (**F**) or Htl^{Act} (**G**), respectively, does not affect PSC size.

(**H**) Downregulation of the Btl ligand *Bnl* in CZ hemocytes does not change PSC size.

(**I-J**) Reducing TORC1 activity in CZ hemocytes by overexpression of Tor^{DN} (**I**) or $Pten^{WT}$ (**J**) does not alter PSC cell number.

(**K**) Downregulation of *Tsc2* in CZ hemocytes does not alter PSC size.

(**L-O**) Although overexpression of $Rheb^{WT}$ (**L**) in CZ hemocytes does not change PSC cell number, $Rheb^{AV4}$ overexpression (**M-O**) robustly increases PSC size (**N-O**) and induces ectopic $Antp^{+}$ expression in medial LG regions (**M** and **O**).

(**P-R**) Altering S6K activity in CZ hemocytes by overexpression of $S6k^{DN}$ (**P**), $S6k^{WT}$ (**Q**) or $S6k^{Act}$ (**R**) does not affect PSC cell number.

Scale bar = 50μm and applies to all panels except for an additional X1.2 magnification for **I**.

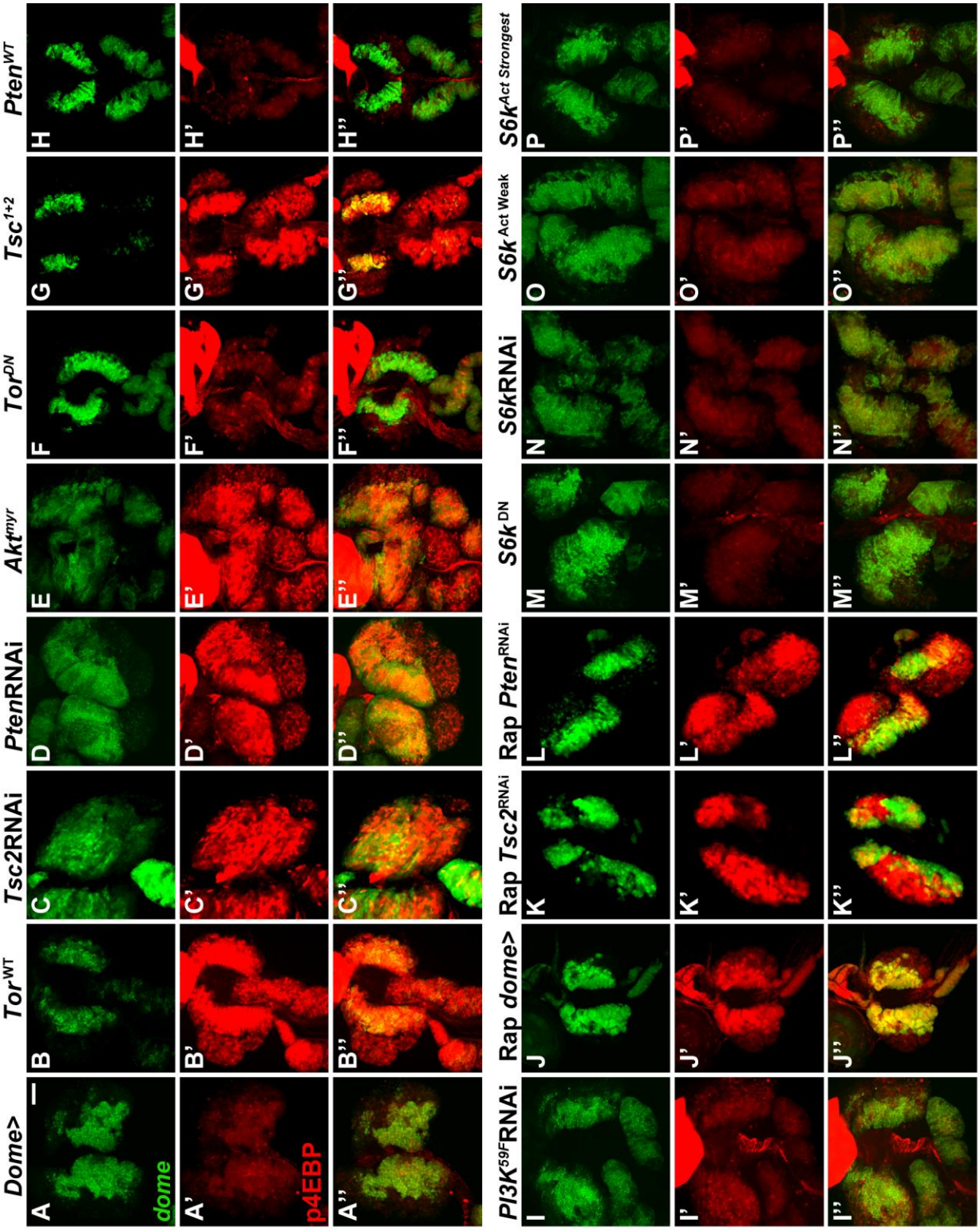


Figure A-11. Differential effects of Target of Rapamycin (TOR) signal pathway components on p4EBP expression in the lymph gland

Figure A-11. Differential effects of Target of Rapamycin (TOR) signal pathway components on p4EBP expression in the lymph gland

All panels represent wL3 LGs. In all panels, *dome-Gal4, UAS-2xEGFP* (WT, **A-A"**) is used to express the specified genetic constructs in hemocyte progenitors (green). The marker of active TOR kinase signaling, phosphorylated translation initiation factor 4E-binding protein (p4EBP) is shown in red.

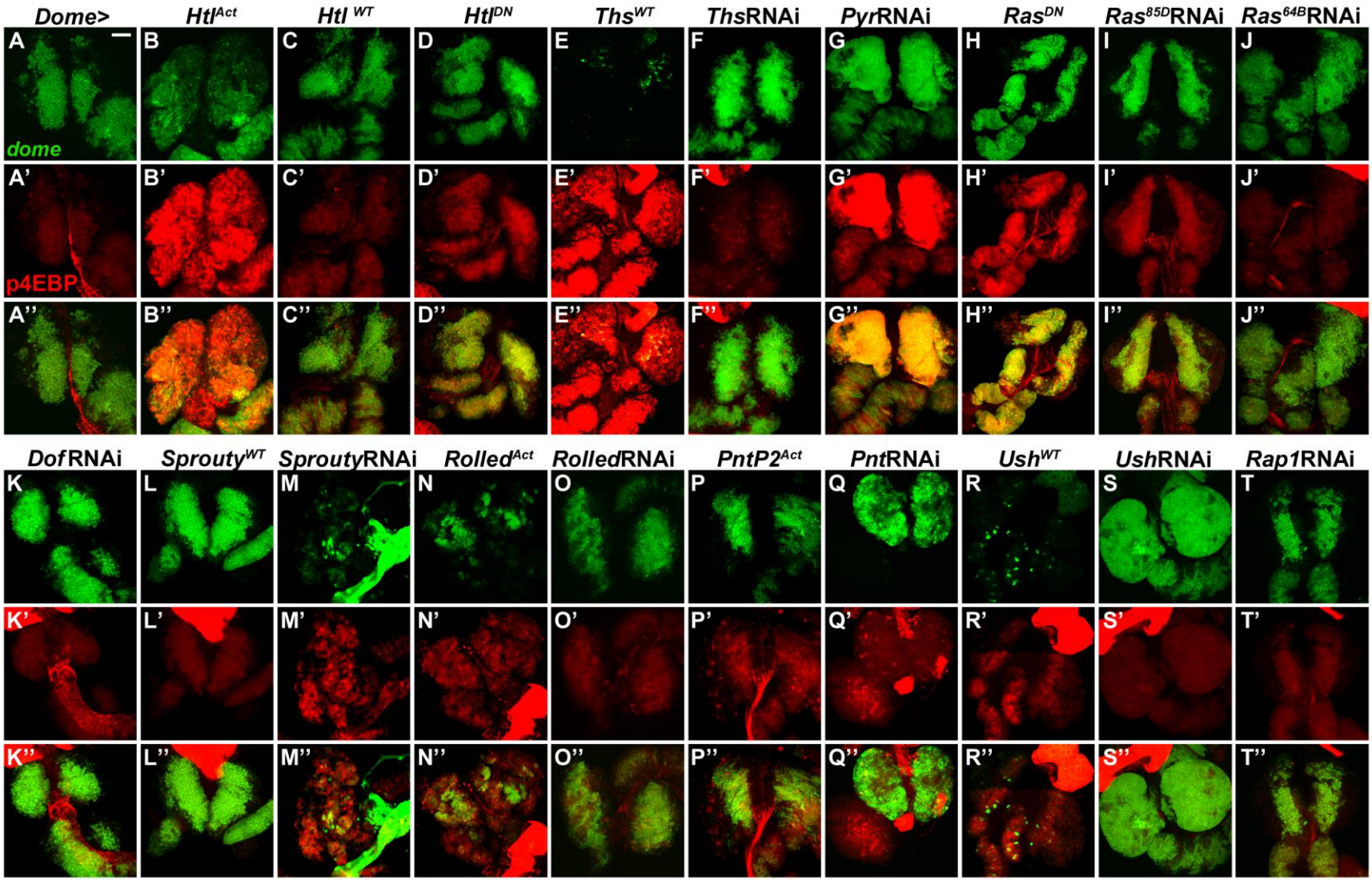
(**A-A"**) WT LG. Expression of p4EBP (red) is highest in *dome*$^{+}$ hemocyte progenitors of the MZ. (**B-E"**) Activation of TOR kinase signaling in hemocyte progenitors via overexpression of *Tor*^{WT} (**B-B"**), downregulation of the pathway inhibitors, *Tsc2* (**C-C"**), or *Pten* (**D-D"**), or overexpression of an activated Akt construct (*Akt*^{myr}, **E-E"**), all robustly increase p4EBP expression throughout the LG.

(**F-J"**) Inhibition of TOR kinase signaling in hemocyte progenitors has distinct effects on p4EBP expression in the LG. While overexpression of *Tor*^{DN} (**F-F"**) or *Pten*^{WT} (**H-H"**) or downregulation of *PI3K59F* (**I-I"**) does not affect p4EBP expression, inhibition of TOR signaling via *Tsc*$^{1+2}$ overexpression (**G-G"**) or systemic Rapamycin treatment (**J-J"**) does increase p4EBP levels in the LG.

(**K-L"**) While Rapamycin treatment of *dome>Tsc2*RNAi and *dome>Pten*RNAi larvae is sufficient to decrease LG size, the elevated p4EBP levels observed upon *Tsc2* or *Pten* downregulation alone (**C'** and **D'**) are not reduced upon Rapamycin treatment (**K-L"**).

(**M-P"**) Neither reducing S6 kinase (S6k) activity [via overexpression of *S6k*^{DN} (**M-M"**) or downregulation of *S6k* (**N-N"**)] nor increasing S6k activity [via overexpression of *S6k*$^{Activated\ Weak}$ (**O-O"**) or *S6k*$^{Activated\ Strongest}$ (**P-P"**)] in hemocyte progenitors affects p4EBP expression in the LG.

Scale bar = 50μm and applies to all panels except for the following additional confocal magnifications: X0.6 (**E-E”**), X0.75 (**D-D”**), X0.8 (**C-C”**, **I-I”**, and **O-P”**), X0.9 (**N-N”**), and X1.2 (**K-K”** and **L-L”**).


Dome>
HtlAct
Htl WT
HtlDN
ThsWT
ThsRNAi
PyrRNAi
RasDN
Ras85DRNAi
Ras64BRNAi
A
B
C
D
E
F
G
H
I
J
dome
A'
B'
C'
D'
E'
F'
G'
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I'
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p4EBP
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I''
J''
DofRNAi
SproutyWT
SproutyRNAi
RolledAct
RolledRNAi
PntP2Act
PntRNAi
UshWT
UshRNAi
Rap1RNAi
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Figure A-12. Differential effects of FGFR-Ras-MAPK signaling pathway components and effectors on p4EBP expression in the lymph gland

All panels represent wL3 LGs. In all panels, *dome-Gal4, UAS-2xEGFP* (WT, **A-A"**) is used to express the specified genetic constructs in hemocyte progenitors (green). The marker of active TOR kinase signaling, p4EBP, is shown in red.

(**A-A"**) WT LG. Expression of p4EBP (red) is highest in $dome^{+}$ hemocyte progenitors of the MZ.

(**B-C"**) Overexpression of Htl^{Act} in hemocyte progenitors increases p4EBP expression throughout the LG (**B-B"**), while overexpression of Htl^{WT} is not sufficient to change p4EBP levels (**C-C"**).

(**D-D"**) Reducing Htl activity in hemocyte progenitors by overexpression of Htl^{DN} does not affect p4EBP levels in the LG.

(**E-E"**) Overexpression of the Htl ligand, *Ths*, in hemocyte progenitors robustly increases p4EBP expression in medial LG regions, including within *dome*-negative hemocytes.

(**F-G"**) While *Ths* downregulation in hemocyte progenitors does not alter p4EBP expression (**F-F"**), *Pyr* downregulation robustly increases p4EBP throughout an expanded population of MZ progenitors (**G-G"**).

(**H-J"**) While overexpression of Ras^{DN} in hemocyte progenitors causes an increase in p4EBP expression in the LG (**H-H"**), downregulation of *Ras85D*RNAi (**I-I"**) or *Ras64B*RNAi (**J-J"**) does not.

(**K-K"**) Downregulation of *Dof* in hemocyte progenitors does not affect p4EBP expression.

(**L-M"**) While overexpression of *Sprouty* in hemocyte progenitors does not alter p4EBP expression in the LG (**L-L"**), *Sprouty* downregulation induces a small increase in p4EBP levels (**M-M"**).

(**N-O”**) While overexpression of *Rolled MAPK*^{Act} in hemocyte progenitors induces a subtle increase in p4EBP expression, extending to *dome*-negative LG hemocytes (**N-N”**), *Rolled MAPK* downregulation does not alter p4EBP expression in the LG (**O-O”**).

(**P-Q”**) Activation (**P-P”**) or downregulation (**Q-Q”**) of *Pointed* (*Pnt*), an ETS domain transcription factor activated downstream of Ras-MAPK signaling, does not alter p4EBP expression in the LG.

(**R-S”**) Overexpression (**R-R”**) or downregulation (**S-S”**) of *U-shaped* (*Ush*) in hemocyte progenitors does not alter 4EBP expression.

(**T-T”**) Downregulation of *Rap1* in hemocyte progenitors does not affect p4EBP expression in the LG.

Scale bar = 50μm and applies to all panels except for the following additional confocal magnifications: X0.8 (**B-B”** and **P-S”**) and X0.67 (**E-E”** and **N-N”**).

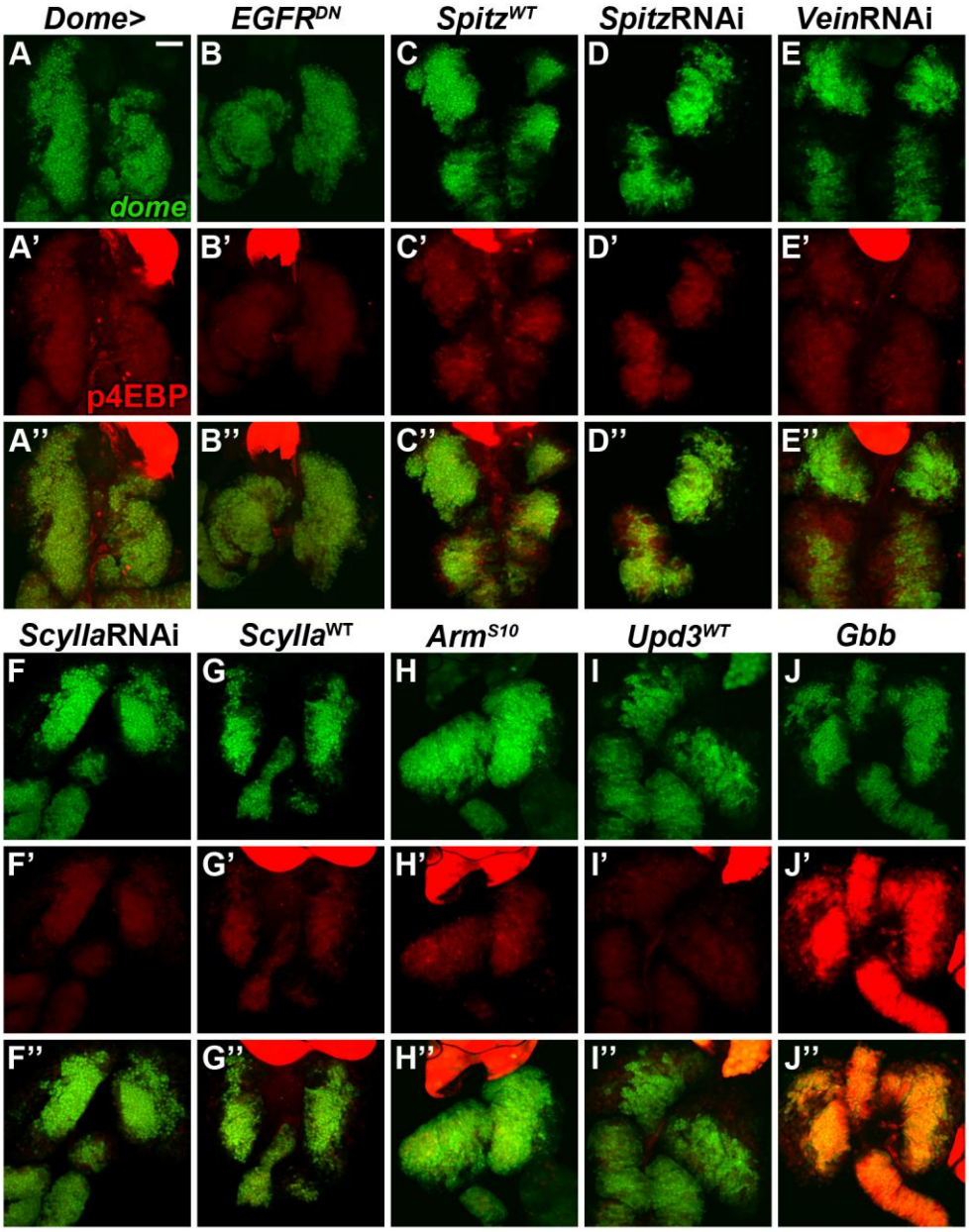

Figure A-13. Regulation of p4EBP expression in LG prohemocytes

All panels represent wL3 LGs. In all panels, *dome-Gal4, UAS-2xEGFP* (WT, **A-A"**) is used to express the specified genetic constructs in hemocyte progenitors (green). The marker of active TOR kinase signaling, p4EBP, is shown in red.

(**A-A"**) WT LG. Expression of p4EBP (red) is highest in *dome*$^{+}$ hemocyte progenitors of the MZ.

(**B-B”**) Inhibition of EGFR signaling in LG progenitors by overexpression of $Egfr^{DN}$ does not change p4EBP expression in the LG.

(**C-C”**) Overexpression of the EGFR ligand, *Spitz*, in LG progenitors does not affect p4EBP levels.

(**D-E”**) Downregulation of the EGFR ligands, *Spitz* (**D-D”**) or *Vein* (**E-E”**), in LG prohemocytes does not affect p4EBP expression.

(**F-G”**) Neither downregulation (**F-F”**) nor overexpression (**G-G”**) of *Scylla* in hemocyte progenitors changes p4EBP expression in the LG.

(**H-H”**) Activation of Wingless/Wnt signaling in hemocyte progenitors via overexpression of the pathway effector, *Armadillo* (Arm^{S10}), does not affect p4EBP expression.

(**I-I”**) Overexpression of the JAK/STAT ligand, *Unpaired 3* (*Upd3*) in hemocyte progenitors, which is endogenously expressed in the MZ and PSC of the LG, does not affect p4EBP levels.

(**J-J”**) Overexpression of the TGF-β factor, *Gbb*, in prohemocytes increases p4EBP expression in the LG.

Scale bar = 50μm and applies to all panels except for the following additional confocal magnifications: X0.8 (**I-I”**) and X1.25 (**F-F”**).

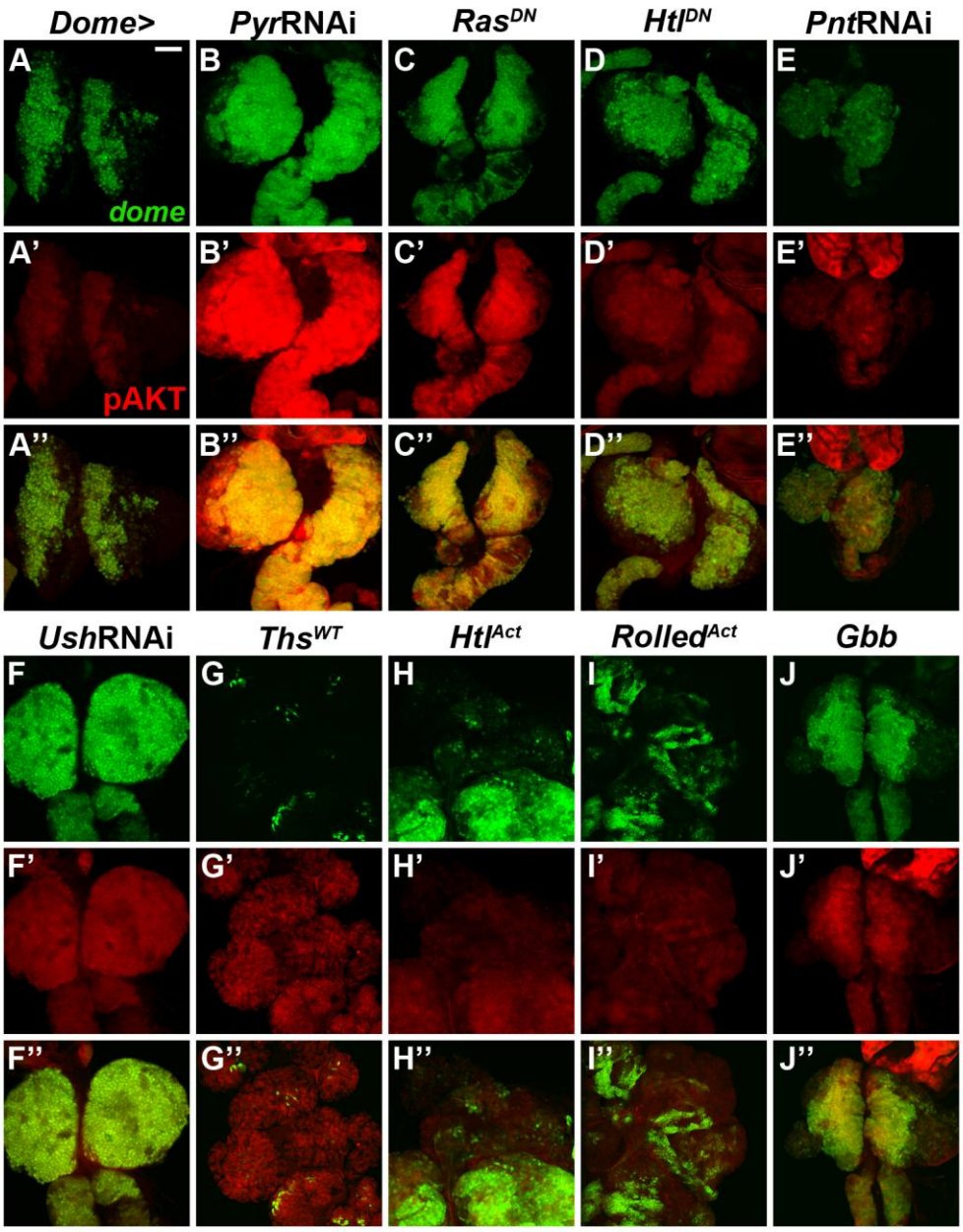

Figure A-14. Regulation of Akt activation in lymph gland prohemocytes

All panels represent wL3 LGs. In all panels, *dome-Gal4, UAS-2xEGFP* (WT, **A-A"**) is used to express the specified genetic constructs in hemocyte progenitors (green). Phosphorylated (activated) Akt (pAkt) is shown in red.

(**A-A"**) WT LG. Expression of pAkt (red) is highest in *dome*$^{+}$ hemocyte progenitors of the MZ.

(**B-C”**) Downregulation of *Pyr* (**B-B”**) or reducing Ras activity in hemocyte progenitors by overexpression of Ras^{DN} (**C-C”**) increases pAkt levels in the LG.

(**D-D”**) Reducing Htl activity in hemocyte progenitors by overexpression of Htl^{DN} does not alter pAkt expression in the LG.

(**E-F”**) Downregulation of the transcriptional regulators, *Pnt* (**E-E”**) or *Ush* (**F-F”**), in hemocyte progenitors does not affect pAkt levels in the LG.

(**G-H”**) Activation of Htl signaling in hemocyte progenitors by overexpression of Ths^{WT} (**G-G”**) or Htl^{Act} (**H-H”**) does not alter pAkt expression in the LG.

(**I-I”**) Activation of MAPK signaling in prohemocytes via overexpression of $Rolled^{Act}$ does not alter pAkt.

(**J-J”**) Overexpression of the TGF-β growth factor, *Gbb*, in prohemocytes does not alter pAkt expression.

Scale bar = 50μm and applies to all panels except for the following additional confocal magnifications: X0.6 (**G-G”** and **I-I”**), X1.1 (**D-D”**) and X1.2 (**C-C”**).

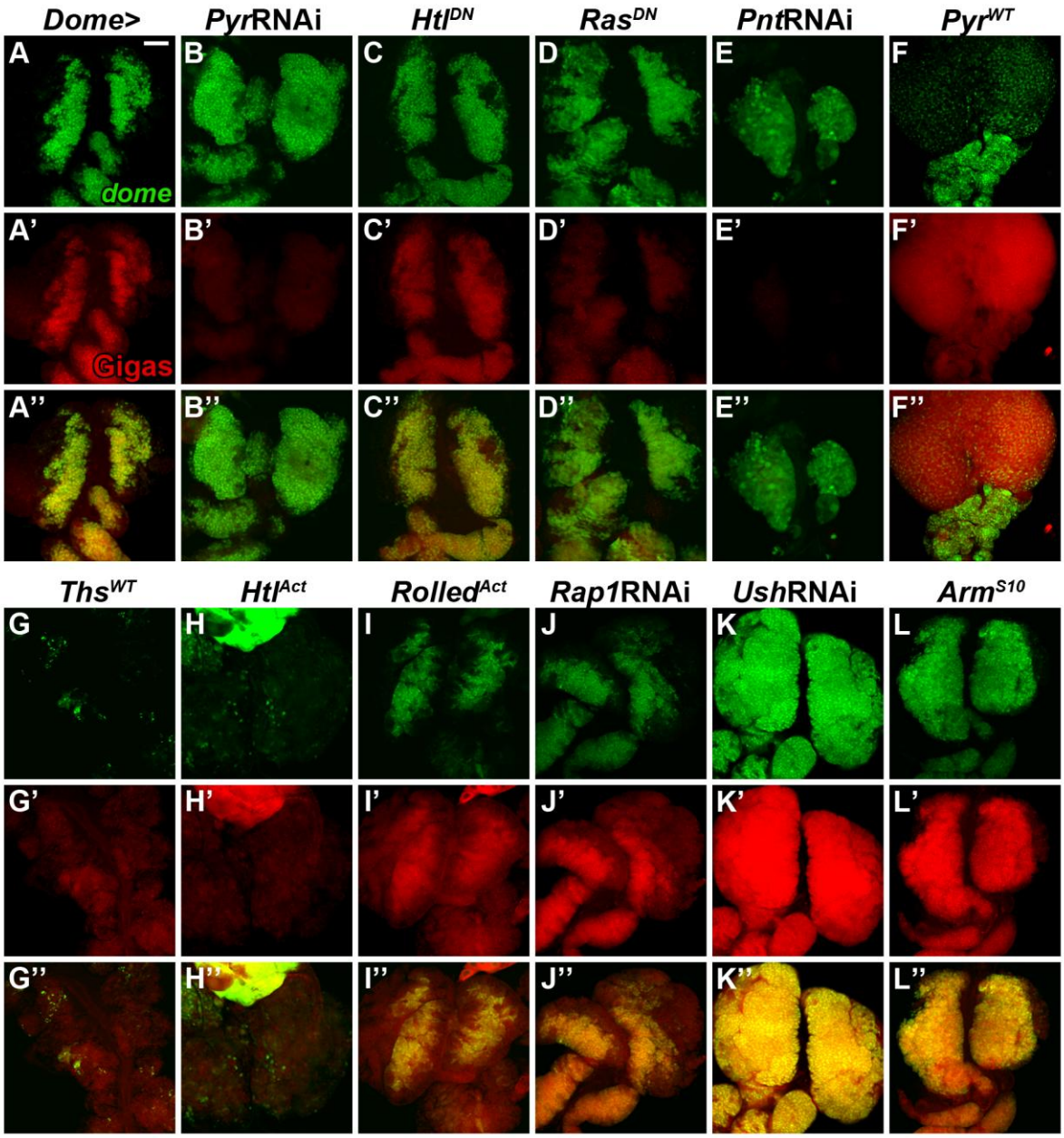

Figure A-15. Regulation of TSC2/Gigas expression in lymph gland prohemocytes

All panels represent wL3 LGs. In panels **A-D"** and **G-L"**, *dome-Gal4, UAS-2xEGFP* (WT, **A-A"**) is used to express the specified genetic constructs in hemocyte progenitors (green). In panels **E-F"**, *HHLT* (*hand-gal4, hml-gal4, UAS-2xEGFP, UAS-FLP; A5CFRT-STOP-FRT-gal4*) is used

to clonally (green) express the specified genetic constructs in the LG. Expression of TSC2/Gigas is shown in red in all panels.

(**A-A"**) WT LG. Gigas expression is highest in the population of *dome*$^{+}$ hemocyte progenitors.

(**B-B"**) Downregulation of *Pyr* expression in prohemocytes autonomously reduces Gigas expression, despite an expansion in the population of *dome*$^{+}$ progenitors.

(**C-C"**) Reducing Htl activity in prohemocytes by *Htl*^{DN} overexpression does not affect Gigas expression.

(**D-D"**) Reducing Ras activity in prohemocytes via overexpression of *Ras*^{DN} autonomously reduces Gigas expression.

(**E-E"**) Clonally downregulating the ETS domain transcription factor, *Pnt*, in the LG significantly reduces Gigas expression.

(**F-F"**) Clonal overexpression of *Pyr*^{WT} in the LG increases the population of Gigas^{+} hemocytes, but does not increase Gigas levels, compared to WT prohemocytes (**A-A"**).

(**G-H"**) Activation of Htl signaling in prohemocytes via overexpression of *Ths*^{WT} (**G-G"**) or *Htl*^{Act} (**H-H"**) reduces Gigas expression, concomitant with a decrease in *dome* expression, compared to WT (**A-A"**).

(**I-J"**) Overexpression of *Rolled*^{Act} (**I-I"**) or downregulation of *Rap1* (**J-J"**) in prohemocytes does not affect Gigas expression in the LG.

(**K-K"**) Downregulation of *Ush* in hemocyte progenitors autonomously increases Gigas expression, concomitant with an expansion in the population of *dome*$^{+}$ progenitors.

(**L-L"**) Activation of Wingless signaling in hemocyte progenitors via overexpression of *Arm*^{S10} autonomously increases Gigas expression, concomitant with an expansion in the population of *dome*$^{+}$ prohemocytes.

Scale bar = 50μm and applies to all panels except for the following additional confocal magnifications: X0.6 (**F-G”**), X0.8 (**J-J”**), X0.9 (**C-C”** and **H-I”**) and X1.2 (**E-E”**).

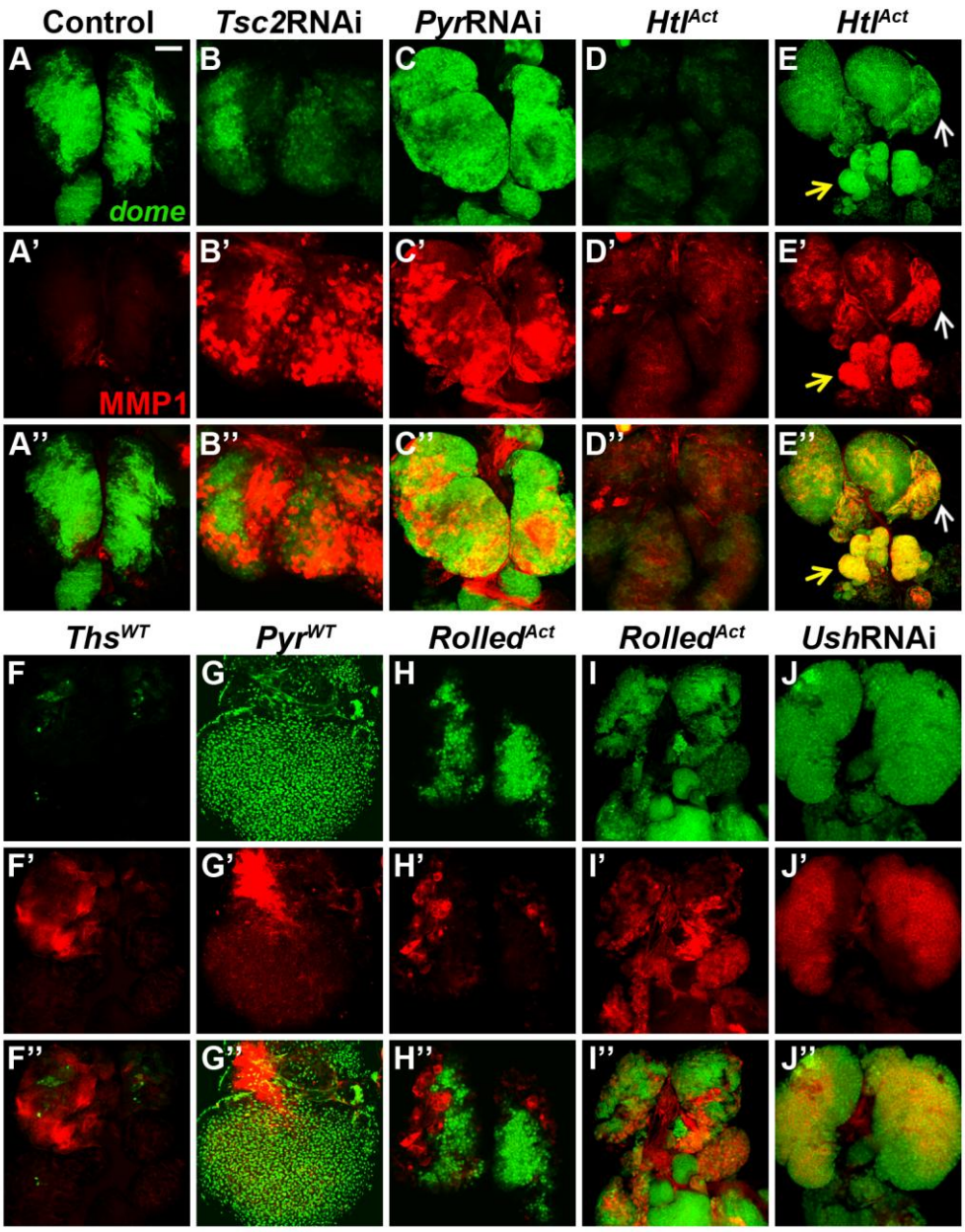

Figure A-16. Changes in Matrix Metalloproteinase 1 (MMP1) expression in the lymph gland

All panels represent wL3 LGs. In panels **A-D"**, **F-F"**, **H-H"** and **J-J"** *dome-Gal4, UAS-2xEGFP* (WT, **A-A"**) is used to express the specified genetic constructs in hemocyte progenitors (green). In panels **E-E"**, **G-G"** and **I-I"**, *HHLT* (*hand-gal4, hml-gal4, UAS-2xEGFP, UAS-FLP;*

A5CFRT-STOP-FRT-gal4) is used to clonally (green) express the specified genetic constructs in the LG. Expression of MMP1 is shown in red in all panels.

(**A-A"**) WT LG. MMP1 is not expressed in the LG, except for low expression detected in a few scattered hemocytes.

(**B-B"**) Increasing TORC1 activation in prohemocytes via downregulation of *Tsc2* significantly increases MMP1 expression in *dome*^{low} LG hemocytes.

(**C-C"**) Downregulation of *Pyr* in prohemocytes significantly increases MMP1 expression in the LG, most noticeably in and around *dome*^{low} LG hemocytes.

(**D-E"**) While progenitor-specific overexpression of *Htl*^{Act} induces a small increase in MMP1 expression (**D-D"**), clonally overexpressing *Htl*^{Act} in the LG (**E-E"**) significantly increases MMP1 levels, especially in lamellocytes (white arrow), identified by their distinct morphology, and in expanded PSC cells (yellow arrow), identified by their higher GFP expression.

(**F-F"**) Overexpression of *Ths*^{WT} in prohemocytes increases MMP1 expression in the LG, in an expanded population of lamellocytes.

(**G-G"**) Clonally overexpressing *Pyr*^{WT} in the LG increases MMP1 expression specifically in a small region of the tissue near the ring gland.

(**H-I"**) While overexpression of *Rolled*^{Act} in prohemocytes induces MMP1 expression in a subset of differentiated hemocytes at the LG periphery (**H-H"**), clonally overexpressing *Rolled*^{Act} in the LG induces MMP1 expression in more medial LG hemocytes (**I-I"**).

(**J-J"**) Downregulation of *Ush* in hemocyte progenitors induces a subtle increase in MMP1 expression throughout most of the expanded prohemocyte population.

Scale bar = 50μm and applies to all panels except for the following additional confocal magnifications: X0.6 (**E-E”** and **G-G”**), X0.8 (**B-B”**, **D-D”**, **F-F”** and **I-I”**), X0.9 (**C-C”**) and X1.2 (**H-H”**).

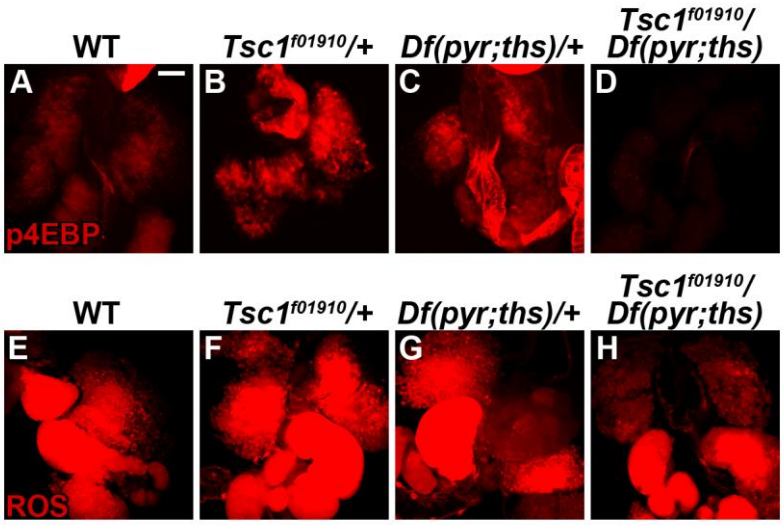

Figure A-17. Interaction between heteroallelic mutations for *Tsc1* and *Pyr/Ths* affects p4EBP and ROS levels in the LG

All panels represent wL3 LGs. Genotypes are listed above corresponding panels. In panels **A-D**, p4EBP is shown in red, whereas in panels **E-H**, ROS levels are shown in red. Scale bar = 50µm. (**A**) WT LG. p4EBP is more highly expressed in medial LG regions, where prohemocytes reside. (**B-C**) Single copy loss of *Tsc1* (*Tsc1*^{f01910}/+, **B**) or single copy of a chromosomal deficiency that includes both *Pyr* and *Ths* [*Df(pyr;ths)*/+, **C**] increases p4EBP expression in the LG. (**D**) Single copy loss of *Tsc1* in combination with a single copy deficiency for both *Pyr* and *Ths* [*Tsc1*^{f01910}/ *Df(pyr;ths)*] significantly reduces p4EBP expression in the LG compared to WT (**A**). (**E**) WT LG. ROS levels are highest in medial LG regions, where prohemocytes reside. (**F-G**) Single copy loss of *Tsc1* (*Tsc1*^{f01910}/+, **F**) or single copy of a chromosomal deficiency that includes both *Pyr* and *Ths* [*Df(pyr;ths)*/+, **G]** does not significantly change ROS levels in the LG, compared to WT (**E**). (**H**) Single copy loss of *Tsc1* in combination with a single copy deficiency for both *Pyr* and *Ths* [*Tsc1*^{f01910}/ *Df(pyr;ths)*] significantly reduces ROS levels in the LG compared to WT (**E**).

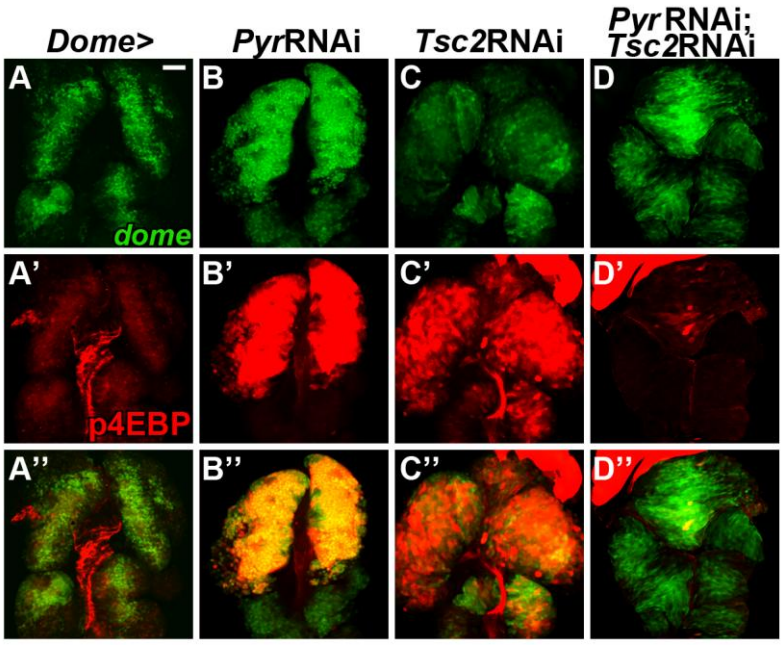

Figure A-18. Interaction between Pyramus and TSC alters p4EBP expression in the lymph gland

All panels represent wL3 LGs. In all panels, *dome-Gal4, UAS-2xEGFP* (WT, **A-A"**) is used to express the specified genetic constructs in hemocyte progenitors (green). p4EBP expression is shown in red.

(**A-A"**) WT LG. p4EBP expression is highest in *dome*$^{+}$ hemocyte progenitors of the MZ.

(**B-B"**) Downregulation of *Pyr* in prohemocytes robustly increases p4EBP expression autonomously within the expanded MZ progenitor population.

(**C-C"**) Downregulation of *Tsc2* in prohemocytes robustly increases p4EBP expression throughout the expanded population of *dome*^{low} hemocytes.

(**D-D"**) Downregulation of *Pyr* upon *Tsc2* downregulation in LG prohemocytes inhibits p4EBP expression in the LG, reducing p4EBP levels lower than in WT (**A-A"**).

Scale bar = 50μm and applies to all panels except for an additional X1.2 magnification in **B-B”** and X0.8 magnification in **D-D”**.

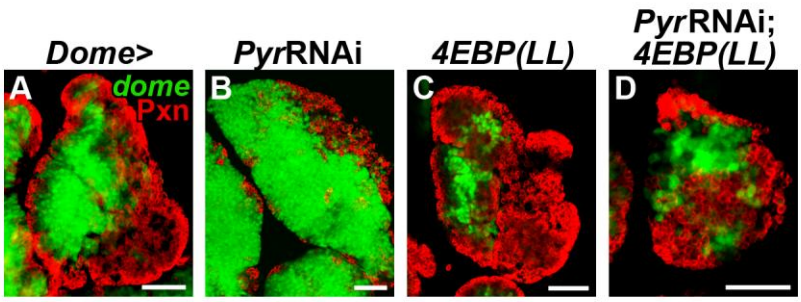

Figure A-19. Effect of 4EBP on Pyramus function in LG prohemocytes

All panels represent wL3 LGs. In all panels, *dome-Gal4, UAS-2xEGFP* (WT, **A**) is used to express the specified genetic constructs in hemocyte progenitors (green). Pxn expression is labeled in red.

(**A**) WT LG. Prohemocytes (green) populate the MZ, whereas differentiated hemocytes (Pxn^{+}, red) populate the CZ.

(**B**) Downregulation of *Pyr* in prohemocytes expands the population of $dome^{+}$ hemocyte progenitors at the expense of the CZ.

(**C**) Overexpression of *4EBP[LL]* in hemocyte progenitors increases differentiation in the LG at the expense of the MZ.

(**D**) Downregulation of *Pyr* upon overexpression of *4EBP[LL]* in prohemocytes increases differentiation in the LG, including within more medial LG regions, where undifferentiated prohemocytes normally reside (compare to **A**).

Scale bars = 50µm.

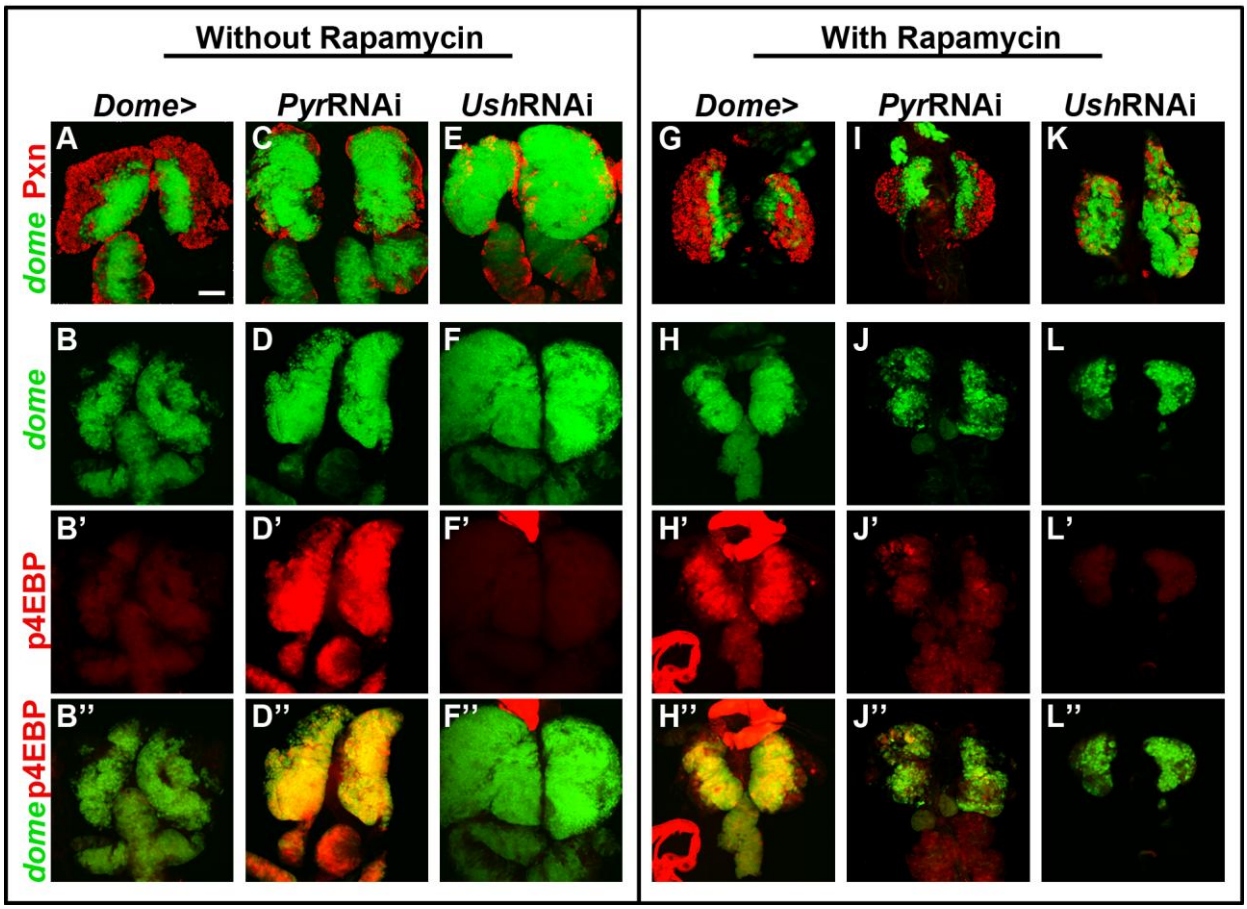

Figure A-20. Effects of Rapamycin treatment upon downregulation of *Pyramus* and *U-shaped* in hemocyte progenitors

All panels represent wL3 LGs. In all panels, *dome-Gal4, UAS-2xEGFP* (WT, **A-B"** and **G-H"**) is used to express the specified genetic constructs in hemocyte progenitors (green). Pxn expression is labeled in red in panels **A**, **C**, **E**, **G**, **I** and **K**. p4EBP expression is shown in red in panels **B-B"**, **D-D"**, **F-F"**, **H-H"**, **J-J"** and **L-L"**. Panels **A-F"** represent conditions of larval growth without Rapamycin treatment, whereas panels **G-L"** represent conditions of larval growth with Rapamycin treatment.

(**A**) WT LG. Differentiated hemocytes of the CZ (Pxn^{+}, red) are labeled.

(**B-B”**) WT LG. p4EBP expression (red) is highest in the population of *dome*$^{+}$ hemocyte progenitors.

(**C-D”**) Downregulation of *Pyr* in hemocyte progenitors restricts differentiation and CZ formation in the LG (**C**) and autonomously increases p4EBP expression (**D-D”**).

(**E-F”**) Downregulation of *Ush* in hemocyte progenitors impairs hemocyte differentiation (**E**) and decreases p4EBP expression in the LG (**F-F”**).

(**G-H”**) Rapamycin treatment of control larvae increases both differentiation (**G**) and p4EBP expression (**H-H”**) in the LG.

(**I-J”**) Rapamycin treatment of *dome>Pyr*RNAi larvae increases differentiation in the LG (**I**) and restricts p4EBP expression (**J-J”**), compared to *Pyr* downregulation alone (**C-D”**).

(**K-L”**) Rapamycin treatment of *dome>Ush*RNAi larvae increases differentiation in the LG (**K**) compared to *Ush* downregulation alone (**E**). p4EBP expression is impaired (**L-L”**), similar to *Ush* downregulation alone (**F-F”**).

Scale bar = 50μm and corresponds to all panels except for the following additional magnifications: X0.8 (**B-B”** and **E-F”**), X1.2 (**K**), and X1.3 (**G** and **L-L”**).

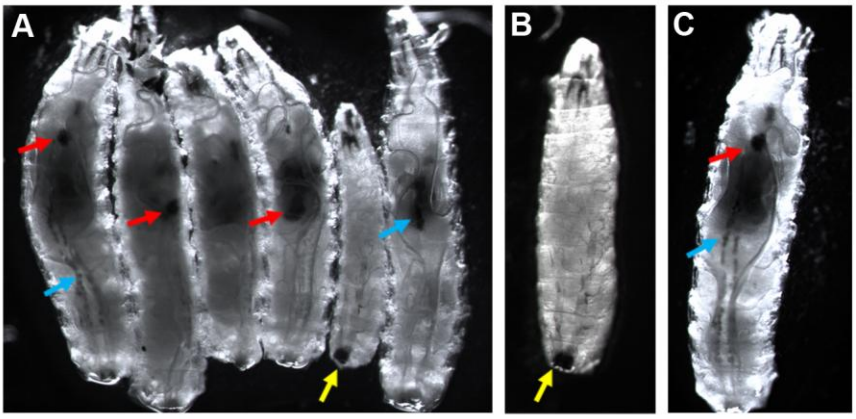

Figure A-21. Systemic Rapamycin treatment upon *Pyramus* downregulation in hemocyte progenitors induces melanotic masses in the larva.

All larvae represented are of the genotype, *dome-Gal4, UAS-2xEGFP; UAS-Pyr*RNAi, and were grown on food plates with Rapamycin to systemically impair active TOR kinase signaling. (**A-C**) Melanotic masses (red arrows, **A** and **C**) develop in *Pyr*RNAi larvae upon Rapamycin treatment. Melanization is also sometimes observed within the trachea (blue arrows, **A** and **C**) and the posterior spiracles (yellow arrows, **A-B**).

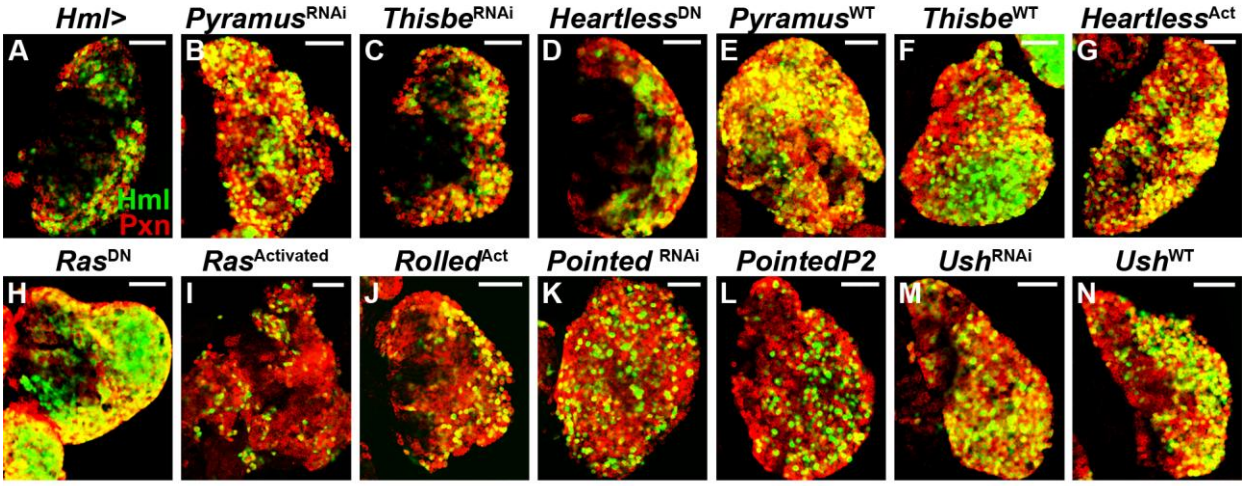

Figure A-22. FGFR-Ras-Pnt-Ush signaling in CZ hemocytes regulates their differentiation

All panels represent wL3 LGs. In all panels, *hml-Gal4, UAS-GFP* (WT, **A**) is used to express the specified genetic constructs in CZ hemocytes (green). The early marker of differentiation, Pxn, is shown in red. MZ prohemocytes are negative for both *hml* and Pxn expression. Scale bars = 50µm.

(**A**) WT LG. CZ hemocytes are labeled in the LG periphery by both *hml* (green) and Pxn (red) expression. Undifferentiated hemocytes in medial LG regions are unlabeled and represent MZ prohemocytes.

(**B-D**) While downregulation of *Pyr* in CZ hemocytes increases differentiation in the LG at the expense of the MZ (**B**), *Ths* downregulation (**C**) or overexpression of Htl^{DN} in CZ hemocytes does not affect differentiation in the LG.

(**E-G**) Activation of Htl signaling in CZ hemocytes via overexpression of Pyr^{WT} (**E**), Ths^{WT} (**F**), or Htl^{Act} (**G**) increases differentiation in the LG at the expense of the MZ.

(**H-I**) Either decreasing or increasing Ras activity in CZ hemocytes by overexpression of Ras^{DN} (**H**) or Ras^{Act} (**I**), respectively, increases differentiation in the LG at the expense of the MZ.

(**J**) Activation of Rolled MAPK in CZ hemocytes by overexpression of *Rolled*^{Act} increases differentiation.

(**K-L**) Both downregulation (**K**) and overexpression (**L**) of the ETS domain transcription factor, *Pnt*, in CZ hemocytes increases differentiation at the expense of the MZ.

(**M-N**) Both downregulation (**M**) and overexpression (**N**) of *Ush* in CZ hemocytes increases differentiation at the expense of the MZ.

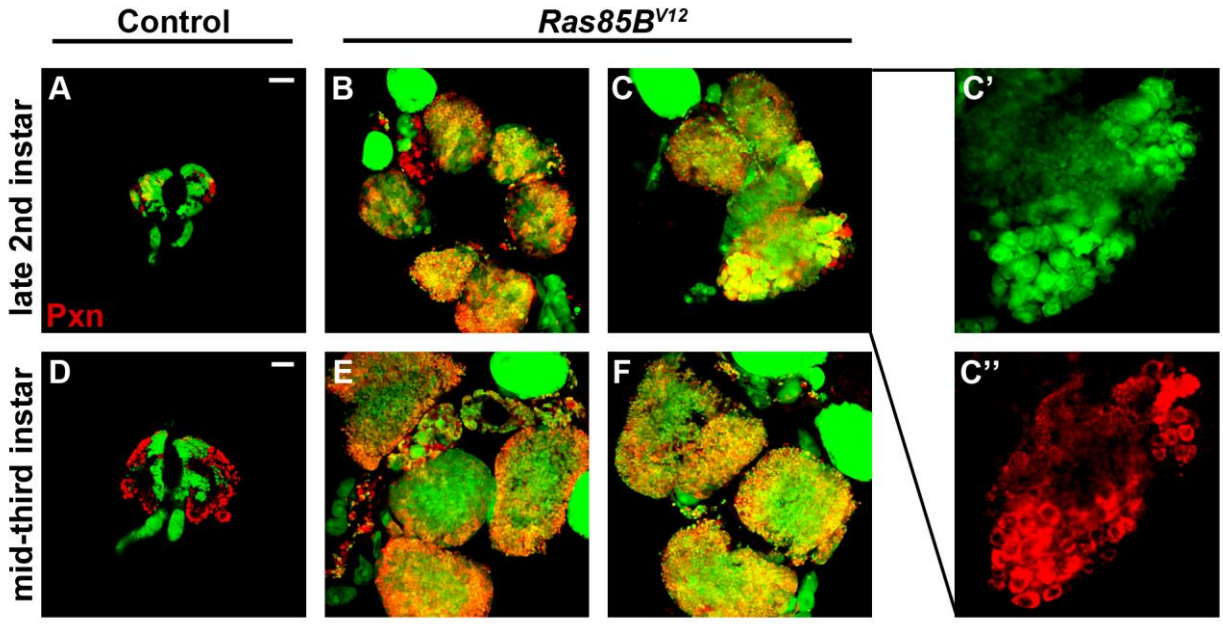

Figure A-23. Early effects of Ras activation on proliferation and differentiation of LG hemocytes

In all panels, *HHLT* (*hand-gal4, hml-gal4, UAS-2xEGFP, UAS-FLP; A5CFRT-STOP-FRT-gal4*) is used to clonally induce Gal4 expression in the LG, such that clonal cells are labeled with GFP (green) and express *UAS-Ras85B^{V12}* (**B-C"** and **E-F**). Pxn expression is shown in red.

(**A**) WT LG at late second instar. The first few differentiating hemocytes localize to the LG periphery.

(**B-C"**) Clonal activation of Ras in LG hemocytes via overexpression of *Ras85B^{V12}* robustly increases LG size and induces differentiation of the expanded hemocytes by late second instar (**B-C**). PSC cells, identified by their higher GFP expression and absence of Pxn expression, are already robustly increased in number at this stage (**B-C**). A population of very large Pxn^{+} cells is also evident (**C-C"**), which are several times larger in size than other adjacent hemocytes.

(**D**) WT LG at mid third instar. A growing population of Pxn^{+} hemocytes expands the early CZ.

(**E-F**) Clonally overexpressing *Ras85B*^{V12} in LG hemocytes robustly increases tissue size/hemocyte number and differentiation by mid third instar stage.

Scale bars = 50μm. Scale bar in **A** corresponds to **A-C**. Scale bar in **D** corresponds to **D-F**.

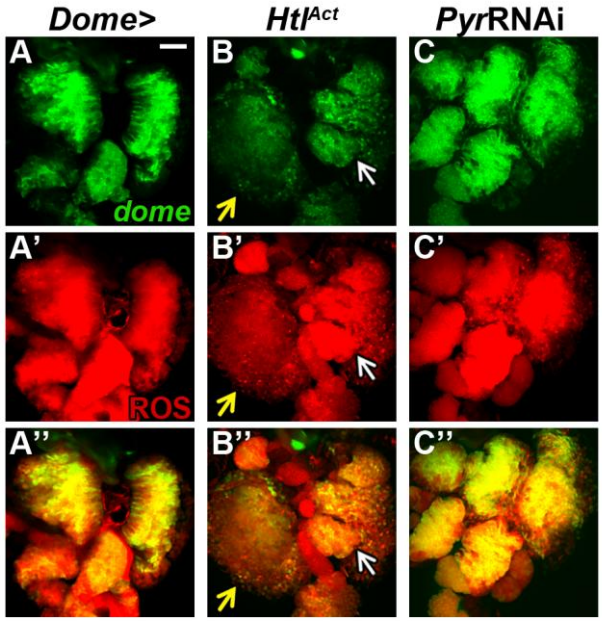

Figure A-24. Regulation of ROS levels in the lymph gland by Heartless FGFR signaling

All panels represent wL3 LGs. In all panels, *dome-Gal4, UAS-2xEGFP* (WT, **A-A"**) is used to express the specified genetic constructs in hemocyte progenitors (green). ROS levels, detected with a dihydroethidium dye, are shown in red.

(**A-A"**) WT LG. ROS levels are highest in *dome*$^{+}$ hemocyte progenitors of the MZ.

(**B-B"**) Activation of Htl signaling in prohemocytes by overexpression of *Htl*^{Act} does not significantly change ROS levels in *dome*$^{+}$ prohemocytes (white arrow). *Dome*^{low} progenitors which are more differentiated have concomitantly lower ROS levels (yellow arrow).

(**C-C"**) Downregulation of the FGF ligand *Pyr* in hemocyte progenitors does not significantly alter ROS levels in the LG, compared to WT (**A-A"**).

Scale bar = 50µm. Panels **B-C"** represent an additional X0.9 magnification.

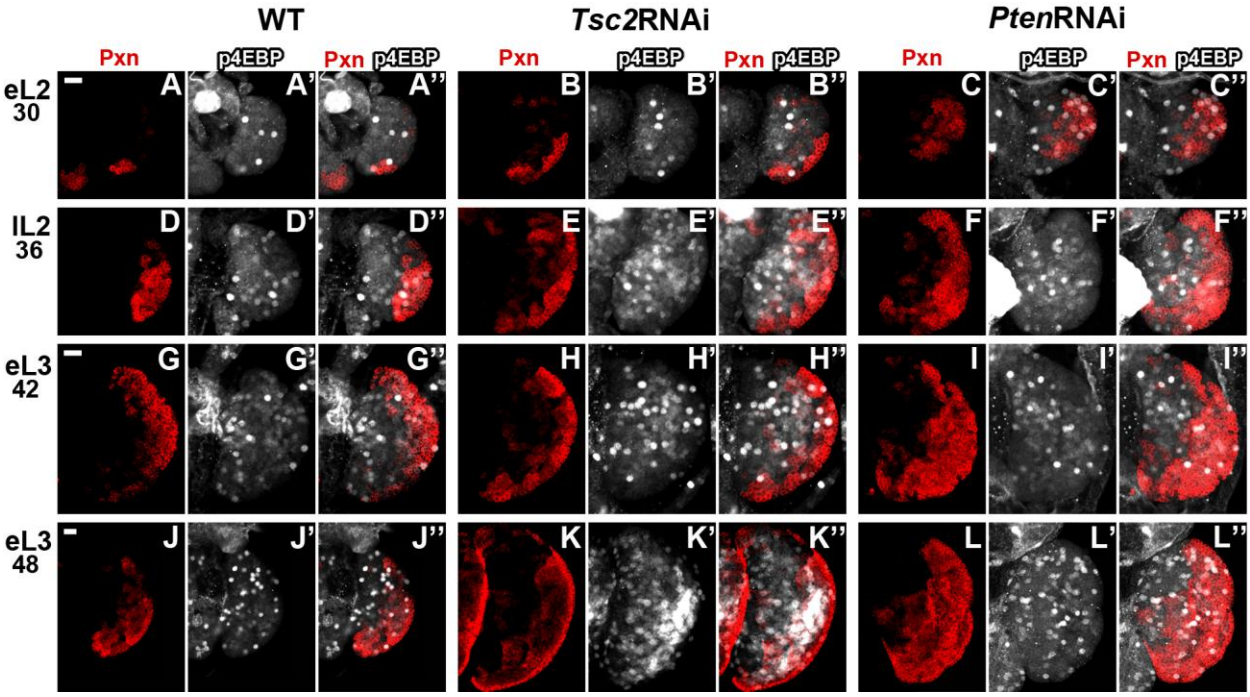

Figure A-25. Dynamic correlation between p4EBP and differentiating hemocytes during early lymph gland development

In all panels *dome-gal4, UAS-2xEGFP* [WT (**A-A", D-D", G-G", J-J"**)] is used to express *UAS-Tsc2*RNAi (**B-B", E-E", H-H", K-K"**) and *UAS-Pten*RNAi (**C-C", F-F", I-I", L-L"**) in prohemocytes. The GFP channel has been omitted for clarity. Staging represents growth at 29°C and each row corresponds to the following selected time-points after egg hatching (AEH): 30hrs (early second instar, el2), 36hrs (late second instar, lL2), 42hrs and 48 hrs (early third instar, eL3). In all panels the early-differentiation marker, Pxn, is shown in red, and p4EBP expression is shown in white.

(**A,D,G,J**) Profile of early differentiating cells (red) and p4EBP expression (white) (**A',D',G',J'**) in WT LGs. p4EBP is expressed at high levels in a small number of differentiating cells (**A",D",G",J"**), increasing in number as the LG increases in size (**J"**).

(**B,E,H,K**) Differentiation initiates and progresses normally upon downregulation of *Tsc2* in prohemocytes. The population of p4EBP^{high} cells (white) increases (**B',E',H',K'**) during development, and although there are some Pxn^{+}/p4EBP^{high} cells present at early stages (**B",E",H"**), a large population of p4EBP^{high} correlates with early differentiating hemocytes by 48hrs AEH (**K"**).

(**C,F,I,L**) Downregulation of *Pten* increases the number of differentiating cells (red) and p4EBP^{high} cells (white) (**C',F',I',L'**). Although Pxn^{+}/p4EBP^{high} cells are observed at early stages (**C",F",I"**), the number of Pxn^{+}/p4EBP^{high} cells increases as the population of differentiating hemocytes increases in the LG (**L"**).

Scale bars = 20µm. Scale bar in **A** corresponds to **A-F"**; scale bar in **G** corresponds to **G-I"**; scale bar in **J** corresponds to **J-L"**.

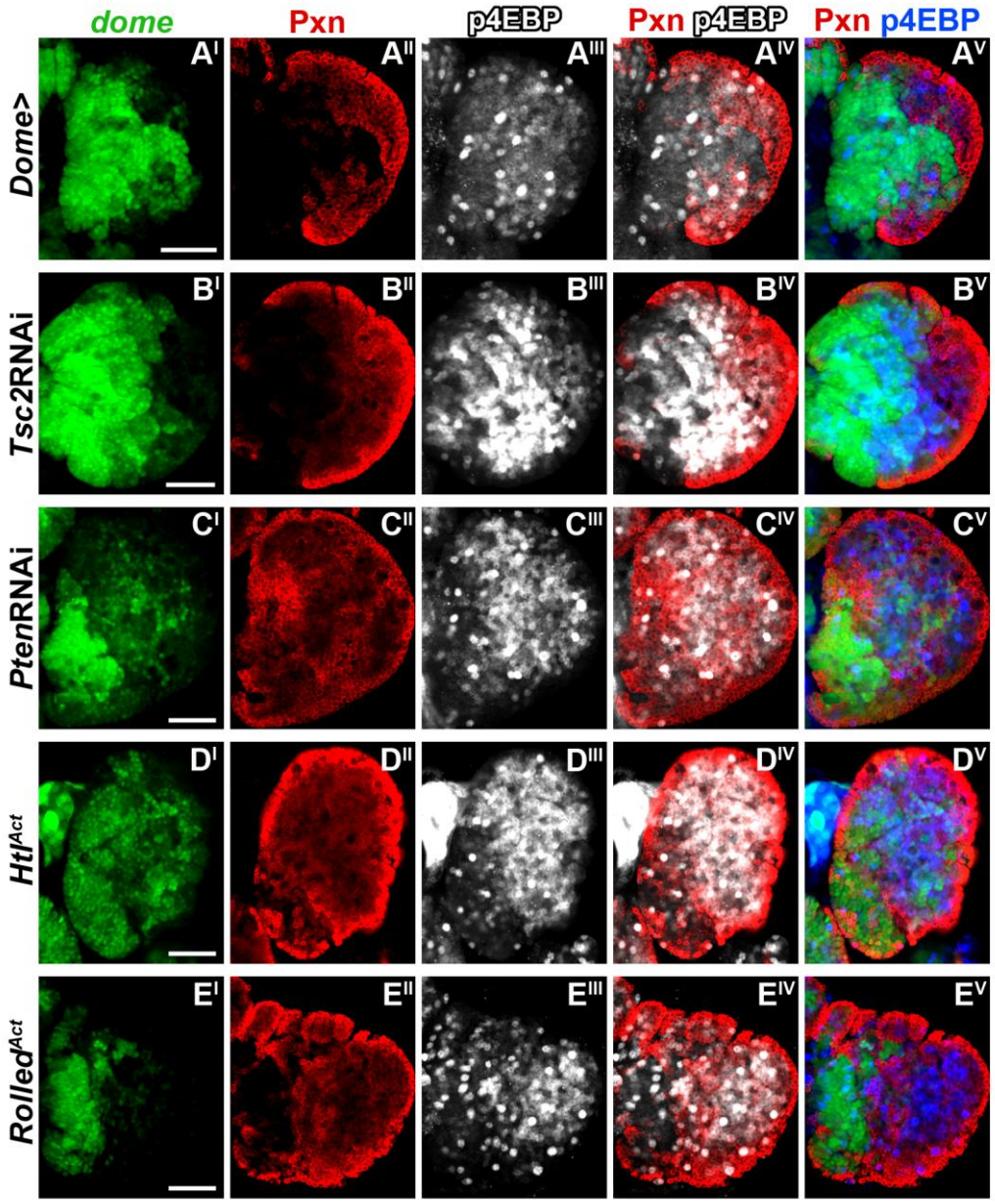

Figure A-26. Accumulation of p4EBP^{high} cells in differentiating progenitors at mid third instar upon both TORC1 and FGFR/MAPK activation

All panels represent mid third instar stage. In all panels *dome-gal4, UAS-2xEGFP* (WT, **A^{I-V}**) is used to drive expression of the specified genetic constructs in prohemocytes. p4EBP is shown in

white in the third and fourth columns, and in blue in the fifth column. Early differentiating hemocytes are marked with Pxn (red).

(**A**$^{\mathbf{I\text{-}V}}$) WT LG. p4EBP is expressed throughout the primary lobe at low levels with some scattered p4EBP^{high}/Pxn^{+} cells (**A**$^{\mathbf{III\text{-}IV}}$**)**.

(**B**$^{\mathbf{I\text{-}V}}$) Hyperactivation of TORC1 signaling in prohemocytes by downregulation of *Tsc2* expands a population of p4EBP^{high} cells along the MZ/CZ boundary in *dome*$^{+}$/Pxn^{+} hemocytes (**B**$^{\mathbf{III\text{-}IV}}$). This cell population is most pronounced in (**B**$^{\mathbf{V}}$), where p4EBP (blue) is best seen between prohemocytes (green) and differentiating cells (red).

(**C**$^{\mathbf{I\text{-}V}}$) *Pten* downregulation also increases the population of p4EBP^{high} cells in the LG, which are positive for (low levels of) both *dome* and Pxn expression.

(**D**$^{\mathbf{I}}$**-E**$^{\mathbf{V}}$) Similar to TORC1 hyperactivation in prohemocytes, activation of Htl or Rolled MAPK signaling in hemocyte progenitors, via overexpression of *Htl*^{Act} (**D**$^{\mathbf{I}}$**-D**$^{\mathbf{V}}$) or *Rolled*^{Act} (**E**$^{\mathbf{I}}$**-E**$^{\mathbf{V}}$), respectively, significantly increases the population of p4EBP^{high}/Pxn^{+} differentiating hemocytes. Scale bars = 50μm.

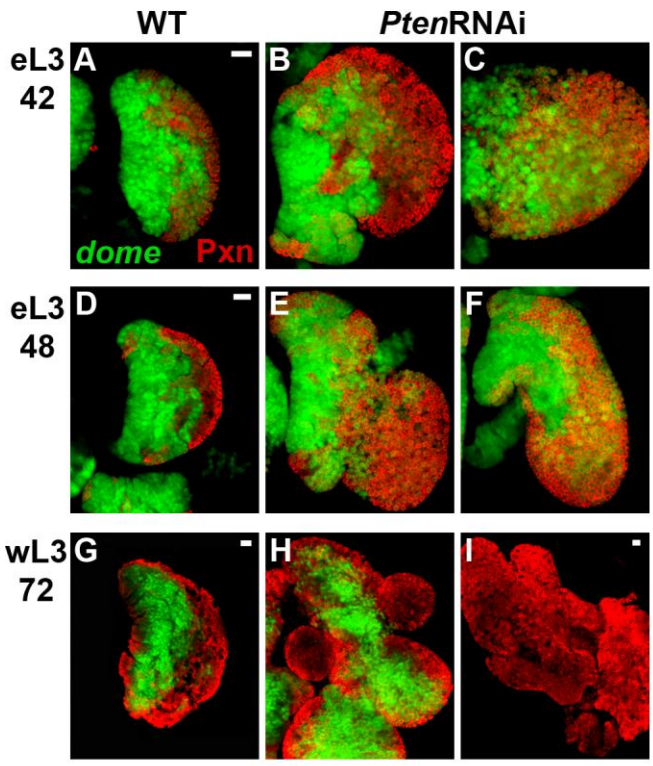

Figure A-27. Early expansion of differentiating hemocytes upon *Pten* downregulation in prohemocytes

In all panels *dome-gal4, UAS-2xEGFP* [WT, (**A**, **D**, **G**)] is used to express *UAS-Pten*RNAi (**B-C, E-F, H-I**). Larval stages and associated time-points AEH at 29ºC are listed to the left of corresponding rows. Pxn is shown in red.

(**A** and **D**) WT LGs at early third instar stage (eL3). A small population of early differentiating cells is located at the LG periphery.

(**B-C** and **E-F**) By eL3, *Pten* downregulation induces an early expansion of Pxn^{+} hemocytes that often form buds of CZ tissues at the LG periphery (**B**, **E** and **F**).

(**G**) WT LG at wL3, demonstrating a mature Pxn^{+} CZ.

(**H-I**) Increased differentiation upon *Pten* downregulation induces buds of CZ tissue (**H**). Large fragments of differentiated hemocytes (*dome* $^{-}$/ Pxn^{+}) also completely detach from the LG (**I**). Scale bars=20μm and apply to corresponding rows, except where specified (**I**).

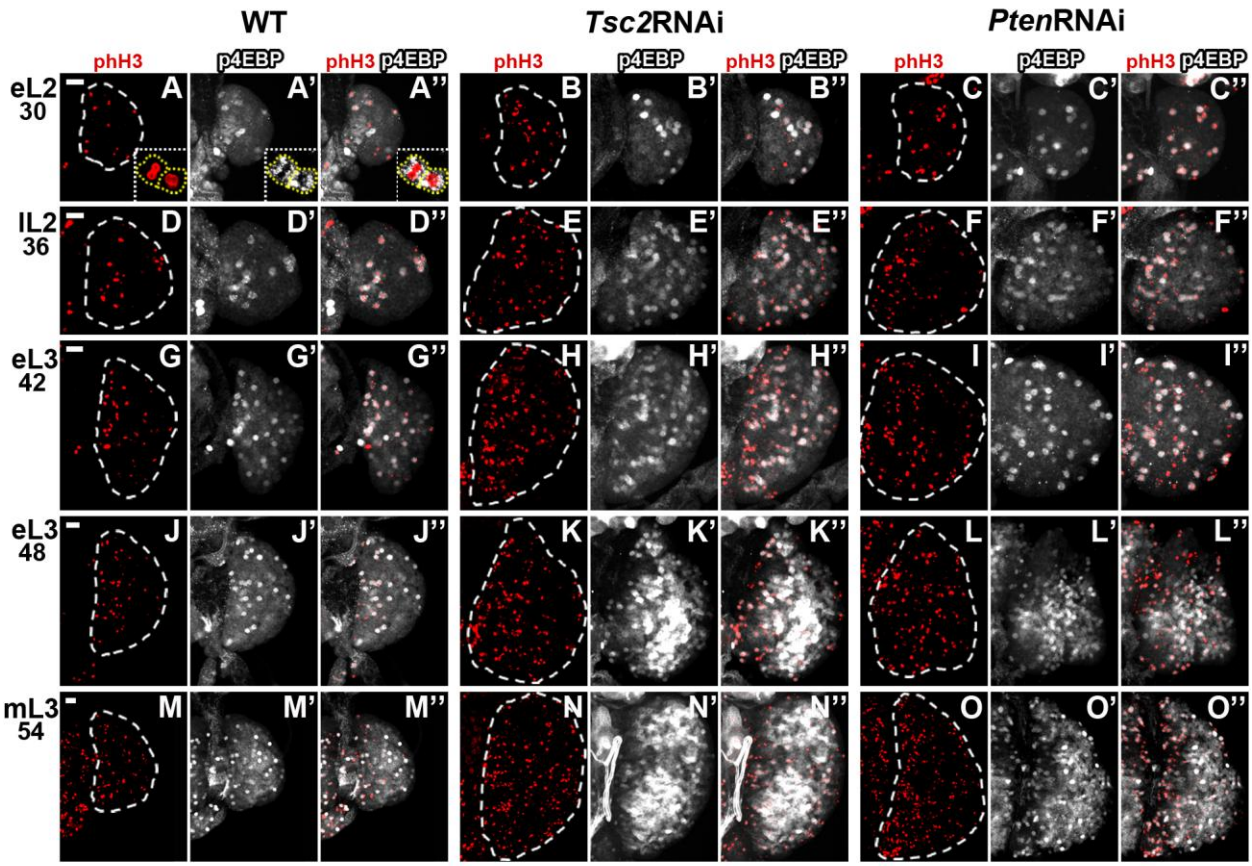

Figure A-28. TORC1 activity correlates with early proliferating prohemocytes and dynamically changes during lymph gland development

In all panels *dome-gal4, UAS-2xEGFP* [WT (**A-A",D-D",G-G",J-J",M-M"**)] is used to express *UAS-Tsc2*RNAi (**B-B",E-E",H-H",K-K",N-N"**) and *UAS-Pten*RNAi (**C-C",F-F",I-I",L-L",O-O"**) in prohemocytes. The GFP channel has been omitted for clarity. Staging represents growth at 29°C and each row corresponds to the following selected time-points AEH: 30hrs, 36hrs, 42hrs, 48hrs and 54hrs AEH.

(**A,D,G,J,M**) Proliferation profile (phH3, red) and p4EBP expression (white) (**A',D',G',J',M'**) in WT LGs. p4EBP is expressed at high levels in a small population of mitotically dividing cells throughout these stages (**A",D",G"**), increasing in number as the LG increases in size (**J",M"**).

[(**B,E,H,K,N**) and (**C,F,I,L,O**)] Downregulation of *Tsc2* or *Pten* increases the numbers of proliferating cells (red) and p4EBP^{high} cells (white) [(**B',E',H',K',N'**) and (**C',F',I',L',O'**), respectively] in the LG. phH3 and p4EBP correlate tightly at early developmental stages [(**B",E",H"**) and (**C",F",I"**)] but by mid 3rd instar stages [(**K",N"**) and (**L",O"**)] a large population of p4EBP^{high} cells is evident, which does not correspond to the population of proliferating cells.

Scale bars = 20μm. Scale bar in **A** corresponds to **A-F"**; scale bar in **G** corresponds to **G-I"**; scale bar in **J** corresponds to **J-L"**; scale bar in **M** corresponds to **M-O"**.

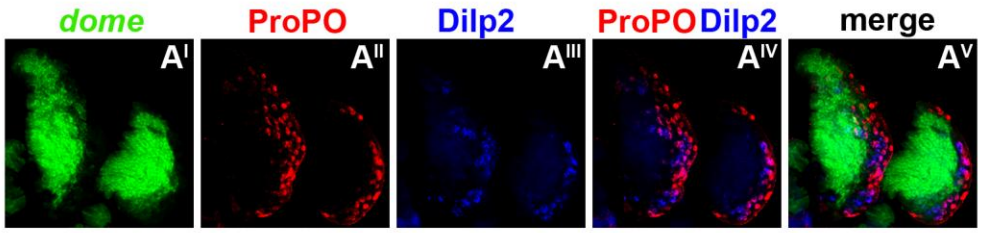

Figure 29. *Drosophila* Insulin-like peptide 2 (Dilp2) is expressed in lymph gland crystal cells

(**A^I-A^V**) WT LG (*dome-gal4, UAS-2xEGFP*) at late third instar. Hemocyte progenitors (green) and peripheral ProPO$^+$ crystal cells (red) are labeled. Dilp2 expression (blue) in the LG is limited to a few scattered hemocytes that often correlate with ProPO$^+$ crystal cells.