Understanding the role of actomyosin complex in the developing lymph gland of *Drosophila melanogaster*

A Thesis

Submitted

By

Shiv Kumar Sharma

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Department of Biological Sciences,

Indian Institute of Science Education and Research (IISER) Mohali

Sector-81, Mohali-140306, Punjab, India

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Dedicated to my parents and sisters for everything…….

Declaration

The work presented in this thesis entitled “Understanding the role of actomyosin complex in the developing lymph gland of *Drosophila melanogaster*.” has been carried out by me under the supervision of Dr. Lolitika Mandal at the Indian Institute of Science Education and Research (IISER) Mohali. This work has not been submitted in part or full for a degree, a diploma, or a fellowship to any university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bona fide record of original work done by me and all sources listed within have been detailed in the references.

Date: Shiv Kumar Sharma

Place: (Candidate)

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

Date: Dr. Lolitika Mandal

Place: (Supervisor)

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LIST OF ABBREVIATIONS

Antp: Antennapedia

CC: Crystal cell

Col: Collier

CZ: Cortical Zone

Diap1: *Drosophila* inhibitor of apoptosis

Dome: Domeless

ECM: Extra-cellular matrix

Hh: Hedgehog

Hml: Hemolectin

Hnt: Hindsight

Hpo: Hippo

HSC: hematopoietic stem cell

HSPCs: hematopoietic stem and progenitor cells

IP: intermediate progenitor

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

JNK: c-Jun N-terminal kinases

LG: Lymph Gland

LT: lineage traced

Lz: Lozenge

MZ: Medullary Zone

PH3: phosphorylated Histone H3

PKA: Protein kinase A

ProPO: Prophenoloxidase

PSC: Posterior Signaling Center

PVF: Platelet Derived Growth Factor/Vascular Endothelial Growth Factor

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

Pxn: Peroxidasin

RNAi: RNA interference

ROS: Reactive Oxygen Species

Trol: Terribly reduced optic lobes

UAS: Upstream Activation Sequence

Ush: U-shaped

Wg: Wingless

WT: wild-type

Wts: Warts

YAP: Yes-associated protein Yki: Yorkie

Yki: Yorkie

Zasp- Z-band alternatively spliced PDZ- motif

Synopsis

Introduction

Cell fate switching must be accurately regulated to generate proper outcomes during organ development. In hematopoiesis, cell fate switching from stem cell to differentiated cells may be tricky as the stem cells goes through various cell fate before it differentiates into mature blood cells. During this process, hematopoietic stem cells or precursor cells respond to external and internal cues that determine the fate of the stem cell by regulating different transcription factors and signalling pathways. With the help of different model systems, it is very well established that all stem cells or progenitor cells have different maintenance and differentiation potency in divergent tissues. Due to the progressive loss of the maintenance capacity and increase differentiation potency, all stem cells or progenitor cells generate different hierarchy. Although, we understand reasonably well how the hematopoietic stem cells or progenitor cells maintain their stemness, it remains to be seen in what way mechanical property or actomyosin activity in progenitor cells or niches regulate different signalling pathways to confer appropriate outputs during organ development. Actomyosin is involved in cellular processes, including cell adhesion, apoptosis, proliferation, differentiation, and collective cell migration in Drosophila, C. elegans, and mammalian system. Studies show that actomyosin assembly also plays a crucial role in cell fate specification and patterning during development via modulating different signalling molecules. The process engaged is highly conserved across taxa. Despite having a wealth of literature illustrating the different role of actomyosin in cell fate specification in divergent cell types, actomyosin components' blood type-specific role is yet to be fully appreciated. The Drosophila hematopoietic system provides an excellent opportunity to tease out the mechanistic basis of blood cell development. Studies in the last decade have shown that Drosophila blood cell development has resounding similarities with

the vertebrate counterparts. Like vertebrates, Drosophila hematopoiesis can be categorized into primitive and definitive hematopoiesis. While primitive hematopoiesis occurs in the Drosophila embryonic head mesoderm, definitive hematopoiesis happens in a multi-lobed organ known as the lymph gland that originates from the cardiac mesoderm in the embryo. By third instar larval stage , the lymph gland's anterior lobe gets organized into three distinct domains. The outer periphery (Cortical zone, CZ) consists of differentiated blood cells, while stem-like progenitors populate the core of the organ (Medullary Zone, MZ). These stem-like cells are regulated through a cluster of cells that forms the Posterior Signalling Centre (PSC) or the hematopoietic niche. This valuable system helped us in elucidating the molecular mechanism essential for progenitor cell maintenance and differentiation. Objectives: Actomyosin activity indicates the mechanical property of a cell. Several studies elucidate the diverse role of actomyosin activity in regulating cell adhesion, apoptosis, proliferation, and differentiation in different cell types. However, blood progenitor specific role of actomyosin complex is not very well characterized. Therefore, the current study aims to understand the role of actomyosin activity in the hematopoietic system using Drosophila as a model.

The outcome of current study:

a. **Cell-cell adhesion mediated actomyosin assembly regulates Cubitus interruptus for the maintenance of hematopoietic progenitor cells in the lymph gland**.

Based on different molecular marker and cell cycle indicators, I have first characterized the Drosophila hematopoietic progenitors at different developmental timepoints. Previous studies have established that early instar progenitor cells are in the proliferative phase. These proliferating progenitors of early stages move into quiescence later in development since they fail to incorporate BrdU. Interestingly, using FUCCI-system, I have found that the quiescent progenitors are arrested in the G2 phase of the cell cycle. Therefore, the previously described “quiescence” in the lymph gland hemocyte progenitors of the late larval stages is actually a

stall in G2. Further, I have done a detailed characterization of the state of both proliferative and quiescent hemocyte progenitors. Using different molecular markers, I have classified Drosophila hematopoietic progenitors into three groups: naive progenitors, primed progenitor, and differentiating Intermediate Progenitors: IPs. Although previous studies have proposed IPs' presence, this study provides the opportunity to identify them by markers. Interestingly, this study also reveals that the maintenance of primed progenitor cells depend on the actomyosin activity. Our analysis revealed that squash (actomyosin component, sqh) and the indicator of activated sqh, pMRLC were highly enriched in the progenitor cells while down-regulated in Intermediate Progenitors of the lymph gland and its loss leads to a reduction in the primed progenitor cells. Our genetic and molecular data suggest that actomyosin positively regulates larval blood progenitors by regulating Cubitus interruptus (Ci, the downstream activator of Hedgehog signalling) via PKA independent regulation. Furthermore, the current work shows how the enrichment of actomyosin is essential for a spatial refinement of Ci-155 expression in primed progenitors crucial for their maintenance. Interestingly, it is clear from our genetic and expression studies that the enrichment of cortical actomyosin progenitor cells is regulated through DE-cadherin-mediated Cell-cell adhesion. This section establishes how actomyosin activity results in the de-sensitization of progenitor cells to Hh signalling, permitting them to differentiate.

b. Ance-shg-actomyosin controls G2/M progression in the proliferative Drosophila hematopoietic progenitor cells.

In the next section of my thesis, I have addressed the role of Ance-shg-actomyosin in the early proliferating blood progenitors of the Drosophila larval lymph gland. I found that knockdown of actomyosin components in early hematopoietic progenitor affects the lymph gland's size. Our detailed analysis reveals that actomyosin assembly is essential for G2/M transition in the proliferative progenitor cells. Interestingly, loss of DE-Cadherin (shotgun) and Angiotensin

Converting Enzyme (Ance) also affect lymph gland size, and progenitor loss of these components leads to a halt in G2. Furthermore, our expression and genetic data implicate that the decrease in proliferation was due to the reduction in string (stg: an essential regulator of G2 to M transition) expression. Overexpression of stg in these genetic backgrounds rescues the cell cycle defect. Therefore, it is quite evident from our analyses that Ance-shg-actomyosin plays a dual role in the progenitors. Early on this axis plays a pivotal role in G2 to M progression of Drosophila early hemocyte progenitors of the lymph gland. Later on in development, this axis is reemployed for the maintenance of the progenitor cells.

c. **The biophysical force generated by neighbouring myo-epithelial cardioblasts is crucial for the hematopoietic microenvironment.**

Apart from intrinsic regulation, hematopoietic progenitor cell maintenance is also regulated through its microenvironment (niche). While many cellular niches derived signalling molecules have been identified, which are essential for progenitor cell maintenance, signals for niche maintenance is still not very well worked out. In this section, using GAL4-UAS and RNAi-based approaches, I have studied the role of Zasp (an essential structural components for muscle integrity) in hematopoietic niche maintenance. My analysis reveals that the down regulation of Zasp in the nearby myo-epithelial cells non-autonomously increases the hematopoietic niche cell number. Employing genetic tools and life imaging, I provide evidence that the myo-epithelial cells physically interact with hematopoietic niche and how upon loss of zasp this physical interaction is affected. This chapter will highlight the possible mechanism that is evoked to increase the cell number of the niche.

Conclusion:

My work unravels an otherwise unappreciated role of actomyosin in patterning and maintaining the Drosophila hematopoietic system via regulating different signalling pathways

in progenitor and niche cells. The current study identifies Ance-actomyosin-Cell adhesion-Hedgehog, as a new axis essential for progenitor maintenance. This study revealed another exciting mechanism where angiotensin-converting enzyme can perform RAAS independent function in the hematopoietic system. That the biophysical force generated by neighbouring myo-epithelial cardioblast crucial for the maintenance of the hematopoietic niche is another exciting outcome of this study. Put together; the current work illustrates how actomyosin activity integrates with patterning, morphogenesis, and maintenance of developing Drosophila hematopoietic organ

Contents

Chapter 1

Drosophila Hematopoiesis – a developmental model to understand the hematopoietic process

1.1 Introduction

The evolving research in basic biology has significant implications in the understanding of the fundamental mechanism required for the development of organs and designing new therapies for disease, including cancer. Over decades, studies in small organisms like *Drosophila* melanogaster, C.elegans and, Zebrafish have made significant contributions to understanding the underlying mechanism of animal development as another promising question related to human health (Wangler et al., 2015; Wangler et al., 2017).

Despite its simplicity, studies on tiny fruit fly, *Drosophila* melanogaster led to discoveries ranging from the identification of phenotype to gene function contributing to our understanding of the developmental instructions that define the body plan. This model system established on the primer of the seminal invention of the 20th century the "chromosome theory of inheritance" by Morgan lab at Columbia University (Benson, 2001) and "the discovery of the production of mutations employing x-ray by Hermann Muller lab (Calabrese, 2018). Apart from easily available mutants, the rapid development of scientific technologies, including the GAL4-UAS (Brand and Perrimon, 1993) and RNA interference (RNAi) (Perrimon et al., 2010) mediated gene silencing, has made the recognition of *Drosophila* an excellent model organism to understand the critical insights the singular function of numerous gene and its targets (St Johnston, 2015). In the recent past, the *Drosophila* model system is moved from just genetic mutational analyses to teasing out complex issues related to animal development, regeneration, aging, and behavior (Green, 2010; Hales et al., 2015)

One of the developmental events that also caught attention is blood cell generation or hematopoiesis (Banerjee et al., 2019; Jung et al., 2005). Work from the last decade has established *Drosophila* as an excellent model system to study blood cell development. It has been shown that several molecular players involve in blood cell development in the vertebrate system also perform similar tasks in *Drosophila* (Banerjee et al., 2019; Hartenstein, 2006; Jung et al., 2005; Rehorn et al., 1996). This considerable homology at the level of transcription factors and signaling molecules, along with diverse genetic tools available, has made *Drosophila* a well-accepted model to understand underlying molecular mechanisms involving in blood cell development.

1.2 *Drosophila* Blood cells development- types, origin and lineage specification

Hematopoiesis is a major developmental process by which the body generates blood cells to defend the pathogens. Understanding how these blood cells develop and function appropriately is a fundamental and vital question. In the vertebrate, a hematopoietic stem cell (HSC) has self-renewal

and long-term multi-lineage activity (Kyba and Daley, 2003; Orkin and Zon, 2008; Riether et al., 2015). They also can produce a hierarchy of hematopoietic progenitor cells and differentiating blood cells. HSC initially gives rise to its lineage-specific progenitor cells, which lost their self-renewal activity but maintained the lineage-specific differentiation program. Differentiation and maintenance of hematopoietic stem and progenitor cells (HSPCs) are tightly regulated by a variety of coordinated signals, which includes HSPCs derived local factors and external cues such as niche derived secreted signalling molecules (Seita and Weissman, 2010)

In recent years, *Drosophila* has emerged as a powerful genetic model system to understand the molecular and genetic programs essential for precursor cell maintenance (Banerjee et al., 2019), blood cell type specification, and generate an immune response against pathogens (Lemaitre et al., 1997; Sorrentino et al., 2002). *Drosophila* HSPCs can produce three types of blood cell types that resemble myeloid lineage of vertebrates and mainly involved in the innate immune response (Banerjee et al., 2019; Jung et al., 2005).

In the subsequent paragraphs, a description is given on blood cell types in *Drosophila*, their origin at different developmental sites, and signaling pathways involved in the maintenance and blood cell specification.

1.2.1 Blood cell types in embryo, larvae and, adult *Drosophila*-

In wild type conditions, *Drosophila* harbors two types of blood cells-phagocytic plasmatocytes and the crystal cells (Banerjee et al., 2019; Jung et al., 2005). These hemocytes originate through the two hematopoietic waves-primitive and definitive. During an immune challenge, the third type of hemocytes, lamellocyte is seen in larvae, which is essential to mount an immune response (Banerjee et al., 2019; Stofanko et al., 2010). Thus, hemocytes in the *Drosophila* can be classified into three broad categories based on their morphology, function, and gene expression profiles

a) Plasmatocytes

b) Crystal cells

c) Lamellocytes

a) Plasmatocytes-

Plamatocytes are the most abundant hemocyte and present in all developmental stages of *Drosophila* (Jung et al., 2005*)*. It is generally appearing as round cells 8-10 μm in diameter and primarily function as professional phagocytes macrophages ((Rizki et al., 1990). The phagocytic role of plasmatocytes is mainly mediated through Croquemort and Draper, which involve in recognize the dying cells ((Franc et al., 1996; Manaka et al., 2004). The Eater (Kocks et al., 2005) and NimrodC1 (Kurucz et al., 2007) also help in recognize the bacteria. Plasmatocytes also involved in the humoral immune response by directly secreting anti-Microbial Peptides (AMPs)

(Samakovlis et al., 1990) and activating the Jak-Stat (Janus kinase signal transducer and activator of transcription pathway in the fat body (Agaisse et al., 2003).

Apart from conventional roles in immunity, plasmatocytes are also involved in apoptosis-induced cell proliferation (AiP) in imaginal disc cells (Fogarty et al., 2016), and injury-induced proliferation in intestinal stem cell (ISC) (Chakrabarti et al., 2016). It is also known that plasmatocytes secrete extracellular matrix (ECM) protein peroxidasin (Pxn), the collagen proteins Dcg1, Viking (Vkg) and Laminin (Bunt et al., 2010; Fessler et al., 1994; Olofsson and Page, 2005; Yasothornsrikul et al., 1997). These proteins are deposited in basement membranes, critical for many morphogenetic processes in the *Drosophila* embryo and adults (Kiger et al., 2001).

b) Crystal Cells

These are nonphagocytic cells that function in the process of melanization, facilitating innate immune and wound-healing responses in the cuticle. Crystal cells can be identifying via para-crystalline inclusions and molecular markers like Lozenge (Lz; *Drosophila* Runx protein), Hindsight (hnt; downstream molecules of Notch pathway), and Pro-phenoloxidase A1(ProPO) (Evans et al., 2014). It is proposed that crystal cell inclusions contain prophenoloxidase enzymes, which is released upon rupture of the crystal cells (Vlisidou and Wood, 2015). During melanization, prophenoloxidase is converted into phenoloxidase that catalyzes the oxidation of phenols to quinones. Quinones are then nonenzymatically polymerized into melanin, which participates in the parasite's encapsulation process (Binggeli et al., 2014; Cerenius et al., 2008). Although it was initially believed that crystal cells are only found in embryo and larvae, a study has demonstrated its presence in adult fly too (Ghosh et al., 2015).

c) Lamellocytes

Drosophila larvae also generates a third type of hemocytes known as lamellocytes during immune challenge which can be recognized by different markers like L1, misshapen (upstream regulator of the c-Jun N-terminal kinase signaling), and Myospheroid (mys; βPS-integrin)(Banerjee et al., 2019; Evans et al., 2014; Jung et al., 2005). These are nonphagocytic and adherent cells encapsulates large object like wasp egg (Nappi, 1975; Rizki et al., 1990). During immune challenge, these cells surround the objects and melanizes them via locally generating cytotoxic compounds thus creating a barrier. Lamellocytes are generate both *de novo* (Lanot et al., 2001) as well as by trans-differentiation of crystal cells (Leitao and Sucena, 2015) and plasmatocytes population (Stofanko et al., 2010).

1.2.2 Understanding of blood cells origin in *Drosophila*; From Embryonic to Adult haematopoiesis

*D*rosophila *melanogaster* is holometabolous and undergoes four stages of the life cycle; Embryo, larvae, pupa, and adult fly. Like the vertebrate's system, *Drosophila* hematopoiesis also occurs at distinct anatomical sites (head mesoderm and cardiogenic mesoderm) in the embryonic stages at different developmental time (Jung et al., 2005)(Figure 1.1A-G). The primitive hematopoiesis occurs in the head region and provides plasmatocytes and the crystal cells in the embryo (Figure 1.1A-C). The second phase occurs in the lymph gland, which originates from the cardiogenic mesoderm and is capable of giving rise to plasmatocytes and crystal cells (Banerjee et al., 2019) (Figure 1.1A-B, D). Lineage tracing experiment (Ghosh et al., 2015) suggest that blood cell originate from the head mesoderm (primitive phase) and lymph gland (definitive phase), home at a specific site in adult known as adult hematopoietic hub and provide hemocytes in the adult *Drosophila* (Figure 1.1G).

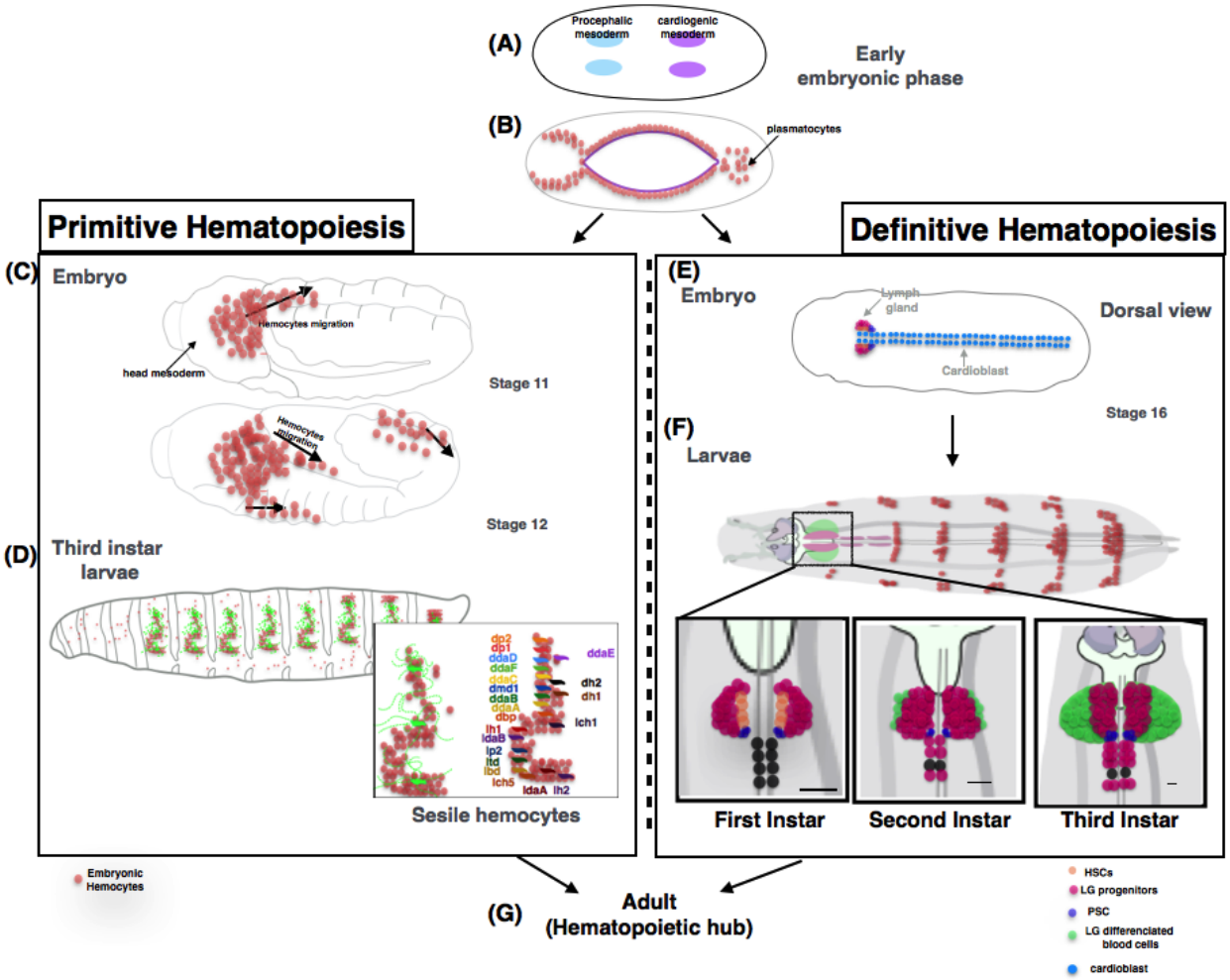

Figure 1.1 Primitive and definitive hematopoiesis in Drosophila: (A-B) Primitive hematopoiesis happens in the procephalic mesoderm (head), whereas definitive hematopoiesis occurs in cardiogenic mesoderm (thorax). (C) At stages 11-12, embryonic hemocytes derived from head mesoderm migrate via several well-defined migration routes (arrow). (D) During the larval stage, head mesoderm derived hemocytes home into hematopoietic pockets under the guidance of signals received from the sensory neurons. (E) Definitive hematopoiesis takes place in the lymph gland, which originates from the cardiogenic mesoderm. In the

embryo, the lymph gland localized in the anterior part of the cardiac tube and dorsal side of the embryo. (F) Schematic diagrams of LG primary lobes from first, second, and third instar larvae. The first instar lymph gland contains niche (blue), hematopoietic stem cells (brown), which give rise to progenitor cells. In the second instar, LG harbors niche cells (blue), progenitor cells (magenta), and in the differentiated cells (green). In the third instar, the overall LG size and the number of differentiated hemocytes (green) increases. (G) Hemocytes originated from the head mesoderm (Primitive hematopoiesis) and the lymph gland (definitive hematopoiesis) home at the specific site in an adult known as the hematopoietic hub. *Modified from (Banerjee et al., 2019; Evans et al., 2003)*

1.2.2.1 Primitive Hematopoiesis in embryonic head mesoderm

The first embryonic wave or primitive phase, which occurs in the embryonic head mesoderm, generates two types of mature blood cells, the plasmatocytes, and the crystal cells, both of which can also be traced in the larvae. The precursor cells which expresses GATA factor Serpent (Srp)(Fossett et al., 2001)) can be first identifiable in embryonic stages 5. They undergo several rounds of cell division before differentiating into either Hemolectin (hml) positive plasmatocytes or Lz positive crystal cells (Lebestky et al., 2000; Rehorn et al., 1996; Tepass et al., 1994). The blood cell fate specification in head mesoderm is regulated through FOG family member U-shaped (Ush) (Fossett et al., 2003),Glial cells missing (Gcm) protein (Bernardoni et al., 1997) and Notch-Serrate pathway (Duvic et al., 2002; Lebestky et al., 2003). It has been demonstrated that the Srp up-regulate Lz expression in hemocytes to induce crystal cell formation (Fossett et al., 2003). In contrast, later in development, Ush along with Gcm and Gcm2 antagonizes crystal cell fate and promotes plasmatocyte fate via repressing Lz (Fossett et al., 2001). At stage 9, plasmatocytes generated in the head region start migrating along specific paths (Figure 1.1C), following cues of the platelet-derived growth factor- and vascular endothelial growth factor-related factors (Pvf) 2 and 3 to populate the entire embryo (Muratoglu et al., 2006; Tepass et al., 1994). Apart from Pvf2, it has also been documented that macrophage invasion required Eiger-Grindelwald-Myosin signaling (Ratheesh et al., 2018), which is needed for cell deformations and reductions in apical tension in the ectoderm, essential for macrophage migration in the germ band.

These head mesoderm derived hemocytes increase their number of approximately 6000 cells by the third larval stage. They are found in circulation as well as in the hematopoietic pockets or sessile clusters attached to the larval cuticle (Lanot et al., 2001). Here in the pocket, they are maintained by signals emanating from the sensory neurons (Makhijani et al., 2011) (Figure 1.1D). In the sessile clusters, hemocytes are found in the aggregation which is necessary to induce crystal cell differentiation from the plasmatocytes (Leitao and Sucena, 2015). It has been demonstrated that new crystal cells in sessile pockets originate through cell contact-mediated Serrete-Notch-dependent plasmatocyte trans-differentiation. These findings highlight the outstanding fundamental

questions of the importance of structure-dependent signaling events essential for cell-fate switching.

1.2.2.2 Definitive hematopoiesis in the embryonic cardiogenic mesoderm-

The second phase or definitive wave of hematopoiesis occurs in a specialized hematopoietic organ known as the lymph gland (LG)(Figure 1.1 F), which originates from the cardiogenic mesoderm (Holz et al., 2003; Rugendorff et al., 1994). The mesoderm gives rise to hemangioblast, a bi-potent progenitor to cardiac and blood lineages(Mandal et al., 2004; Medvinsky and Dzierzak, 1996)

1.2.2.2.1 Induction of the hematopoietic program in the embryonic cardiogenic mesoderm-

Several studies have identified various signaling molecules involved in the specification of different lineage from the cardiac mesoderm. Interestingly, these signaling pathways also perform a similar task in the vertebrate system (Evans et al., 2003; Fossett et al., 2001). GATA factor Pannier (Pnr) (Klinedinst and Bodmer, 2003) and Nk2-homeobox-containing transcription factor Tinman (tin) required for specification of cardiogenic mesoderm (Mandal et al., 2004) while TGF protein Decapentapelagic (dpp), Wingless signaling (Wg), Notch, FGF, Htl essential for its maintenance (Klinedinst and Bodmer, 2003) (Figure 1.2A-C). Moreover, Notch signaling plays a dual role; early on, it is required for restricting cardiogenic fate, while later in development, it ensures restriction of cardioblast fate and facilitates the specification of the lymph gland (Mandal et al., 2004).

There are some key stages to the embryonic lymph gland (LG) development programs in the cardiogenic mesoderm which specify the hematopoietic stem cell and niche (Figure 1.1 D-J):

(1) During germ band retraction (8−10 hours after fertilization), Tin and Pnr in the cardiogenic mesoderm become restricted to the cardioblasts. While some cells cluster which gives rise to LG precursor start expressing the zinc finger protein Oddskipped (Odd), Serpent (Srp) and Homothorax (Hth) and become negative for Tin and Pnr (Figure 1.2D). So, establishment of the hematopoietic program in these cardiogenic mesoderm cells relies on the Odd, Srp, and Hth.

(2) Once the hematopoietic fate is established at embryonic stages 11-12, a fraction of Srp positive cells in the third thoracic segment (T3) start down-regulating Hth and initiates the expression of homeobox gene Antennapedia (Antp) (Figure 1.2E-J). This group of cells is specified as the cellular niche (Posterior Signaling Center, PSC) for hematopoietic stem cells. The down-regulation of Hth expression is essential for the specification of PSC, as over-expression of Hth causes loss of PSC cells (Mandal et al., 2007).

(3) Collier (Col), an Early B-cell Factor like transcription factor initially expresses in the entire embryonic lymph gland, but later on, at stage 16, it gets restricted only in Antp positive PSC. Genetic epistasis and expression analysis revealed that Antp is genetically upstream of the Collier in the PSC (Crozatier et al., 2004).

Thus, the PSC cells are specified distinctly from the rest of the lymph gland primordia. So, the embryonic lymph gland is made of hematopoietic precursor cells and their cellular niche.

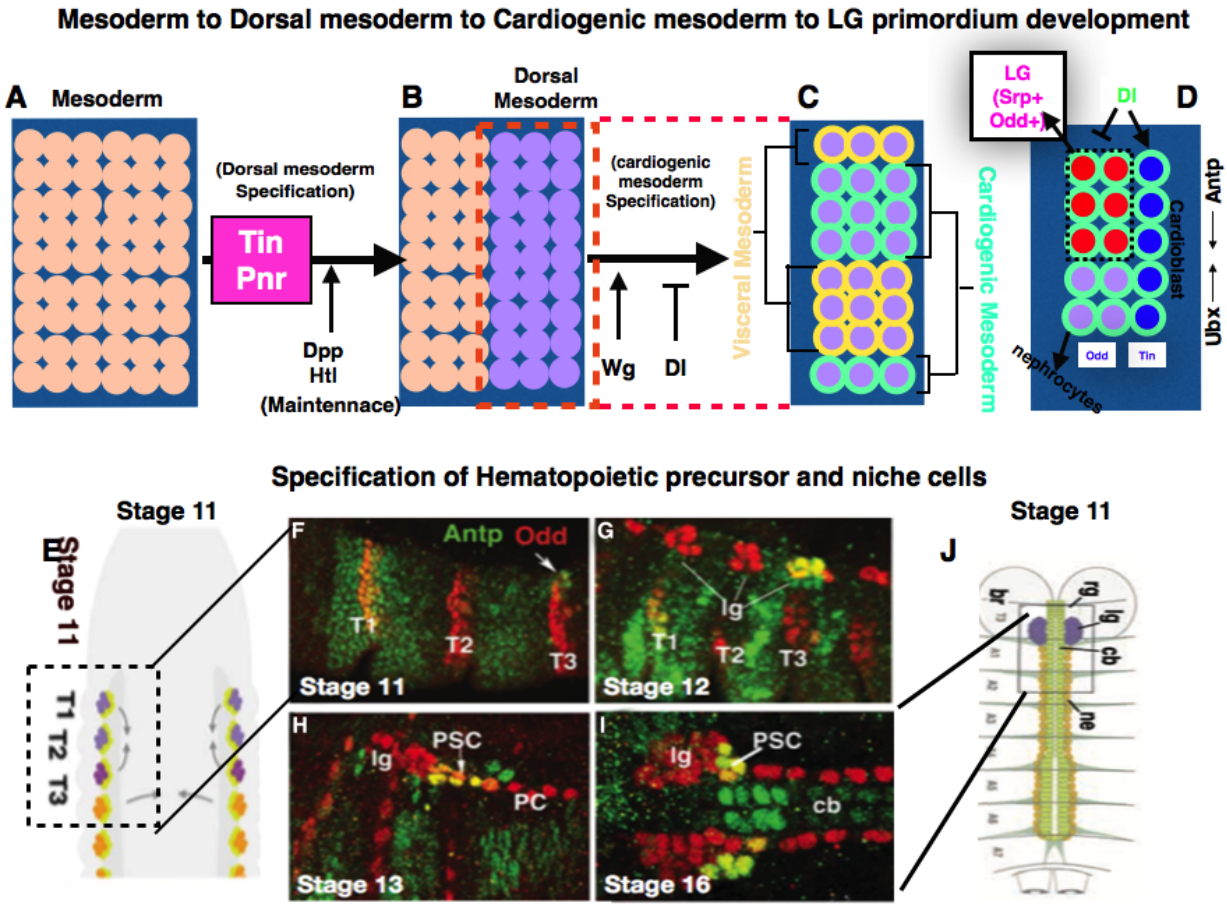

Figure 1.2 Specification of the lymph gland from the cardiogenic mesoderm. (A-B) Scheme showing the generation of dorsal and cardiogenic mesoderm. Dorsal mesoderm from mesoderm is specified through Tinman (Tin) and Pannier (Pnr) while it maintains though heartless (Htl) and decapentaplegic (Dpp) signaling. (C-D) Cardiogenic mesoderm specification required input from wingless and Notch signaling. Cardiogenic mesoderm is further specified in lymph gland precursors, cardioblasts, and nephrocytes. (E-J) Srp+ Odd+Lymph gland is specified in the T1-T3 thoracic segments via Antennapedia (Antp) (stage 11) and is repressed by ubx. At stage 12-16, Antp expression only restricted to the niche cells (PSC) in the T3, and other odd+ cells give rise to for hematopoietic stem cells. Modified from Mandal *et al.* 2004.

1.2.2.2.2 Hematopoietic stem cells in the *Drosophila* larval system

It was believed that there are no hematopoietic stem cells in the fly, but Dey et al. 2016 challenged this notion. Employing lineage-tracing and genetic ablation strategies, they found the existence of Notch expressing HSCs in the first instar larval lymph gland. Furthermore, using a loss of function

and gain of function experiment, they identified Dpp as the signal from the hematopoietic niche required for HSCs maintenance (Dey et al., 2016). These Notch expressing HSCs cells give rise to Dome positive hematopoietic progenitor cells. This model system can further be explored to understand the developmental program essential for hematopoietic stem cell maintenance.

1.2.2.2.3 Hematopoietic progenitor cells in the *Drosophila* larval system

Before identifying hematopoietic stem cells, the lymph gland was used as a progenitor cell model system to understand various fundamental questions related to progenitor cell fate specification and niche-progenitor interactions.

In the third instar larvae, the lymph gland is composed of a pair of anterior (primary) lobes and several small posterior (secondary) lobes, which contain immature precursor cells (Jung et al., 2005; Krzemien et al., 2010). The anterior lobe of the lymph gland is organized into different distinct domains (Fig 1A-A'). The outer periphery (Cortical zone, CZ) consists of differentiated blood cells, while progenitors populate in the core of the organ (Medullary Zone, MZ). Posterior to these domains, a cluster of cells forms the Posterior Signaling Center which serves as the hematopoietic niche (Krzemien et al., 2007; Mandal et al., 2007), crucial in maintaining the progenitor cells through Hedgehog signaling (Mandal et al., 2007). Upon pupation, progenitor cells of the lymph gland differentiate into plasmatocytes and crystal cells and are released into circulation. Apart from the primary lobe, the lymph gland is also made of secondary, tertiary and, sometimes additional lobes that line the dorsal vessel. It was demonstrated that progenitor's cells from the secondary and tertiary lobe home into the hematopoietic hub of adult fly (Ghosh et al., 2015).

1.2.2.2.4 Hematopoiesis in Adult Drosophila- Hematopoietic hub, a source of new blood cells in the fly

Primitive hematopoiesis generates hemocytes for the embryo, larvae, and adult system, whereas definitive hematopoiesis provides hemocytes for the larval and adult system. It was believed that hematopoiesis does not happen in the adult, and its survival depends on the contribution of both embryonic and larval hemocytes. However, Ghosh et al. provided evidence of Notch positive progenitor cells and new blood cell development in the dorsal side of the adult fly abdomen. It was found that progenitors and differentiated hemocytes are embedded in the functional extracellular matrix of Laminin A and Pericardin (Ghosh et al., 2015). Interestingly, these clusters are capable of crystal cell development and proliferation in response to bacterial challenges. This adult model system can further dissect the fundamental questions related to hematopoiesis during normal development, cancer, infection, wound healing, and aging.

1.3 Drosophila larval Lymph gland A model for understanding blood cells development, maintenance, and differentiation

The definitive hematopoiesis happens in the lymph gland that specified in the embryonic phase and persisted till pupation. The lymph gland is a multi-lobed structure, which stays in close association with the cardiac tube or dorsal vessel. During metamorphosis, the lymph gland disintegrates and releases hemocytes into circulation. It has been demonstrated that the larval lymph gland's blood precursors are the source of precursor cells in the adult hematopoietic hub to generate both plasmatocytes and crystal cells (Ghosh et al., 2015).

1.3.1 Development, pattering, and cellular organization of the lymph gland

During the embryonic stage, some of the Pnr and Tin positive cardioblast mesoderm cells start expressing Odd, Srp, and Hth, the Notch positive hematopoietic stem cells in the embryo. In the first instar, Notch positive stem cells divide and give rise to Dome expressing progenitor cells. The lineage tracing experiment suggests that Notch expressing stem cells generate all types of differentiated cells in the third instar lymph gland (Dey et al., 2016). The first instar lymph gland is made up of three different cell types Notch expressing stem cells, Dome expressing progenitor cells, and PSC that serve as the niche that secretes Dpp. At the beginning of the second instar, none of the Notch expressing stem cells can be seen (Dey et al., 2016), implicating that the Notch positive stem cells eventually become Dome positive progenitor cells.

In the late second instar, progenitor cells proliferate (Jung et al., 2005; Mondal et al., 2011) as reported by the high number of BrdU as well as PH3 labeling (Figure 1.3A-C), to generate sufficient progenitor cells. In the late second instar stages, when differentiation in the lymph gland initiates in the periphery (Figure 1.3D), the third zone Cortical Zone (CZ) of differentiated cells (Hml+ Pxn+) is defined and can be visualized thereafter(Jung et al., 2005).

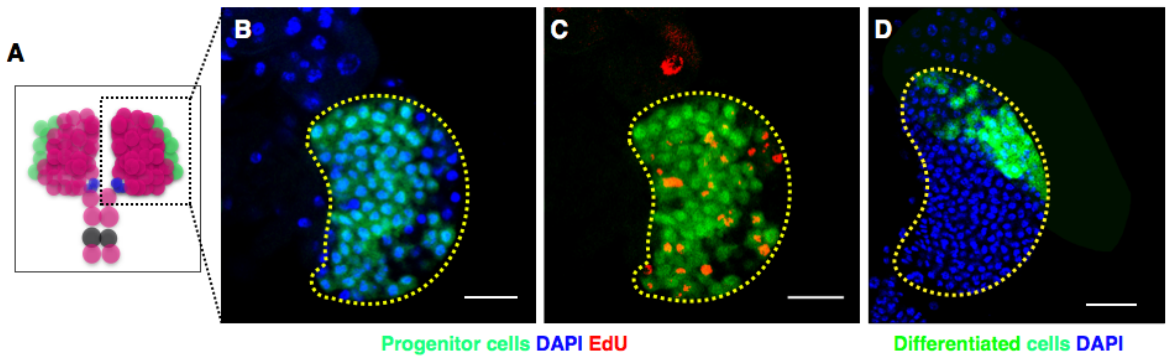

Figure 1.3 Proliferation and differentiation in second instar lymph gland. (A). Schematic diagrams of second instar LG primary lobes. (B-C) Second instar LG progenitor (green), are proliferative (EdU; red) in

nature. (D) The majority of cells in the late second-instar primary lobe are undifferentiated, while few cells in the periphery initiate the expression of differentiated cell marker (Pxn).

The third instar lymph gland is a multi-lobed structure and stays in close association with the heart (or dorsal vessel) (Figure 1.4A). The standard axis accepted is with respect to dorsal vessel (Banerjee et al., 2019): the region near the dorsal vessel is medial, and the opposite is distal (Figure 1.4B). While the niche side is referred to as posterior, and opposite to the niche is the anterior side. In third instar lymph glands, the blood progenitors and the differentiated cells can easily be distinguished by the DIC microscopy based on the morphological differences Figure 1.4C or by the differential distribution of extracellular matrix protein Perlecan (also know as *terribly reduced optic lobe*; *trol*) (Figure 1.4D) (Grigorian et al., 2013; Jung et al., 2005). The progenitor cells show a smooth appearance of tightly packed cells, while differentiated cells exhibit a granular appearance. (Figure 1.4F). At this time-point, the BrdU or PH3 incorporation can only be seen in the CZ while the MZ lacks BrdU labeling (Jung et al., 2005; Mondal et al., 2011) (Figure 1.4E). A narrow rim of cells (IZ, intermediate zones) between MZ and CZ defines cells that are about to differentiate. Thus, the primary lobes of the lymph gland broadly can be divided into four distinct zones (Jung et al., 2005; Krzemien et al., 2010)

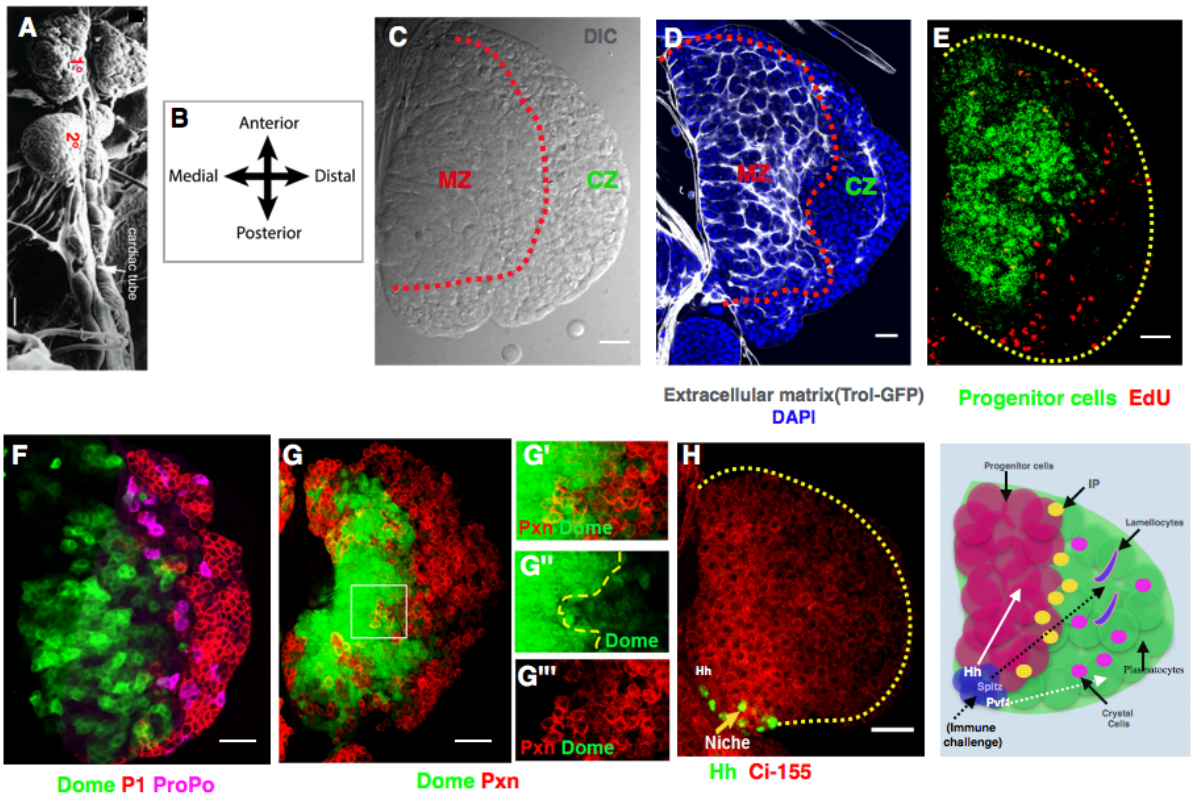

Figure 1.4 Pattering and cellular organisation in third instar lymph gland (A) A scanning electron micrograph showing LG and cardiac tube (dorsal aorta) (Meister, M et al., 2003) (B) The region near the dorsal vessel is medial, and the opposite is distal to follow the convention nomenclature. In contrast, the niche side is posterior and the opposite side as an anterior side. (C) DIC image shows the morphological difference between the MZ (medial) and the CZ(distal). (D) A denser network of ECM made of Terribly reduced optic lobe (trol; grey) is seen in the MZ (medial) while the ECM network lost in the CZ populations (distal). (E) The tightly packed MZ (green) cells are in quiescence, while CZ cells proliferate, as evident by EdU (red) incorporation. (F) The primary lobe of the third larval lymph gland showing progenitor cells (green), P1, a plasmatocyte specific marker (red), and ProPO, a crystal cell-specific marker (magenta) in in the CZ. (G) A population (box) exists at the junction of the medullary zone and cortical zone, which co-express Dome (green) and Pxn(red). (H) The niche express Hh (green), while progenitor cells highly enriched with Ci-155(white arrow). Schematic representation of different cell types in the LG. niche (blue), progenitor cells (red), IP cells(yellow)

(A) Outermost zones (mature differentiated types of blood)

(B) Innermost zone (MZ) (precursor cells that lack any of the differentiated markers)

(C) Intermediate zone (IZ) (Intermediate progenitor cells expressing both progenitor and differentiated cell marker)

(C) Posterior signaling centre (cellular microenvironment regulates precursor cells)

The outermost zones (Cortical Zone; CZ)

It houses mature differentiated blood; crystal cells and plasmatocytes (Figure 1.4F). Crystal cells can be visualized by Hnt, Lz, and ProPo. Plamatocytes can be marked by P1, Pxn, Hml, similar to embryonic plasmatocytes(Evans et al., 2014)

The innermost zone (Medullary MZ)

It lacks any of the differentiated markers and expresses Domeless (Dome; a cytokine receptor for Jak-Stat pathway)(Jung et al., 2005) (Brown et al., 2001) (Figure 1.4F), Thioester protein IV (TepIV; a thioester protein responsive to Jak-Stat signaling) (Krzemien et al., 2007), and Cubitus Interruptus (Ci; transcription activation for *Hh* pathway(Mandal et al., 2007) (Figure 1.4H)

Intermediate zone (IZ):

This population exists at the junction of the medullary zone and cortical zone. Interestingly, this population co-express progenitor cell marker, Dome, and early differentiation markers Hml and Pxn (Figure 1.4G-G"'). The cells of this zone lack mature markers for plasmatocytes (P1) and crystal cells (Lz), suggesting they are in an intermediate state between progenitor and differentiated cells (Krzemien et al., 2010). Lack of available progenitor and IP cell-specific marker, these intermediate progenitors are not yet fully characterized.

Posterior signaling center (PSC):

It is composing of 40-50 Antp positive cells that work as a cellular microenvironment for the hematopoietic precursor cells and express Serrate (Ser), PDGF- and VEGF-related factor 1 (Pvf1), and Hedgehog (Hh) (Figure 1.4H-I) (Crozatier et al., 2004; Lebestky et al., 2003; Mandal et al., 2007). It acts as a hematopoietic niche capable of maintaining these progenitors in a stem-like state by Hh signaling (Mandal et al., 2007). During the immune challenge, niche senses the immune response and increases the levels of Reactive Oxygen Species (ROS) in the niche cells which results in the activation and secretion of Spitz (Spi) (Louradour et al., 2017) (Figure 1.4I). Spitz activates the epidermal growth factor receptor (EGFR) pathway in the progenitor cells, promoting lamellocyte formation. Thus, the hematopoietic niche is an essential entity during normal development as well as during immune challenges (Louradour et al., 2017).

1.3.2 Molecular regulators of blood progenitor cells expansion, maintenance and differentiation in *Drosophila* lymph gland

1.3.2.1 Expansion, maintenance and differentiation of progenitor cells of lymph gland

A variety of coordinated signals are essential for the development of tissue. Understanding these signals and molecular mechanisms involved in tissue development is crucial to unravel the cause of diseases like cancer. Studies in many organisms have pointed out the coordination of extrinsic and intrinsic signals during blood cell development to ensure enough blood cell development associated with the needs of an organism (Blank et al., 2008). Broadly, these signals can be grouped in systemic and local signals. Systemic signals provide information about organism status, including nutrition, developmental, and external environment status. Local signals include the intrinsic regulator, including transcription factor, cell-cell contact, cell-ECM adhesion, and niche-derived signals. The vital key to tissue development regulation is coordinated orchestration and reception of these systemic and local extrinsic signals. In the lymph gland, both systemic and local signals that include insulin signaling, gamma aminobutyric acid (GABA) signaling, calcium signaling, hippo pathway, wingless signaling, and dpp signaling have also been shown to crosstalk each other (Banerjee et al., 2019; Milton et al., 2014; Shim et al., 2013a; Sinenko et al., 2009).

A few local and systemic signaling involve in *Drosophila* blood progenitor cell maintenance and development is covered here (Figure 1.5A-B).

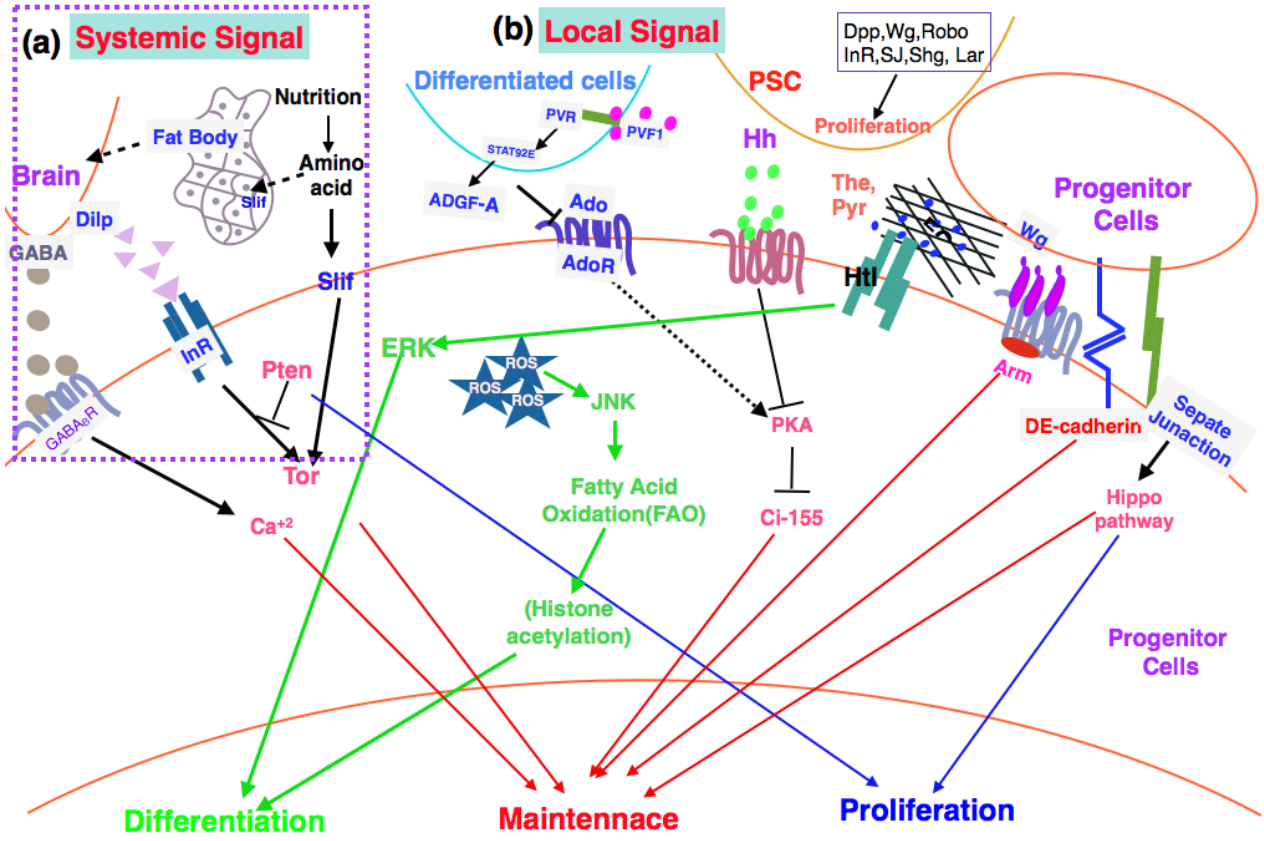

Figure 1.5 (A-B) Local and systemic signals required for proliferation (blue arrows), maintenance (red arrows), and differentiation (green arrows) in blood progenitor cells in *Drosophila* lymph gland.

1.3.2.2 Local signals involved in proliferation, maintenance and differentiation of progenitor cells-

1.3.2.2.1 DE-cadherin mediated cell-cell adhesion involve in blood progenitor cell maintenance

The lymph gland progenitor cells are highly enriched in DE-cadherin (Shg) (Jung et al., 2005). Loss of Shg reduced progenitor numbers and increased lamellocyte differentiation, while over-expression of Shg blocks the differentiation of plasmatocytes and crystal cells(Gao et al., 2009). *Drosophila* GATA transcriptional co-factor Ush maintains DE-cadherin expression by binding SrpNC and inhibiting its ability to repress DE-cadherin expression (Gao et al., 2013). This study highlights the importance of DE-cadherin mediated cell-cell adhesion in blood progenitor maintenance, regulated through the GATA-FOG complex.

1.3.2.2.2 Septate-junction mediated Hippo pathway regulates proliferation and blood progenitor maintenance-

The Salvador-Warts-Hippo (Hippo) signaling pathway is a well-conserved pathway and regulates organ growth and cell fate(Milton et al., 2014). In the *Drosophila* lymph gland, the Hippo pathway regulates the proliferation and differentiation of the progenitor cells. Overexpression of Yorkie (Hippo Pathway Transcriptional Regulator, Yki) or inhibition of Warts (wts) leads to a reduction in proliferation and maintenance of the progenitor cell (Milton et al., 2014). It is found that the Septate-junction components Coracle(Cora) and Neurexin IV (NrxIV) promote Hippo signaling in the lymph gland, and depletion of septate-junction components in progenitor cells replicates the phenotypes associated with Hippo pathway mutants, including increased differentiation of blood cells (Khadilkar and Tanentzapf, 2019).

1.3.2.2.3 Wg signaling essential for blood progenitor and niche cell maintenance-

Wingless (Wg) and its receptors, Frizzled (Fz2) express in the progenitor cells and the niche throughout the development and plays an essential role in the lymph gland (Sinenko et al., 2009). In the progenitor cells, the inhibition of Wg pathway via expression of dominant-negative versions of both Fz and Fz2 in the progenitor cells leads to disorganization of the boundary between progenitor cells and differentiated cells and increment of the intermediated progenitor cells expressing both MZ and CZ markers (Sinenko et al., 2009). Opposite to that, hyperactivation of the Wg pathway by either overexpression of Wg or a constitutively active form of β-Catenin results in an increase of the progenitor cell number and reduction of the CZ. The cell-cell adhesion molecule Shg (or DE-Cadherin) is down-regulated in the progenitor cells when Wg signaling decreases, which can explain the mis-organization of different zones. In the niche, Wg signaling regulates the PSC cell number and its function. Inhibition of Wg signaling leads to a decrease in the PSC cell number and affects prohemocyte maintenance. Thus, Wingless signaling plays a dual role in hematopoietic progenitor cell maintenance via autonomous acting in the progenitor cells and positively regulating the development of the niche.

1.3.2.2.4 Calcium signaling involve in blood progenitor maintenance

The calcium/calmodulin pathway plays a vital role in progenitor cell maintenance in the lymph gland (Shim et al., 2013b). Inhibition of calcium signaling via knockdown of the inositol trisphosphate receptor (IP3R) and ryanodine receptors (RyR) receptor required for release of Ca^{+2} from the endoplasmic reticulum (ER) to cytoplasm or inhibition of downstream components like calmodulin lead to a decrease in the progenitor cell number and increase in differentiated cells. Hyper-activation of calcium signaling in the progenitor cells leads to their increased number at the expense of differentiated cells (Shim et al., 2013b) . Put together, it can be concluding that calcium in the progenitor cells is essential for its maintenance.

1.3.2.2.5 Cellular niche derived Hedgehog sense by the progenitor population essential for their maintenance

Hedgehog (Hh) is a critical morphogen that guides the patterning of both invertebrate and vertebrate development (Lee et al., 2016). It was first discovered in *Drosophila* embryonic epidermis segments, where Hh signalling activation maintains the wingless expression, which is involved in pattering the segments (Ingham and Placzek, 2006)). Later on, the gain of function and loss of function studies in the *Drosophila* larval wing disc provides a much fundamental understanding of Hh signaling. It functions in a context-dependent manner and required for proliferation, differentiation, and patterning. Hh works as a morphogen and regulates downstream signaling through binding with its twelve-pass transmembrane receptor Patched (Ptc). Binding of Hh with Ptc relieves its negative interaction with smoothened (Smo) and inhibits proteolytic cleavage of Cubitus Interruptus (Ci), a transcription activator of Hh signaling. In the absence of Hh, Ptc inhibits the activity of Smo leading to the activation of protein kinase A (PKA) complex, which facilitates the degradation of Ci from its activator form (Ci-155) into its repressor form (Ci-75).

It has been shown that Ci-155 and the receptor Patched (Ptc) are enriched in the hematopoietic progenitor cells (Mandal et al., 2007), demonstrating active Hh signaling therein. Interestingly, overexpressing the PKA or dominant-negative form of Ci in the MZ blocks Hedgehog signaling and causes precocious loss of hematopoietic progenitor cells (Mandal et al., 2007; Mondal et al., 2011). Additionally, inhibition of Smoothened receptor through human Smoothened inhibitor: PF-04449913 also induces differentiation of Hh dependent progenitors, clearly implicating the importance of Hedgehog signaling in hematopoiesis (Giordani et al., 2016).

It was demonstrated that Hh is expressed explicitly in the PSC(niche) and secreted through thin cellular extensions (cytoneme) into the medullary zone (Mandal et al., 2007). Analysis of temperature-sensitive mutant Hh^{ts} (Mandal et al., 2007) or driving Hh dsRNA in PSC shows increased differentiation (Baldeosingh et al., 2018), suggesting the involvement of niche derived Hedgehog signaling in blood progenitor maintenance. Additionally, driving Col dsRNA in PSC or analysis of *antp* hypomorphic allele shows a considerable decrease in Hh expression and an increase in differentiation, indicating that Hh in the PSC indirectly affects lymph gland homeostasis. Later on, Srp is identified as a positive regulator (Tokusumi et al., 2012) and Su (Hu) as well as Ush as a negative regulator of Hedgehog expression in the niche (Tokusumi et al., 2010).

1.3.2.2.6 Pvr-Stat-Adgf-A Mediated Equilibrium signal

Apart from Hedgehog, PSC also secretes the Pvf1 (a ligand for Pvr), which regulates progenitor cell maintenance through a complex mechanism that involves the Pvr-Stat-Adgf-A pathway (Mondal et al., 2011). The niche derived Pvf1 is sensed by the differentiated cells that lead to activation of Pvr signaling therein. Activation of Pvr pathway induces expression of the secreted protein Adgf-A through non-canonical activation of the Stat pathway in differentiated cells. Adgf-A is a secreted enzyme and sends out a backward signal to the MZ via conversion of adenosine, a proliferative signal into inosine. In absence of Adgf-A, adenosine bind with its adenosine receptor (AdoR) present in the progenitor cells and activates PKA (cAMP-dependent protein kinase) which mediates the conversion of Ci from its full-length active (Ci-155) to a repressive cleaved form (Ci-75). Interestingly, the timing of lymph gland differentiation (source of Adgf-A) and the onset of quiescence in the progenitor population are closely matched, indicating that the two events are probably interlinked.

In summary, the stabilization of maintenance signals, Ci-155 in the progenitor cells, is regulated through two mechanisms. First, Hh-dependent signal regulation (niche mediated) (Mandal et al., 2007) and second Pvr-Stat- Adgf-A (differentiated cells) (Mondal et al., 2011)mediated inactivation of PKA, leading to increased progenitor cell maintenance. Thus, these two signals Hedgehog (niche derived) and Adgf-A (differentiated cells derived) provide equilibrium to maintain the progenitor cell's maintenance and quiescence.

1.3.2.2.7 Jak-Stat pathway

The Jak-Stat pathway is known to regulate progenitor identity and prevent differentiation (Jung et al., 2005; Krzemien et al., 2007). The cytokines Unpaired 1-3 binds with its receptor Dome, expressed by the progenitor cells, thus activating the downstream genes through Stat (Krzemien et al., 2007). Although, loss of Dome or Stat specifically within the progenitor cells does not affect the progenitor maintenance (Mondal et al., 2011), suggesting that non-autonomous involvement of Stat activity in the progenitor cells maintenance. It is also demonstrated that Latran (encoded by eye transformer), which is express in the progenitor cells, can heterodimerize with Dome and inhibit its function (Makki et al., 2010). Interestingly, loss of Latran has an effect on lamellocyte formation upon wasp parasitization but does not affect normal differentiation. Further, it is demonstrated that the Jak-Stat pathway regulates Ush, which promotes DE-Cad and Ptc expression in the progenitor cells. Additionally, Jak-Stat pathway also regulates protein Asrij (Arj) (Kulkarni et al., 2011), the *Drosophila* Homolog Ovarian carcinoma immunoreactive antigen domain-containing, and inhibition of Arj function cause reduction in the number of progenitor cells.

1.3.2.2.8 Reactive Oxygen Species (ROS) mediated JNK signaling in the progenitors is essential for progenitor cells priming for differentiation- include FAO

Like mammal progenitors, *Drosophila* hematopoietic progenitors have high reactive oxygen species (ROS) to prime them towards differentiation (Owusu-Ansah and Banerjee, 2009). It has been demonstrated that genetic manipulation that increases the ROS level in progenitor pushes the precursor cells towards aberrant differentiation while decrease in the ROS level halts the differentiation program (Owusu-Ansah and Banerjee et al., 2009). Additionally, it has also been shown that increase ROS activates JNK mediated FoxO activation and Polycomb (PcG) down-regulation, and this regulation was correlated with the differentiation in the *Drosophila* lymph gland. Thus, the above study provided novel insights into the relationship between physiological ROS and hematopoietic differentiation program. Recently, it has been demonstrated that JNK regulates the expression of CPT1/whd (withered), the rate-limiting enzyme of Fatty Acid Oxidation (FAO), and inhibition of key regulators of FAO leads to halt in the differentiation of the progenitors (Tiwari et al., 2020). Furthermore, it has been shown that FAO regulates the histone acetylation in a Histone Acetyl Transferases (HAT) dependent manner and inhibition of HAT genes phenocopies the FAO loss phenotypes (Tiwari et al., 2020). Altogether, the above study reveals a genetic cascade that links JNK (cellular signaling), FAO (metabolism), and histone acetylation (epigenetic modification), which is essential for the differentiation of hematopoietic progenitor cells. Reactive Oxygen Species (ROS) mediated JNK signaling in the progenitors is essential for progenitor cells priming for differentiation.

1.3.2.2.9 Extracellular matrix (ECM) regulates blood progenitor cells differentiation:

The extracellular matrix (ECM) is an essential component of the tissue that provides the structural support and defines the tissue boundary, regulating cellular signals' availability. The heparan sulfate proteoglycan (HSPG), Perlecan, is well conserved in *Drosophila* (also known as a trol: terribly reduced optic lobe). Trol forms a thin layer in the periphery of the lymph gland. The MZ progenitor's population is tightly packed in the dense Trol-positive extracellular matrix (Grigorian et al., 2013). Loss of trol in the whole animal leads to a drastic reduction in the proliferation, which resulted in a significant reduction in the lymph gland size. Importantly, progenitor specific down-regulation or trol mutant lymph glands also show a decrease of the progenitor population and increase differentiation, suggesting that trol in the progenitor is essential for lymph gland progenitor maintenance (Grigorian et al., 2013). In addition to trol, the Collagen IV subunits Viking (Vkg) and Cg25C are also expressed in the lymph gland (Jung et al., 2005), but their

functional roles are not well documented. Together, these studies highlight the importance of different ECM proteins in the distribution of signaling molecules and the progenitor cells' maintenance.

1.3.2.2.10 Heparan sulfate proteoglycan regulates heartless signaling essential for hematopoietic progenitor differentiation.

The Fibroblast Growth Factor Receptor Heartless (Htl) express in the progenitor cells, and inhibition of receptor causes an expansion of the progenitor population with loss of differentiated cells (Dragojlovic-Munther and Martinez-Agosto, 2013). Apart from htl, its two FGF-8 like ligands Thisbe (Ths) and Pyramus (Pyr) are also implicated in the differentiation of the progenitor cell population within the MZ. Furthermore, it has been found that over-activation of the FGF pathway or RAS in progenitor cells leads to increment in the differentiation. Downstream to the FGFR-RAS pathway, pointed (Pnt) and Ush are involved in progenitor differentiation (Dragojlovic-Munther and Martinez-Agosto, 2013). Further studies have found that ECM regulates the sequestration of FGF signaling ligand and maintains the progenitor cells. Altogether, these data suggest that Htl, Ras/MAPK, Pnt, and Ush involve in the hematopoietic progenitor's differentiation in the lymph gland.

1.3.2.2.11 Notch signaling essential for differentiation, specification, expansion, and survival of crystal cells.

In the lymph gland, Serrate mediated Notch signaling is involved in promoting Lz expression in the lymph gland and initiates the differentiation program essential for the specification of crystal cell (Terriente-Felix et al., 2013). It has been demonstrated that Yki and Scalloped (Sd) dependent Serrate expression can be detected in scattered cells known as lineage specifying cell (LSCs) very near to the crystal cells (Ferguson and Martinez-Agosto, 2014). As a result, the inhibition of Yki or Sd in these cells leads to a significant decrease in ProPO+ cells. Later in development, Notch activity is also controlled through the ligand-independent mechanism, which is required for the expansion and maintenance of crystal cells both in hypoxic stress and physiologic conditions (Mukherjee et al., 2011). Lz expression induces nitric oxide (NO) synthase, which is essential for the production of NO in the developing crystal cell. NO stabilizes hypoxia-inducible factor-a (Hif-a), Sima, via inhibiting the protein involved in Sima degradation. Stabilized Sima forms a complex with full-length Notch and activates downstream targets involved in expansion, maintenance, and survival of crystal cell.

1.3.2.3 Systemic signaling essential for progenitor maintenance-

1.3.2.3.1 Linking nutritional status to the progenitor cell maintenance-

Dietary regulation of lymph gland blood cell development is mainly regulated by Insulin-Like Peptides 2 (dILP2) and amino acid (Shim et al., 2012). In *Drosophila*, dILP2 is secreted from the insulin-producing cells (IPCs) in the brain into the circulation, and the release of Dilp2 is dependent on the organism's nutritional status (Ikeya et al., 2002). It has been found that reduced dilp2 level through depletion of nutrition via starvation or genetic ablation of IPCs causes increased differentiation in the lymph gland (Dragojlovic-Munther and Martinez-Agosto, 2012; Shim et al., 2013a). Thus, it can be concluding that nutrition status is an essential aspect of blood cell development in *Drosophila*. Dilp2 activates downstream signaling cascade PI3K-Akt-Tor or S6K via binding with Insulin Receptor (InR). The depletion of InR, Akt, and Tor, specifically in progenitor cells, causes increased differentiation, similar to starvation (Benmimoun et al., 2012; Shim et al., 2012)

In contrast, overexpression of TSC or Rheb increases the progenitor population, suggesting the decisive role of the insulin pathway in progenitor cells. Finally, it was determined that Insulin Receptor signaling through TOR directly modulates levels of Wg in prohemocytes, and over-expression of Wg can rescue both the starved and InR depletion phenotypes (Shim et al., 2012). Besides insulin signaling, essential amino acids present in the hemolymph are also directly sensed by progenitor cells and is critical for their maintenance.

In addition to directly regulating the progenitor population, insulin signaling also regulates progenitor maintenance through positive regulating PSC cell number and size (Benmimoun et al., 2012). Inhibition of positive regulators of insulin signaling like InR, PI3K, Akt, and Tor drastically decreases the PSC cell number. In contrast, activation of Insulin signaling by depletion of Pten or overexpression of PI3K in the PSC all cause an increased number of PSC cells. Interestingly, decreasing the niche cell number via inhibition of insulin signaling directly influences blood progenitor maintenance. Interestingly, insulin signaling in the niche is regulated through Leukocyte-antigen-related-like (*Lar)* receptor, a receptor protein tyrosine phosphatase (Kaur et al., 2019). Loss of Lar in the hematopoietic niche leads to the up-regulation of insulin signaling and ectopic progenitor differentiation. It has been shown that Lar physically associates with the insulin receptor and negatively regulates insulin signaling (Kaur et al., 2019). Thus, Lar tightly regulates the insulin signaling, in the hematopoietic niche and maintains the Lymph gland homeostasis.

1.3.2.3.2 GABA in progenitor cells - Linking olfaction to blood cell development.

In the *Drosophila* brain, GABA is released from neuro-secretory cells and it serves as the primary inhibitory neurotransmitter. It has been found that GABA signaling is involved in blood progenitor maintenance (Shim et al., 2013b). Inhibiting GABA synthesis in the Kur6 neurons decreases the GABA in the circulation and in the lymph gland, leading to increased hemocyte differentiation. GABA exerts influence on the lymph gland through its metabotropic GABA-B receptors, which is expressed in the lymph gland progenitors. Interestingly, loss of GABA receptor and its signaling cascade decreases cytosolic calcium levels and induces a differentiation phenotype similar to that observed upon blocking Calcium signaling.

The secretion of GABA into hemolymph from neurosecretory cells depends on the sense of smell through olfactory receptor neurons (ORNs). In the odorant receptor mutant, the level of GABA in Kur6 neurons, in circulation and in the lymph gland, decreased, displaying a remarkably similar lymph gland phenotype identical to those observed upon loss of GABA. Rearing larvae on odorant molecules less synthetic food also increases the differentiation in the lymph gland that can be rescued by adding small odorant molecules into the larval environment (Shim et al., 2013b). Put together, the above study suggests that smell can regulate blood development via secretion of GABA neurotransmitters.

1.4 Summary and Aim of the study-

The delicate balance between biochemical and mechanical signals required for maintenance and differentiation of stem cells and the progenitors is essential for tissue homeostasis . *Drosophila melanogaster* remains one of the most commonly used model organisms for a deeper understanding of essential developments and biomedical science. Signaling molecules necessary for the development of the hematopoietic system are conserved in *Drosophila* and higher vertebrates (Evans et al., 2003; Fossett and Schulz, 2001). The discovery of these signaling molecules and the availability of advanced molecular tools in *Drosophila* led to a boom in the hematopoietic field to understand the basic development of the hematopoietic system. Studies in the *Drosophila* lymph gland (LG) unravel several interesting signals essential for blood cell development, which perform a similar task in vertebrate hematopoiesis (Evans et al., 2003; Fossett and Schulz, 2001). In *Drosophila*, LG contains a large pool of multipotent blood progenitors maintained by a complex network of signaling pathways.

Despite extensive studies in the LG during recent years, several important questions remain unanswered. One aspect that is essential to consider is the involvement of mechanical cues such as actin cytoskeleton-mediated intracellular tension in the lymph gland development. It has been shown in other systems that stem cell maintenance, differentiation, and cell fate determination tissue can be regulated through differential mechanical tension or actomyosin activity. Several in vitro and in vivo studies have identified the cellular tension or actomyosin participation in the regulation of tissue development in fruit fly as well as in other model systems. Growing evidence suggest the involvement of actomyosin in cell proliferation, cell fate specification, and cellular migration. Despite substantial progress in our understanding of the involvement of actomyosin in the regulation of the diverse cellular process, including in cell adhesion, proliferation, and differentiation (Munjal and Lecuit, 2014; Murrell et al., 2015; Rauskolb et al., 2014) but the blood specific role of actomyosin is not very well worked out.

Interestingly, actomyosin activity can be controlled through differential cell-cell adhesion. It is evident the progenitor cells have high DE-cadherin-mediated cell-cell adhesion, and loss of it leads to a reduction in the progenitor cells (Gao et al., 2009, 2013).While it has been found that there is differential cell-cell adhesion in the lymph gland, no direct proof of the role of actomyosin in the lymph gland development has been demonstrated.

Another important aspect is to understand the major regulator of cellular niche maintenance. Although it has been demonstrated that niche sends out a plethora of signaling ligands that activate the downstream signaling pathway essential for the development of the lymph gland, it is still

unclear what molecular regulator is essential for maintaining the size and function of the hematopoietic niche.

Thus, the specific aims of this study were:

1. To investigate the role of actomyosin, if any, in the development of lymph gland hematopoiesis.
2. To unravel the molecular player involved in hematopoietic niche maintenance.

Chapter 2

Materials and methods

2.1 Fly husbandry

All flies were raised on standard corn meal medium containing maize powder, sugar, yeast and agarose. Propionic acid and Nepagin were used as a preservative and antifungal agents. All fly stocks were grown at 25°C in standard bottles/vials except for those used in RNAi and *GAL4/UAS* expression experiments. In those cases, crosses were maintained at 29°. For *GAL80ts* experiments, crosses were initially maintained at 18° (permissive temperature) for seven days and 2 hr (equivalent to 60 hr at 25°) AEL and then shifted to 29° till dissection.

For synchronization of larvae, flies were allowed to lay eggs for 3 hr. Newly hatched larvae within a 1-hr interval were collected and transferred onto fresh food plates and aged for specified time periods at 25°.

2.1.1 Fly strains

While most of the *Drosophila* lines used in this study were procured from Drosophila Stock Center at Bloomington, Illinois, USA, others have been a kind gift from various scientists of the fly community. Transgenic and mutant alleles used are listed in Table 2.1, and full genotypes for each experiment are listed in respective figures.

The following fly strains were used for this project:

2.1.1.1 Gal4 lines, LexA line, and Gal80 lines:

	Fly Lines	Genotypes	Chromosome	Bloomington number/DGRC Reference
1	Antp-Gal4	w1118; +/+;Antp-Gal4/Tm2.	3	kind gift from S. Cohen, European Molecular Biology Laboratory, Heidelberg, Germany (Mandal et al., 2007)
2	pCol-Gal4	w1118;Pcol85/Cyo^Tb	2	a kind gift from M. Inamdar, JNCASR, India (Krzemien et al., 2007)
3	TepIV-Gal4	y*w1118;P{w+mW.hs=GawB}NP7379/CyO,P{w-=UAS-lacZ.UW14}UW14	2	DGRC(105442) (Shim et al., 2013b)
4	Hml-Gal4	w1118;Hml-Gal4/Hml-Gal4 ;+/+	2	BL30139 (Goto et al., 2003)
5	Pxn-Gal4	w; pxn-Gal4/pxn-Gal4;+/+	3	Kind gift from Dr. Utpal Banerjee, UCLA,USA (Stofanko et al., 2008)

	Fly Lines	Genotypes	Chromosome	Bloomington number/DGRC Reference
6	Mef2-Gal4	w;+/+;mef-Gal4/ mef2-Gal4	3	(BL 27390) (Bour et al., 1995)
7	Dome-Gal4	dome-Gal4/ FM7; Gal80ts/+; +/+	X	a kind gift from S. Noselli, CNRS, France (Makki et al., 2010)
8	Hand-Gal4	w;Hand-Gal4/Hand Gal4; +/+	2	(Han et al., 2006)
9	*ay-GAL4*	w[1118];P{w[+mC]=AyGAL4}25/ CyO	2	*BL3953 (Ewen-Campen et al., 2017)*
10	Trio-LexA	w[1118]; P{y[+t7.7] w[+mC]=GMR18B03-lexA}attP40	2	BL61517
11	Mhc-Gal80	y w; +/+; Mhc-Gal80/Mhc-Gal80	3	Liqun Luo (Pauli et al., 2008)
12	LexAOP-Gal80	w[+mC]=8XLexAop2-IVS-GAL80-WPRE}su(Hw)attP5	3	BL32216 (del Valle Rodriguez et al., 2011)
13	Tub-Gal80ts(II)	w[*]; P{w[+mC]=tubP-GAL80[ts]}20; TM2/TM6B, Tb[1]	2	*BL7019 (Yi et al., 2013)*
14	Tub-Gal80ts (III)	w[*]; P{w[+mC]=tubP-GAL80[ts]}2/TM2	2	*BL7017(Zhan et al., 2016)*

Table 2.1: Gal4, LexA and Gal80 fly lines Used in this Work

2.1.1.2 Reporter lines:

	Fly Lines	Genotypes		Bloomington number/DGRC Reference
1	Zcl-1973(x) or Trol-GFP	w1118; P{w[+mC]=PTT-un1}Zcl1973	x	kind gift from U. Banerjee, UCLA, USA. (Grigorian et al., 2013)
2	ROS reporter	w1118;gstD-GFP; +/+	2	kind gift of Richa Rikhy, IISER Pune, India (Sykiotis and Bohmann, 2008)
3	Hh-GFP	w1118;P{hhF4f-GFP}	2	kind gift from R. Schulz, Notredame, USA (Tokusumi et al., 2010)

	Fly Lines	Genotypes		Bloomington number/DGRC Reference
4	Dome-meso-LacZ	domeMESO-LacZ; +/+;+/+	x	kind gift from J.C Hombria, Spain (Makki et al., 2010)
5	Sqh-GFP	y1 w* cv1 sqhAX3; P{sqh-GFP.RLC}2	2	*BL57144 (Munjal et al., 2015)*
6	hsFlp	P{ry[+t7.2]=hsFLP}12, y[1] w[*]; sna[Sco]/CyO	x	BL1929 (Bohm RA et al.,2010)
7	GRASP	w[*]; P{y[+t7.7] w[+mC]=CoinFLP-LexA.GAL4}attP40 P{w[+mC]=lexAop-rCD2.RFP}2; P{w[+mC]=UAS-CD4-spGFP1-10}3, P{w[+mC]=lexAop-CD4-spGFP11}3/TM6C, Sb[1]	2;3	58755 (Bosch JA et al.,2015)
8	TRE-GFP	w[*]; P{y[+t7.7] w[+mC]=TRE-EGFP}attP16	2	59010 (Chatterjee and Bohmann, 2012)
9	Dome-meso-EBF2	Dome-MESO-EBF2/Fm7i; +/+;+/+	x	kind gift from U. Banerjee, UCLA, USA.(Evans CJ et al., 2014)
10	zasp-GFP	w[1118]; P{w[+mC]=PTT-GB}Zasp52[G00189]	2	BL6838 (Lin et al., 2014)
11	CycB-GFP	y1 w*; P{PTT-GC}CycBCC01846	2	BL51568 (Bandura et al., 2013)
12	MOE-GFP	w*; P{His2Av-mRFP1}III.1, P{sGMCA}3.1	2	BL59023 (Edwards et al., 1997)
13	DIAP1-GFP	y1 w*; Diap1-GFP/cyo:+/+	2	(Zhang et al., 2008)
14	*Pxn-YFP*	w[1118]; PBac{802.P.SVS-2}Pxn[CPTI003897]	3	DGRC 11545
15	Pvf2-LacZ	P{Pvf2-lacZ.C}	3	(Choi et al., 2008)

Table 2.2: Reporter Transgenes used in this Work

2.1.1.3 UAS lines:

	Fly Lines	Genotypes	Chromosome	Bloomington number/DGRC Reference
1	UAS-GFP.nls	w1118; P{UAS-GFP.nls}14	2	BL4775
2	2xGFP	w1118; UAS-2xEGFP; +/+	2	*BL6874*
3	GTRACE	w1118; UAS-RFP,UAS-FLP,Ubip63FRT-STOP-FRT-GFP/Cyo;+/	2	kind gift from U. Banerjee, UCLA, USA (Evans et al., 2009)
4	FUCCI (Cell Cycle reporter line)	w1118;P{w[+mC]=UAS-GFP.E2f1.1-230}32P{w[+mc]=UAS-mRFP.NLS. CycB.1-266}19/ Cyo,P{ry[+t7.2]=en1}wg[en11]; MKRS / TM6B,Tb	2	BL55121 (Zielke et al., 2014)
5	UAS-stg-GFP	y[1] w[1118]; P{w[+mC]=UAS-stg-GFP}	3	Kind gift from Stefano Di Talia
6	UA-ROCK	y[1] w[*]; P{w[+mC]=UAS-Rok.CAT}7.1	2	*BL6668* (Zhang et al., 2018)
7	UAS-hh.GFP	y[1] w[*]; P{w[+mC]=UAS-hh.GFP}	2	Kind Gift from S. Mayor (Vyas et al., 2008)
8	UAS-ci.HA	w*; P{UAS-ci.HA.m1-4}2; hhIJ32, P{GAL4-prd.F}RG1/TM2	2	*BL32569*
9	UAS-Inr	y[1] w[1118]; P{w[+mC]=UAS-InR.Exel}2	2	BL8262 (Chatterjee et al., 2014)
10	UAS-Yki	w[*]; P{y[+t7.7] w[+mC]=UAS-yki.V5.O}attP2	3	BL28819 (Zhang et al., 2015)
11	UAS-smo	y1 w*; P{UAS-FLAG-smo.act}2	2	BL44621 (Galasso A et., 2019)
12	UAS-Yki GFP	y[1] w[*]; P{w[+mC]=UAS-yki.GFP}4-12-1	2	BL28815 (Francis et al., 2019)
13	UAS CD tomato cherry	w[*]; P{y[+t7.7] w[+mC]=10XUAS-IVS-myr::tdTomato}attP2	2	BL32222 (Segal et al., 2016)

	Fly Lines	Genotypes	Chromosome	Bloomington number/DGRC Reference
14	UAS smo RNAi	y1 v1; P{TRiP.JF02363}attP2	3	BL27037 (Tian et al., 2015)
15	UAS-stg RNAi	y1 sc* v1 sev21; P{TRiP.HMS00146}attP2	3	BL34831 (Inaba et al., 2011)
16	UAS-sqh RNAi	w1118; P{GD1695}v7916	2	GD 7916 (Aranjuez et al., 2016)
17	UAS-zip RNAi	y1 sc* v1 sev21; P{TRiP.HMS01618}attP2	3	*BL36727 (Rothenbusch-Fender et al., 2017)*
18	UAS-ance RNAi	y1 sc* v1 sev21; P{TRiP.HMS03009}attP2	3	*BL36749 (Kim et al., 2017)*
19	UAS-inr RNAi	y1 v1; P{TRiP.HMS03166}attP40	3	BL51518 (Shim et al., 2012)
20	UAS-Hh RNAi	y^1sc v^1sev21;p{TRIP.HMS00492} attP2/TM3,Sb^1	3	BL32489 (Liu et al., 2015b)
21	UAS-Yki RNAi	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF03119}attP2/TM3, Sb[1]	3	BL31965 (Sun et al., 2015)
22	UAS-wts RNAi	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00026}attP2	3	BL34064 (Sun et al., 2015)
23	UAS-sav RNAi	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00760}attP2	3	BL32965 (Sun et al., 2015)
24	UAS-ryr RNAi	y1 v1; P{TRiP.JF01101}attP2	3	BL31695 (Xu et al., 2017)
25	UAS-blw RNAi	y1 v1; P{TRiP.JF02896}attP2	3	BL28059 (van den Ameele and Brand, 2019)
26	UAS-jub RNAi	y1 sc* v1 sev21; P{TRiP.HMS00714}attP2	3	BL32923 (Jia et al., 2015)
27	UAS-zasp RNAi	y1 v1; P{TRiP.JF01133}attP2	3	BL31561 (Liu et al., 2013)
28	*UAS-shgRNAi*	y1 sc* v1 sev21; P{TRiP.HMS00693}attP2	3	*BL 32904* (Sumi et al., 2018)
29	UAS Irsp53 RNAi	y1 sc* v1 sev21; P{TRiP.HMC02920}attP2		BL44526 (Segal et al., 2016)

	Fly Lines	Genotypes	Chromosome	Bloomington number/DGRC Reference
30	UAS-ajuba RNAi(vdrc)	P{KK108565}VIE-260B	2	VDRC kk/101993 (Rauskolb et al., 2011)
31	UAS yki RNAi(VDRC)	P{KK109756}VIE-260B	2	VDRC kk/104523 (Vissers et al., 2016)

Table 2.3: UAS Transgenes Used in this Work

2.1.1.4 Mutant lines

	Fly Lines		Genotypes	Bloomington number/DGRC Reference
1	Ance mutant	Ance[34Eb-2] Adh[D] pr[1] cn[1]/CyO, Adh[nB]	2	*Ance*34Eb2(BL3584) (Hurst et al., 2003)
2	Ance def. line	Df(2L)b88f32/In(2L)Cy, In(2R)Cy, Duox[Cy] dpy[lvl] b[1] pr[1] Bl[1] L[4] sp[2]	2	BL3897 (Hurst et al., 2003)
3	Sqh E21	y1 w*; P{sqh-E21}109	2	BL25763 (Winter et al., 2001)
4	Sqh A2021	y1 w*; P{sqh-A20,A21}1	2	BL25764 (Jordan and Karess, 1997)
5	Loh	w1118; Mi{ET1}lohMB05750	2	BL25461 (Drechsler et al., 2013)
6	Yki^{B5}	l(2)gl4 ykiB5/CyO	2	BL36290 (Milton et al., 2014)

Table 2.1: Mutant Alleles Used in this Work

Generation of Antp-Gal4, which express only in Cardioblast or Hematopoietic niche-

The available Antp-Gal4 is expressed both in the cardioblast and hematopoietic niche. To create cardioblast-specific Antp-Gal4 lines, we use a combination of Gal4 and LexA-AOP systems. LexA-AOP was used to suppress the Antp-Gal4 line in the hematopoietic system using LexA-AOP-Gal80. Upon analyzing different LexA available in the Bloomington stock center, we have identified Trio-LexA, which express only in the lymph gland. The re-combination of Trio- lexA with LexAOP-Gal80 gives hematopoietic specific Gal80 expression. The expression of Trio-LexA, AOP-Gal80 with Antp-Gal4, leads to the suppression of Antp-Gal4 in the hematopoietic niche.

To create a hematopoietic niche-specific Antp-Gal4, we suppressed the cardioblast-specific Antp-Gal4 expression using the Mhc-Gal80 system, which is known to express in the cardioblast. The re-combination of Mhc-Gal80 with Antp-Gal4 leads to the suppression of Antp-Gal4 in the cardioblast.

2.1.2. Culture Conditions for Clonal analysis experiment-

The following strategy was adopted to induce zip RNAi clones: the first instar larvae of genotype *hsFLP; ay-GAL4/tub- GAL80ts*; *UAS-zip RNAi* were placed in a 37°C water bath 45 min for heat-shock and then transferred to 18° to suppress Gal4 activity until the early third instar. After that, to express the zip RNAi, third instar larvae were reared at 29° till dissection.

The following strategy was adopted to induce ROCK overexpression clones: early third instar larvae of genotype *hsFLP*; *ay-GAL4/UAS-ROCK* were placed in a 37°C water bath 45 min for heat-shock and transferred to a temperature of 29°C till dissection.

2.1.3 Culture Conditions for Lineage tracing experiment-

For lineage-tracing experiments, the Gal4 technique for real-time and clonal expression (GTRACE) was used. *GTRACE* combines the Gal4-UAS system, FLP recombinase-FRT, and fluorescent reporters to generate cell clones that provide information about the origins of individual cells in *Drosophila* or trace the lineage of the cell expressing a gene of interest. *GTRACE* in a Gal4 dependent manner not only marks the current or the real-time expression of the gene driving the Gal4 with RFP but also independently marks all the cells arising from the same gene lineage with a GFP. To initiate lineage tracing, FLP recombinase, upon being activated in a Gal4 dependent manner, flips out FRT-flanked transcriptional termination cassette between *Ubi-p63E* promoter and EGFP construct. This brings EGFP directly under the Ubi-p63E promoter, thereby labelling all the progeny cells arising thereafter with EGFP.

To check the expression of *Trio-LexA, LexAOP-Gal80; Antp-Gal4* in the lymph gland, *UAS-GTRACE/Cyo* stock was crossed to *Trio-LexA, LexAOP-Gal80; Antp-Gal4 and* crosses were kept at 29ºC till dissection.

To check the expression of Antp Gal4, Mhc-Gal80 in the lymph gland, *UAS-GTRACE/Cyo* stock was crossed to *Antp-Gal4, Mhc-Gal80* fly line*, and* crosses were kept at 29°C till dissection.

2.1.3 Starvation, protein-rich, Sucrose-only, lipid-only Culture, and amino-only Conditions

To analyse the systemic effect of nutrition on Ance expression following strategy was adopted;

For the starvation experiment, 48 hrs AEH larvae were transferred to agar plates instead of yeasted cornmeal plates and dissected at 60 hrs AEH. For the recovery phase experiment, 48 hrs AEH larvae were transferred to agar plates and then transfers the larvae to standard dissection (12 hrs AEH). To see the effect of Yeast on Ance expression, the larvae were grown in the high yeast containing food after 48 hrs AEH. For only sucrose and Amino acid condition, 48 AEH larvae were transferred to agar plates with 20% sucrose solution, or EAA instead of yeasted cornmeal plates, and dissected at 72 hrs AEH.

2.2 Immunohistochemistry and imaging

Lymph glands from synchronized larvae of the desired developmental stage were dissected in cold phosphate-buffer saline (1× PBS, pH 7.2) and fixed in 4% paraformaldehyde prepared in 1× PBS (pH 7.2) for 45 min at room temperature (RT). Tissues were permeabilized by 0.3% PBT (0.3% triton-X in 1× PBS) for 45 min (3 × 15 min washes) at RT. Blocking was then done in 10% normal goat serum (NGS) for 30–45 min at RT. Tissues were next incubated with the respective primary antibody with appropriate dilution in 10% NGS for 18–20 hr at 4°. Postincubation with the primary antibody, tissues were washed thrice in 0.3% PBT for 15 min each. Next, the tissues were incubated in secondary antibody for 18–20 hr at 4°. The tissues were then subjected to four washes in 0.3% PBT for 15 min each, followed by incubation in DAPI solution (Invitrogen, Carlsbad, CA) for 1 hr at RT. Excess DAPI was washed from the tissues using 1× PBS before mounting in Vectashield (Vector Laboratories, Burlingame, CA).

Detection of pMRLC expression in lymph gland:

pMRLC staining was performed with a slight modification of the above protocol. Lymph gland from synchronized larvae was dissected in ice-cold PBS and fixed in 4% paraformaldehyde prepared in ice-cold 1× PBS (pH 7.2) for 5 hr at 4°. Tissues were then permeabilized with 0.3% PBS-T (0.3% Tween 20 in 1× PBS). Primary antibody and secondary antibody incubations were performed following the standard protocol.

Detection of DE-cadherin expression in lymph gland:

To detect DE-cadherin, the lymph glands were incubated in DE-cadherin antibody (1:50 in PBS) before fixation (Langevin et al., 2005)for 1 hr at 4°. Tissues were then fixed in 4% paraformaldehyde prepared in ice-cold 1× PBS (pH 7.2) for 5 hr at 4°. Post incubation primary antibody was washed in 0.3% PBT for 30 min (thrice, 10 min each). Secondary antibody incubation, washes, and mounting were performed following the standard protocol.

Detection of extracellular Hh in lymph gland:

Synchronized larvae were dissected in cold PBS and fixed in 4% paraformaldehyde prepared in ice-cold 1× PBS (pH 7.2) for 5 hr at 40°. Tissues were washed in 1× PBS for 20 min (twice, 10 min each). Blocking was done in 10% NGS (in 1× PBS) for 30–45 min. Tissues were then incubated with the Hh antibody with appropriate dilution in 10% NGS (1× PBS) for 18–20 hr at 4°. Postprimary-antibody incubation tissues were washed with 0.3% PBT for 45 min (thrice, 15 min each). The standard protocol was followed for the rest of the experiment.

2.2.1 Primary antibodies for immunostaining

The Primary antibodies used in this study are as follows:

	Antigen	Species	Concentration	Source
1	P1	Rabbit	1:50	gift from I. Ando
2	Pxn	Mouse	1:500	gift from J. Fessler
3	Ance	Rabbit	1:500	Gift from A. D. Shirras
4	ProPO	Rabbit	1:2000	gift from M. Kanost
5	Ci-155	Rat	1:5	Developmental Studies Hybridoma Bank (DSHB)
6	Hh	Rabbit	1:1000	Gift from P. Ingham
7	Ptc	Mouse	1:30	DSHB APA1,
8	DE-cadherin	Rat	1:100	DSHB DCAD2
9	pMRLC	rabbit	1:50	Cell Signaling
10	β galactosidase	Mouse	1:100	Promega, Madison, WI)
11	GFP	Mouse	1:100	Sigma-Aldrich Cat# G6539
13	PH3	Rabbit	1:200	Cell Signaling Technology Cat# 3642S
14	Antp	Mouse	1:30	DSHB 4C3
15	Stg		1:500	Gift from Dr. Stefano Di Talia

2.2.2 Secondary antibodies for immunostaining- The secondary antibodies used in this study are as follows:

CyTM3-Conjugated affinipure donkey anti rabbit IgG (H+L) (Catalog # 711-165-152 Jacksons Immuno Research Laboratories, USA). This affinity purified secondary antibody conjugated with cyanine CyTM3 dye (absorption maxima/ emission maxima is 550 nm/ 570 nm) was used at a dilution of 1:500 to detect primary antibodies raised in rabbit.

FITC-Conjugated affinipure goat anti rabbit IgG (H+L) (Jacksons Immuno Research Laboratories, USA # 711-096-152): This affinity purified secondary antibody conjugated with Fluorescein FITC dye (absorption maxima/ emission maxima is 492 nm/ 520 nm) was used at a dilution of 1:600 to detect primary antibodies raised in rabbit.

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

(FITC)-Conjugated affinipure goat anti mouse IgG (H+L) (Jacksons Immuno Research Laboratories, USA # 115-095-166): This affinity purified secondary antibody conjugated with Fluorescein FITC dye (absorption maxima/ emission maxima is 492 nm/ 520 nm) was used at a dilution of 1:600 to detect primary antibodies raised in mouse.

CyTM5 649-Conjugated affinipure goat anti rat IgG (H+L) (Jacksons Immuno Research Laboratories, USA # 712-495-153): This affinity purified secondary antibody conjugated with cyanine 649 dye (absorption maxima/ emission maxima are 650 nm/ 670 nm) was used at a dilution of 1:600 to detect primary antibodies raised in rat.

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

(FITC)-Conjugated affinipure donkey anti rat IgG (H+L) (Jacksons Immuno Research Laboratories, USA # 712-165-153): This affinity purified secondary antibody conjugated with Fluorescein FITC dye (absorption maxima/ emission maxima is 492 nm/ 520 nm) was used at a dilution of 1:600 to detect primary antibodies raised in rat.

2.2.3 Stains:

Phalloidin- Texas Red (Molecular Probe, Catalogue # T7471): Phalloidin is basically a toxin isolated from poisonous fungus *Amanita phalloides*, which specifically binds to polymeric filamentous actin inside cell and prevents its de-polymerization. This F-actin probe is conjugated with red fluorescent Texas red dye (excitation maxima: 591nm, emission maxima: 608). The

DAPI (4‟,6-diamidino-2-phenylindole dihydrochloride, Molecular Probe, Catalogue # ZB1130). This is a blue fluorescent dye which binds to A-T rich region of double stranded DNA. It is generally used to stain nuclei in live as well as fixed tissues. Its absorption maxima/emission maxima are 351nm/461nm. The working dilution was 1µg/ml prepared in 1X PBS.

TO-PRO-3 iodide (Molecular Probe, Catalogue # T3605): This is a highly sensitive carbocyanine nucleic acid stain with far red fluorescence. It is used as counter-stain for nucleus. Its absorption maxima/emission maxima are 642nm/661nm. The working dilution was 1:500 in 1X PBS.

Phalloidin staining

Dissected samples were fixed in 4% paraformaldehyde prepared in ice-cold 1× PBS (pH 7.2) for 45 min at RT on a shaker. Post fixation, three washes of PBT were done before incubating the tissues in rhodamine-phalloidin (1:100 in PBS) (Molecular Probes, Eugene, OR) for 1 hr. Post incubation, three PBS washes for 10 min each were carried out. Mounting in DAPI Vectashield was done for subsequent imaging.

EdU incorporation

A Click-iT EdU (5-ethynyl-2'-deoxyuridine, a thymidine analog) kit from Life Technologies was used to perform the DNA replication assay. Lymph glands were dissected in EdU solution (1:1000 in PBS) and kept for 36–40 min at RT for EdU incorporation. The next step was fixation in 4% paraformaldehyde prepared in 1× PBS (pH 7.2) for 45 min at RT. Tissues were then permeabilized in 0.3% PBT (0.3% triton-X in 1× PBS) for 45 min (thrice, 15 min each) at RT. Blocking was then done in 10% normal goat serum (NGS), for 30–45 min at RT. For detecting the incorporated EdU in cells, an azide-based fluorophore was used as described in the manufacturer's protocol.

2.3 Imaging and statistical analyses

Images were captured using Zeiss LSM 780 and Leica SP5 confocal microscopes. All images were captured as confocal Z sections using the same settings within each set of experiments.

Each experiment was repeated at least thrice to ensure reproducibility. Data are expressed as mean ± SD of values from three sets of independent experiments. Usually, 10 lymph glands were analyzed per genotype and all statistical analyses performed employed a two-tailed Student's *t*-test. *P* values of <0.05, <0.005, and <0.0005 are considered as statistically significant.

Quantitative analysis of cell types in lymph gland

For quantifying the percentage of differentiated cells or progenitor cells, three middle stacks from confocal *Z* stack were merged into a single section for each lymph gland using ImageJ/Fiji (NIH) software as previously described (Shim *et al.* 2012). This merged section provides the clearest view of progenitor cells of MZ and the neighboring differentiating cells of the CZ. For images with >1 wavelength, each channel/marker was separately analyzed. Following Shim *et al.*, for estimation of differentiation, P1 positive areas were recalibrated into an identical threshold by using the Binary tool (Process–Binary–Make binary, Image J). The Wand tool automatically captured the area with identical threshold, whereas the size was measured using the Measure tool (Analyze–Measure). To measure the total size of the lobe, recalibration of the DAPI area was done by the Threshold tool until it was overlaid with an identical threshold color. Wand tool was used for selecting this total area for measurement. Percentage of differentiation was estimated by dividing the size of the P1-expressing area by the total size of the lobe (DAPI area). In all cases, per genotype, 10 randomly selected lymph glands were analyzed, and a standard *t*-test was done to evaluate the statistical significance.

Quantification of the number of Hh+ niche cells-

For quantitation of the Hh positive niche cells in the developing lymph gland, the total number of hhF4-GFP cells in the lymph gland at different developmetal time was counted using spot module of Imaris software. Ance-expressing progenitor cells in lymph glands were counted using the surface and spot module of Imaris software. The Ance + progenitor cells were separated using surface modules and then Ance+ cells were counted using spot modules. Fucci+ progenitors were similarly counted.
http://www.bitplane.com/download/manuals/QuickStartTutorials5_7_0.pdf.

Quantification of the number of Ance+ and Fucci + progenitors:

Ance-expressing progenitor cells in lymph glands were counted using the surface and spot module of Imaris software. The Ance + progenitor cells were separated using surface modules and then Ance+ cells were counted using spot modules. Fucci+ progenitors were similarly counted.
http://www.bitplane.com/download/manuals/QuickStartTutorials5_7_0.pdf.

Quantification of intensity of protein:

To quantify the expression of proteins in progenitor cells of different genotypes of the lymph gland, we followed the protocol adapted by Shim *et al.* (2012) from http://sciencetechblog.files.wordpress.com/2011/05/measuring-cell-fluorescence-using-imagej.pdf.

To analyze the intensity of Ci-155 in the progenitor cells, a define regions of interest (ROIs) corresponding to progenitor cells were created using ImageJ/Fiji software, and then the mean intensity of each ROI was quantified. For each genotype, at least three ROIs in a lymph gland were defined, and 10 biological samples were quantified.

To analyze Ci intensity profiles in lymph gland with respect to Hh-expressing niche, intensities in four different regions in Ance-positive and Ance-negative progenitors were measured. (Three points equidistant from each other in the Ance-positive domain compared with one in the Ance-negative domain.

Sqh-GFP, F-actin, and DE-cadherin intensity analysis:

To measuring the intensity of Sqh:: GFP, F-actin, and DE-cadherin (Shg, a defined line was drawn along the plasma membrane using ImageJ/Fiji software in Ance-positive and Ance-negative cells (after background correction) (adapted from (Roubinet et al., 2017). For each genotype at least five such measurements were done. Biological replicate was 10.

2.4 Quantitative analysis of *ptc* transcript

The transcript level of *ptc* was analyzed by quantitative RT-PCR (qRT-PCR). RNA was isolated from 150 to 200 whole lymph glands of third instar larvae.

2.4.1 Isolation of total RNA from lymph gland (LG)

Appropriate number of (200 LG) synchronized larvae were dissected on ice cold 1X PBS and is such a way that only the lymph gland was isolated. Samples were homogenized in TRiZOL (Invitrogen # 15596018). After homogenization sample was kept at room temperature for 10 minutes for complete tissue dissociation. 200µl of chloroform was added and incubated at room temperature for 10-15 minutes. The resulting mixture was then centrifuged at 4°C for 15 minutes at 2000 rcf. Three layers were separated out, uppermost aqueous layer containing RNA, middle layer containing proteins and lowermost organic layer containing DNA. The upper layer was carefully taken out in to a separate RNase-free tube so that middle layer remains unaffected. The sample was then incubated with equal volumes of isopropanol for 10 minutes at room temperature to precipitate out the RNA and then centrifuge at 13,000 rpm for 15minutes at 40C to pellet out the RNA. RNA pellet was washed with 70% ethanol twice and dissolved in DEPC water. Quantification of RNA was performed by using nanodrop spectrophotometer.

2.4.2 Synthesis of c-DNA

c-DNA was synthesized using Verso c-DNA synthesis kit (Molecular Probe # AB1453A) following the manufacturer‟s recommended protocol. 500 ng of RNA was used for c-DNA synthesis. RNA was incubated with cDNA reaction mix at 42˚C for 30 min and then kept on 95˚C for 2 min. c-DNA mix was prepared using 5X RT buffer, 500µM dNTP mix, OligodT and random Hexamer primer mix, RT Enhancer to remove contaminating DNA and Verso enzyme mix which acted as reverse transcriptase for c-DNA manufacture. c-DNA mix was added to the RNA and the reaction was mixed properly by pipetting and by short spin. This mixture was then incubated at 42˚C for first 30 min. Reaction was terminated by incubating at 95˚C for last 2 min c-DNA samples were stored at -20˚C.

Constituents	**Final Concentration**	**Volume For 20µl reaction**
5 X RT Buffer	1X	4µl
dNTPs Mix (500µM)	50 µM	2µl
RT Enhancer	1.5 mM	1µl
RNA Primer	1X	2µl
Verso Enzyme Mix	1 unit	1µl
RNA (500ng/µl)	500 ng	1-5µl
Nuclease-free water	**---**	9-13 µl
TOTAL		**20µl**

2.4.3 qRT PCR

The 20 µl reaction mixture was prepared by adding reagents in the following sequence: PCR water; 10X Taq Buffer; dNTPs mix; $MgCl_2$; Forward Primer; Reverse Primer; and Taq Polymerase.

Preparation of PCR Reaction Mixture:

Constituents	**Final Concentration**	**Volume for 20µL reaction**
10 XTaq Buffer	1X	2.0µl
dNTPs Mix (10mM)	0.2mM	0.4µl
MgCl2	1.5 mM	0.8µl
Forward Primer (10µM)	1µM	0.4µl

Reverse Primer (10µM)	1µM	0.4µl
Taq Polymerase (3 unit/ µl)	0.6 units	0.2µl
DNA (500ng/µl)	500ng	1 µl
PCR water		14.8 µl
Total		**20µl**

The amplification reaction consisted of 35 cycles with initial denaturation at 94˚C for 5 minutes, followed by 39 cycles of denaturation at 95˚C for 30 seconds, annealing at 59˚C (Variable for different set of primers) for 45 seconds and extension at 72˚C for 30 seconds. Reaction was terminated after final extension for 10 minutes at 72˚C. 10µl of PCR products were loaded on 1% agarose gel along with 50bp DNA ladder as the marker to check the amplification.

2.4.4 Real Time PCR

Real time qRT-PCR was performed using SYBR green mix Biorad on Biorad CFX96 instrument, following the instructions provided in the manual. After setting up the reactions with SYBR Green, plate form was made on instrument and reaction was set up at 950c for 15 sec and 590c for 15 sec and 720c for 15 sec for 40 reactions. Melting curve was performed from 950c to 720c for 5 min, to analyse Tm of the amplicon. Expression analysis was performed on instrument using ΔΔCt method.

In both control and experimental analyses, expression levels of the *ptc* transcript were normalized with respect to *rp49* expression. Primers were designed from NCBI. We specifically designed primers from exon-exon junction to avoid any misinterpretation from DNA contamination. Specificity of primers were checked with primer blast. The control and experiment analyses were repeated thrice, using triplicates each time. The list of primers used is as follows:

rp49-forward, 5′-CTAAGCTGTCGCACAAATGGC-3′.
rp49-reverse, 3′-TTCTGCATGAGCAGGACCTC-5′.
ptc-forward, 5′-TGCACCTCTACGACACCGAA-3′.
ptc-reverse, 3′-GGATCTTTACATACGCACCAGC-5′.

Chapter 3

Cell adhesion mediated actomyosin assembly regulates activity of Ci for maintenance of hematopoietic progenitor cells in the lymph gland.

3.1. Introduction

The development process of an organism involves a start from a single cell to a multicellular cell type with diverse function (Arakaki et al., 2013; Furusawa and Kaneko, 2012). The patterning, and binning of these specialized cell types into different tissue is the most exciting event in developmental biology. The development of tissue depends on the diverse cellular processes, which include cell proliferation, differentiation, migration, pattering, and cell-type specification (Dzierzak and Bigas, 2018; Weaver, 2017). Understanding these processes and their regulators can provide the necessary information helping us to design therapeutic interventions. Several biochemical signaling pathways have identified, which operate during development to dictate gene expression essential for diverse cellular processes. It has been demonstrated that activation of Hedgehog, Wnt, Notch, Hippo, TGFß, and EGFR biochemical pathways in the different cell types at a different developmental time can elicit the diverse cellular responses (Chaudhuri et al., 2016; Hansson et al., 2004; Pan, 2010; Rahman et al., 2015; Sever and Brugge, 2015; Steinhart and Angers, 2018; Wee and Wang, 2017). Thus, the regulation of these cellular signaling is essential to maintain the homeostasis in a tissue. The cross-talks of these biochemical signaling with each other serves as major regulators that coordinate the activation of the biochemical signaling in time and cell-type-specific manner (Ammeux et al., 2016; Sever and Brugge, 2015))

In addition to biochemical signals, cells in the tissue also responded to mechanical cues mediated by actomyosin assembly, which is known to drive the tissue morphogenesis (Munjal and Lecuit, 2014) (Heer and Martin, 2017; Lecuit et al., 2011). Recent studies have demonstrated how the actomyosin mediated mechanical forces have direct effects on proliferation, differentiation, lineage specification, and morphogenesis during tissue development (Rauskolb et al., 2014; Shin et al., 2014; Trappmann et al., 2012; Vicente-Manzanares et al., 2009). Actomyosin is a highly dynamic structure that is composed of actin, myosin, and their associated proteins. Actin combines with the myosin to form actomyosin networks capable of generating contractile forces and intercellular tension. The animal uses contractile forces or intercellular tension in a wide variety of cellular processes, ranging from the subcellular to the tissue level (Munjal and Lecuit, 2014). Studies in different organisms illustrate its involvement in cytokinesis during cell division (Meitinger and Palani, 2016), in cellular migration during gastrulation (Behrndt et al., 2012), and support that it renders to the syncytial germline architecture (Priti et al., 2018). The primary function of actomyosin has been implicated in cytokinesis or during cell migration, but recent works suggest that it also involve in diverse cellular process including specification (Martin et al., 2010; Nie et al., 2014), cell adhesion, apoptosis, proliferation, and differentiation in Drosophila, C.elegans, zebrafish as well as in higher vertebrates (Munjal and Lecuit, 2014; Murrell et al., 2015; Rauskolb

et al., 2014; Vicente-Manzanares et al., 2009).Although actomyosin in all non-muscle cells comprises the same actin and non-muscle myosin, they differ in their size, location, and the associated proteins in different cell types. This association of actomyosin with different proteins might account for the diversified function of actomyosin.

Assembly and contractility of actomyosin structures can be regulated through actin polymerization, myosin activation, and anchoring of actomyosin rings to the plasma membrane. Actin polymerization in cells is regulated through arp2/3 complex, formins, and Ena (Enabled), which are involved in nucleation, elongation, and branching of F-actin (Arber et al., 1998; Scott et al., 2006). The actomyosin assembly can also regulate though well conserved Rho kinase (ROCK) or myosin light chain kinases (MLCK), which involve in phosphorylating myosin regulating light Chain (MRLC) (Munjal and Lecuit, 2014; Vicente-Manzanares et al., 2009). Apart from these factors, cell-cell and Cell-ECM adhesion also play a crucial role in regulating actomyosin assembly via anchoring of actomyosin rings to the plasma membrane (Chugh and Paluch, 2018; Flores-Benitez and Knust, 2015; Goodwin et al., 2017) (Figure 3.1).It has been demonstrated that cell-cell adhesion and cell-ECM adhesion can effect on cellular fate or behaviour by controlling the intracellular tension that directly regulate the cell shape (Mui et al., 2016). Recently, another mode of action of actomyosin was demonstrated in the *Drosophila* as well as in higher vertebrates. The cell-cell adhesion mediated actomyosin assembly involves in modulating several signaling pathways via tethering the different enzyme or signaling molecules near the plasma membrane (Moujaber and Stochaj, 2020; Rauskolb et al., 2014). In the mammalian system, actomyosin regulator, ROCK, and ECM negatively involve in adipogenic, chondrogenic, and neurogenesis differentiation (Hyvari et al., 2018; Yim and Sheetz, 2012; Zhou et al., 2019), while it involves positively in oestrogenic differentiation (Arnsdorf et al., 2009). Thus, the above evidence suggests that actomyosin can participate in modulating the maintenance and differentiation of precursor cells.

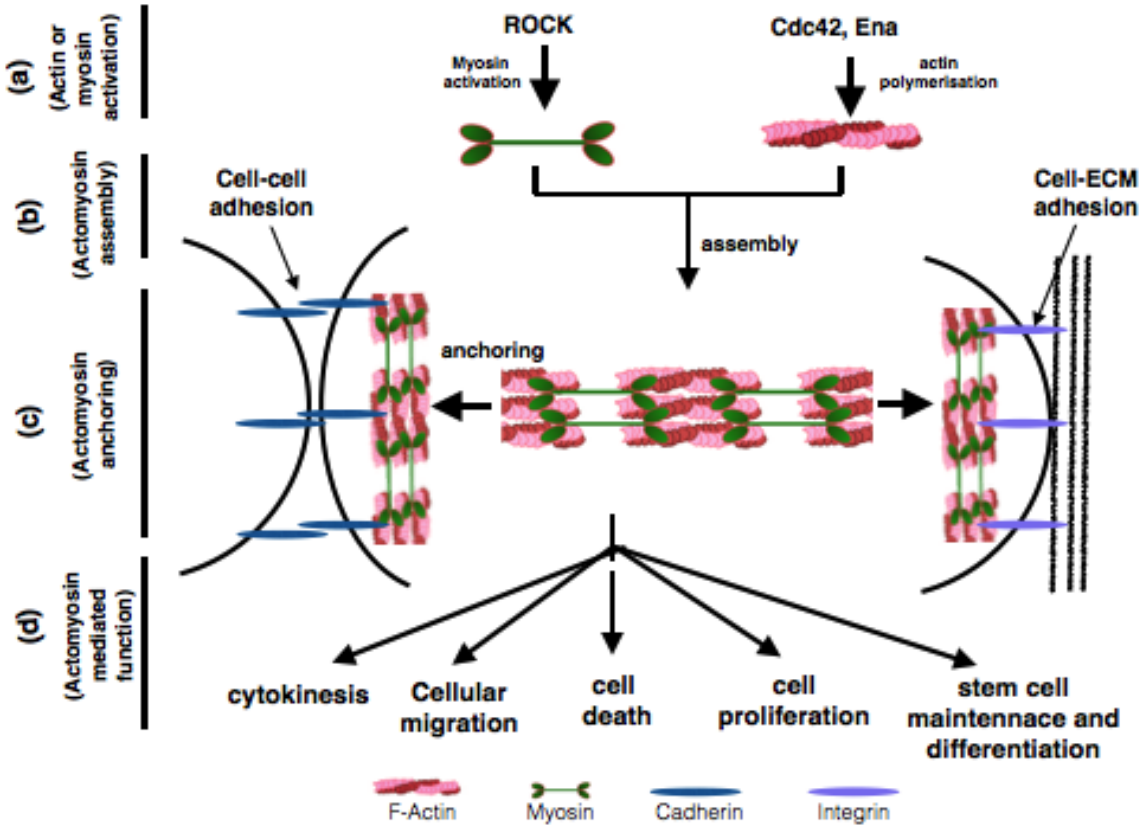

Figure 3.1 Cellular regulation of actomyosin assembly and contractility and its diverse role in tissue development. (a-b) Schematic of actin polymerization, myosin activation, and assembly of the actomyosin structures. Actin polymerization is mediated by the Ena, Arp2/3, Cdc42, and Formin, which involve nucleation and elongation of the actin. Myosin activation depends on the Rho kinase (ROCK) or myosin light chain kinases(MLCK), which involve phosphorylating myosin regulating light chain leading to the formation of actomyosin structures. (c) Schematic diagram showing anchoring of actomyosin rings to the plasma membrane. The localization of extracellular matrix (ECM), E-cadherin and integrin regulates the actin and myosin's anchoring at the cell surface. (d) Cells sense their mechanical environment through cell-cell and cell-ECM adhesion and modulate actomyosin activity and can function differently depending on context and cell type. Apart from its role in cytokinesis, actomyosin can also regulate cell migration, cell death, proliferation, and stem cell maintenance and differentiation depending on context and cell type. (Crawford et al., 1998;Mizuni et al., 1990;Duque j et al., 2016;Flores-Benitez and Knust 2015; Goodwin K et al., 2017).

Although cell-cell adhesion and ECM is known to influence lymph gland hematopoiesis in *Drosophila (Gao et al., 2013; Grigorian et al., 2013)*, the involvement of actomyosin in hematopoiesis is not worked out.

Current chapter describes the involvement of actomyosin in blood cell development in the *Drosophila* lymph gland. It remains to be seen if the circuit identified in this study is active in vertebrates hematopoiesis.

3.2. Results

3.2.1 *Drosophila* hematopoietic progenitors are heterogeneous

It is generally considered that lymph gland progenitors are not homogenous as they have different proliferation and differentiation capacities at different developmental stages (Banerjee et al., 2019; Jung et al., 2005). Although, the majority of the blood progenitors within the lymph gland can be visualized by Dome expression, few in the innermost core lack Dome. Additionally, the progenitors that lie adjacent to the CZ zone (IP) co-express Pxn, and Dome (Banerjee et al., 2019) . Despite this apparent heterogeneity evident in the progenitors, due to lack of specific markers, none of these cell types have been fully characterized in the lymph gland.

The two bona fide reporters, Domeless (Jung et al., 2005) and TepIV (Shim et al., 2013b) expression can be used to visualize hematopoietic progenitors residing in the lymph gland. Domeless (Dome) is an essential receptor for upd, JAK/STAT signaling (Brown et al., 2001) while TepIV is a downstream responder of the same JAK/ STAT signaling (Irving et al., 2005). Both Dome and TepIV are expressed in LG hematopoietic progenitor cells and majorly overlap (Figure 3.2A-A').

Previously, it has been found that second instar progenitor cells are proliferative as demonstrated by Bromodeoxyuridine BrdU (5-bromo-2'-deoxyuridine) incorporation. While late third instar progenitor cells devoid of BrdU, suggesting that progenitor cells differ in their proliferation status depending on their developmental stage (Jung et al., 2005). EdU (5-ethnyl-2᾽-deoxyuridine)) incorporation in the progenitor cells of second and third instar lymph glands demonstrate that early on, they are proliferative **(**Figure 3.2B) while late in development they lose their proliferative capacity (EdU; Figure 3.2C).

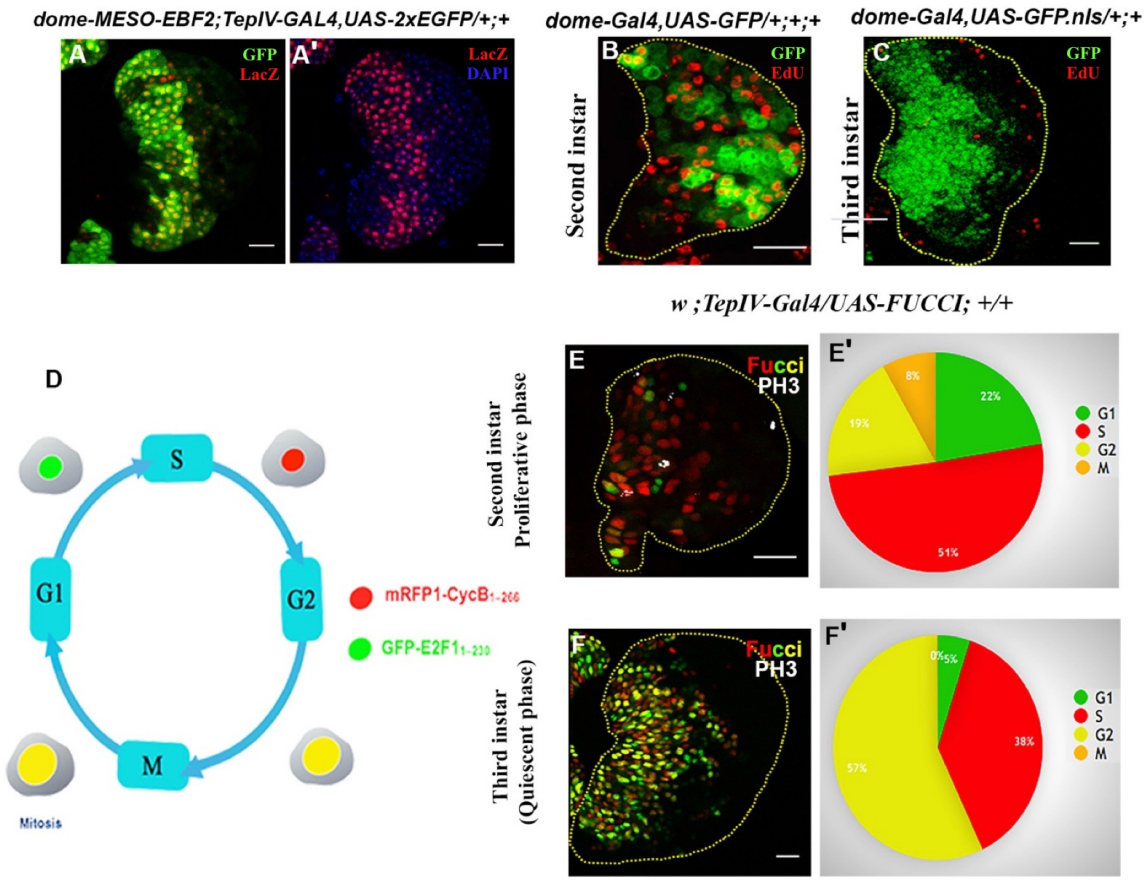

Figure 3.2 Cell cycle analysis of Drosophila hematopoietic progenitor cells using G1, G2, S, and M phase markers. (A)The bonafide hemocyte progenitor specific Gal4 drivers: Dome (red) and TepIV (green) expresses in overlapping domains. (B-C) At second instar (B) Dome expressing lymph gland progenitor cells(green) exhibit high proliferation capability (EdU: red) during third instar (C) lymph gland progenitor (green) lack EdU (red) incorporation. (D)Scheme for Fly-Fucci-fluorescent ubiquitination-based cell cycle indicator (Zielke N et al., 2012). This construct uses two probes; the first one is E2F moiety fused to GFP. Since Cdt2 degrades E2F during S, the GFP marks cells in G1, G2, and M phases of cell cycle only. The second probe, coupled with this system, is CycB moiety fused to mRFP.This moiety is susceptible to APC/C degradation during the G1 phase, as an outcome of which the RFP tagged to it, marks cells in S and those undergoing G2/mitosis in yellow**.** (E-F) Expression of Fly-Fucci using progenitor specific GAL4: TepIV-Gal4. Early on **(E)** progenitors in the lymph gland are in S-phase (red), while few of them are in G1 (green) and G2 (yellow). **(F)** In the late third instar lymph gland, the progenitor (green) lack EdU (red)/PH3 (E, grey) incorporation is paused in G2 (Fucci: yellow). **(E'** and **F')** Quantitative analyses of the Cell Cycle of the progenitors in the two stages.

It has been demonstrated that late third instar progenitors are in the quiescence stage, as they do not incorporate BrdU (Jung et al., 2005; Mondal et al., 2011). EdU incorporation analysis revealed a similar result, suggesting that late third instar progenitor cells of LG might have exited the cell cycle. To determine cell cycle status of the progenitor cells, UAS-FUCCI system was employed.

(Figure 3.2D). Monitoring of the progenitor specific FUCCI expression by TepIV-Gal4 in second instar reveals that the proliferative progenitors were primarily in S phase, while a few were in G1 and fewer were in G2/M(Yellow/PH3)(Figure 3.2E- E'). In the third instar, it was found that majority of progenitor cells were in G2 phase (yellow), which also lack PH3 labeling (Figure 3.2F with quantification in Figure 3.2F'). Therefore, these results revealed that *Drosophila* hematopoietic progenitor cells of the lymph gland are arrested in the G2 phase in the late larval stages. The above results suggest that although these progenitor cells of the lymph gland lack EdU incorporation, they have not exited the cell cycle rather are halted in G2-M phase.

These data suggest that early and late *Drosophila* blood progenitor cells are different as they have differential proliferative potential. These results prompted us to perform detailed marker specific characterization of both second and third instar hematopoietic progenitors of the lymph gland. Analysing the expression of Dome-Gal4, UAS-GFP constructs (a validated marker for third instar blood progenitors) in the second instar lymph gland revealed a variable expression of Dome. While the peripheral progenitor cells of second instar exhibited high levels of Dome, the expression was hardly detectable in the inner core (medial) progenitor cells of the lymph gland (Figure 3.3A-B). Thus, *Drosophila* hematopoietic progenitor cells can be categorized into two subtypes based on Dome expression; Dome^{high} and Dome^{low}.

Furthermore, these two subtypes could also be appreciated by monitoring the appearance of Pvf2, a ligand for VEGF signaling (Munier et al., 2002). PVF2 expressed in the Dome^{low} progenitor cells of the lymph gland while the Dome^{high} cells lack Pvf2 expression {(visualized by the Pvf2-LacZ, a transcriptional fusion construct (Choi et al., 2008) (Figure 3.3C-D')}. It has been demonstrated that Pvf2 expression in the lymph gland is essential for normal lymph gland proliferation during early larval stages. Its expression is regulated through TEAD family transcription factor Scalloped (Sd) (Ferguson GB et al., 2017). This finding further highlights the importance of the Pvf2 expressing domain in hematopoiesis.

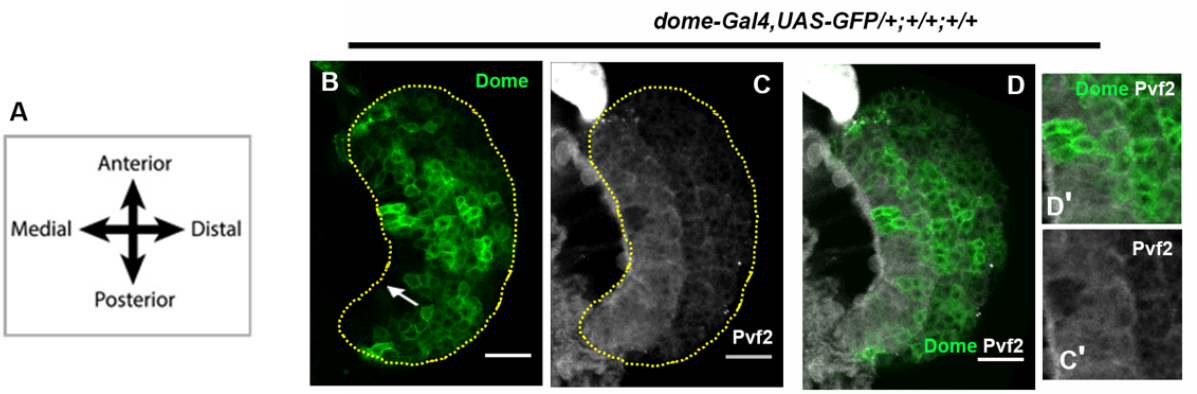

Figure 3.3 Pvf2 expressed in the subpopulation of progenitor cells. *(A)Representation of lymph gland direction with respect to the dorsal vessel. The region near the dorsal vessel is medial, and the opposite side as distal(A). In the second instar larval lymph gland, few cells near the dorsal vessel (medial side) are low in dome-GFP* ***(B,*** *arrow****),*** *which also co-localize with Pvf2 lacZ (grey,* ***C-C'*** *and* ***D-D'****). While other population which expressed high dome lack Pvf2 expression.*

Next explored was the expression of angiotensin-converting enzyme (Ance) (Tatei et al., 1995), which has been previously reported in third instar blood progenitors (Benmimoun et al., 2012). \ Interestingly, mammalian angiotensin converting enzyme (ACE) is also expressed in the hematopoietic stem and progenitor cells. In *Drosophila* quiescent hematopoietic progenitor cells of LG, Ance expression (red) is restricted to the tightly-packed progenitor cells {visualized by the heparin sulfate proteoglycan, trol (Terribly Reduced Optic Lobes; green) (Gregorian et al., 2013) expression (Figure 3.4A-A")}. Quite intriguingly, temporal analysis of Ance revealed that early in development, Ance is ubiquitously expressed in both subtypes (Dome^{high} and Dome^{low}) (Figure 3.4B-B') or (Pvf+ and Pvf2-)(Figure 3.4C-C') of hematopoietic progenitor cells

Later in the development, the Ance can be seen in most of the MZ population. In contrast, Pvf2 expression was restricted to a few of the blood progenitor cells observed near the dorsal vessel (Figure 3.4D-E'). Thus, Ance is present in both the Pvf2- Dome^{high} progenitors as well as the Pvf2+ Dome^{low} domain (Figure 3.4 E').

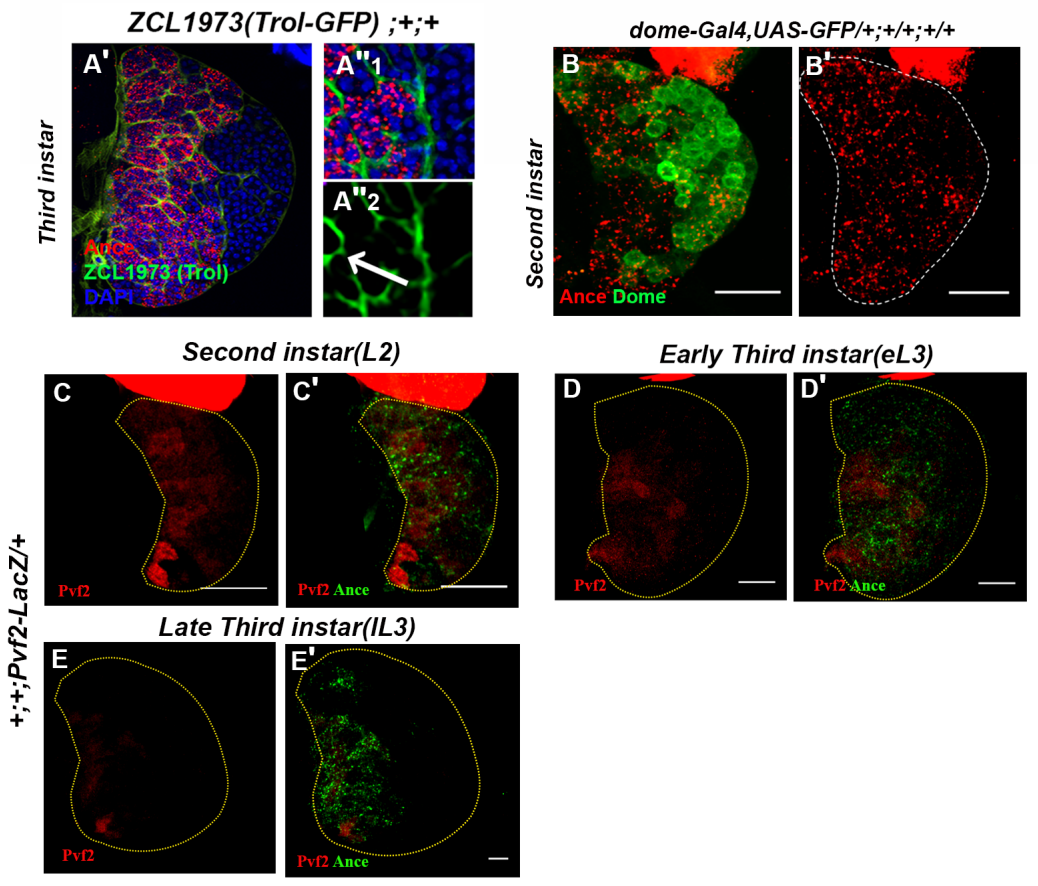

Figure 3.4 Ance expressed in the Pvf2^{+} dome^{low} and Pvf2^{-} dome^{high} subpopulation. (A-A''2) Tightly packed third instar hematopoietic progenitor cells (visualized by trol-GFP expression) expressed Ance (red). **(B-B')** Ance expression (red) marks both Dome^{low} and Dome^{high} population in the early second instar. **(C-E')**. Pvf2 expression (red) in the developing lymph gland can be detected in PSC and progenito cells (Ance in green) in the inner core of the MZ through development.

Interestingly, temporal analysis of Ance in the early second instar lymph gland (where differentiation is yet to begin) reveals a group of Dome expressing progenitor pool at the periphery of MZ that is negative for Ance. Later in development (in third instar lymph gland), most of the domain of Dome expression in the lymph gland overlaps with Ance expression except in the distal progenitor cells of the MZ (**Figure 3.5A-B'2)**. Thus, based on these marker analyses, three types of progenitor cells were identifed. The first one is naïve progenitor: which expresses Pvf2, Ance, and are Dome^{low}, while the Dome^{high} primed progenitors are negative for Pvf2 but expresses Ance . The third population which expresses Dome but devoid of Ance is present in the periphery of the MZ.

It was previously shown that there is a small Dome+ population of progenitor cells positioned between MZ and the CZ that also express differentiated markers Pxn and Hml. These were described as Intermediate progenitor cells as these Dome+ cells express the earliest differentiation markers Hml and Pxn, but lack mature markers for plasmatocytes (P1) and crystal cells (Lz) (Krzemien et al., 2010). The current study identifies this as Ance^{-}Dome^{+} intermediate progenitor population. Co-expression analysis revealed that the Ance expression is mutually exclusive with Pxn as reported via Pxn-Gal4, UAS-GFP (Nelson et al., 1994) that further supports this hypothesis (**Figure 3.5C-C'')**. These IP cells (Ance^{-},Dome^{+}, Pxn^{+}) could also be visualized by simultaneous labeling of LG progenitor cells with Pxn, Dome, and Ance. It could be seen in the peripheral area of the MZ as a fraction of population which co-express Dome and Pxn (Figure 3.5DK-E') (Sinenko et al., 2009) but lack Ance expression (Figure 3.5D-G'2). These data validates the above hypothesis that Ance^{-}Dome^{+} Pxn^{+} cells represent the previously identified IP cells (Krzemien et al., 2010; Sinenko et al., 2009).

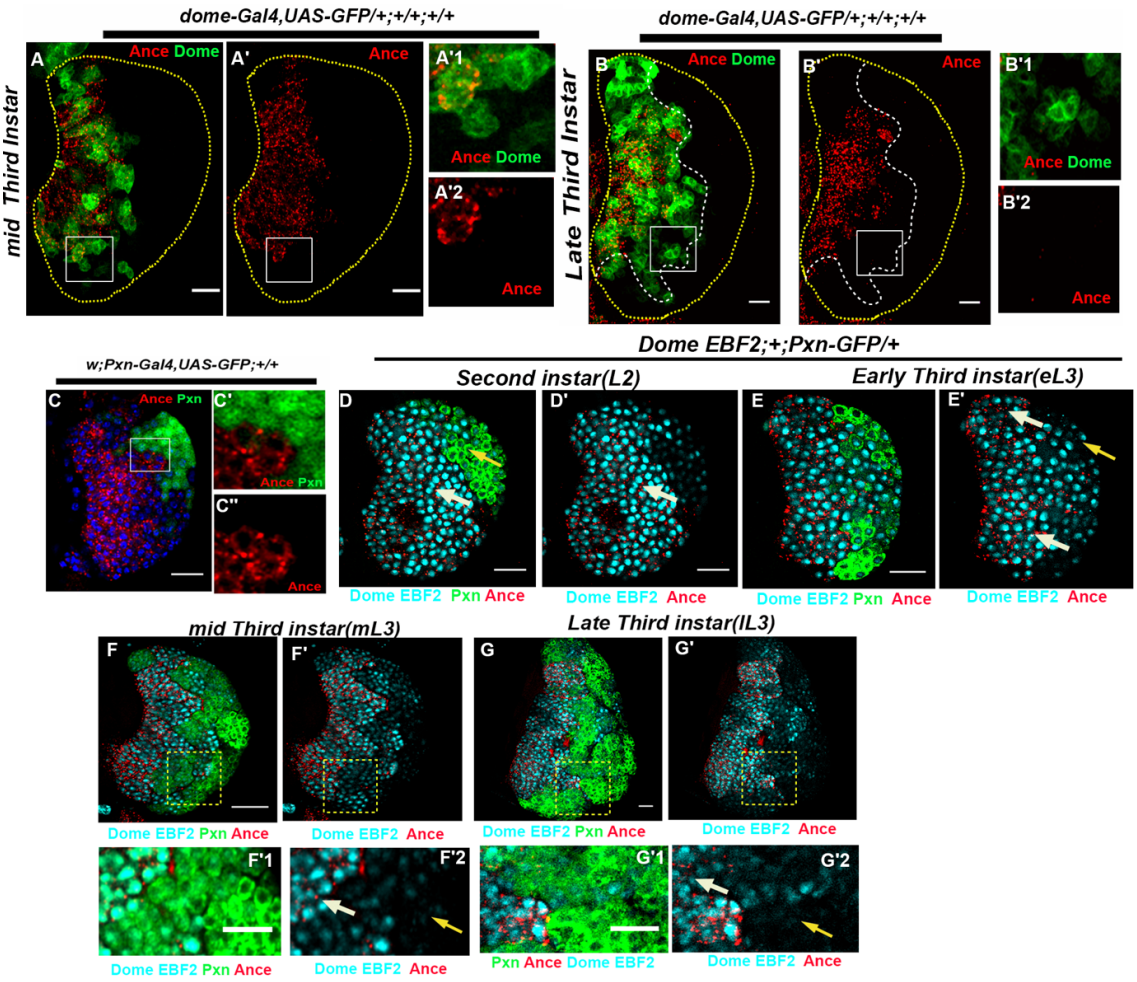

Figure 3.5 Expression of Ance is restricted to the progenitor cells, while IP cells lack Ance expression. (A-B'2) Ance expression (red) marks a restricted population of Dome-GFP cells in the mid-third instar lymph gland. Higher magnification of the selected area in A and B shows that cells in the periphery of MZ, which express Dome, lack Ance expression (A-A'2, B-B'2). **(C-C'')**Ance is excluded from the PxnGFP positive cells. **(D-G'2)** Co-labeling of PxnYFP, Dome, and Ance throughout development reveals that the Ance expression marks the inner core of the MZ (progenitor cells) while dome^{+} Pxn^{+} IP at the periphery of the MZ lack ance expression (yellow arrow).

Based on PH3 patterns, it was suggest that this dome+/Pxn+ intermediate progenitor cells are proliferative (Krzemien et al., 2010). To analyze the cell cycle status in details, the fly FUCCI construct was over-expressed using TepIV-Gal4, which expresses both in Ance+ progenitor and Ance- IP cells. Upon visualizing IP cells using co-labeling with Pxn and Tep-IV, it was clear that the majority of the IP cells (TepIV+Pxn+) are cycling (**Figure 3.6A-A''),** while the progenitor's cell (TepIV+Pxn-) are G2 arrested in the lymph gland.

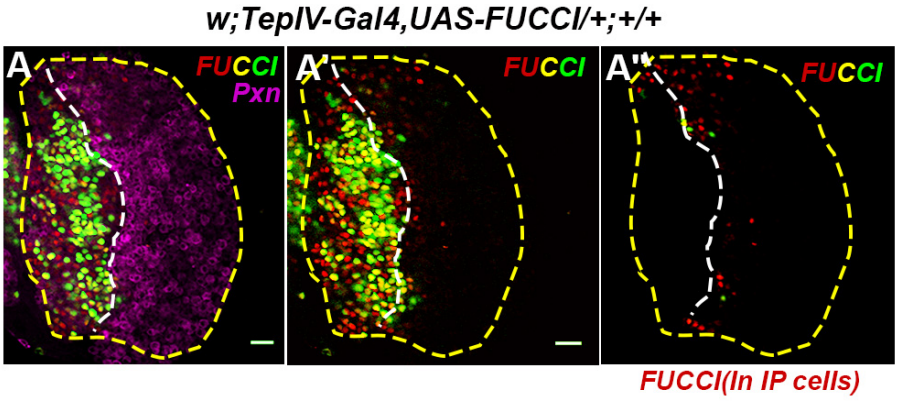

Figure 3.6 IP cells are cycling while progenitor's cell arrested in the G2 phase. (A-A") The intermediate progenitors (Tep IV+Pxn +) are cycling as revealed by FUCCI labeling (red: indicating S phase*)*.

In summary, *Drosophila* hematopoietic progenitors can be classified into three groups: naive progenitors ($Pvf2^{+,}$ $Ance^{+}$, $Dome^{low}$), primed progenitors ($Pvf2^{-}$, $Ance^{+}$, $Dome^{high}$) and differentiating IP cells ($Ance^{-}$, $Dome^{high}$, Pxn^{+}) (**Figure 3.7A-A"**). The current study identifies a suitable marker for the IP cells, which was previously proposed but lacked specific markers (Krzemien et al., 2010; Sinenko et al., 2009).

The above data also revealed that the IP cells also differ in the cell cycle status from the progenitor's cells. The IP cells are cycling while the progenitors are stalled at G2. Similar to *Drosophila* hematopoietic progenitor cells, the pause at G2 is also seen in *Drosophila* germline (Morris and Spradling, 2011), intestinal stem cells, neuronal stem cells (Otsuki and Brand, 2018), sensory organ precursors of *Drosophila* (Meserve and Duronio, 2017), primordial germ cells in C.elegans (Seidel and Kimble, 2015) and tailbud progenitors in zebrafish (Bouldin et al., 2014). The G2 cell cycle arrest seen in diverse tissue in various organisms suggests that it can be a preferred cell cycle arrest adopted by these stem cells/progenitor cells. Interestingly, G2 cell cycle arrest also essential for activation and enhance response of a specific signaling pathway in these tissues. So, it can be hypothesized that G2 arrest in hematopoietic progenitor cells might be required for activation or increase response to explicit cellular signaling, which is essential for lineage commitment or maintenance of progenitor cells. Interestingly, the G2 arrested *Drosophila* hematopoietic progenitor cells can also provide a unique opportunity to use third instar lymph gland as a model system to uncouple signals relevant for maintenance vs. differentiation independent of cell cycle intervention.

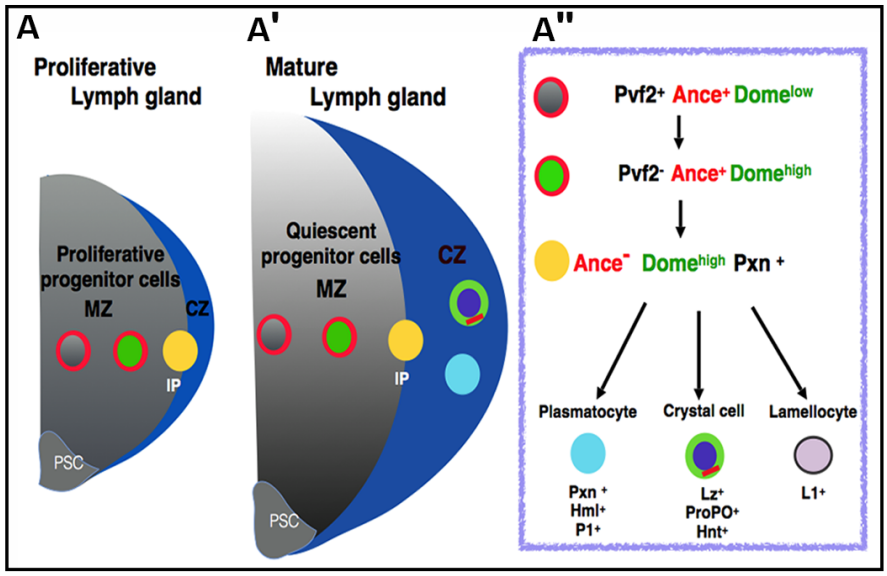

Figure 3.7 Drosophila hematopoietic progenitors are heterogeneous (A-A") A Scheme based on above results describing the heterogeneous progenitors of MZ in the larval lymph gland.

Actomyosin is enriched in Ance+ *Drosophila* hematopoietic progenitor

To understand the requirement of actomyosin activity in the development of the lymph gland, the myosin expression was analyzed. Non-muscle myosin II heavy chain is a complex protein, is encoded by *zipper* (*zip*) while the regulatory light chain is encoded by *spaghetti squash* (*sqh*) (Edwards and Kiehart, 1996). To visualize the spatial and temporal expression of myosin in the lymph gland, Sqh-GFP (regulatory myosin light chain tagged to GFP) was used in the different time windows. In order to understand whether actomyosin expresses differentially in the heterogeneous progenitor cells, actomyosin expression was mapped with respect to the Ance domain in early proliferative and late G2 arrested progenitor cells. Interestingly, in the third instar lymph gland, sqh-GFP expression was highly enriched in Ance positive progenitor cells (**Figure 3.8A-A''**). In contrast, nearby Ance negative cells exhibited a sharp decline in sqh GFP expression. This result indicated that Ance positive progenitor cells have an inherent high actomyosin activity.

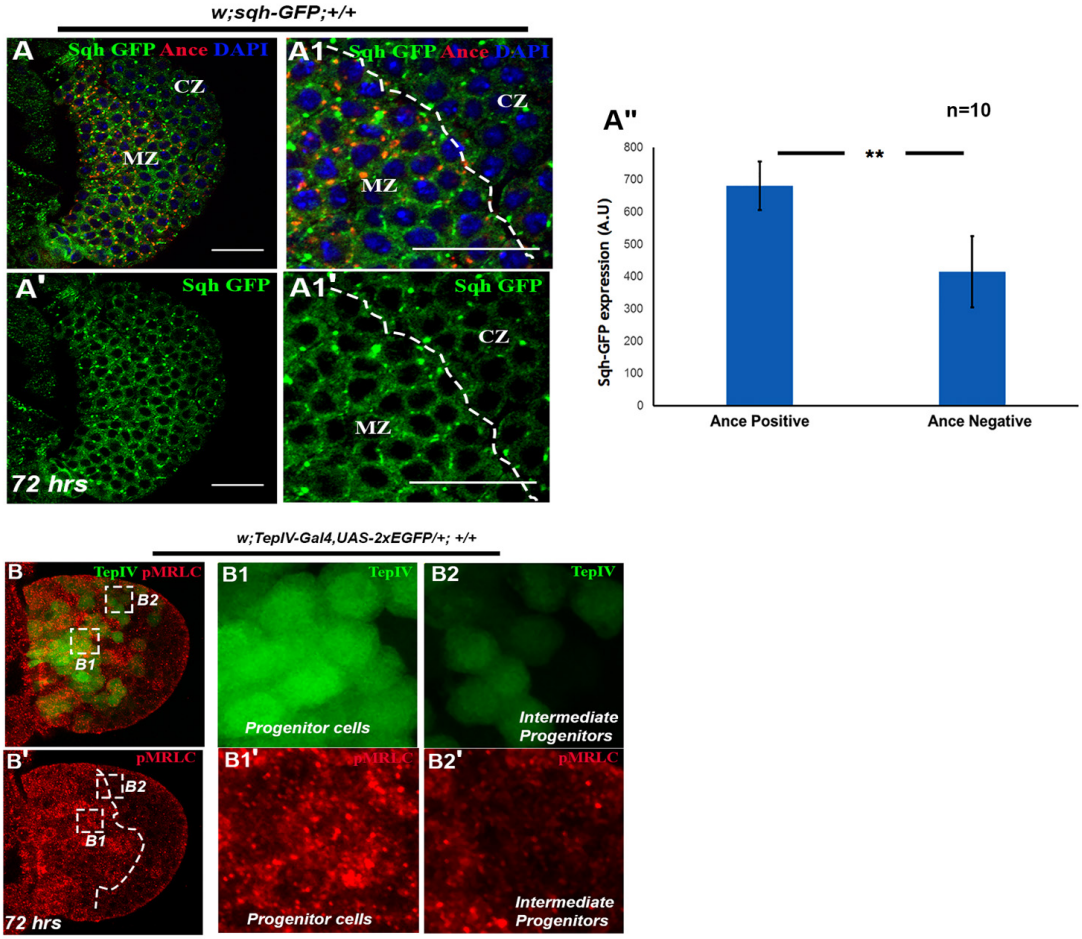

Figure 3.8 Ance positive progenitors are enriched in actomyosin components. (A-A") Ance (red) positive progenitor cells of the third instar LG are enriched in Sqh (green) (A"). (A") Quantitative analyses of the above result P-value: 5.213×10^{-3}. (B-B2') pMRLC (red) is also notably enriched in the progenitor population (green, B1), while down-regulated in progenitor cells which are in the MZ periphery (IPs, green low, B2). B1 and B2 are the high magnification of B representing two regions that are distinguished by high and low TepIV-Gal4, UAS-2xEGFP indicating inner and intermediate progenitors (IP).

It is established that phosphorylation of sqh is essential for its function, and impairment in this process leads to the reduction in actomyosin assembly (Martin et al., 2009; Royou et al., 2002) To visualize phosphorylated sqh, pMRLC staining was performed in early and third instar progenitor cells in TepIV-GAL4, UAS-GFP background. Interestingly, the indicator of activated sqh, pMRLC, is highly enriched in the progenitor cells while down-regulated in IP cells (**Figure 3.8B-B'2)**. The above results suggest that Ance+ progenitor cells have high actomyosin activity compare to IP cells.

3.2.2 Actomyosin modulates the maintenance of G2 arrested progenitor cells

It is important to note that stem cells or precursor cell's proliferation and differentiation are interrelated (Bylund et al., 2017; Mondal et al., 2011). Since actomyosin is known to regulate the cytokinesis, the downregulation of actomyosin will potentially affect the above two processes. In order to dissect the cell cycle independent role in hematopoietic progenitor cells, it is important to avoid impairment in cytokinesis. Interestingly, late *Drosophila* hematopoietic progenitor cells are arrested in the G2 phase, thereby providing an excellent model system to understand the cytokinesis independent role of actomyosin in these precursor cells.

To test the potential role of the actomyosin cytoskeleton in *Drosophila* hematopoietic progenitor cells, knockdown of *sqh,* and *zipper* were performed in G2 arrested progenitor cells using GAL4-GAL80 (Brand and Perrimon, 1993)based TARGET) system that allows the spatial and temporal knockdown of the gene (McGuire et al., 2003). To achieve spatial control, progenitor cells specific TepIV-Gal4 was used to knock down the target gene using RNAi. The temporal control over Gal4 expression was achieved by expressing a temperature-sensitive (ts) allele of Gal80 (a known repressor of Gal4) under the ubiquitous tubulin promoter. In the progeny, the $Gal80^{ts}$ molecule remains active at 18^0C, thereby keeping the, Gal4 inactive. $Gal80^{ts}$ activity can be stopped at 29^0C, resulting in the activation of Gal4 that drives the expression of respective RNAi in a cell-type specific manner (**Figure 3.9A)**.

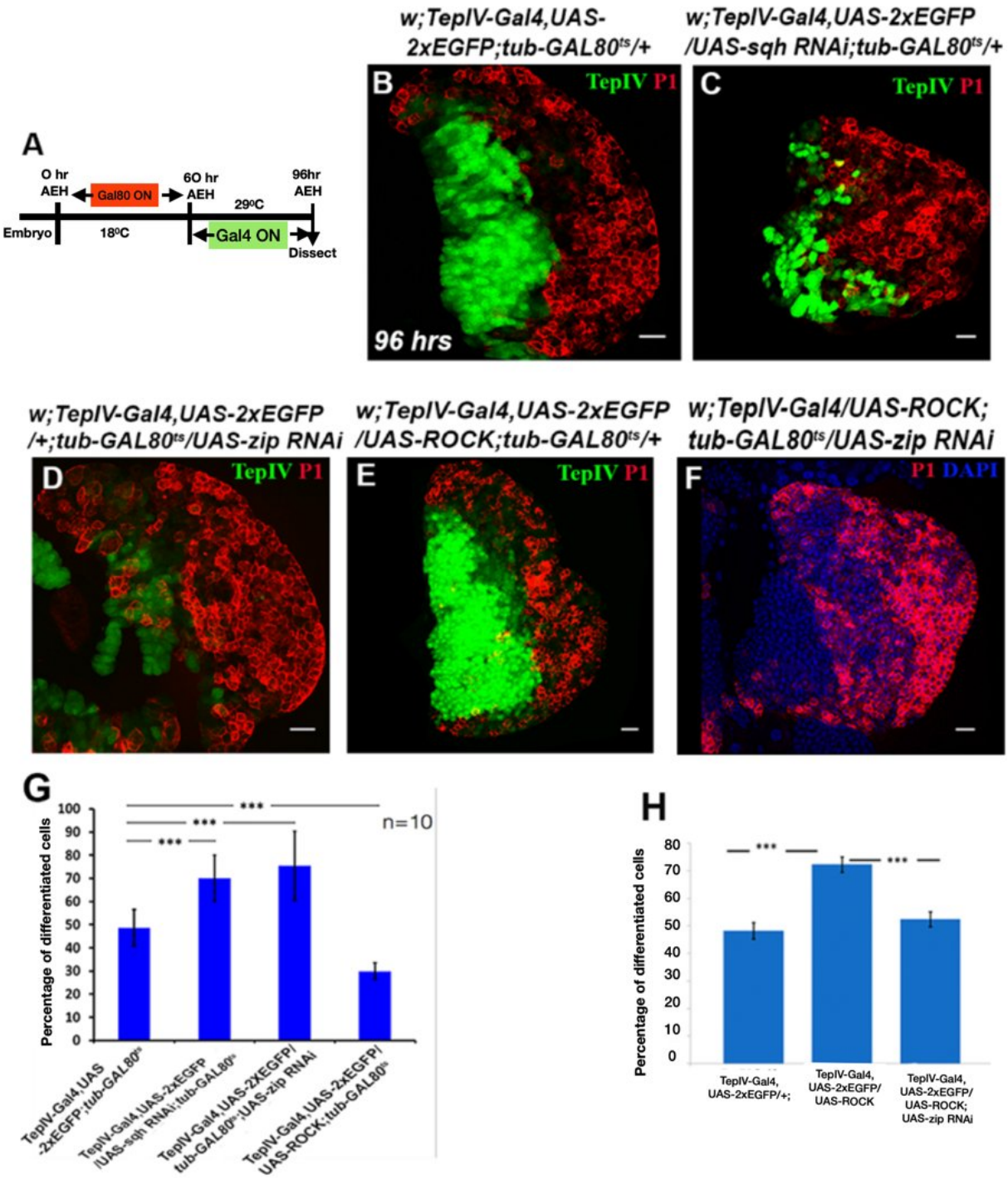

Figure 3.9 Actomyosin modulates the maintenance of quiescent progenitor cells. (A)Scheme illustrating the timeline adopted for all our studies. The synchronized larvae collected from the crosses were kept at 18ºC to keep the GAL80 repression on till 7days AEL (equivalent to 60hrs AT 25ºC). Following this, the vials were shifted to 29oC to downregulate the desired gene function. (B-C) Down-regulation of actomyosin components affects progenitors. Compared to Control (B)RNAi mediated down-regulation of Sqh (C) or Zip (D) function affects progenitor number (visualized by TepIV-Gal4, UAS-2xEGFP). (E) Over-expression of ROCK in the progenitors results in an increases in the number of progenitors (TepIV+). (F) Co-expression of UAS-zip RNAi with activated ROCK fails to sustain the progenitors. (G) Quantitative analyses of the differentiation index in G. P-value: TepIV-Gal4,UAS2xEGFP/ UAS-sqh RNAi; tub-GAL80^{ts} =1.256x10^{-6}, TepIV-Gal4, UAS2xEGFP;tub-GAL80^{ts}/UAS-zipRNAi=3.192x10^{-7}, TepIV-Gal4,UAS 2xEGFP/UAS-ROCK; tub-GAL80^{ts} = 6.128x10^{-6}.(H) Quantitative Analyses of P1 Compared to control P-value for TepIV-Gal4,UAS-2xEGFP/UAS-ROCK; tub-GAL80^{ts} = 5.865x10-12. While compared to TepIV Gal4, UAS-

2xEGFP/UAS-ROCK; tub-GAL80^{ts}, the P-value for TepIV-Gal4 /UAS ROCK; tub-GAL80^{ts}/ UAS-zip RNAi= $5.891x10^{-11}$ Error Bars: S.D, Scale bar: 20 μm

In an attempt to bypass the requirement of sqh or zip in cytokinesis, expression of these components were down-regulated in the quiescent progenitors (post 60 hrs AEH following the Scheme in Figure 3.9A). Larvae were then dissected and analysed in 96 hours AEH. Interestingly, sqh knockdown from the quiescent progenitor cells exhibited an increase in the total number of differentiated cells (P1 positive) in the late third instar lymph gland (Figure 3.9B-C). To analyze the sqh loss phenotype in more details, the differentiated population was quantified using Imaris software (based on spot quantitation, quantified P1 positive cell number). Quantitation of the P1 positives cells number revealed that differentiated cells in wild type were around 40 ± 6 %. In contrast, it was drastically increased (75 ± 8%) in the lymph gland of TepIV-Gal4/UAS-sqh RNAi larvae (Figure 3.9G). Likewise, zipper knockdown was also performed in the quiescent population following the same time-line. Similar to sqh loss, knockdown of the Zipper from the progenitor cells also leads to increment in the differentiation of progenitor cells (Figure 3.9D). It is clearer from the quantitation of the differentiated cells that an increase in P1 positive area occurs upon loss of actomyosin components.

Actomyosin contractility is regulated through ROCK Rho-associated protein kinase, ROCK: comprising the catalytic domain, ROCK), which phosphorylates the regulatory light chain of Myo II (Sqh), an event essential for the activity of myosin heavy chain, Zipper (Amano et al., 1996). Therefore, over-expression of ROCK leads to an increase in phosphorylation of Sqh, which can lead to an increase in progenitor maintenance. Progenitor specific overexpression of activated ROCK resulted in a reduction in differentiated cells and increment in progenitor cell number (Figure 3.9E)**.** Quantitation of the differentiated cell area in overexpression ROCK revealed that in control, the % of differentiated cells was around 40 ± 6 **(Figure 3.9E)**. While in the lymph gland of TepIV-Gal4/UAS ROCK larvae differentiation drastically decreased (28±4). ROCK is known to be a multifunctional kinase involved in cell behavior, motility, centrosome positioning through modulating various signaling pathways which can be independent of its effect on actomyosin (Bryan et al., 2010; Noma et al., 2012). To analysis whether the increase maintenance of progenitor cells seen in overexpression of ROCK was through actomyosin activity, *zip* function was down-regulation in the progenitors which had higher activity of ROCK.

In contrast to ROCK overexpression, where the percentage of differentiation was significantly less compared to control, a *zip* loss in the same genotype resulted in rescuing this phenotype (Figure 3.9F and H). These experiments indicate the direct involvement of actomyosin activity in the *Drosophila* hematopoietic progenitor cell's maintenance. Next, it was analysed how actomyosin activity regulates progenitor cell maintenance.

3.2.3 Actomyosin assembly modulates Ci level in progenitor cells-

It has been previously demonstrated that hematopoietic progenitors required Ci-155 mediated signaling for their maintenance (Mandal et al., 2007; Mondal et al., 2011). Interestingly, Ci-155 has been shown to be regulated through *sqh* in *Drosophila* wing imaginal discs (Liu et al., 2015a). To determine a possible link between Actomyosin function and Ci-155 in hematopoietic progenitors, the level of Ci-155 was analyzed in the lymph gland where *sqh or zip* was downregulated in the quiescent progenitors using *TepIV-Gal4*. Interestingly, a significantly reduced level of Ci-155 expression is evident in the progenitor cells which lack Zipper (Figure 3.10A-B').

Furthermore, progenitor specific overexpression of activated ROCK leads to increase expression of Ci-155 when compared to control (Figure 3.10C- B'). The quantitative analysis also revealed an increase in the Ci-155 level (Figure 3.10D), which implicated a positive relation between actomyosin assembly and Hh signaling. This elevated Ci-155 in the ROCK-activated progenitors restored to the wild-type level (Figure 3.10E-E') upon expression of zip RNAi in same genetic background (Figure 3.10F-G). The recovery in the Ci-155 level also leads to the rescue of stalled differentiation seen in progenitors on ROCK overexpression.

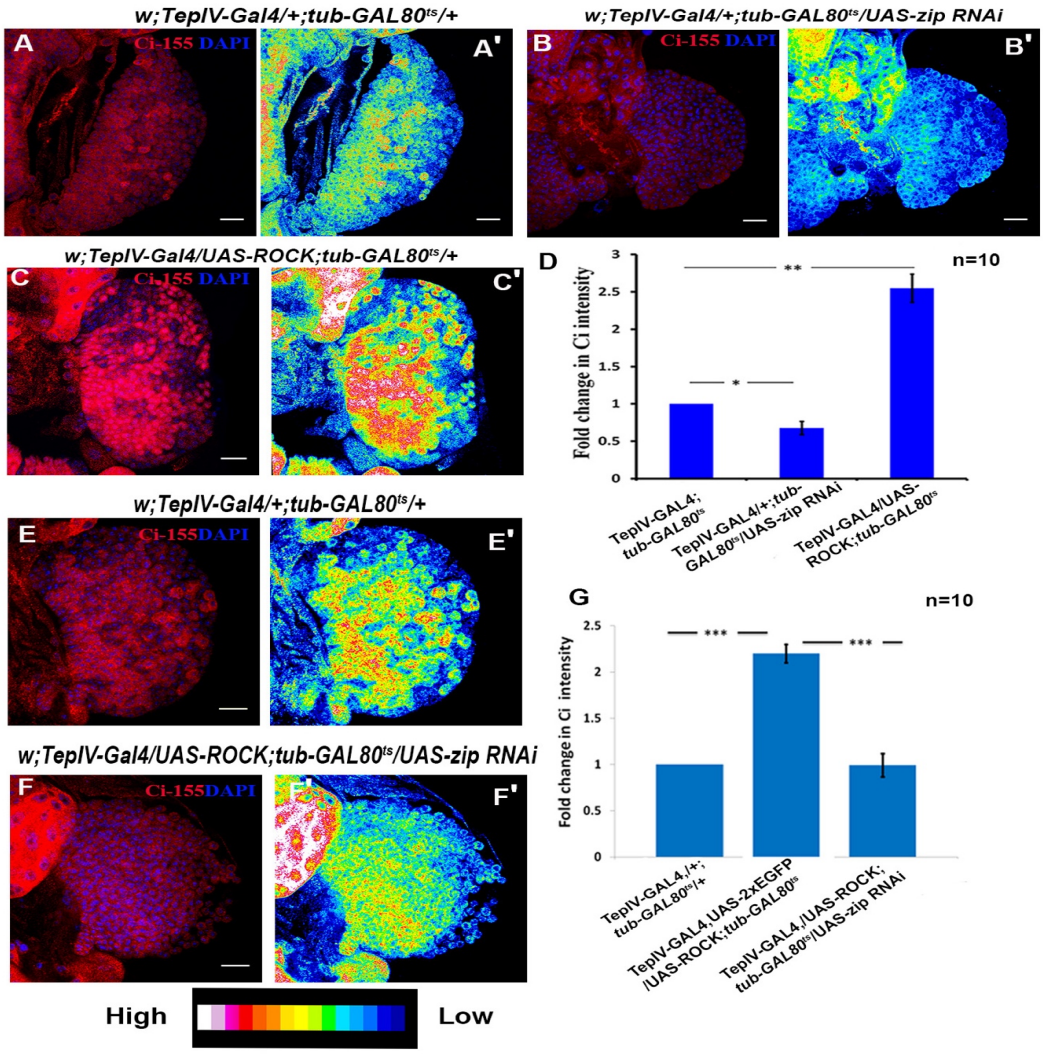

Figure 3.10 Actomyosin assembly modulates the Ci level in progenitor cells. *The genotypes are mentioned on the top of the relevant panels. Scale bar: 20 µm. (A-D) RNAi mediated down-regulation of Zip (B-B') results in lowering of the Ci-155 level overexpression of ROCK increases the Ci-155 in the progenitors ((C-C'). (D) Quantitative analyses of the above result. P-value: TepIV Gal4; tub-GAL80^{ts}/UAS-zipRNAi = 2.146x10^{-3} and TepIV-Gal4 /UAS-ROCK; tub-GAL80^{ts} =4.97x10-3. (E-G) Co-expression of zip RNAi with activated ROCK in progenitors fails to sustain the Ci-155 expression (F-F') compare to control (E-E'). (G) Quantitative Analyses of Ci-155 in the above experiment. P-value for TepIV Gal4, UAS-2xEGFP/UAS-ROCK; tub-GAL80^{ts} = 4.614x10^{-3} compared to control and P-value for TepIV-Gal4/UAS-ROCK; tub-GAL80^{ts}/ UAS-zipRNAi= 2.63x10^{-4}compared to TepIV-Gal4, 2xEGFP /UAS-ROCK; tub-GAL80^{ts}*

The above results indicated that the actomyosin network essential for maintenance of higher Ci-155 level in progenitor cells is a positive regulator of hematopoietic progenitor maintenance.

3.2.4 RNAi mediated clonal analysis revealed an autonomous regulation of Ci-155 activity through actomyosin activity-

To determine whether the actomyosin component cell-autonomously regulates Ci-155 expression in the lymph gland, zip *RNAi* clones were generated using Ay-GAL4 (Ito et al., 1997). This technique combines the Act5C-Gal4/UAS system and the hsp70-flipase (flp)/FRT system. Act5C promoter-Gal4 fusion gene is made non-function by the insertion of transcriptional termination signals. Upon heat-shock, the hsp70 promoter drives the flp gene that encodes flippase, which induced recombination at FRT sites and removes the termination signals, generating a functional Act5C-Gal4 fusion. Thus, cells that experienced recombination and their progeny constitutively activate the Gal4 under the act5C promoter, which derives the UAS linked transgene, including GFP and RNAi. Inducing the mock clone, which has only UAS-GFP transgene, generates two types of clones; GFP+ and GFP-. No differential Ci-155 expression was evident in lymph glands with the mock clones (Figure 3.11A-A'). However, *zip RNAi* clones (marked by GFP) exhibited compromised Ci155 levels compared to the nearby wild-type cells (Figure 3.11B-B'). Quantitative analyses also revealed that in most *zip RNAi* clones, there is an approximately 60% reduction in Ci-155 expression (Figure 3.11C). Furthermore, up-regulated Ci-155 expression could be seen in the ROCK overexpressed clones compared to the neighboring wild-type cells (Figure 311D-D'). It has been demonstrated that the increased activity of Ci-155 can lead to the activation of a downstream gene like *ptc* (Figure 3.11E). Therefore, if *actomyosin* potentiates Hh signaling via modulating the Ci-155 level, it is expected that ROCK's overexpression should enhance signaling levels and increase the *ptc* transcription/translation. Indeed, similar to Ci-155, Patched expression was also increased in the ROCK-overexpressing clones (Figure 3.11F-F'"), suggesting increasing Ci-155 expression in ROCK overexpression is sufficient to activate the downstream Hh target genes (Chen

and Struhl, 1996). Moreover, the patched transcript level also increases upon progenitor-specific ROCK overexpression compared with the wild-type lymph gland (Figure 3.11G).

Put together; these results demonstrate the cell-autonomous regulation of Ci activity by actomyosin in lymph gland progenitors.

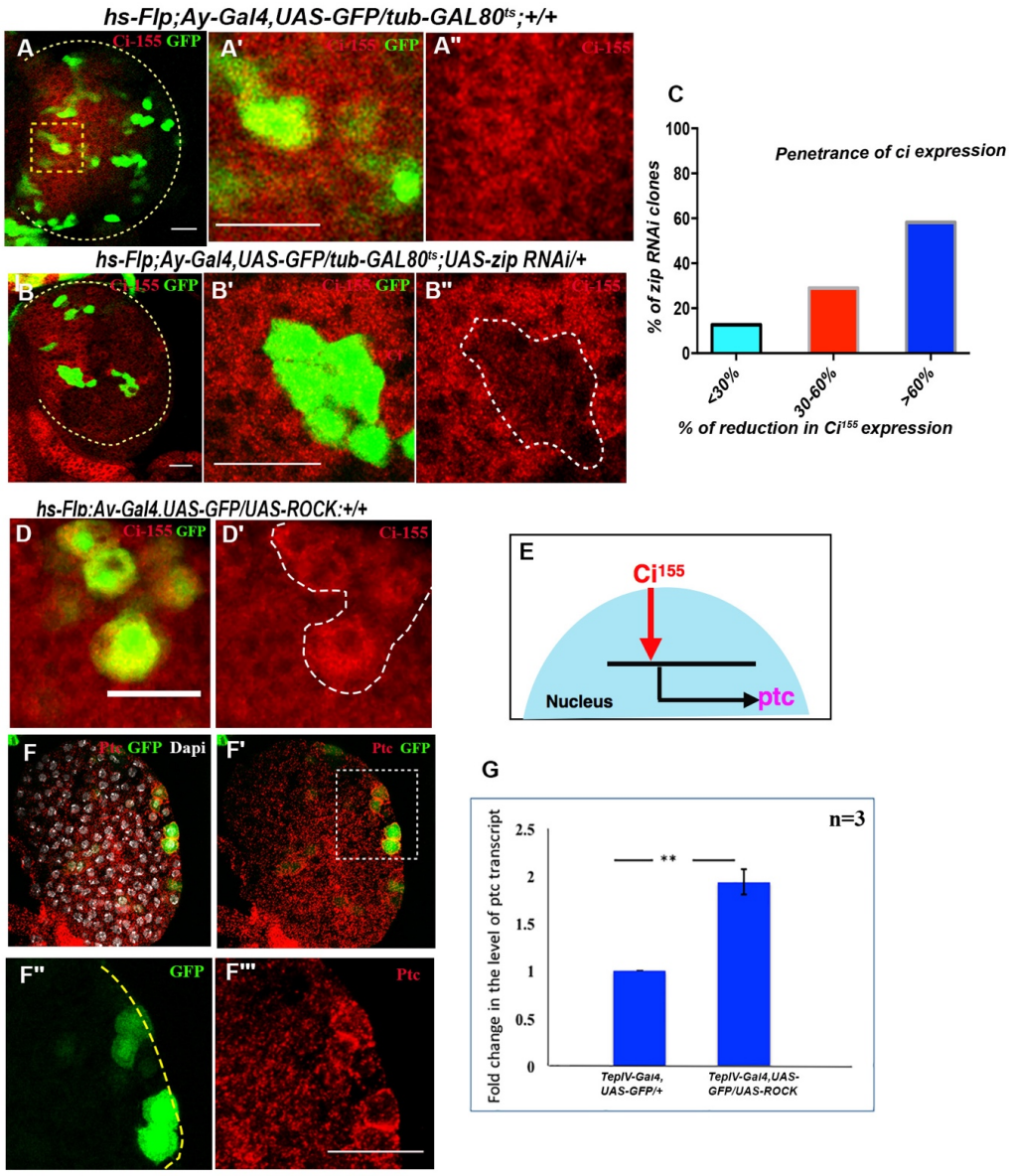

Figure 3.11 Actomyosin activity cell-autonomously regulates the Ci-155 activity.

(A-C) Ci-155 expression decreases in hsFLP; ay-GAL4/tub-GAL80ts; UAS-zip RNAi clones (green) (B-B") compared to mock clones (A-A") and neighbouring cells. (C) Penetrance of the phenotype (loss of Ci-155 expression) in hsFLP;ay-GAL4/tub- GAL80^{ts}; UAS zip RNAi clones. (D-D') hsFLP; ay-GAL4/UAS-ROCK clones (green) have up-regulated Ci-155(red) as well as up-regulated downstream target gene(E) Ptc expression (red: F-F'") expression compared to the neighboring wild-type cells. (F" and F'" are the higher magnification of F denoted by the box in F'). (G) A two-fold increase in ptc transcript level seen upon overexpression of ROCK in the progenitors. P-value: 6.832622X 10^{-3}

3.2.5 Actomyosin mediated Ci regulation through PKA independent mechanism-

Canonical Hh signaling regulates the cell-autonomous Ci-155 level by inhibiting PKA (protein kinase A) dependent phosphorylation of Ci-155. When Hh is available, it blocks PKA-dependent phosphorylation of Ci-155 and proteolytic processing of Ci-155 into the repressor form Ci-75 (Chen and Jiang, 2013; Ranieri et al., 2014) Whether the regulation of the Ci level through actomyosin also depends on canonical PKA-mediated regulation was next addressed. In ordered to investigate this possibility, a Ci variant (ci.HA) that lacks PKA phosphorylation sites (Chen et al., 1999) was co-expressed with zip RNAi in the progenitors. Interestingly, despite the constitutive expression of activated Ci under these conditions, the progenitors could not maintain a high Ci-155 level (Figure 3.12A-B' and with quantification in Figure3.12C). These data suggest that activation of the actomyosin network in the progenitors is essential for fine-tuning of Ci which is necessary for their maintenance (Figure3.12D). These data also show that the above regulation is independent of the canonical PKA activation.

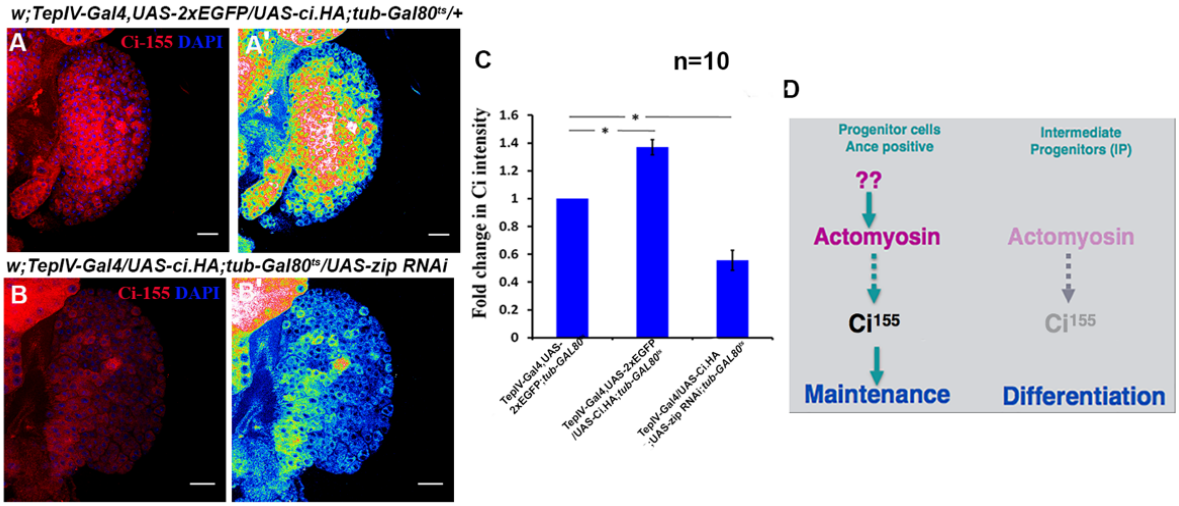

Figure 3.12 Actomyosin regulates Ci-155 *through PKA independent mechanism.(A-B) Overexpression of activated Ci is unable to maintain the Ci-155 level in zip loss from progenitors compared to control. (C) Quantitative analysis of Ci expression. P- value: TepIV-Gal4, UAS2xEGFP/UAS-ci.HA, tub-GAL80^{ts} = 5.512x^{-2}. TepIV-Gal4/UAS-ci.HA; tub-GAL80^{ts}/UAS-zipRNAi = 7.371x10^{-3}. (D) A scheme based on our findings.*

3.2.6 Actomyosin activity regulates the enrichment of Ci-155 in the progenitor cells of the lymph gland

It has been demonstrated that niche derived Hh regulates Ci-155 in the hematopoietic progenitor cells (Mandal et al., 2007). Hh expression in the niche starts after 18hr AEH (Dey et al., 2016).

Expression profiling of Hh in the lymph gland revealed that the number of Hh-expressing niche cells increased with larval developmental (visualized by hhF4: a transcriptional reporter of *hh*) (Figure 3.13A-E). However, the expression of Ci-155 in the developing lymph gland revealed an interesting observation. The increase in number of Hh-expressing niche cells (Figure 3.13A-E) correlates with an increased expression of Ci-155 (Figure 3.13A-B' and quantitative studies in Figure 3.13F) in progenitor cells till mid-third instar. Although, after mid-third instar, a further increment in the Hh-expressing cells occurs, it was not sufficient to increase the level of Ci-155 in the progenitor cells (Figure 3.13C-D'). Instead, a significant drop of Ci-155 expression beyond 72 hr AEH was seen. The above observation suggested that although Hh signaling's strength increases at the source, it does not reflect at the level of Ci-155 in the progenitors. It can hypothesize that there might be another developmental regulation on the level of Ci-155 expression in the hematopoietic progenitors that might be required for progenitor homeostasis.

Interestingly, analysis of Ci-155 expression with respect to Ance expression revealed that Ci-155 expression was restricted to Ance expressing progenitors (Figure 3.13G-I) with a sharp decline beyond that domain (IP cells, Figure 3.13G"). The quantitative analysis of Ci-155 in the lymph gland also validated the above observation (Figure 3.13H-I). Further, expression study revealed a spatial refinement of Ci-155 expression limited to the Sqh^{High} progenitors (Figure 3.13J-J") and decreased drastically in Ance- IP cells. Quantitative analysis also validated above observation that there was a sharp decline in the level of Ci-155 beyond Ance's positive domains, which is also low in sqh (Figure 3.13K). Down-regulation of Ci-155 in the IP cells suggests that a sharp decline in Ci level might allow progenitor differentiation (Figure 3.14A). Therefore, it can hypothesize that decreased cortical actomyosin in the IP cells might involve in a sharp decline in the Ci-155. To validate the above hypothesis, the sqh was hyperactivated via overexpression of ROCK in this population, and Ci-155 was analyzed. Indeed, Ci-155 levels were higher in the IP cells upon ROCK overexpression (Figure 3.14B-C') via Pxn-Gal4 (Pxn-Gal4, UAS-GFP/UAS-ROCK construct)(Figure 3.14C-C') compared to the low levels observed in the IP cells of the control lymph gland (Figure 3.14B-B'). However, lack of Ance labeling suggests that sustained activity of Ci-155 failed to revert these IP cells into the progenitor state (Figure 3.14C1").

Interestingly, sustained Ci activity in the differentiated and intermediate progenitors enhances the proliferation (EdU incorporation, (Figure 3.14E-F') and disturbs the normal lymph gland patterning. However, Ci-155 activation in IP cells does not affect cell-fate specification assayed by differentiation markers for plasmatocyte (P1) and crystal cell (Propo) (Figure 3.14G-L').

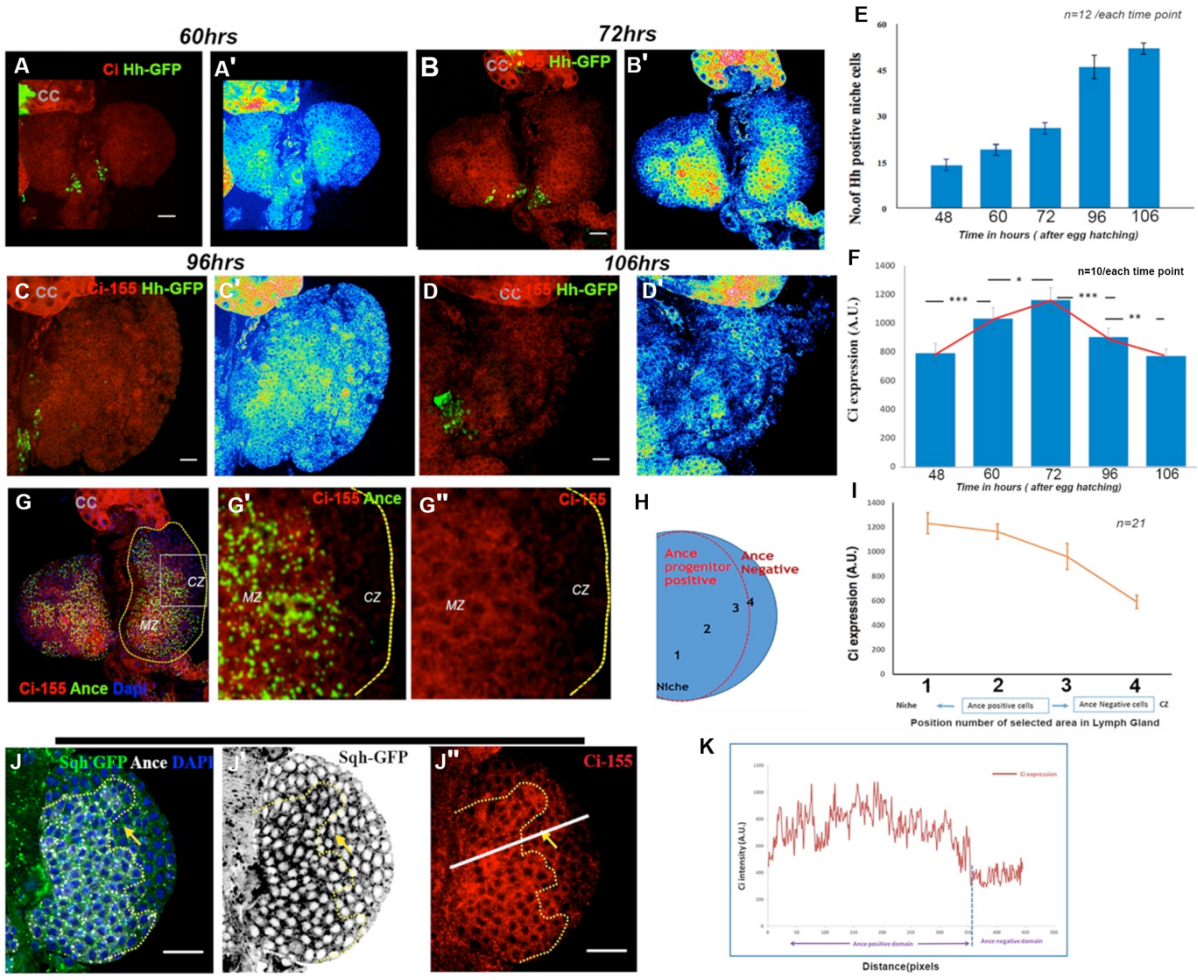

Figure 3.13 Developmental regulation of Ci-155 in the the ance+ progenitor cells. (A-E) Ci-155 (activated Ci) profiling in 48, 60, 72, 96, and 106-hour AEH. (F) The quantitative analysis of the Ci-155 in developing lymph gland. An increase in Ci-155 level occurs with the increment in niche number (E) until 72hrs AEH. Despite the increase in the number of Hh expressing cells post 72AEH (E), a decline in Ci-155 expression is evident in the progenitors (F). P value for 60hrs compared to 40hrs =$4.128x10^{-5}$, P value for 72hrs compared to 60hrs= $8.521x10^{-2}$, P value for 96hrs compared to 72hrs =$4.108x10^{-7}$ and P value for 106 hrs compared to 96 hrs= $6.019x10^{-3}$. Ci-155 expression (red) is limited to Ance (green) expressing progenitor cells. A drastic reduction in Ance or Ci-155 can be seen in the CZ. (J-J") Co-labelling of Sqh, Ance and Ci-155 reveal a drastic decline in the Ci-155 level in the IP cells (arrow, IP). (K) The intensity profile of Ci-155 in Ance positive and Ance negative progenitors along the line drawn in J" reflecting the stark decline in the level in later.

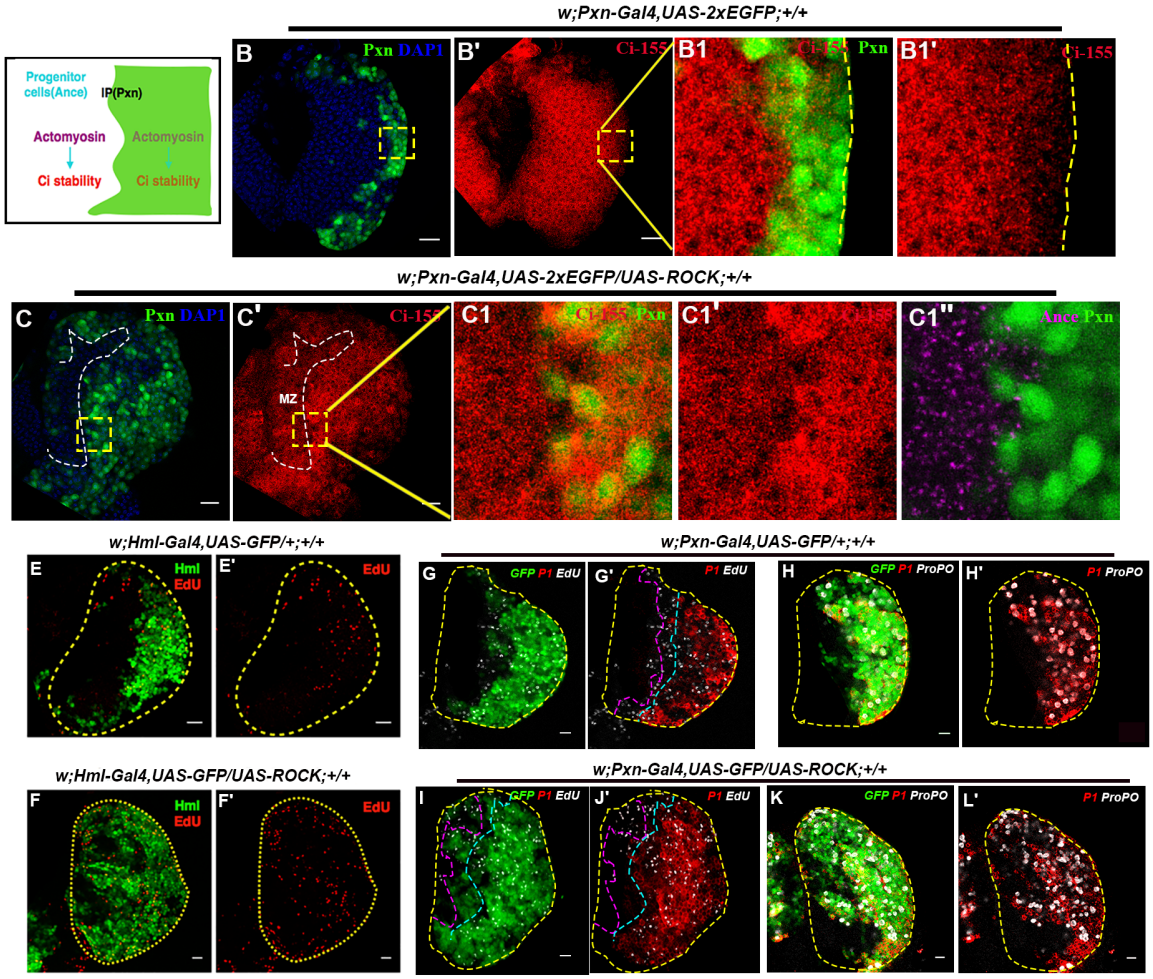

Figure 3.14 ***R*egulation of actomyosin mediated Ci-155 in progenitor cells essential for LG homeostasis** *(A) A scheme based on our hypothesis. (B-C1') Sustained activation of sqh in IP stabilizes Ci-155 (C-C1') compared to control (B-B1'). (C1") This sustained Ci-155 expression in IP cells does not revert them to the progenitor state (evidenced by lack of Ance: purple). (B1-B1') are the higher magnification of an ROI selected from B' and (C1-C1') are the higher magnification of regions selected from C'. (E-L') Overexpression of ROCK leads to increase proliferation (EdU) (E-F') of IP and disturbed zonation in the lymph gland without affecting terminal differentiation (G-L') (assayed by P1: Plasmatocytes and ProPO: Crystal Cells).*

Collectively, these data demonstrated that spatial and temporal orchestration of Ci-155 level in the lymph gland is essential for lymph gland homeostasis. This Ci-155 level relies on the actomyosin activity in the lymph gland. The increase in actomyosin activity in the Ance positive progenitor cells is associated with a higher Ci-155 level. Next, an attempt was made to understand the regulation of actomyosin assembly in Ance positive progenitor cells.

3.2.7 DE-cadherin mediated cell-cell adhesion regulates enrichment of actomyosin components in progenitor cells.

Next, it was addressed how actomyosin is enriched in the Ance positive progenitor cells. Studies have elucidated that various factors control cortical actomyosin assembly, including cell-cell adhesion (Millan et al., 2010) (Hunter et al., 2015; Jurado et al., 2016). Interestingly, hematopoietic progenitors in the lymph gland express high cell-cell adhesion molecules, DE-cadherin (Jung et al., 2005)), which is shown to be involved in progenitor maintenance (Gao et al., 2013). However, how this DE-cadherin regulates progenitor cell maintenance is not very well elucidated. In ordered to understand the link between high DE-cadherin mediated cell adhesion and actomyosin activity in *Drosophila* hematopoietic progenitor cells, the spatial and temporal expression of DE-cadherin/Shotgun (Shg) was analyzed in Ance expressing progenitors. Interestingly, the expression study revealed that Ance-positive progenitors are enriched with DE-cadherin/Shg (**Figure 3.15A–B"'** with the quantification in **Figure 3.15**D**),** while Ance-Pxn+ IP cells down-regulate shg expression. It is evident from the expression studies that the inner core of Dome-GFP expressing progenitor cell population is enriched with Shg while peripheral D*ome-GFP* expressing progenitors downregulated its expression. (Indicated by the arrow in **Figure 3.15C-C')**. The above expression pattern suggests that Shg is highly enriched in the progenitor cells while downregulated in IP cells, which might be necessary for differentiation.

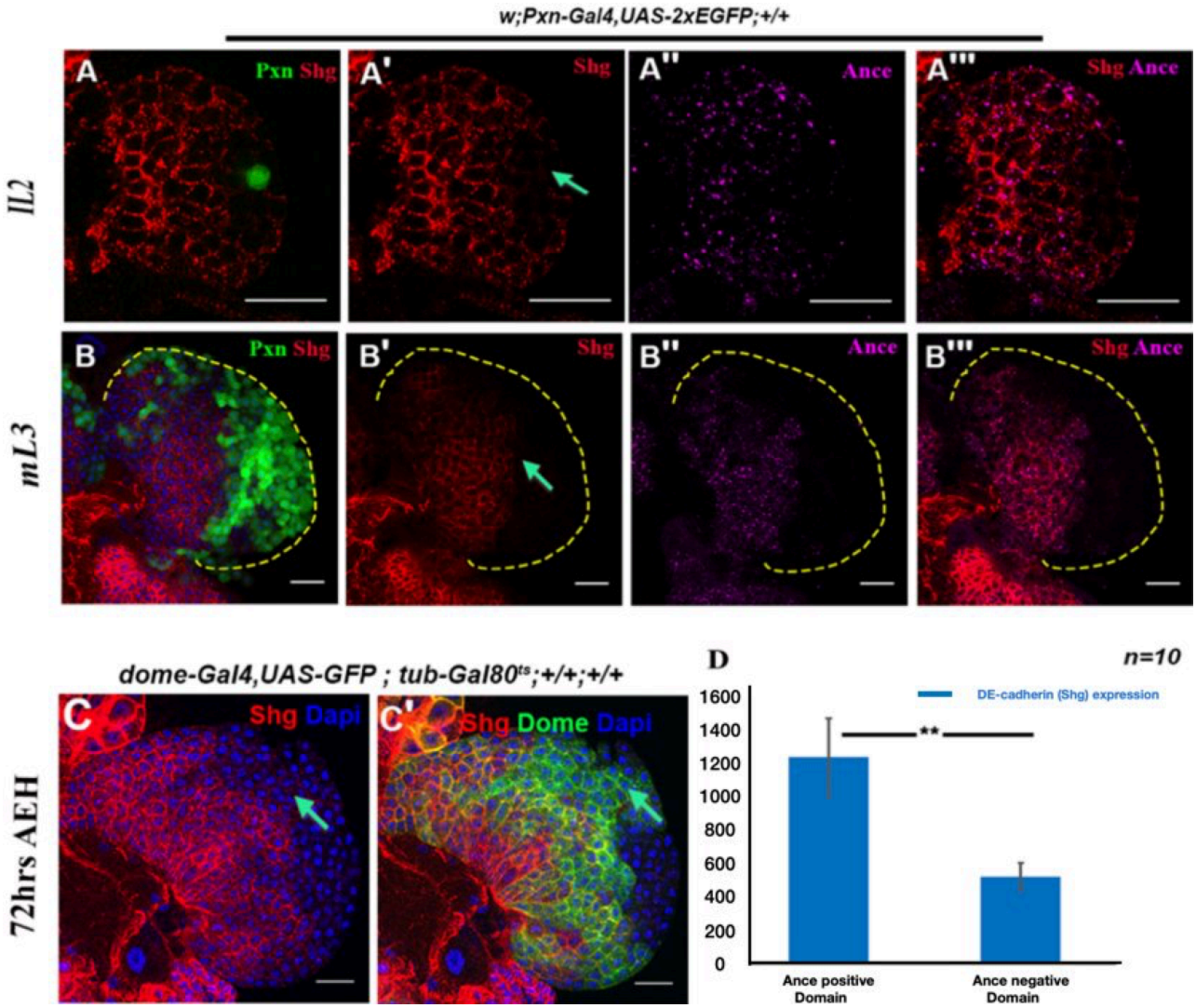

Figure 3.15 DE-cadherin (Shg) enriched in ance+ progenitor cells. The genotypes are mentioned on the top of the relevant panels. (A-B") Ance positive progenitor cells (magenta) are highly rich in DE-cadherin. Throughout development DE-cadherin expression (arrow in A' and B') is down-regulated in the Intermediate Progenitor (visualized by Pxn GFP). In A-A", the age of the larvae is late second instar (lL2) whereas B-B" it is mid third instar (mL3). (C-C) The inner core of Dome (green) expressing progenitors are rich in Shg (red). Dome positive progenitors in the periphery of the MZ have down-regulated Shg expression (green arrow in C'). (B) Quantitative data are illustrating the DE-cadherin/Shg expression level in Ance positive and Ance negative progenitors. P value: 2.187×10^{-3}. All images analyzed are from 72 hrs AEH. (C-D') Validation of both Shg antibody expression and the UAS-shgRNAi construct in the lymph gland. Error Bars: SD

To reveal the role of shg mediated cell-cell adhesion in quiescent progenitor cells, down-regulation of Shg from the progenitor cells was performed in the same timeline used for actomyosin studies. Interestingly, upon down-regulation of Shg from the progenitor cells (Figure 3.16A-B'), the progenitor number drastically decreased while the number of differentiated cells increases (Figure 3.16C-D). Quantification revealed a 30% increase in the differentiation index (Figure 3.16D') of the above genotype. Further, the Ci-155 level in the progenitor cells was significantly reduced upon shg down-regulation (Figure 3.16E-F') compared to the control lymph glands. This mimic the phenotypes seen with the loss of actomyosin components.

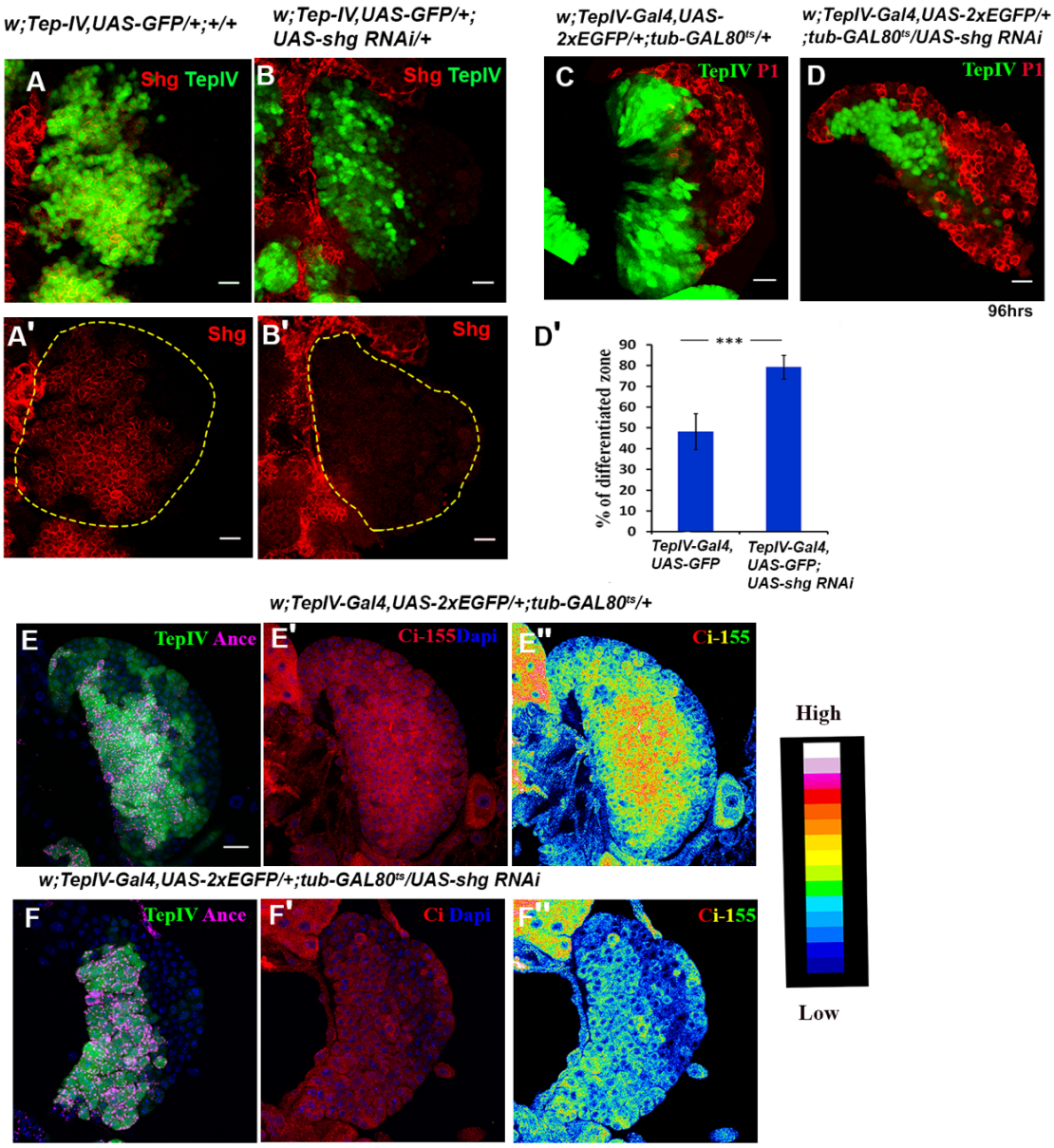

Figure 3.16 DE-cadherin regulates Ci-155 activity essential for hematopoietic progenitor cell maintenance. (A-B) Validation of both Shg antibody expression and the UAS-shgRNAi construct in the lymph gland.(C-D) Down-regulation of Shg function in the progenitors affects their numbers compared to control. Progenitors marked by TepIVGFP (green) and differentiated cells by P1 (red). (D') Quantitative analyses of the above result reveal 30% increment in differentiation index. P-value=3.126×10^{-7}. (E-F") Compared to Control (E-E") Ci-155 level is reduced in the progenitors upon loss of Shg (F-F"). Progenitors are visualized by TepIV-Gal4, 2xEGFP (green) and Ci-155 in red.

The Cadherins mediated cell-cell adhesion involved in tethering of the actin cytoskeleton to the plasma membrane which recruits non-muscle Myosin II and forms a mechanically active actomyosin network (Chen et al., 2015; Jodoin et al., 2015; Kasza and Zallen, 2011; Nelson and Weis, 2016). Next, it analyzed whether the loss of shg from progenitors affects the actin cytoskeleton (visualized by the F-actin expression: Figure 3.17A-B'). Upon knockdown of shg in the progenitor cells, F-actin expression drastically altered in the progenitors. Higher magnification

images also showed that F-actin organization near the plasma membrane drastically affected upon Shg downregulation (Figure 3.17A1–A2' and B1–B2'). Quantitation analysis of the cortical F-actin level (assayed by drawing a line along the plasma membranes of progenitors) (Cartagena-Rivera et al., 2016) also revealed that Shg downregulation in the hematopoietic progenitors affects the level of F-actin (Figure 3.17C Phal).

The above results suggest that both DE-cadherin mediated cell-cell adhesion and actomyosin network regulates progenitor maintenance signal Ci-155 (Figure 3.17D).

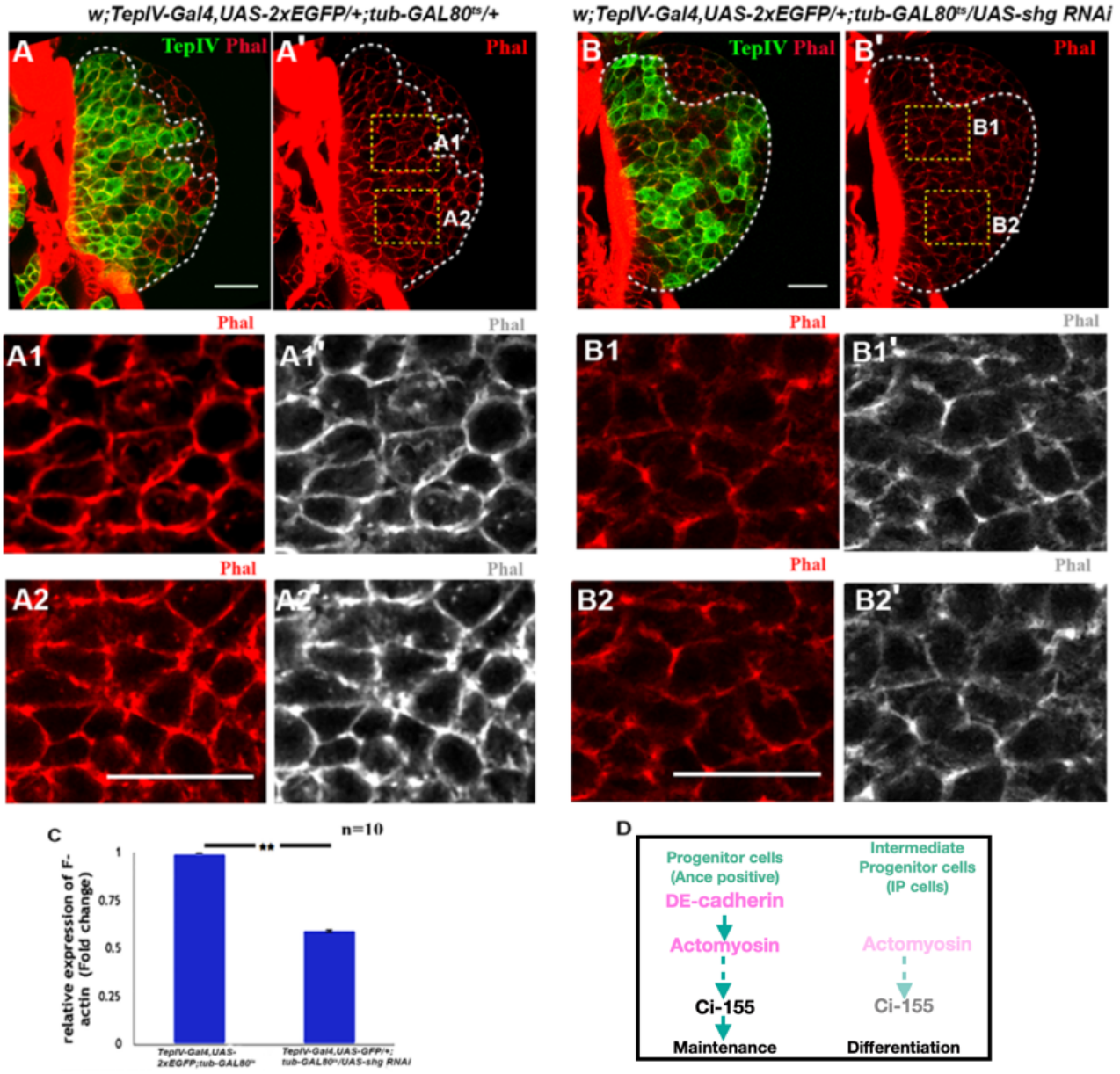

Figure 3.17 Loss of DE-cadherin affect F-actin organization in lymph gland. (A-B2') F-actin assembly in progenitors is compromised upon down-regulation of DE-cadherin (shg) when compared to control **(**compare **A-A2'** with **B-B2').** Progenitors are marked by TepIV-GFP (green) and Phal (red and gray). A1 and A2 are areas selected for higher magnification A' while B1 and B2 are selected area of B' for higher magnification. **(C)** Quantitative analyses of the above result show a noticeable decline in F actin expression level. P value=8.319 x10^{-3}. (D) A scheme based on our above finding linking shg genetically to Ci-155

expression via actomyosin and bringing about progenitor maintenance in MZ whereas down-regulation of the entire network facilitates progenitor differentiation. All Figures lymph gland are from 72 hr AEH. Error Bars=S.D

3.2.8 Ance is a genetic modifier of Shg-actomyosin-Ci network essential for progenitor maintenance.

Apart from actomyosin components and Shg, the progenitor population is also enriched with *Ance* throughout development. Interestingly, studies in the mammalian endothelial cell (Kohlstedt et al., 2006)have found a strong association of actomyosin (MYH9: myosin heavy chain) with Angiotensin-Converting Enzyme (ACE), which is essential for activation of MYH9. Therefore, it can be hypothesized that Ance might genetically interact with actomyosin in the lymph gland, and this association is necessary for the maintenance of progenitor.

ACE (Angiotensin Converting Enzyme) is a component of a renin-angiotensin-aldosterone system (RAAS), a dedicated signaling pathway responsible for regulating blood pressure. ACE also expressed in the vertebrate hematopoietic stem and progenitor cells (Bernstein et al., 2013). Studies done to date in vertebrate have teased out the requirement of ACE in development and the other components of RAAS (Jokubaitis et al., 2008; Rousseau-Plasse et al., 1998; Sinka et al., 2012). However, it has been proposed that ACE can function beyond its complex collaboration with the RAAS (Kohlstedt et al., 2006). Thus, the blood-specific role of ACE beyond RAAS in hematopoiesis is yet to be elucidated. Since *Drosophila* has Ance despite having no RAAS mediated blood pressure system, the *Drosophila* lymph gland can be used as a model system to the elicited blood-specific role for ACE.

Ance mutant (heteroallele: *Ance34Eb2*/ Df (2L) b88f32) (Hurst et al., 2003) shows a sharp increase in the differentiated cells (P1 marker for the plasmatocytes, (Figure 3.18A-B) and a concomitant decrease in progenitors, compared to the control lymph gland. Quantification of the differentiation index reveals a 30% increase in (Figure 3.18C) in differentiation. To confirm the above results, RNAi-mediated down-regulation of *Ance* was performed in the progenitor cells at the same time frame used in the Shg and actomyosin experiments (Figure 3.9A). Such down-regulation of Ance in progenitor cells resulted in a drastic decline in the number of progenitors (visualized by *GFP*) and increased differentiation (P1: red, Propo: magenta marking the crystal cells), mimicking both *shg* and actomyosin loss phenotype (Figure 3.18D-E'). Quantification of the above result reveals a 32% increase in the differentiation index (Figure 3.18F).

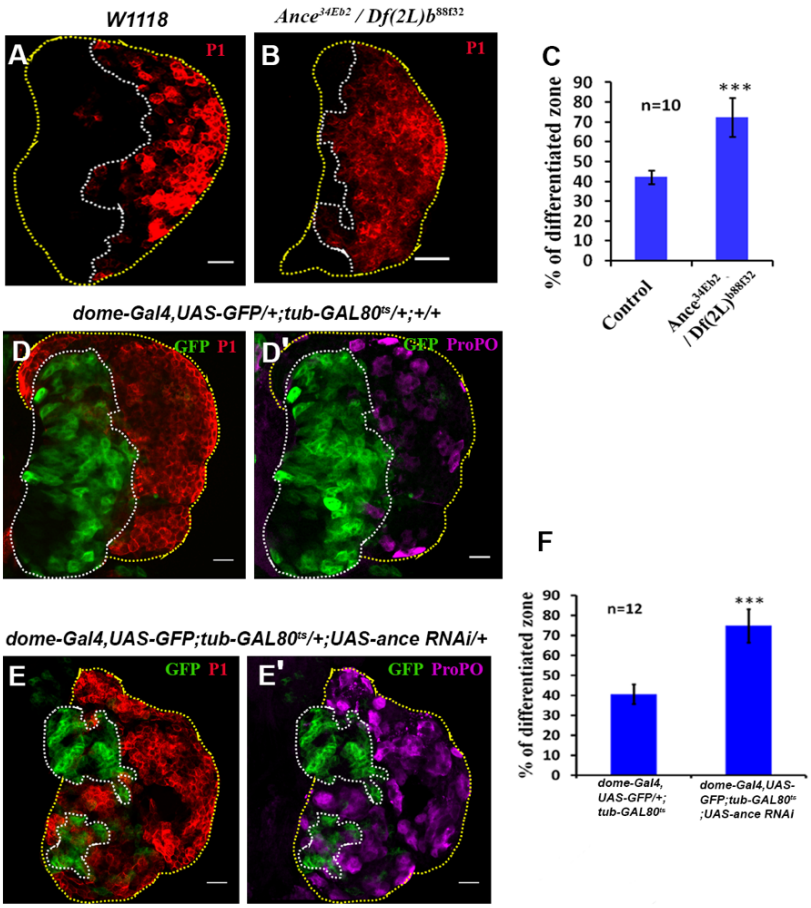

Figure 3.18 Ance regulates maintenance of Hematopoietic progenitor cells. **(A-C)** Hetero-allelic combination of Ance34Eb2/Df(2L)b^{88f32} **(B)** shows a significant increase in differentiation when compared to the wild-type **(A)**. **(C)** Quantitative analysis of differentiating cells in wild type and Ance34Eb2/ Df(2L)b^{88f32} (P=1.58x10-6). **(D-F)** RNAi mediated downregulation of Ance **(E)** using Dome-Gal4 in quiescent progenitor leads to increase in plasmatocytes (in red) compared with control **(D)**. Crystal cells also increase upon Ance downregulation (**E'** compare with **D'**, control). **(F)** Quantitative analysis of differentiating cells in control and upon Ance down-regulation by RNAi (P-value =4.22x10-10).

Next, Ci-155 expression was assayed in the Ance knockdown lymph gland at 72h AEH as a complete absence of Dome*GFP* expression due to ectopic differentiation occurs by 96h AEH upon Ance loss. At this time (72h AEH), progenitors could still be seen with ectopic differentiation due to Ance down-regulation. The level of Ci-155 was significantly reduced in this genotype (Figure 3.19B-B') compared to the control lymph gland (Figure 3.19A-A'). It was next asked whether overexpressing ci.HA in progenitors carrying the *TepIV-Gal4*; *tub-GAL80ts/UAS-Ance RNAi* construct might rescue this phenotype. Indeed, this genetic manipulation resulted in a robust increment in the domain of Ci-155 expression (Figure 3.19D-E'), which increases the progenitor's cell maintenance. However, overexpressing ci.HA in progenitors carrying the *TepIV-Gal4*; *tub-GAL80ts/UAS-Ance RNAi* construct, a drastic decline in Ci-155 expression (Figure 3.19F-F') with a reduction in progenitor number and increase in differentiation was observed (**Figure 3.19C-C').**

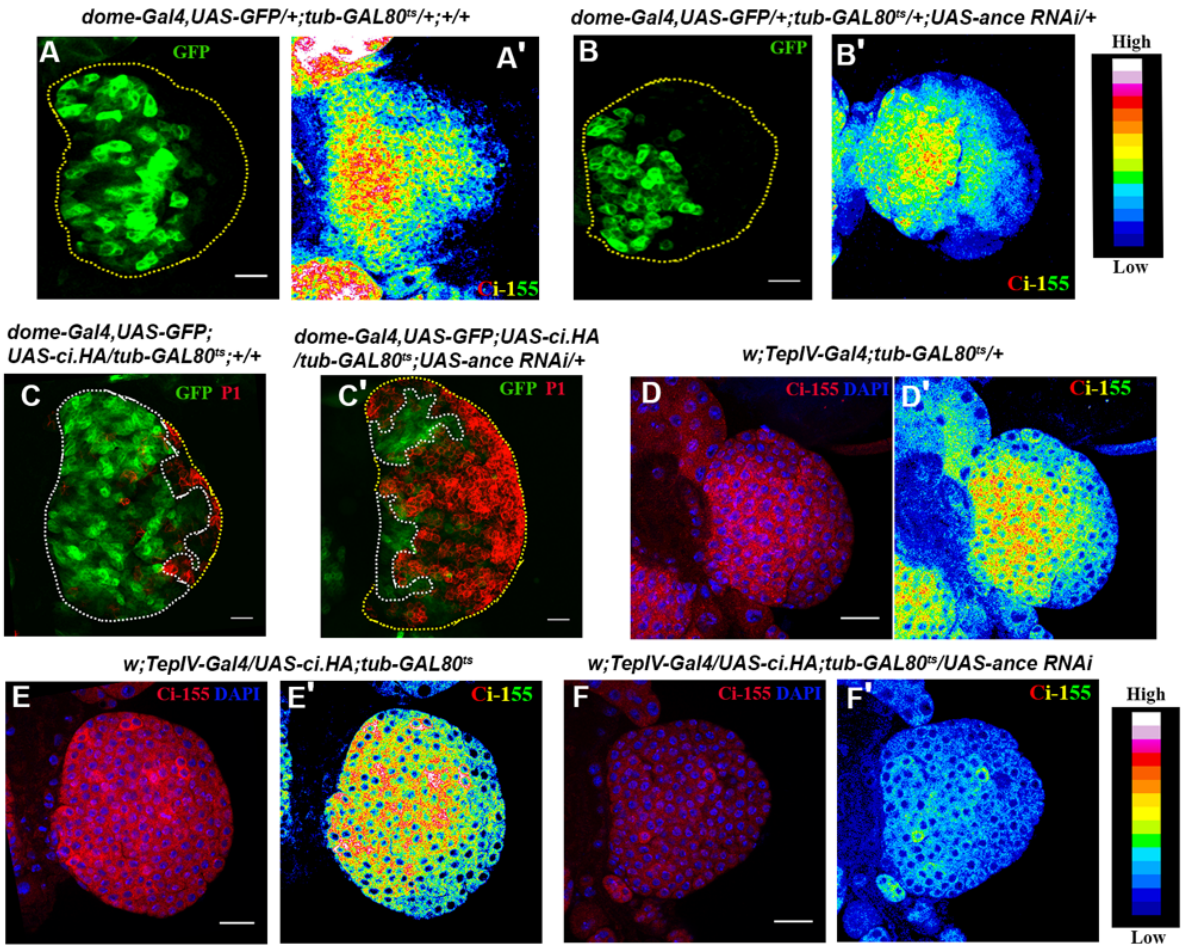

Figure 3.19 Ance regulates Ci-155 through PKA independent mechanism in hematopoietic progenitor cells. (A-B') Upon Ance down-regulation from the progenitors, the *C*i-155 expression is significantly reduced even at 72 hrs AEH compared to control. Please note: Since at 96hrs the entire lymph gland is differentiated upon Ance loss, 72 AEH larvae were where few progenitors can still be detected.(C-C') Compared to control, overexpression of Ci-HA in the progenitor (I) post- hatching, led to their increment in number (*dome*-GFP), (C') shows that simultaneous removal of Ance Function overrides Ci -HA activity.(D-F') Ci-HA overexpression in progenitors results in robust up-regulation of Ci^{155} (E-E') when compared to control (D-D'). However, when Ance is down-regulated in the progenitors of the above genotype, they are unable to sustain high Ci-155 level (F-F').

The above genetic and expression data indicate that Ance and the Shg-actomyosin network might interact to ensure hemocyte progenitor homeostasis.

To understand the link between ance, actomyosin, and shg, the expression of shg was analyzed in knockdown of *Ance* condition. The accumulation of Shg on the progenitors' plasma membrane was drastically decreased upon loss of Ance (**Figure 3.20A-B'** and quantitative estimation **Figure 3.20C)**. Interestingly, sqh-GFP expression was also reduced after Ance knockdown in the progenitors as visualized by Sqh-GFP expression (**Figure 3.20D-E'** and quantitative estimation in **Figure 3.20E'')**. Moreover, a decline in the F-actin level was also observed upon hematopoietic progenitor specific down-regulation of Ance. (**Figure 3.20F-H'**).

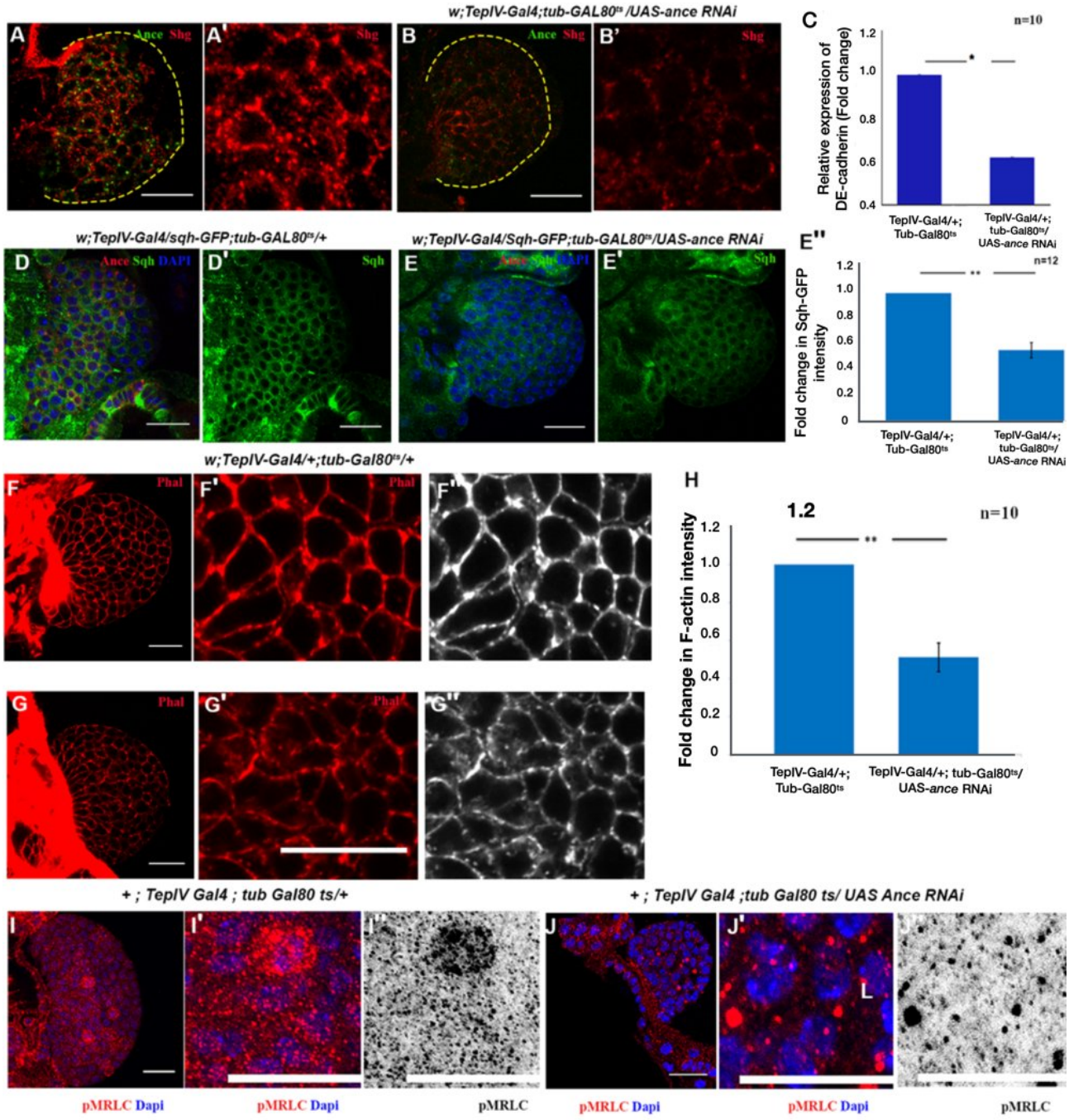

Figure 3.20 Loss of Ance affect DE-cadherin (Shg) and actomyosin in hematopoietic progenitor cells. (**A-B'**) Shg expression (red) is drastically compromised in the plasma membrane of Ance knockdown progenitors compared to control. **(C)** Quantitative analyses of the above result validate the down-regulation of Shg upon loss of Ance function. P value = 9.43×10^{-3}. **(D-G'')** Reduction in sqh GFP expression **(D-E')** and cortical F-actin level **(F-G'')** are evident upon Ance downregulation from the progenitor cells. **(H)** Quantitative analyses of the F-actin level upon Ance loss, P- value=7.781×10^{-3}.

Next, the pMRLC antibody was used to investigate the sqh phosphorylation status in progenitors with impaired Ance function. A drastic reduction in the level of pMRLC expression was observed upon RNAi-mediated downregulation of Ance (**Figure 3.20I-J''**) compare to wild type progenitor. Interestingly, sustained activation of sqh via over-expression of ROCK was able to rescue the Ci-

155 expression in the progenitors that lacked Ance (**Figure 3.21A-B'**). Quantitative analysis of the intensity profile of the Ci-155 level in the above experiment was indeed comparable with control (**Figure 3.20C).** This overexpression of ROCK was also able to rescue the maintenance defect seen upon Ance loss (**Figure 3.21D-E**).

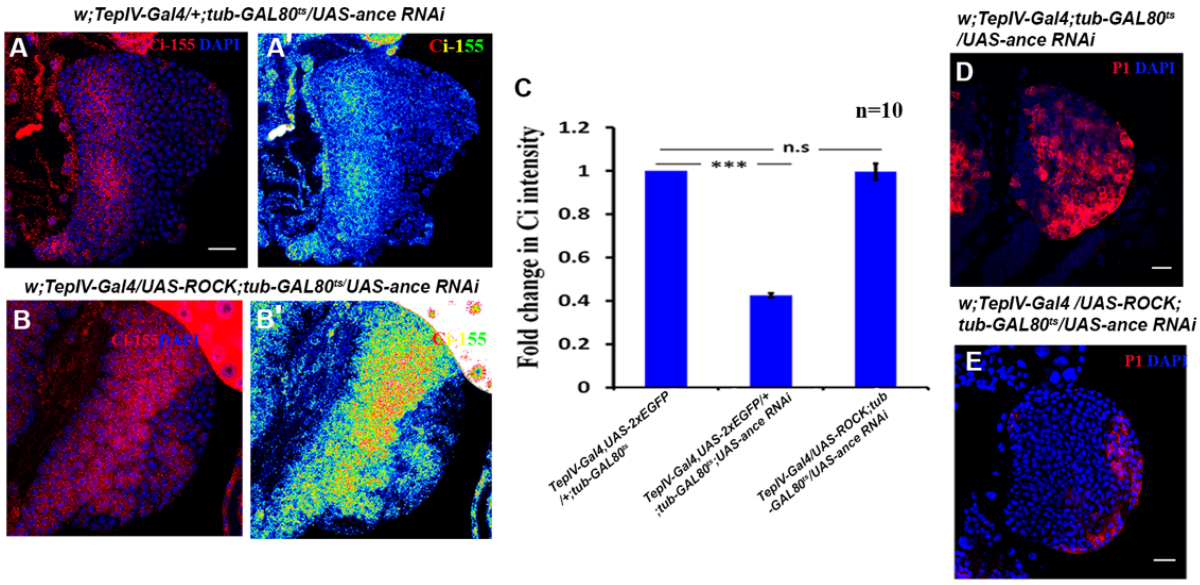

Figure 3.21 Ance genetically interacts with actomyosin.(A-B')Loss of Ance from progenitors affects Ci-155 level. However, the expression of UAS-ROCK in this genotype can rescue the Ci-155 expression **(D-F)** and thereby the maintenance defect seen upon loss of Ance**. (C)** Quantitative analyses, P value for TepIV-Gal4; tubgal80^{ts}; UAS-AnceRNAi=6.35x10^{-5} and P value for TepIV-Gal4; tubgal80^{ts}/UAS-ROCK; UAS-AnceRNAi is 9.003x10^{-1}.

Using these results in conjunction with the above findings that both down-regulation of Shg and actomyosin components show phenotypes identical to Ance loss from progenitors, it can be rationalized that Ance, Shg, and actomyosin are genetically interacting to bring about hemocyte progenitor maintenance. The above results suggest a strong link between the Ance function and increased actomyosin activity in hemocyte progenitors, reflecting a similar relationship to that reported in endothelial cells (Kohlstedt et al., 2006).

3.2.9 Niche derived Hh does not regulate cell adhesion or actomyosin assembly in hematopoietic progenitor cells.

Studies on mammalian and *Drosophila* tissues have demonstrated that Hh signaling can also regulate cell adhesion and actomyosin contractility both positively (Jarov et al., 2003; Schlichting et al., 2005) as well as negatively (Lai et al., 2017). In zebrafish, a positive feedback loop between cell-cell contact duration and Nodal signaling is essential for meso-endodermal fate segregation (Barone et al., 2017). Based on the above findings, it can hypothesize that a feedback loop between Hh signaling and actomyosin contractility might be operating in LG to stabilize the pattern formation. Thus, Hh signaling can employ a second axis through cell adhesion and actomyosin to fine-tune Ci protein's activity. Although the loss of Hh from the niche **(Figure 3.22 B-B')** or Smo from progenitors **(Figure 3.22 C-C') affects** the cell number, the level of Shg expression in the residual progenitors was comparable to control **(Figure 3.22 A-A'** and **Figure 3.22 E)**.

Moreover, on enhancing Hh signaling in the progenitor cells via overexpression of *smo,* resulted in the increment in progenitor cells number, yet the level of Shg expression is analogous to wild-type **(Figure 3.22 D-D'** and **(Figure 3.22E)**.

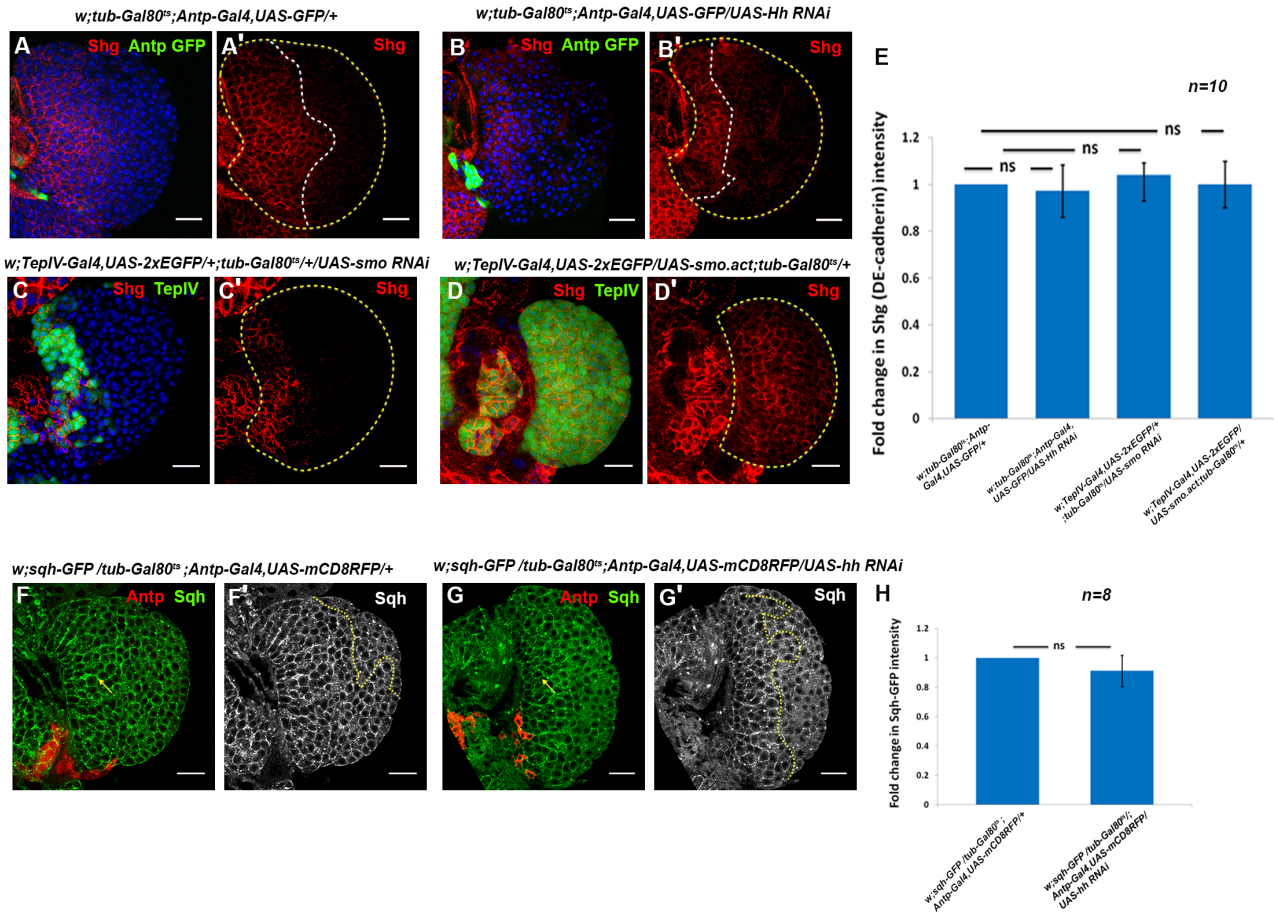

Figure 3.22 Hh signaling does not regulates DE-cadherin (Shg) and actomyosin in hematopoietic progenitor cells. (A-B') Although the Shg domain (progenitor cells) is drastically affected, the expression of Shg expression is not affected upon Hh downregulation from the niche **(C-D')** donw-regulation of smo in

the progenitors affect their number, but the expression of Shg remains comparable to wild type. **(D-D')** Overexpression of smo in the progenitors enhances their number, but the expression Shg expression is simmilar to wild type progenitor cells. **(E)** Quantitative analyses of the above results P values: (tubgal80ts, AntpGal4-UASGFP> UAS-HhRNAi): $0.71x10^{-1}$; TepIV-Gal4;tubgal80ts>UAS-SmoRNAi: $6.82\ x10^{-1}$; (*Te*pIV-Gal4;tubgal80ts> UAS-Smo.act): $9.79\ x10^{-1}$ **(F-G')** The loss of Hh signaling from the niche affects the progenitor number, but does not affect the expression of sqh expression in the residual progenitors. **(H)** Quantitative analyses of the above results. P-value: $2.838x10^{-1}$. The yellow dotted line mark whole of the lymph gland in A', B',C' and D' while white dotted line marks the progenitor domain in A' and B'. However, yellow dotted line mark progenitor domain in F' and G'. Age of the larvae=72hr AEH.

Thus, Hh signaling does not affect Shg levels in the progenitor cells. Next, it was assayed whether the Hh signaling can modulate the actomyosin assembly in the progenitor cells by assaying sqh GFP expression. Upon loss of the Hh from the niche, the expression of sqh-GFP was also comparable to wild-type **(Figure 3.22F-G'** and **(Figure 3.22H).** From these results, we can infer that Hh signaling does not regulate cell-cell adhesion nor actomyosin assembly in hematopoietic progenitor cells.

3.3 Discussion

Understanding the precursor's cells' ability to differentiate into various types of cells is crucial to unravel the underline mechanism essential to maintain tissue homeostasis. This question in tissue biology is more complicated when dealing with the heterogeneous cell population. It is widely known that progenitor cells in the tissue are not homogeneous, and they can be categorized based on their commitment to differentiation (Banerjee et al., 2019; Jung et al., 2005).

The current study found that *Drosophila* hematopoietic progenitor cells are also heterogeneous and can be categorized into different subpopulations based on different molecular markers. Interestingly, LG consist of a well-defined pattern of different cell types, including various types of precursor cells and differentiated cells, which occupies definite position within this organ. The tightly packed Ance+ progenitor cells found in the core of the LG also express high levels of the maintenance factor Ci-155. It was previously known that the high levels of Ci-155 is sustained by the Hh signaling from the niche and ADGF signaling from the differentiated cells (Mandal et al., 2007; Mondal et al., 2011). However, the sharp gradient observed within the progenitors and a drastic fall in the differentiated cells cannot be accounted for only to these two inputs. Thus, it needs to resolve how this noisy morphogen gradient of Ci-155 is transformed into its progenitor specific enrichment in the developing lymph gland.

Cell–cell adhesion and actomyosin activity is essential for lymph gland homeostasis

Interestingly, Ance's positive progenitor cells have high actomyosin, and DE-cadherin mediated cell-cell adhesion, which drastically decreases in the Ance- cell at the periphery (IP cells). The loss of cell-cell adhesion in the Ance- cells leads to increase granularity as well as spacing, which might be essential to switch on the differentiation program (Banerjee et al., 2019) .

This study reveals that the DE-cadherin function is linked to actomyosin activity in the Ance progenitors since its down-regulation alone affects actomyosin assembly and thereby the progenitor maintenance. Herein, cell-cell adhesion and actomyosin together act as a sensor in progenitor identity, ensuring their homeostasis**.** The current study also found that the actomyosin network regulates the morphogen-dependent patterning in the developing lymph gland progenitors in *Drosophila*. In developing the lymph gland, the cooperation between actomyosin and cell-cell adhesion regulates the hierarchy in the hematopoietic progenitor cells at different stages of maturation. Like the vertebrate neural tube patterning where cadherin 7 increase the SHH signaling (Kawano et al., 2017), in *Drosophila* hematopoietic progenitor cells also, cell adhesion or the activity of the actomyosin network might c regulate the activation of Hh signaling in the progenitors of the lymph glan

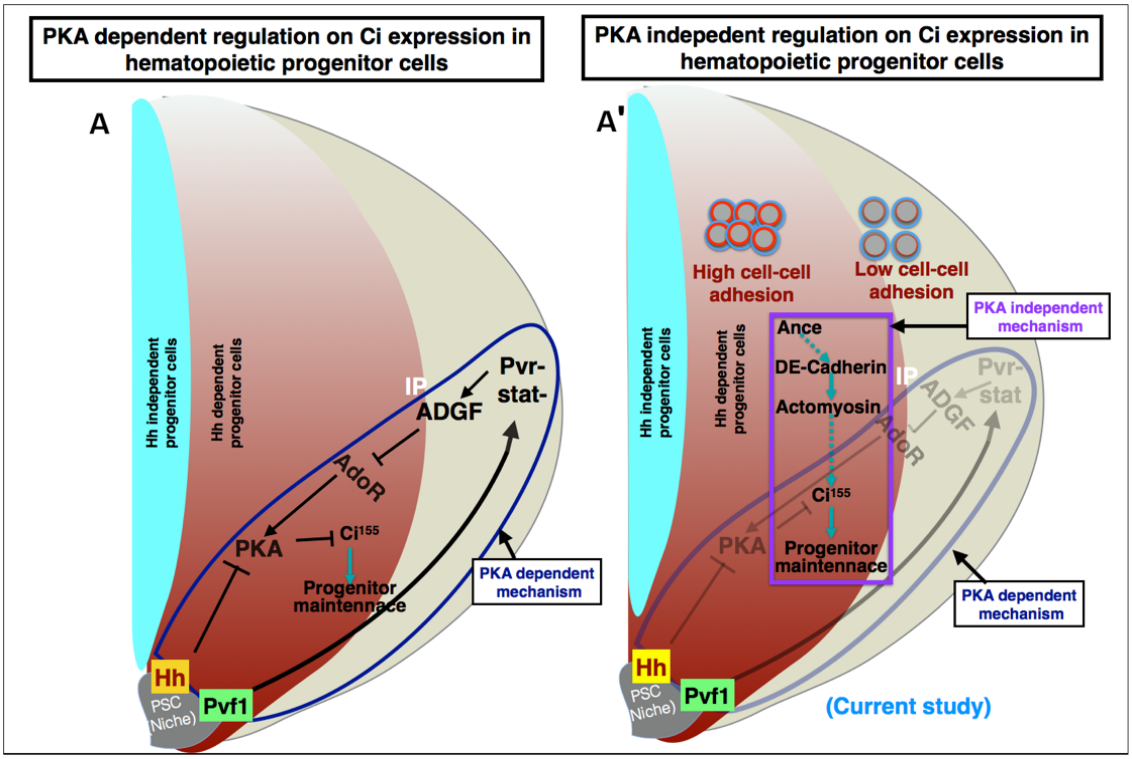

Figure 3.22 A Scheme based on the above findings:(A) Based on the recent results, the MZ of the late larval lymph gland consists of two distinct domains. While one domain (cayenne) is sensitive to Hh signals received from the PSC/niche, the other domain (cyan), which is juxtaposed on the dorsal vessel is Hh-insensitive. Within the Hh sensitive domain, two distinct pathways primarily regulate the maintenance of Ci expression in the MZ. Hh and ADGF signaling regulate the Ci (Ci-155) expression in a PKA-dependent manner. (B) Here, this study demonstrates the third axis of Ci regulation, which is independent of PKA coupled with cell adhesion and actomyosin activity essential for progenitor homeostasis. It is also found that Ance is a genetic regulator of this pathway described. Dotted arrows indicate mechanisms yet to be understood (Sharma et al., 2019)

This study also suggests that compartment formation between Ance+ progenitor and Ance- IP cells due to high cell-cell adhesion and high actomyosin activity (higher tension) is essential for lymph gland homeostasis.

Interestingly, higher actomyosin and cell-cell contacts also associated with tissue patterning in many systems suggesting that higher actomyosin and high cell-cell contact is a hallmark of many compartmental and tissue boundaries formation. The formation of a distinct pattern during development necessitates cells to recognize their position within the tissue and then differentiate accordingly. The tissue patterning is primarily based on the positional information and reaction-diffusion models (Green and Sharpe, 2015; Wolpert, 2016). However, cytoskeletal forces, cell adhesion, polarity, and tissue-scale mechanical signals have emerged as valuable player required for tissue patterning(Chan et al., 2017; Heller and Fuchs, 2015; Meilhac et al., 2009; Xiong et al., 2014). These factors, along with various signaling pathways, coordinate the underline gene expression essential for tissue patterning.

PKA independent regulation of Ci activity in hematopoietic progenitor essential for their maintenance and pattering of the lymph gland

It has been demonstrated that MZ of the late larval lymph gland is made of two distinct domains. While one domain present near the dorsal vessel is insensitive to Hh, the other domain is sensitive to Hh signaling (Baldeosingh et al., 2018; Banerjee et al., 2019). In the Hh-sensitive domain, Hh signaling from the niche and ADGF signaling from the differentiated cells regulates Ci-155 in a PKA-dependent manner (Figure 8A).

This study elucidates the third axis of Ci regulation is through cell adhesion, and high actomyosin, and is independent of PKA mediated Ci-155 regulation. The cell-cell adhesion regulates the assembly of cortical actomyosin in progenitor cells, modulating Ci's activity, thereby maintaining the progenitor status. An elegant study(Ruel et al., 2003) has revealed that the Cos2-Fu-Ci complex needs to be translocated to the plasma membrane, where the activation of Ci takes place through Hh signaling (Ruel et al., 2003). Based on these results, it can be proposed that this complex is supported by actomyosin meshwork near the plasma membrane facilitating the activation/stabilization of Ci. Altogether, it can hypothesize that the downregulation of cell-cell adhesion and actomyosin is required to de-sensitize progenitor cells to Hh, which is essential for their differentiation (**Figure 3.22 A-A').**

Angiotensin-converting enzyme is a genetic modifier for Shg-actomyosin mediated Ci regulation

The involvement of Cell-cell adhesion and actomyosin has previously been reported in vital cellular processes, including morphogenesis, proliferation, differentiation, and collective cell migration in Drosophila, C.elegans and mammalian system (Mammoto and Ingber, 2010; Rauskolb et al., 2014). Various *in vitro* studies in the mammalian system has implicated the diverse role of actomyosin derived biophysical forces in Hematopoietic stem cells- progenitor expansion, maintenance, and homing and differentiation, survival, and lineage specification (Adamo et al., 2009) as well as the expansion of adult HSCs and progenitors (Shin et al., 2014). It also has been shown that high levels of actomyosin contractility essential for tumor progression, suggesting actomyosin as a crucial regulator for the maintenance of tissue homeostasis. Although it is clear that actomyosin dynamics is the critical regulators for the maintenance of tissue homeostasis, how spatiotemporal regulation of actomyosin is maintained during different cellular contexts is still not very well documented.

Ance as a genetic modifier for the Shg-actomyosin-Ci network revealed. Although how Ance mechanistically interacts with Shg or actomyosin is not clear, this study spells out a strong genetic link between Ance (ACE homolog), DE-cadherin, and actomyosin network, crucial for progenitor maintenance. This enzyme's presence throughout the evolutionary ladder indicates its specific

requirement beyond the robust RAAS system (Fournier et al., 2012). A RAAS independent role of ACE has been proposed in mouse and *Drosophila*, regulating male fertility (Fuchs et al., 2005; Krege et al., 1995; Lambert et al., 2010). Interestingly, ACE can perform its function independently of its well-known peptidase activity through directly regulating gene expression (Fuchs et al., 2004; Fujihara et al., 2013). This implicating that its first function might as a signaling molecule while its enzymatic activity is a moonlighting activity acquired with evolution. This study will be the starting point to tease out the RAAS independent role of ACE indirectly regulating the signaling pathway using the *Drosophila*, a model system.

Interestingly, ACE was shown to be associated with actin and the non-muscle myosin light chain IIA in endothelial cells. Signaling through ACE therein results in phosphorylation of non-muscle myosin heavy chain. The current study also demonstrates a genetic relationship between Sqh and Ance. Both of these facts attribute that in the blood progenitor Ance and squash collaborates to maintain the Ci-155 level in the larval lymph gland. Current work has evoked several stimulating cell biological queries. It has been demonstrated that in endothelial cells, the signaling via the angiotensin-converting enzyme results in the phosphorylation of the non-muscle myosin heavy chain IIA (Kohlstedt et al., 2006). The exact molecular mechanism by which Ance regulates actomyosin activity in the progenitor is an important question that needs to be addressed.

Thus, employing *Drosophila*, a genetically amenable model, where the known components of complex Renin-Angiotensin System are absent, opens a new avenue to understand the cell-specific role of ACE.

Chapter 4

Ance-Shg-actomyosin controls G2/M progression in the *Drosophila* hematopoietic progenitor cells.

4.1 Introduction-

Stem cells and progenitor cells continuously generate mature differentiated cells to maintain the homeostasis of an organ. The dynamic regulation of precursor cell numbers and progeny involves a complex intrinsic signaling cascade, strongly influenced by the external factors that include cell-cell adhesion and matrix stiffness (Qiao et al., 2014; Sanchez-Romero et al., 2019) (Mih et al., 2012). It is widely accepted that in addition to a biochemical signaling cascade, mechanical cues also impact tissue growth rate to a large extent. Stem or precursor cells respond to through the process of mechanosensing, which can regulate proliferation, self-renewal, and differentiation (Mammoto and Ingber, 2010; Meng et al., 2018). These mechanical cues are mainly sensed by adhesion complex that can interact with the actomyosin cytoskeleton to regulate proliferative signals such as Rho kinase (RhoA) (Arnold et al., 2017; Dupont et al., 2011; Mih et al., 2012) and extracellular signal-regulated kinase (ERK) (Hirata et al., 2015)) and Yes-associated protein (YAP) (Sun and Irvine, 2016).

The influence of actomyosin-mediated mechanical on cell proliferation was proposed in the past (Huang and Ingber, 1999) and has been demonstrated recently in cultured cells (Aragona et al., 2013; Dupont et al., 2011) as well as *in vivo* (Hufnagel et al., 2007; Rauskolb et al., 2014). Interestingly, the involvement of actomyosin in cell proliferation is somewhat complicated; it depends on the cell type and the extracellular matrix's rigidity. It is demonstrated in specific scenarios/cases that inhibiting actomyosin contractility leads to a robust decrease in cell proliferation ((Assoian and Klein, 2008) (Mammoto and Ingber, 2010) While several lines of evidence suggest that inhibition of myosin (Mammoto et al., 2004; Yu et al., 2012).

In *Drosophila* wing disc, genetic perturbations or pharmacological inhibition of the actomyosin cytoskeleton led to decreased wing disc growth, affecting the final wing size in adults (Rauskolb et al., 2014). Whereas activation of myosin or inducing actin formation by the loss of actin capping proteins also stimulate wing disc growth and lead to increased wing size in adult *Drosophila*. Analogous experiments in mammalian and other vertebrate systems replicated the above results (Ibar et al., 2018; Wang et al., 2019). Although the different roles of actomyosin in various precursor cells have been widely demonstrated, involvement in hematopoietic tissue growth is not very well understood. It has been shown in vertebrates that Hematopoietic Stem Cells (HSCs) require the differential actomyosin forces essential for asymmetric cell division and regulation of HSCs fate (Shin et al., 2014; Shin et al., 2013). However, direct visualization of the involvement of actomyosin in the hematopoietic stem or progenitor cell proliferation is not worked out. Based on the findings of the current study that Ance-Shg-actomyosin is crucial for the maintenance of *Drosophila* hematopoietic progenitor cells (cited in chapter 3), it was

hypothesized that this cascade might also have additional role in *Drosophila* hematopoietic progenitor cells.

Drosophila blood progenitor cells are derived from hematopoietic stem cells in the early larval stage (Dey et al., 2016). These progenitor cells proliferate continuously until the late third instar stages, wherein they move into quiescence (Banerjee et al., 2019; Mondal et al., 2011). The temporal coordination between organism development and cell cycle withdrawal is critical for the maintenance of tissue homeostasis. It is known that premature quiescence in the progenitor cells leads to decreased lymph gland size and halted differentiation. In contrast, failure to arrest proliferation can lead to loss of maintenance of progenitor cells and along with increased differentiation (Banerjee et al., 2019; Mondal et al., 2011). Therefore, to understand progenitor homeostasis, it is essential to understand the molecular mechanism that regulates progenitor cells' proliferation.

To understand the involvement of the Ance-Shg-Actomyosin pathway in the maintenance of *Drosophila* hematopoietic organ size, Actomyosin components/Shg/Ance was knockdown in second instar progenitor cells using the TARGET system. During the second larval instar, the progenitor cells within the MZ extensively proliferate and increase the size of the organ, while late third instar progenitor cells show low proliferative state (Jung et al., 2005; Krzemien et al., 2010; Mondal et al., 2011). Thus, the growth of the MZ extensively depends on the proliferation of the early progenitor cells. This chapter will reveal the requirement of Ance-Shg-actomyosin in the proliferation of early hematopoietic progenitor cells.

4.2 Results-

4.2.1 Loss of actomyosin components in early proliferative progenitor cells reduced the size of the lymph gland

To investigate the effect of myosin knockdown on the lymph gland, proteins essential for actomyosin assembly were downregulated using RNAi, employing hematopoietic progenitor specific TepIV-Gal4 a timeline shown in scheme **(Figure 4.1A)** that overlaps with the self-renewal phase of the progenitors. Sqh was downregulated post first-instar larval stages (24 AEH), and lymph glands were analyzed around 96hr. The tissues were counter stained with DAPI to visualize the nucleus. Interestingly, upon loss of Sqh from the proliferative progenitor cells the lymph gland size decreases **(Figure 4.1 B-C).** This is further evident upon quantifying the total size of the lymph gland, and comparing it with the wild type **(Figure 4.1E)**. Quantifying the size of the lymph gland in Sqh RNAi revealed a 50% decrease in the lymph gland size.

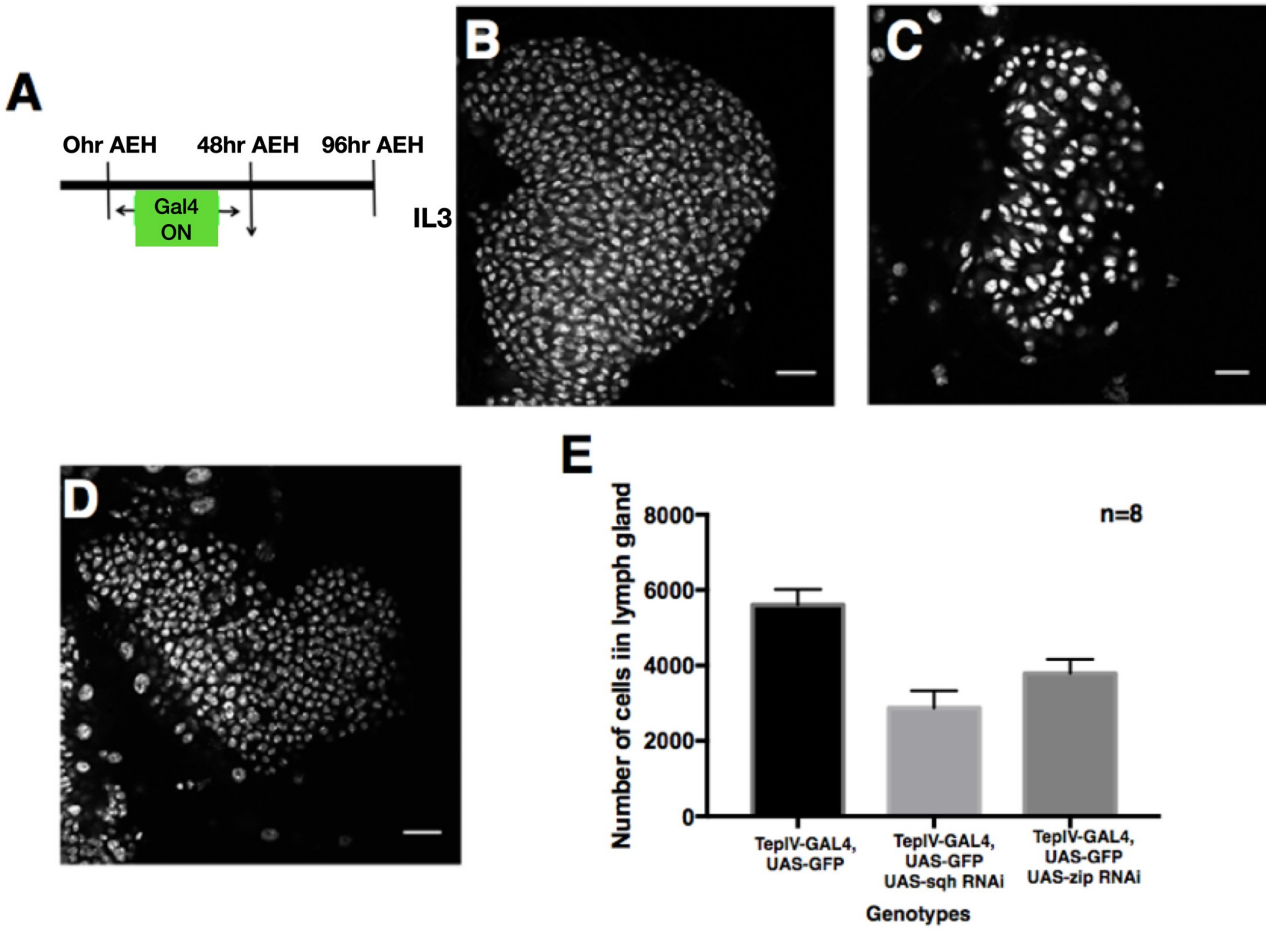

Figure 4.1 Inhibition of myosin affect LG size. Compare to wild type (A), the size of LG drastically reduced in sqh RNAi (B) and zip RNAi form the progenitor. (d) Quantification of number of cells in Sqh and Zip knockdown in the LG.(P-value TepIV-Gal4,UAS-2xEGFP/UAS-sqh RNAi=$1.5623x10^{-7}$; TepIV-Gal4,UAS-2xEGFP/+; UAS-zip RNAi = $9.782x10^{-3}$. Genotypes are shown on top of corresponding panels. Scale bar=20µm. DAPI marks the nucleus.

This result was further confirmed by RNAi mediated downregulation of Zipper (myosin heavy chain) using RNAi in the same time window **(Figure 4.1A)**. Analogous to Sqh loss, downregulation of Zipper from the progenitors also gave a smaller lymph gland **(Figure 4.1C-D)**. Quantifying the total size of the lymph gland in Zipper loss also reveal a 45% decrease in the lymph gland size compared to control lymph gland. Together, these results establish that the lymph gland size is regulated through actomyosin activity **(Figure 4.1E)**.

4.2.2 Actomyosin components regulate proliferation in Ance positive progenitor cells independent of its role in cytokinesis.

In order to understand the role of Sqh in the Ance positive proliferating progenitor cells, EdU incorporation was carried out in the progenitors where Sqh function is down regulated. Compared to control, a reduction of EdU incorporation in the progenitors is evident upon Sqh loss **(Figure 4.2A-B')**. Quantitative analysis also suggests a 60% percent reduction of EdU incorporation in loss of Sqh compared with the control **(Figure 4.2E)**. This result is further supported by analyzing the proliferation ability of the hematopoietic progenitor cells in the loss of Zip from the progenitor cells. Zip was knockdown using RNAi via TepIV-Gal4 in the same timeline used for Sqh knockdown. Indeed, knockdown of the Zip from the progenitor cells reduced the progenitor cells' proliferation capacity as seen via reduced EdU incorporation **(Figure 4.2C-C' and E)** compared with wild-type cells **(Figure 4.2A-A')**, mimicking Sqh knockdown from progenitor cells **(Figure 4.2B-B' and E)**. Quantitative analysis also indicates a drastic reduction in EdU incorporation in knockdown of Zip from the progenitor cells.

Next, the effect of increased myosin activity was analyzed via overexpression of an activated form of ROCK comprising the catalytic domain (ROCK) known to increase the activity of actomyosin (Rauskolb et al., 2014). When the ROCK was overexpressed in progenitor cells, EdU incorporation in the progenitor cells was increased; suggesting that actomyosin positively regulates the progenitor cell proliferation **(Figure 4.2D-D, and E)**.

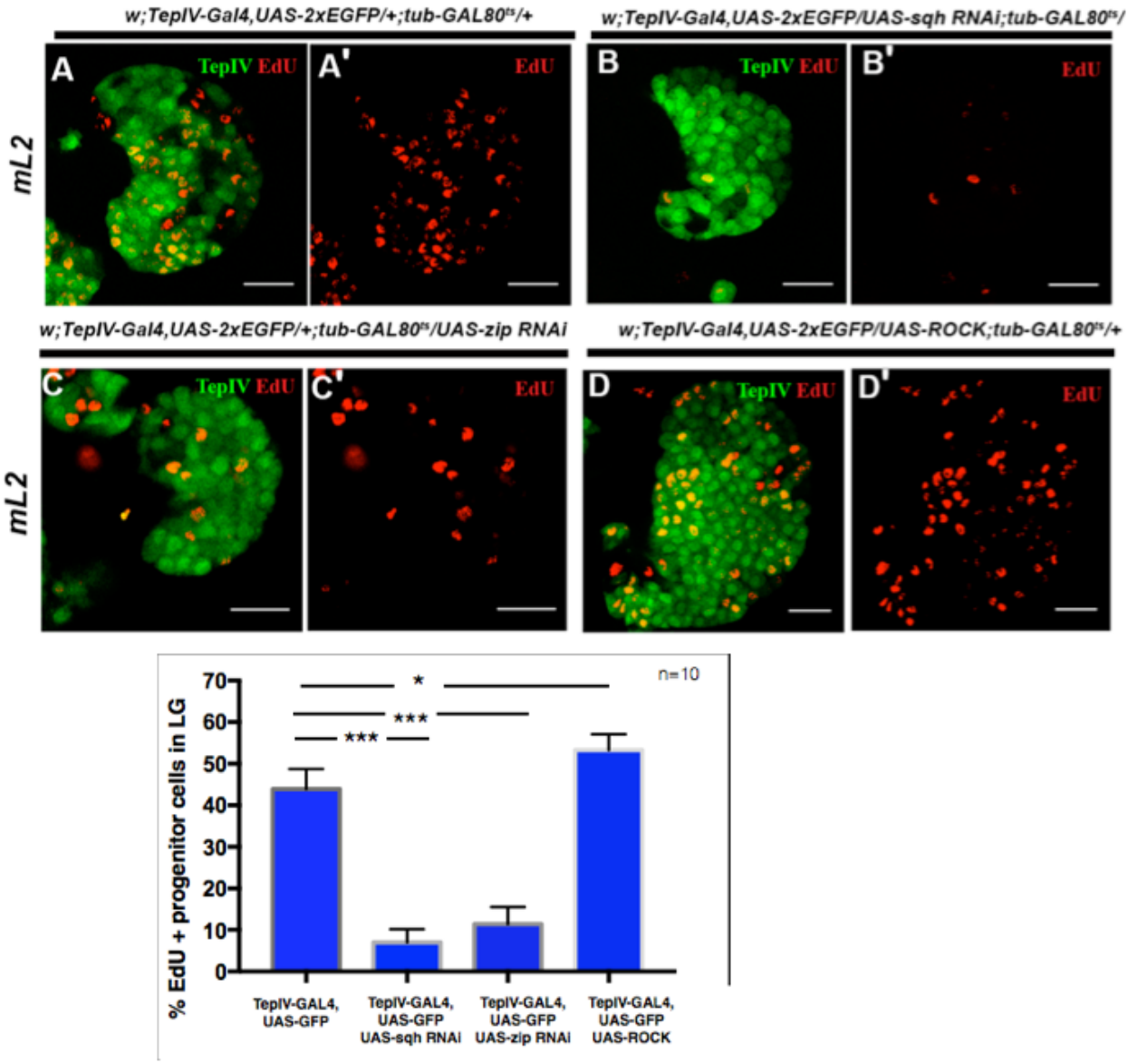

Figure 4.2 Actomyosin is essential for early progenitor proliferation. (A-B') Upon down-regulating Sqh (B-B') or Zip (C-C' activity in proliferative progenitor, a strong reduction in EdU incorporation was seen compared to control lymph gland progenitor cells (A-A'). (D-D') Overexpression of ROCK resulted in increment of self-renewal in the progenitors. P- value TepIV-Gal4 ,UAS-GFP/UAS-sqh RNAi= $2.451X10^{-7}$;TepIV-Gal4,UAS-GFP;UAS-zip RNAi=$6.104X10^{-5}$; TepIV-Gal4,UAS-GFP/UAS-ROCK= $9.267X10^{-3}$ Genotypes are shown on top of corresponding panels. Scale bar=20µm. DAPI marks the nucleus.

Actomyosin components play a distinct yet vital role in cytokinesis via providing the local force required for separation of the two daughter cells (Mendes Pinto et al., 2012). Therefore, it was essential to analyze whether this reduced progenitor cell proliferation is due to impairment in cytokinesis and hence impaired the cell cycle. Analyzing the PH3 (phosphorylate histone H3), which marks the mitotic cells, suggests that Sqh loss from the progenitor cells leads to a reduction in mitotically active cells compared to control **(Figure 4.3A-C)**.

In the cytokinesis, which occurs at the end of the mitosis during the cell cycle, myosin involved in the physical separation of the two daughter cells arise from the mitotic division (Mendes Pinto et al., 2012). In developing organs, cytokinesis failure leads to a bi-nucleate tetraploid (polyploid) cell that halts the cell division and affects the growth of an organ. To further test whether the reduce proliferation seen in sqh knockdown can be attributed to defects in cytokinesis, Sqh was down-regulated by TepIV-Gal4 in the early third instar. It is to be noted that TepIV-Gal4 is active in both Ance expressing (arrow) as well as Ance^{-} (arrowhead) hemocyte progenitors. Strikingly, Sqh knockdown using TepIV-Gal4 leads to cytokinesis defects in TepIV^{+} Ance^{-} IP cells. In contrast, Ance^{+} progenitor cells do not seem to be arrested in the cytokinesis (Figure 4.3D-D2'). These results reveal that actomyosin have a cytokinesis independent role in *Drosophila* hematopoietic progenitor cells (Figure 4.3D-D1').

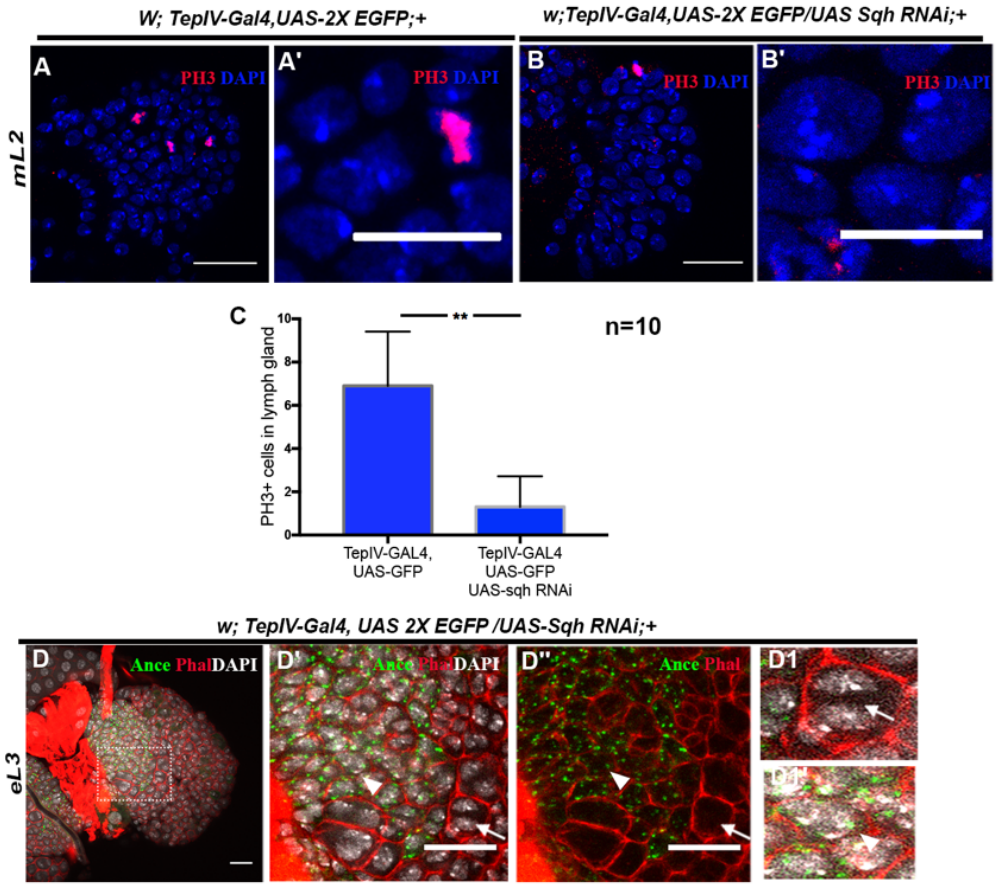

Figure 4.3 Actomyosin is essential for early progenitor proliferation. (A-B') Down regulation of Sqh activity from progenitors leads to mitotic defect visualized by PH3 labeling (red).(C) Quantification of PH3 positive cells in wild type and Sqh RNAi($p=2.7845\times10^{-4}$). (D-D2') loss of Sqh can cause mitotic arrest. On Sqh down regulation by TepIV-Gal4, from both Ance expressing (arrowhead) as well as non-expressing (arrow) hemocyte progenitors (red), a defect in mitoic entry was seen in Ance positive cells (arrowhead, D1') whereas cytokinesis defect was encountered in Ance negative population (arrow in D1). Genotypes are shown on top of corresponding panels. Scale bar=20µm. DAPI marks the nucleus.

4.2.3 Ance-Shg-Actomyosin regulates proliferation in *Drosophila* hematopoietic progenitor cells

Results described in Chapter 3 illustrate the crucial role of actomyosin in the maintenance of hematopoietic progenitor cells and how it is controlled through DE-cadherin mediated cell-cell adhesion and angiotensin-converting enzyme (Ance). Next, goal was to know whether the proliferation defects seen in early progenitors are specific to reduced actomyosin or reduction in upstream components; Shg and Ance which can also affect proliferation.

EdU incorporation in Shg deficient progenitor cells was next carried out to assay the involvement of DE-cadherin in the proliferation of *Drosophila* hematopoietic progenitor cells. The knockdown of Shg was performed using RNAi mediated approach employing hematopoietic progenitor specific TepIV-Gal4 in a timeline shown in scheme (Figure 4.1A). In control lymph glands, uniform EdU incorporation could be seen in progenitor cells **(Figure 4.4A-A')**. Upon knockdown of Shg in these TepIV positive progenitor cells, a dramatic decrease in the number of EdU incorporated cells in progenitor **(Figure 4.4B-C)**. The above results demonstrate that the DE-cadherin mediated cell-cell adhesion is essential for early hematopoietic progenitor cells in *Drosophila*. Disruption in the cell-cell adherence leads to compromised proliferation in the lymph gland.

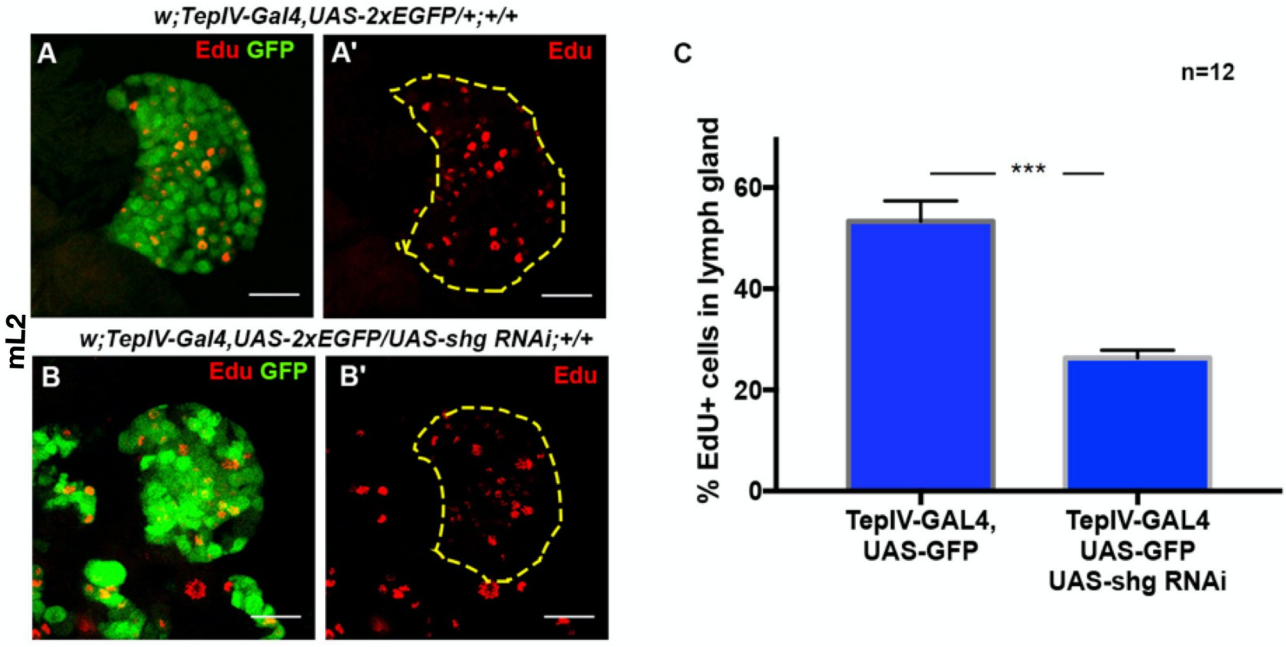

Figure 4.4 Cell-cell adhesion regulates proliferation of *Drosophila* hematopoietic progenitor cells. (A-B') Down-regulation of Shg leads to a strong reduction in EdU incorporation (B-B') in proliferative progenitor compare to wild type LG (A-A'). (C) Quantification of EdU incorporation in wild type and *shg* RNAi($p=3.7845\times10^{-5}$). Scale bar=20µm. DAPI marks the nucleus.

Next, to ascertain whether Ance is also involved in the proliferation of early hematopoietic progenitors, Ance deficient progenitor cells were analysed for their proliferation capacity.

As mentioned previously, the Ance mutant (heteroallele: $Ance^{34Eb2}$/ Df (2L) b88f32) shows a drastic reduction in larval lymph gland size (Figure 4.5A-C). Since the final size is achieved by high self-renewal potential of early instar progenitors, proliferation profile of Ance mutant lymph glands of 36 hours (second instar) and 54 hours (third instar) after egg hatching (AEH) was subjected to detail analysis. In comparison to the wild type lymph glands of similar age, the incorporation of EdU was drastically affected in the mutant (Figure 4.5D-H'). The above result establishes that Ance is required for progenitor cells' proliferation, and its absence thus affects the organ size.

Next, it was analyzed whether Ance can autonomously affect cell proliferation via modulating actomyosin in progenitor cells. To perform this experiment, Ance expression was modulated spatially and temporally using dome-Gal4. As mentioned in the previous section, the appearance of dome-Gal4 is not ubiquitous in progenitors of the early instar lymph gland, and dome-gal4 expresses in naive progenitor cells weakly. The knockdown with this driver created patches of Ance negative and Ance positive cells as visualized by labeling these tissues with an anti-Ance antibody. (Figure 4.5J-J'"). At this time point, in control lymph glands, EdU incorporation was comparable in dome GFP^+ and dome GFP^- cells indicating a phase of active proliferation (Figure 4.5K-K'). However, with the loss of Ance, there was a strong decrease in EdU incorporation in dome-GFP^+ cells, indicating a severe compromise in proliferation capacity of the blood progenitor (Figure 4.5L-L' and Figure 4.5M). The above results are further endorsed when EdU incorporation in such lymph glands were restricted to the dome GFP^- cells, where the Ance function was not perturbed. These results were validated by another independent progenitor driver, TepIV-Gal4 (Figure 4.5N-N'). Similar response in both cases demonstrates the role of Ance in proliferation.

The above results establish that Ance-Shg-actomyosin activity is mandatory for sustenance of the proliferation seen in the early progenitor cells.

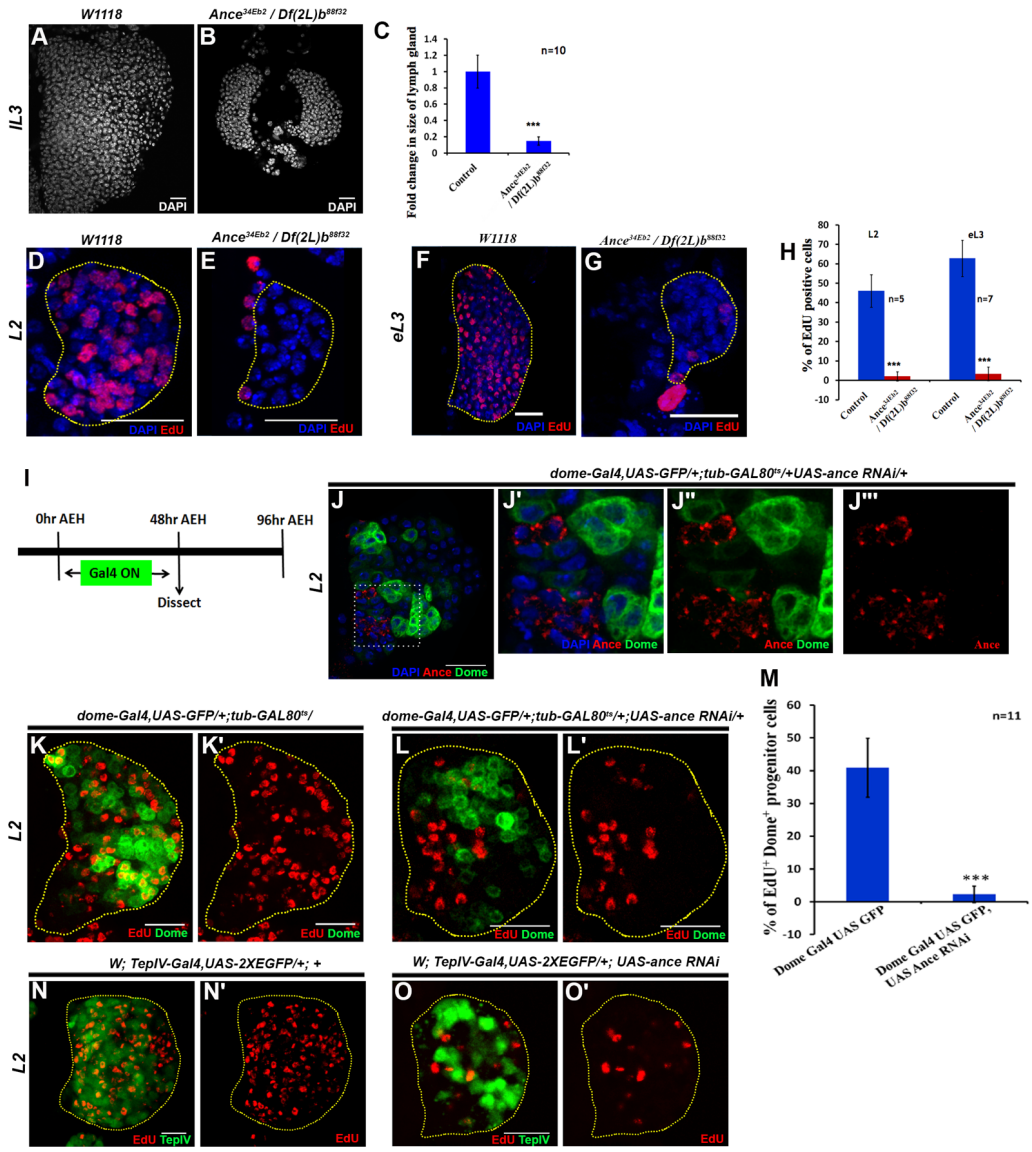

Figure 4.5 Ance is essential for proliferation of *Drosophila* hematopoietic progenitor cells (A-C) Lymph gland size decreases drastically in Ance mutant (B) compare to wild type (A). Nucleus is marked with DAPI (grey). (C) Quantification of larval lymph gland size reveals stark decrease in Ance mutant compared to control (P-value= 1.23×10^{-7}). (D-H) At second instar (d) or third instar (f) lymph gland progenitor cells exhibit high proliferation capability (EdU: red), which is drastically reduced in loss of Ance (E and G). (H) Quantification of EdU incorporation in wild type and Ance mutant (36h, P= $4.44 \times 10^{-4}$and 54h, P= 3.846×10^{-7}). (I) Scheme describing the experimental timeline used for Ance knockdown in early larval lymph gland. (J-J''') Ance labeling (red) in domeGal4-UAS GFP; UAS AnceRNAi (J) sufficient knockdown of the Ance protein in dome-GFP population on activation of Ance RNAi, visualized by Ance staining. Ance is present in non-GFP population (J''') validating the RNAi construct. (K-L') EdU incorporation is uniform in both the population (GFP+ and GFP-) of L2 control larvae (K-K', N-N'). Upon Ance knockdown by dome-Gal4 (L-L') and Tep4-Gal4 (O-O') a dramatic reduction of EdU incorporation is observed. EdU however was incorporated in non-GFP cells. (M) Quantification of proliferation capacity

(EdU incorporation) in progenitor specific knockdown of Ance (L2) when compared to control ($P= 1.63x10^{-8}$). Genotypes are shown on top of corresponding panels. Scale bar=20µm. DAPI marks the nucleus.

4.2.4 Ance-DE-cadherin-Actomyosin cascade regulates G2-M transition in *Drosophila* hematopoietic progenitor cells-

Interestingly, the nuclear size (visualized by DAPI staining) of the progenitor cells upon Ance down regulation is bigger than wild type progenitor cells **(Figure 4.6A-B')**. It is known that during the late S or G2 phase, DNA contents increases (Rabinovitch, 1994). Based on the above fact, it can be hypothesized that Ance knockdown might increase the number of progenitors that are in halted either in G2 or late S cell cycle.

The cell cycle status of such progenitors was assayed via expressing the UAS-Fucci system (UAS-GFP-E2f11-230 UASmRFP1NLS-CycB1-266) (Zielke and Edgar, 2015) expression in the progenitor cells by TepIV-Gal4 **(Figure 4.6C)**. This construct marks cells in the G1 phase as green, those in the S phase as red, whereas cells in the G2/M phase appear yellow **(Figure 4.6D-D'')**. To distinguish between G2 and M, Phospho Histone 3 (PH3) can be done to preferentially mark cells in mitosis. Histone H3 is phosphorylated at M phase, and phosphorylated histone H3 can be used as a validated marker of M-phase cells. As mentioned previously, in proliferating progenitors of the second instar, there are a large number of cells in the S phase (red). In contrast, very few cells are in G1 (green), and G2/M (yellow) phase (**Figure 4.6D**) can be seen in the lymph gland. Interestingly, Ance loss from the progenitors resulted in a dramatic increment in yellow cells (G2) that lacked PH3 expression, demonstrating an arrest in mitotic entry (**Figure 4.6E-E''**). Therefore, it is evident from our analyses that Ance plays a pivotal role in G2 to M progression of *Drosophila* early hemocyte progenitors of the lymph gland.

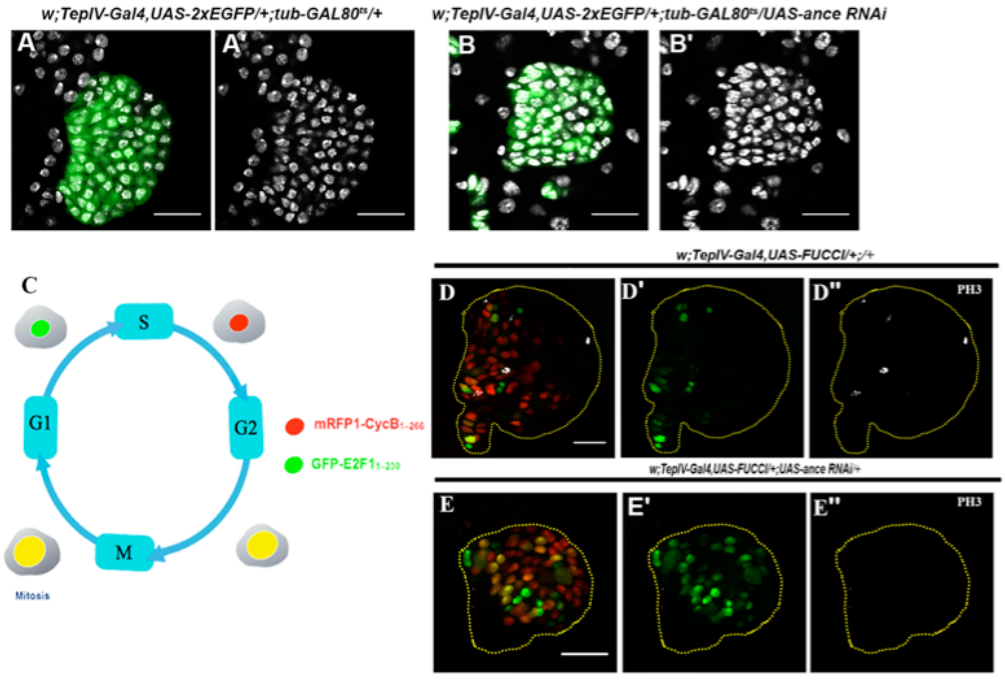

Figure 4.6 Ance might regulate String/Cdc25 activity essential for G2/M Transition. (A-B) LG size drastically decreases progenitor specific loss of Ance (B) compare to wild type (A). Nucleus is marked with DAPI (grey). (C) Fly-Fucci-fluorescent ubiquitination-based cell cycle indicator (Zielke N et.al 2012) system relies on degradation of two reporters, GFP-E2F11–230 and mRFP1-CycB1–266 that degrade during S and G1 phase respectively. Cell labeled with green, red and yellow are in G1, S and G2 / M-phases. Phospho-histone 3 (PH3) was used to distinguish between G2 and M-phase. (D-D") Expression of Fly-Fucci using progenitor specific GAL4 reveals that most of the cells are in S phase (red), while few cells are in G1 (green) and G2 (Yellow; negative for PH3) or M phase (PH3). On attenuation of Ance function (E-E") with Fly-Fucci in the background demonstrate cell cycle arrest in G2 phase visualized by increase in number of Yellow cells that lack PH3 (E"). Genotypes are shown on top of corresponding panels. Scale bar=20μm. DAPI marks the nucleus.

The kinase activity of the Cyclin B–Cdk1 is essential to promote the G2-M transition. It has been reported that CycB mutant cells have reduced cell proliferation. To investigate whether CycB is affected in an Ance-Shg-Actomyosin knockdown, the first expression of CycB was examined during hematopoietic progenitor development using the CycB-GFP protein trap line, which reports the endogenous CycB expression. In early proliferative progenitor cells, the CycB level increases in the G2 phase which reaches a peak in late G2. Its expression starts to decline at metaphase and is again reduced in early anaphase (**Figure 4.7A-A2"**). This expression profile suggests that, as expected, CycB accumulates in the G2 phase to promote the G2/M transition and is degraded in anaphase to facilitate an exit from mitosis.

Interestingly, CycB expression was affected in knockdown of Ance. Compared with the control, the expression of CycB was not reduced in progenitor cells that lacked Ance. Instead, CycB is accumulated in every cell of the progenitor, and there are no oscillations of CycB (**Figure 4.6B-B2"**). These results suggested that Ance does not regulate G2-M transition through regulating CycB expression. To further confirm that accumulation of cyclin B in the Ance RNAi was not due to cells remaining at early M-phase, PH3 expression was investigated in the Ance knockdown condition. In contrast, the number of cells stained by anti-phospho-histone H3 antibody in the progenitor cells of the TepIV>UASAnce-RNAi and the wild-type lymph glands indicating that the populations of M-phase cells reduced in the TepIV>UAS-Ance RNAi compare with the wild type lymph gland.

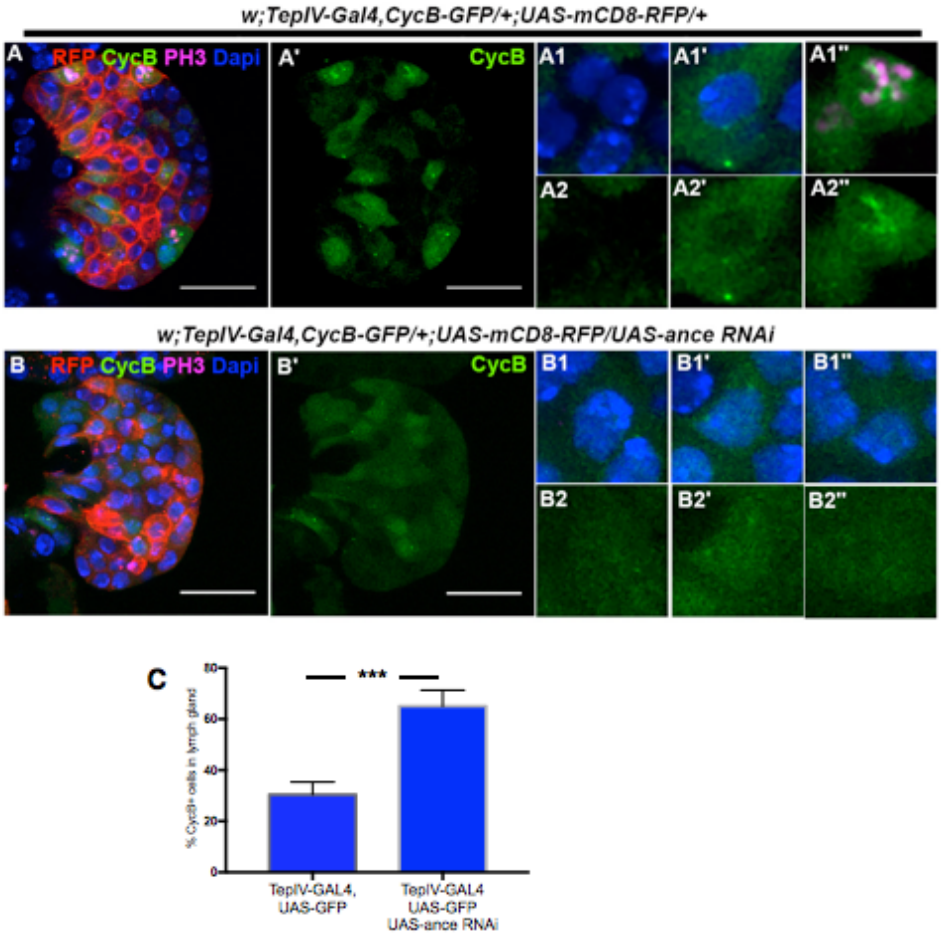

Figure 4.7 Ance loss did not affect CycB expression in lymph gland but increase number of CycB positive cells. (A) CycB GFP (green) is expressed in self-renewing progenitors (red) in G2 (arrowhead in h'-h3') and enriched in Mitotic cells (arrow: magenta, PH3+,A-A2'). This enrichment is lacking in Ance knockdown because of the lack of mitotic cells but the number of CycB expressing cells was increased in Ance loss from the progenitor cells compare with control. Genotypes are shown on top of corresponding panels. Scale bar=20µm. DAPI marks the nucleus.

Further, the CycB expression was also analyzed in Sqh or Shg knockdown cells **(Figure 4.8A-C2').** Similar to *Ance* RNAi, knockdown of Sqh or Shg using TepIV-Gal4 in progenitor cells also show an increasing number of CycB expressing cells, and these cells lack PH3 expression **(Figure 4.8A1-A2', '. Figure 4.8B1-B2' and Figure 4.8C1-C2').** So, it can be concluded that disruption in cell-cell adhesion or actomyosin assembly in *Drosophila* hematopoietic progenitor cells leads to G2 arrest.

These results suggest that Ance-Shg-Actomyosin does not regulate CycB expression in *Drosophila* hematopoietic progenitor for G2-M transition.

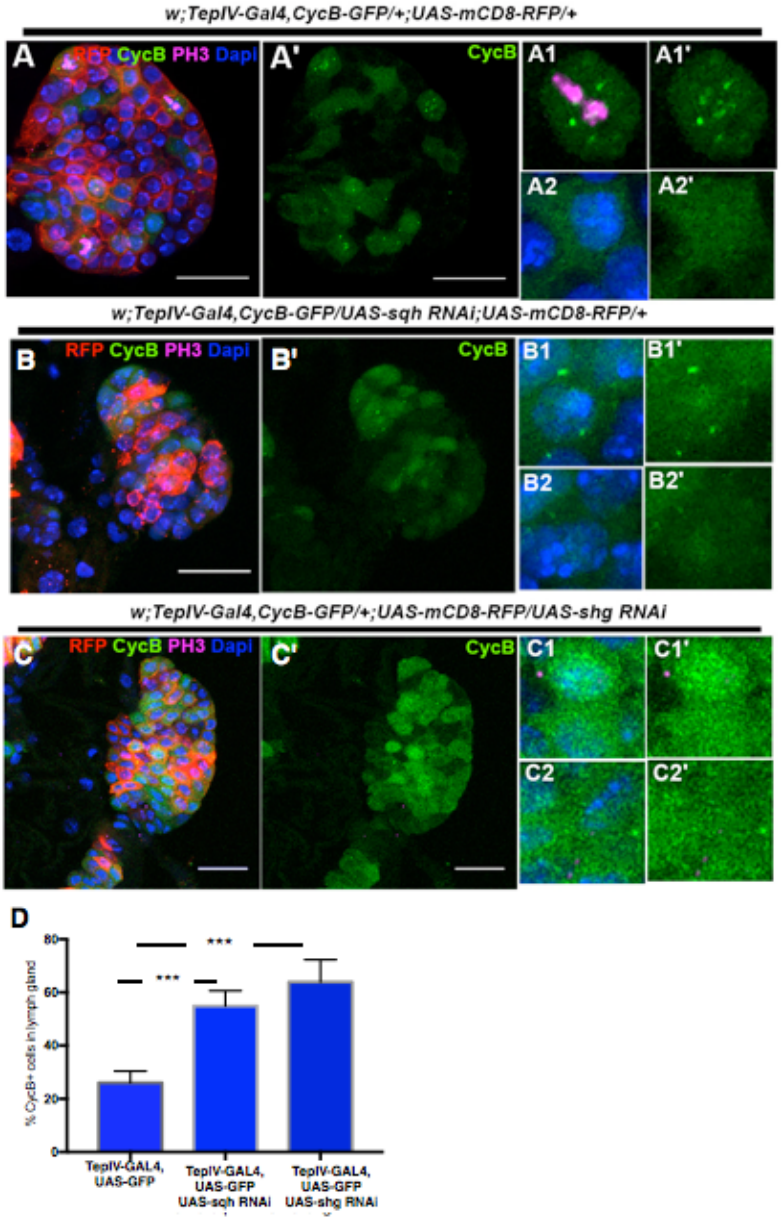

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Figure 4.8 Loss of Cell-cell adhesion and actomyosin activity increase number of CycB positive cells. (A-B2') Elevated CycB (green) expression in mitotic cells (PH3, pink) seen in control (A-A2') is lacking in Sqh (B- B2') or Shg(C-C2') loss from progenitors, even though there is an increment of the number of cells expressing CycB indicating a G2 arrest (B-B2'). Zoom image of random area from B1 and C1 indicates the CycB expression (arrows, B'-B2', C'-C2') in Sqh or Shg loss from the progenitor cells compared with control A'-A2'). Mitotic cell are visualized by PH3 (magenta) staining (A1'to C2').

4.2.5 Downregulation of Ance-Shg-Actomyosin assembly regulates string expression in hematopoietic progenitor cells.

It was very well established that for cells to enter into mitosis, the CDK1– CyclinB complex activity is indispensable (Pines, 1999). Although, this complex form late in S-phase, it remains inactive because of the phosphorylation of CDK1 at its key residues. The Cdc25 gene encodes the phosphatase, which removes the inhibitory phosphate groups and thereby elicits mitosis. In *Drosophila*, the Cdc25 homolog is String (Stg) (Di Talia and Wieschaus, 2012; Edgar et al., 1994;

Edgar and O'Farrell, 1990), which has also been shown to be mandatory to initiate mitosis. It has been demonstrated that misexpression of can trigger mitosis in G2 arrested sense organ precursors (SOP) and wing disc cells (Swaminathan and Pile, 2010) (Negre et al., 2003). Conceivably, inhibition of Stg expression in eye disc and wing disc cells leads to suppression of proliferation and the cells are stalled at the G2 phase (Johnston and Edgar, 1998). As expected, inhibition of String in the proliferative progenitor cells in the lymph gland reduces proliferation as evident from the scarcity of EdU incorporation **(**Figure 4.9A1-B'**).**

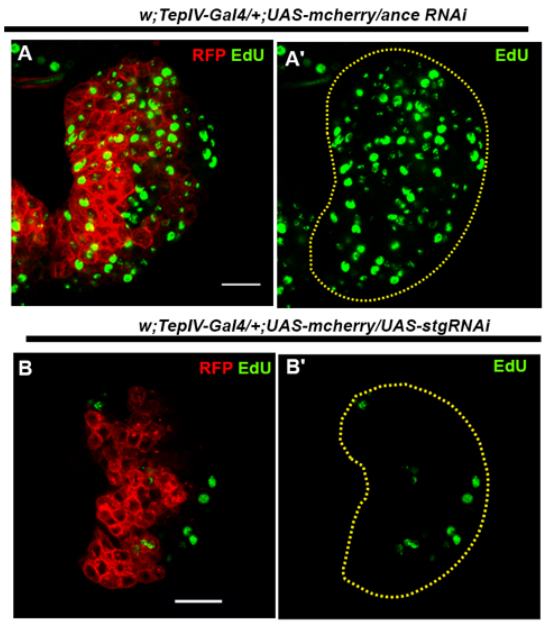

Figure 4.9 Loss of Stg affect progenitor cells proliferation in LG. (A-B') Expression of stg RNAi (B-B') leads to reduced EdU (green) incorporation when compared to control (A-A') phenocopying Ance knockdown. Genotypes are shown on top of corresponding panels. Scale bar=20µm. DAPI marks the nucleus.

Along with previous studies, the current observations suggest an essential role of CDC25 (String) in the G2-M transition. Next, it was asked whether the loss of Myosin or Ance affects String and inhibits CDK1 activity. Consistent with this idea, String expression was analyzed in the progenitors from where Sqh was downregulated. As shown in the wild type lymph gland, String is expressed in few mitotic cells **(Figure 4.10A-A1')**, however, the expression disappears from the progenitors wherein in Sqh expression was attenuated **(Figure 4.10B-B1')**. These results suggest that myosin regulates G2-M transition in *Drosophila* hematopoietic progenitor cells via modulating Stg.

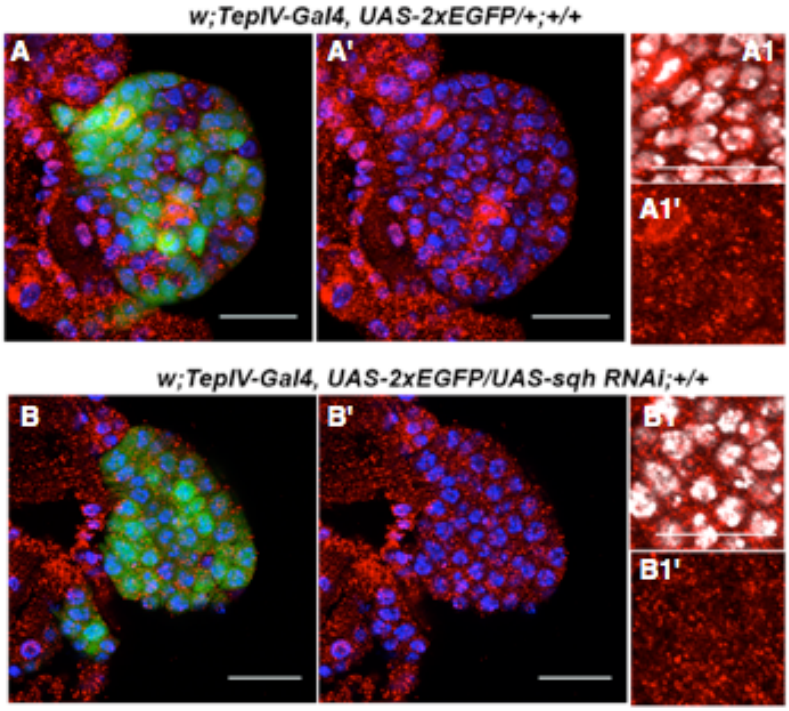

Figure 4.10 loss of Sqh affect Stg expression in the hematopoietic progenitor cells. Compared to control (A-A1') compromised Stg (red) expression is evident in early progenitors that lack Sqh function only (B-B1'). Zoom image of random area from B' indicates decline in Stg expression (arrows, B'-B2'") compared with control A. Mitotic cell was visualized by DAPI (grey) staining (A1'to B2'"). Genotypes are shown on top of corresponding panels. Scale bar=20µm. DAPI marks the nucleus.

To resolve whether *String* expression in the progenitors is also affected upon Ance knockdown, status of String was assayed. In the wild type LG, the String is accumulated in the G2, and mitotic cells. However, a drastic reduction of its expression was observed in the lymph gland where Ance is downregulated (Figure 4.11A-B2"). This becomes evident when the higher magnification images are compared. Ance knockdown cells lacked string expression (Figure 4.11B-B2") compared to the wild type progenitor cells (Figure 4.11A1-22").

Next, it was analyzed whether overexpression of String in Ance knockdown condition can suppress the phenotype. Compare to the wild type lymph gland (Figure 4.12A-A'and E), overexpression of String (Figure 4.101B-B'and E) in the lymph gland leads to increment in PH3 positive cells while Ance knockdown (Figure 4.12C-C' and E) decreases PH3 positive cells. To establish the genetic relation between Ance and String, the overexpression of String was performed in the Ance knockdown condition. Over-expression of Stg in Ance RNAi resulted in an increment of PH3 positive cells (Figure 4.12D-D' and E) indicating suppression of the mutant phenotype. The above results further consolidated our hypothesis that Ance and actomyosin act upstream of String in hematopoietic progenitor cells. This set of data connects

Ance/Shg/Actomyosin and Stg in a genetic pathway that affects cell cycle progression in the developing hemocyte progenitors.

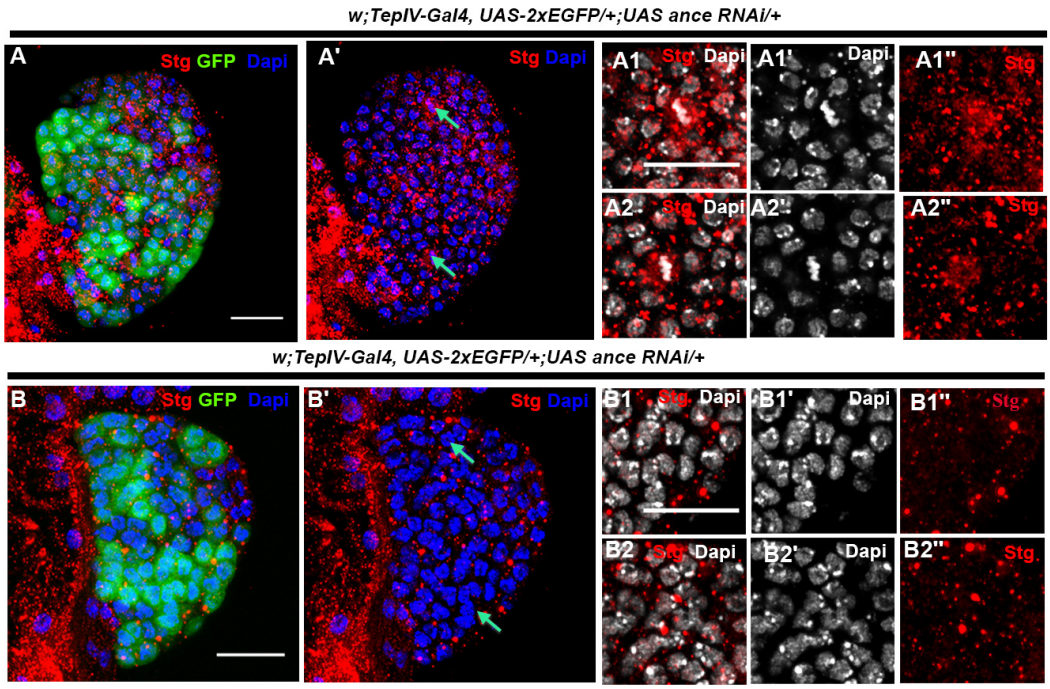

Figure 4. 11 Ance regulates string in the Drosophila hematopoietic progenitor cells. (A-B2''') String (red) expression in early lymph gland of control (A'). String /cdc25 expression increase from late G2 and is highly enriched in mitotic cells (arrows, A1'-A2'''). Down regulation of Ance in progenitor cells leads to drastic reduction in Stg expression (B-B'). Zoom image of random area from B' indicates decline in Stg expression (arrows, B'-B2''') compared with control A. Mitotic cell was visualized by DAPI (grey) staining (A1'to B2'''). Genotypes are shown on top of corresponding panels. Scale bar=20µm. DAPI marks the nucleus.

The results described above do not claim direct regulation of String on Ance/Shg/Actomyosin cascade. Instead, it provides a genetic link between String and Ance/Shg/Actomyosin cascade. Further analyses are required for understanding the precise role of Ance/Shg/Actomyosin cascade in G2 to M cell-cycle transition.

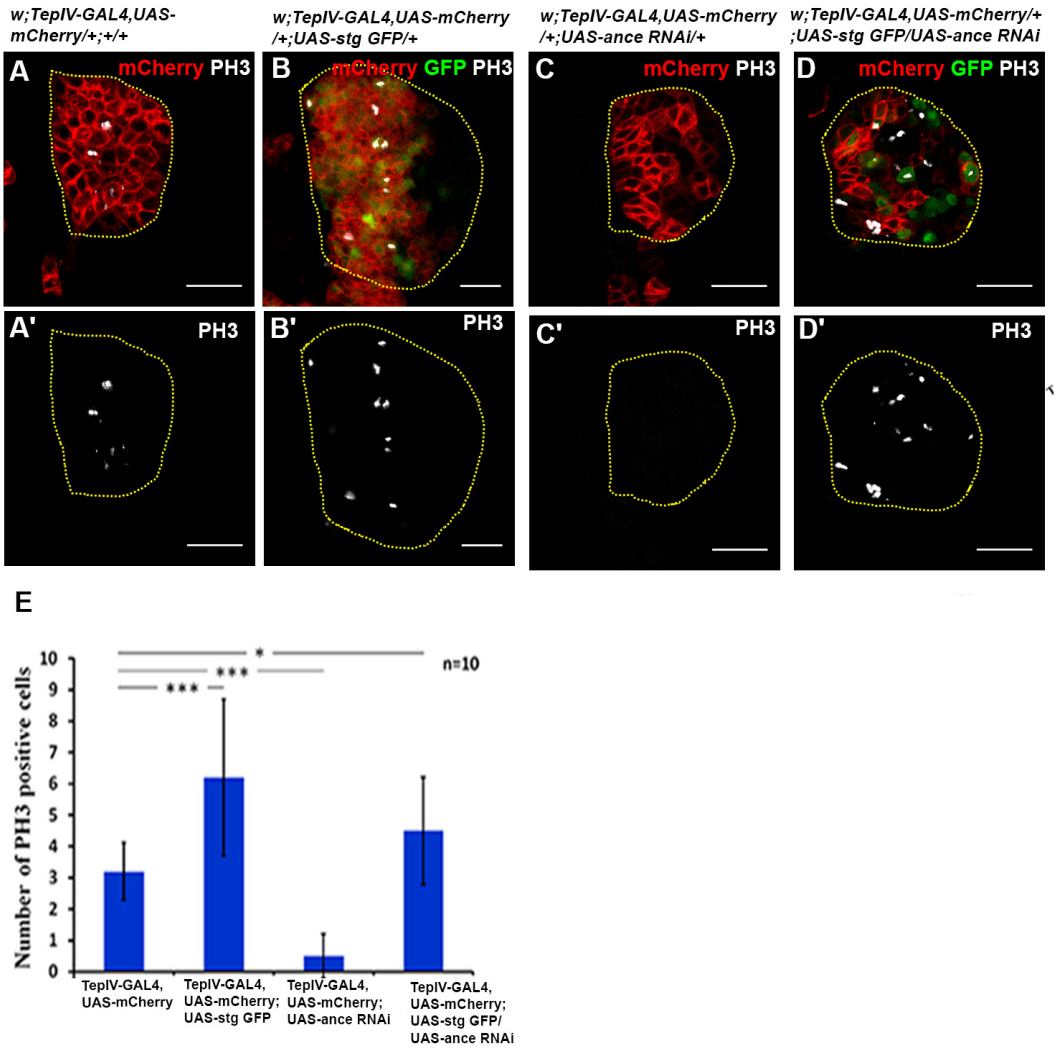

Figure 4.12 Overexpression of Stg rescue the proliferation defects seen in Ance loss from the progenitor cells. (A-E) Over expression of Stg (B) in the progenitors leads to an increase in the mitotic index (PH3; grey, B') compared to control (A and A'). Overexpression of Stg (D) from the progenitors where Ance is downregulated, rescues the G2 arrest otherwise seen upon Ance loss (C-C'). (E) Quantification of mitotic index (PH3 staining) in wild type, Stg overexpression (P=0.00408), Ance knockdown (P=1.15x10-6) and Stg over-expression in Ance knockdown background (P=0.05346). Genotypes are shown on top of corresponding panels. Scale bar=20µm. DAPI marks the nucleus.

4.2.6 **Amino acid deprivation affects the Ance expression in *Drosophila* hematopoietic progenitor cells.**

This study indicates that the expression of DE-cadherin-actomyosin components is highly enriched in the progenitor cells and required for their proliferation and maintenance. The genetic analysis suggests that Ance is required for progenitor specific enrichment of DE-cadherin and Actomyosin complex and it regulates string, which is the major player of G2-M transition. In the third instar,

this complex also regulates Ci-155, an essential player for progenitor maintenance. However, how Ance expression regulated in the progenitor cells remains to be elucidated. An effort to understand this aspect led us to unexpected link of dietary conditions and Ance expression.

Drosophila Larval hematopoietic system is highly sensitive to nutrition conditions (Shim et al., 2012). Previous work has shown a reduction in progenitor maintenance and LG growth during starvation (Shim et al., 2012). It has been shown in a mammalian system that starvation affects the ACE expression (Komaki et al., 1988) and can indirectly affect blood pressure. Still, hematopoietic specific relation between diet and ACE expression have not been worked out. Whether nutritional status controls the expression of Ance in hematopoietic progenitor cells was next investigated. To analyse the effect of diet on Ance expression in the hematopioetic progenitor cells, larvae were starved in agar plates and the expression of Ance were analysed. The strategy adopted for starvation involved the rearing the newly hatched larvae in regular food for 48 AEH then transfer the larvae for 12 AEH in the agar plates for starvation (Figure 4.13A). As expected, the growth of the LG and TepIV positive MZ was drastically affected upon starvation (Figure 4.13B-D). Interestingly, the expression of Ance in hematopoietic progenitor was significantly reduced (Figure 4.13C') due to starvation (Figure 4.13B').

Moreover, the decline in Ance expression was rescued in the progenitor cells when larvae were shifted to standard food (Figure 4.13F-F'). The above results suggest that diet can control Ance's expression in the progenitor cells, and dietary effect on Ance expression is reversible (Figure 4.13G).

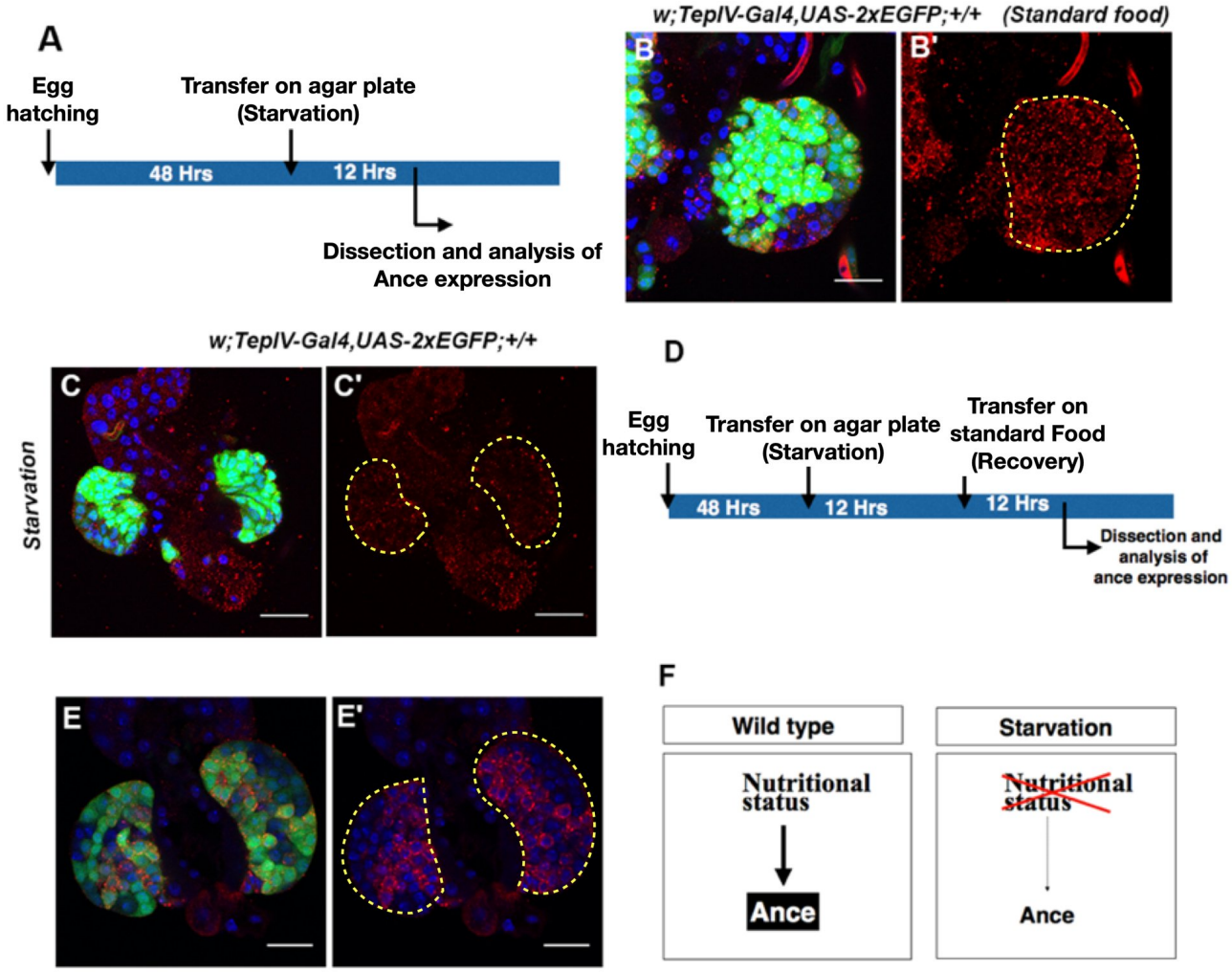

Figure 4.13 Nutritional status regulates Ance expression in *Drosophila* lymph gland. (A)Scheme of starvation of larvae followed in this panel. Wild type Ance (red;B-B')) expression in the LG progenitor cells. Down-regulation of Ance (red; B-B') protein in the progenitor cells in the starved larvae. (D) Quantification of TepIV positive MZ in the control and starve larvae ($P=6.304x10^{-5}$). (E) Strategy depicting the steps employed to analyse Ance expression in starved larvae vs larvae reared in normal diet. (F-F') A recovery of Ance expression was evident post transferring the starved larvae in the normal diet. (G) Model describing the nutritional status controls Ance expression in *Drosophila* hematopoietic progenitor cells. Genotypes are shown on top of corresponding panels. Scale bar=20µm. DAPI marks the nucleus.

Insulin signaling is a highly conserved mediator of diet and can regulate the underlying gene expression in response to organismal nutritional status (Figure 4.14A). Whether insulin signaling can control the Ance expression in progenitor cells was next addressed. Inhibition of insulin signaling in the progenitor cells did not affect Ance expression (Figure 4.14B-C). Additionally, over-expression of insulin signaling did not increase the Ance expression in the progenitor cells. These results suggest that insulin signaling does not affect the Ance expression in the hematopoietic progenitor cells.

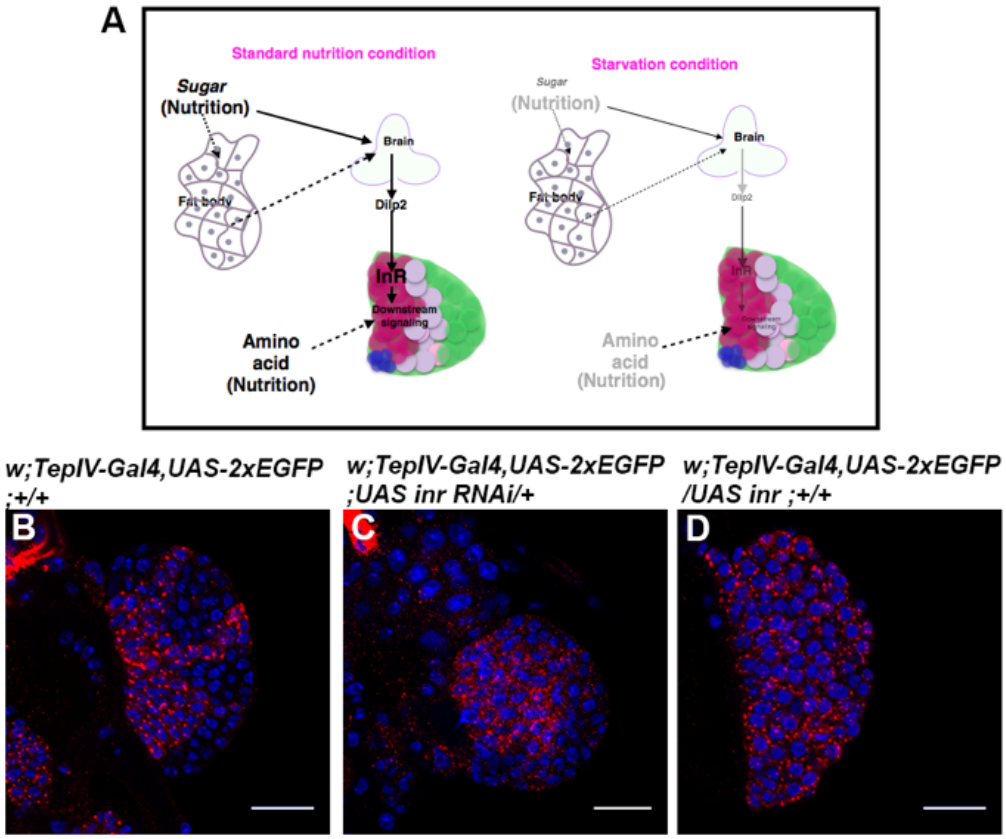

Figure 4.14. Ance expression does not depend Insulin signaling in Drosophila hematopoietic progenitor cells. (A) Model describing the nutritional sensing in LG progenitor cells, InR signaling activation in progenitor cells regulates through brain derived dilp2 which regulates through nutritional (sugar) sensing in brain and fat body. During Starvation, dilp2 secretion from brain reduced which affect the InR signaling activation in progenitor cells. Another nutritional component, Amino acid also senses by slimfast receptor and activate the Tor pathway in the progenitor cells. Wild type Ance (red;B-B')) expression in the LG progenitor cells. Downregulation of InR (C) or overexpression of InR (D) did not affect the Ance expression in the progenitor cells. Genotypes are shown on top of corresponding panels. Scale bar=20µm. DAPI marks the nucleus.

Studies have demonstrated that apart from the insulin signaling, amino acid also affect the progenitor cells maintenance (Shim et al., 2012). The loss of amino acid transporter Slimfast in the progenitor cells affects hematopoietic progenitor maintenance. Since InR has no effect on Ance expression, it was hypothesized that tinkering with the amino acid diet might affect Ance expression. In *Drosophila melanogaster*, yeast in the food is source of essential amino acid as well as most other noncaloric nutritional requirements. The removal of the yeast from the food, deficient the amino (Steck et al., 2018). Ance expression was analyzed in the larvae grown on food with yeast and without yeast (Figure 4.15A). Interestingly, the removal of yeast from the food drastically reduced the Ance expression (Figure 4.15C-C') compared to LG of larvae grown in a standard diet (Figure 4.15B-B'). These results suggest that amino acid regulates Ance expression in the *Drosophila* hematopoietic progenitor cells. To directly visualise the involvement of amino acid

in regulation of Ance expression, yeast diet was provide to the starved larva, which exhibited reduced Ance expression in the lymph gland. Since yeast is a rich source of amino acid and protein providing only yeast to starve larvae can reverse the effect of starvation on Ance expression. The protocol was standardized, as shown in Figure 4.15A. Ance expression drastically reduced in starved larvae compared to larvae grown in standard medium-yeast (Figure 4.15C-C'). When larvae were shifted back to yeast (protein-rich medium), after 12 hours of starvation noticeable recovery of Ance expression can be seen in the lymph gland (Figure 4.15D-D'). Thus, the above result indicates that Ance expression is dependent on the diet. Since starvation affects Ance expression which can be rescued by protein-rich food, it can be safely inferred that Ance expression is dependent on amino acid.

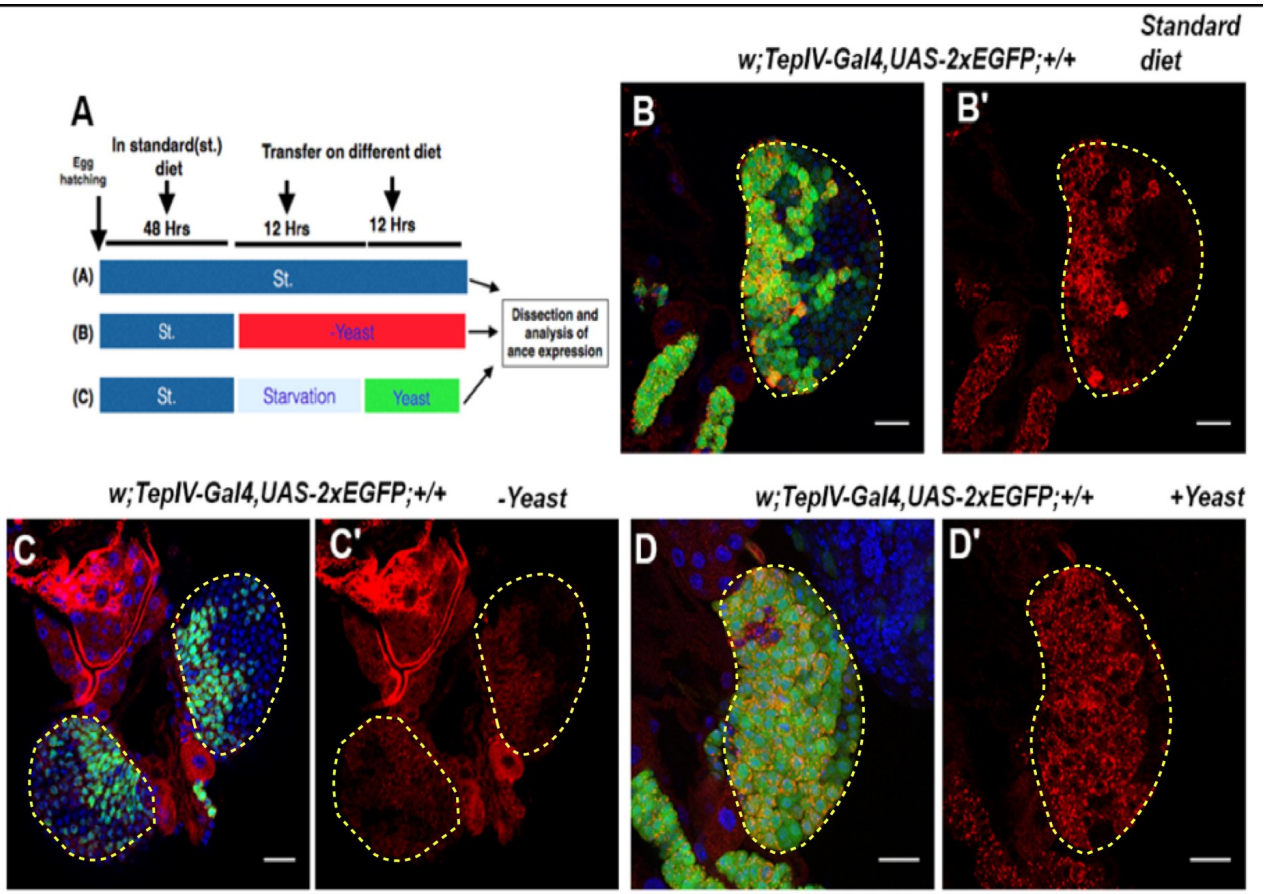

Figure 4.15 Amino acid regulates Ance expression in *Drosophila* lymph gland. (A) Scheme of protocol adopted for starvation, standard diet-yeast diet, and recovery of starved larvae on yeast supplement. (B- B') Wild type Ance (red;B-B')) expression in the LG progenitor cells. Downregulation of Ance (red; C-C') expression in the progenitor cells (GFP) of the larvae fed on standard–yeast diet. (D-d') A recovery of Ance expression was seen after providing yeast supplement to starved larvae. Genotypes are shown on top of corresponding panels. Scale bar=20µm. DAPI marks the nucleus.

To get a better insight on the effect of amino acid diet on Ance expression, different diet supplements were provided to the starve larvae in the recovery phase and the restoration of Ance expression in the progenitor cells was assayed. The larvae fed on sucrose (Figure 4.15B), or lipids (Figure 4.16C) did not show a rescue in Ance expression in the progenitor cells compared with larvae grown in the standard diet (Figure 4.16A). However, supplements of essential amino acid recovered the Ance expression in hematopoietic progenitors of starving larvae (Figure 4.16D). The above results suggest that EAA regulates Ance expression in the progenitor cells.

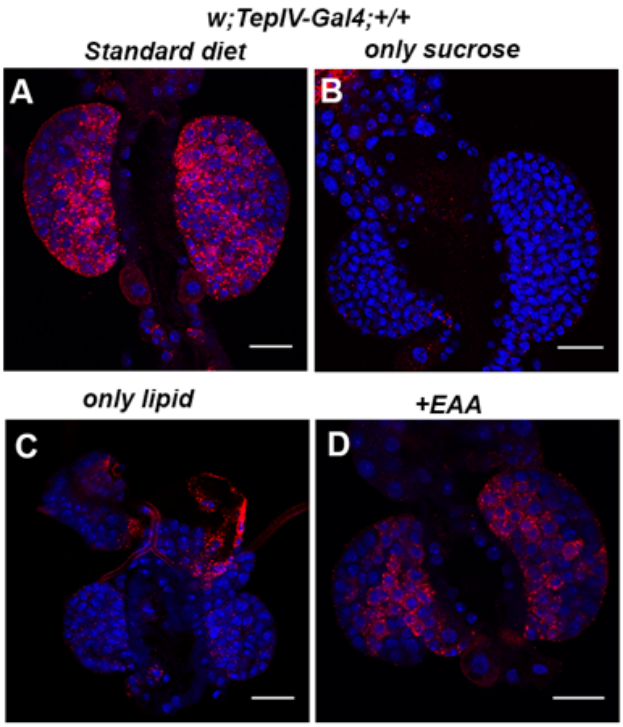

Figure 4.16 Essential Amino acid (EAA) regulates Ance expression in *Drosophila* lymph gland. (A) Wild type Ance (red;B-B')) expression in the LG. Ance (red) expression in the progenitor cells reduced in the larvae fed on only sucrose (B) or only lipid (C). (D) Ance expressed in the larvae fed on the EAA diet. Genotypes are shown on top of corresponding panels. Scale bar=20µm. DAPI marks the nucleus.

In sum, the above results demonstrated that the nutrition status of the organisms controls the angiotensin-converting enzyme expression in the *Drosophila* hematopoietic progenitors. It has been shown in the murine intestine that Angiotensin converting enzyme 2 is required for amino acid absorption, but whether amino acid regulates Angiotensin converting enzyme 2 expression (Hashimoto et al., 2012), is yet to be demonstrated. A link between angiotensin-converting enzyme expression and essential amino acid thus established through this study can be a start point to investigate the molecular pathway by which EAA regulates Ance expression in the lymph gland using powerful genetic tools that this model offers.

4.3 **Discussion-**

Three major conclusions can be drawn from the above study:

(a) Ance-Shg-Actomyosin regulates the proliferation of early hematopoietic progenitor cells.

(b) Ance-Shg-Actomyosin regulates proliferation through modulation of G2 to M transition.

(c) Ance-Shg-Actomyosin and string genetically interact to regulate G2 to M progression.

(d) Essential amino acid (nutritional status) regulates Ance expression in *Drosophila* hematopoietic progenitor cells.

A growing body of research shows the involvement of actomyosin components in modulating different signaling components. Different cellular response mediated through has been documented in cell culture cells (Dupont et al., 2011), *Drosophila* zebrafish, C. elgans, and in higher vertebrates (Goupil et al., 2017; Heer et al., 2017; Rauskolb et al., 2014). Although fundamental roles of the actomyosin pathway in cytokinesis (Mendes Pinto et al., 2012), migration (Gupton and Waterman-Storer, 2006; Pandya et al., 2017), and apoptosis (Domnina et al., 2004) have been well studied, its involvement in tissue growth is yet to be fully understood. Of note, both positive and negative effects of actomyosin modulation on proliferation in different model systems have been reported (Assoian and Klein, 2008; Mammoto et al., 2004; Mammoto and Ingber, 2009; Mih et al., 2012). Although several studies connect actomyosin with proliferation, the precise role of ROCKs in cell proliferation is yet clear: some reports suggest inhibition of ROCK can affect G1-S cell cycle transition in the proliferative cell (Croft and Olson, 2006), but others suggest that inhibiting myosin activator (ROCK) via a Y-27632 stimulate proliferation (Vishnubhotla et al., 2012). However, treatment with several other ROCK inhibitors (H1152, GSK269962A, AT13148, GSK429286A, and chroman1) decreases the progenitor cell proliferation (Kumper et al., 2016). This may be due to low inhibition rate of Y-27632 as a ROCK inhibitor, since it was found that AT13148, GSK429286A could mimic the genetic loss of Rock1 and Rock2 (Kumper et al., 2016; Wilkinson and Frame, 2016). It can be hypothesized that differential rate of myosin inhibition can leads to various effect of myosin inhibition on proliferation. An in-vivo study indicates that overexpression of the kinase domain of ROCK2 in mouse skin that overactive ROCK boost proliferation ((Samuel et al., 2011). Altogether, these results suggest that actomyosin can modulate different cellular responses, dependent on the cell type and downstream signaling effectors.

From this chapter, it can be concluded that actomyosin regulates the G2-M transition in the proliferative hematopoietic progenitor cells in the larval hematopoietic organ of *Drosophila*. In general, inhibition of myosin components in proliferative cells leads to a defect in cytokinesis. It is found that inhibition of actomyosin in Ance expressing blood progenitor cells leads to arrest in the G2 phase. However, their immediate neighboring hemocyte progenitor cells (Ance negative but

TepIV positive) have cytokinesis defects. These results signify that actomyosin's unique involvement in G2-M progression is specific and can happen only in Ance positive hemocyte progenitor cells. Therefore, it is likely that inhibition of actomyosin assembly in different cell types can give rise to cell cycle arrest in a cell type-specific manner. In line with these results, it is shown that inhibition of Rho GTPase with toxin B or C3 exoenzyme (Ando et al., 2007) in Hela cells halts cells at G2 phase. It has been reported that S. pombe cells treated with two different actin inhibitors: latrunculin B and latrunculin A has differential effects on the cell cycle(Rupes et al., 2001). Inhibition with latrunculin B, cells can enter mitosis but shows cytokinesis defects, while inhibition with latrunculin A, cells are arrested at the G2 phase. The possible reason behind the discrepancy might be because of different specificities of the two drugs that can inhibit differential actomyosin assembly.

Moreover, similar defects can also be seen in cells treated with cytochalasin D. The C3 treatment also disrupts the actin cytoskeleton, indicating that myosin in conjugation with actin cytoskeleton is involved in G2/M transition (Rupes et al., 2001). Furthermore, (Schmidt et al., 2007) reported that inhibition of a Rho effector, PRK2/PKN2 also leads to impaired Cdc25B phosphorylation and cells arrested at the G2 phase. Our results in conjugation with established literature suggested the involvement of actomyosin in the G2/M transition.

It is also demonstrated that the upstream regulator of actomyosin assembly is also involved in this process, as inhibition of Shg or Ance also affect proliferation. E-cadherin is a crucial regulator of human and mouse stem cell pluripotency and self-renewal through modulation of different signaling pathways. It is required for cell adhesion and cell junction formation, and it binds with the cytoskeleton through various catenins. Interestingly, similar to actomyosin, cadherin also performs a different function in tissue growth in multiple systems, where it shows negative and positive regulators of proliferation (Benjamin et al., 2010; El-Amraoui and Petit, 2010; Jeanes et al., 2008; Stepniak et al., 2009). It is reported in *Drosophila* that DE-cadherin positively regulates proliferation (Dumstrei et al., 2003). In the *Drosophila* embryo, it is proposed that DE-cadherin regulate proliferation through tribble, an essential regulator of CDK1 activation, clearly suggesting that DE-cadherin mediated cell-cell adhesion can regulate G2-M transition (Mata et al., 2000).

Interestingly, in saccharomyces cerevisiae, Trbl homolog Hsl1 activity depends on its physical association with the cytoskeleton (Barral et al., 1999; Grosshans and Wieschaus, 2000). It has been shown that Hsl1 regulates Swe1, which negatively regulates mitosis. It will be exciting to see how DE-cadherin mediated actomyosin activity modulates proliferation in *the Drosophila* lymph gland.

β-catenin can be a missing link between actomyosin and G2-M transition

The exact nature of the molecular signal linking cell-cell adhesion mediated actomyosin activity to G2/M transition remains elucidated. Further studies on the relationship between Ance, DE-cadherin, actomyosin, and G2-M phase transition will likely elucidate alternative pathways important in regulating progenitor cell proliferation. Possible candidates include β-catenin, members of Wnt signaling cascade known to have a role in regulating CDC25 (Suzuki et al., 2015), and are known to be controlled through cell-cell adhesion-actomyosin assembly (Brembeck et al., 2006{Hall, 2019 #185)}. The involvement of *Drosophila* β-catenin homolog armadillo has been reported in cell proliferation. In addition to its function in cell-cell adhesion, β-catenin is also involved in the Wnt signaling pathway. A central event in this pathway seems to be the accumulation of unbound cadherin cytosolic β-catenin. Interestingly, wingless signaling mediated β-catenin activation negatively regulates cell proliferation in *the Drosophila* wing disc at the dorsoventral boundary (Johnston LA et al., 1998). It is seen that reduction of E-cadherin/actomyosin activity can increase the cytoplasmic pool of β-catenin that can be activated by wingless signaling, thereby increasing the downstream pathway (Hall et al., 2019). So, cell-cell adhesion can negatively affect wingless signaling. In the conjugation with these results, it can be hypothesized that actomyosin can affect β-catenin mediated G2-M transition. It will be fascinating to see whether the above signaling pathway can affect the cell cycle in the progenitor cells. Its reduction can rescue the proliferation of defects observed in the altered cell-cell adhesion or actomyosin assembly.

ACE, E-cadherin, and actomyosin in cancer cell

A growing body of research shows that tumor development is associated with cell-cell adhesion and tissue stiffness (Gkretsi and Stylianopoulos, 2018; Handorf et al., 2015; Paszek et al., 2005). Generally, it has been assumed that the increased cellular myosin via ROCK activity observed in cancer cells is required for promoting invasion and metastasis (Olson and Sahai, 2009). However, recent studies in cell line and various model systems, including *Drosophila*, suggest that increased cell adhesion or actomyosin assembly can regulate cell proliferation (Croft and Olson, 2006; Mammoto and Ingber, 2009{Mammoto, 2010 #127; Rauskolb et al., 2014)}. Therefore, it can be hypothesized that increase actomyosin activity seen in cancer conditions may promote tumor growth by increasing cell proliferation.

An important question remains on how cell-cell adhesion mediated actomyosin activity regulates multiple cell cycle progression to accelerate tumor cell proliferation. This study shows that knockdown of the Shg/Sqh/Zipper leads to reduced cellular proliferation and altered tissue growth while overexpressing ROCK increases tissue growth via accelerated proliferation via regulating

G2-M transition. Therefore, altered cell adhesion seen in cancer conditions might contribute to cellular proliferation observed in the cancer condition.

The angiotensin-converting-enzyme(ACE) is expressed in HSPCs and renal progenitor cells (Lin et al., 2011{Jokubaitis, 2008 #191)}. Interestingly, inhibiting Angiotensin-Converting Enzyme in HSCc and Renal Progenitor Cell affects their proliferation, suggesting that ACE can regulate cell proliferation apart from being an essential regulator of blood pressure maintenance through the RAAS system. Our finding also demonstrated that Ance could regulate proliferation through modulation of cell-cell adhesion mediated actomyosin assembly. The ACE is highly expressed in diverse cancer conditions (Rosenthal and Gavras, 2009). The usage of ACE inhibitors can reduce tumor growth and metastasis both in vitro and in vivo (Lindberg et al., 2004). Numerous studies from the human cohort have shown that long-term administration of ACE inhibitors and Angiotensin receptor blockers (ARB) reduced the cancer progression in multiple cancers, including leukemia (Rosenthal and Gavras, 2009; Yoon et al., 2011). Quite strikingly, high actomyosin activity and Cdc25 activity are also an integral part of cancer (Boutros et al., 2007). Thus, it would be interesting to see whether the genetic link between Ance-Shg-Actomyosin-String unrevealed here, is an identical route that cancer cells adopt for their progression. It will be exciting to understand the signaling cascade involved in cellular hematopoietic progenitor cell proliferation due to increased cell-cell adhesion or Actomyosin activity that can be useful for cancer studies and designing therapeutic targets.

Chapter 5

Biophysical force generated by neighboring myoepithelial cardioblast is crucial for the *Drosophila* hematopoietic microenvironment maintenance.

5.1 Introduction

Tissue homeostasis requires a precise balance between the number of precursor cells and differentiating cells(Biteau et al., 2011; Blanpain and Fuchs, 2009). One of the major regulators that orchestrate this balance is specialised microenvironments, also called niches, which provide signals for stem cells/progenitor cell maintenance and their proliferation (Banerjee et al., 2019; Decotto and Spradling, 2005; Ge et al., 2020). The concept of a stem cell niche was first proposed in vertebrate by Schofield in 1978. However, the first in vivo demonstration of it came much later from invertebrate systems like Drosophila germline and the distal tip of Caenorhabditis elegans (Decotto and Spradling, 2005; Ferraro et al., 2011; Xie and Spradling, 1998) (Kimble and White, 1981). Till now, various stem cell niche has been identified in different tissue including the germline, hematopoietic, skin, hair follicles, and nervous systems from invertebrates to higher vertebrates (Xie and Spradling, 1998) (Decotto and Spradling, 2005; Kimble and White, 1981);(Mandal et al., 2007) (Morrison and Spradling, 2008; Nilsson et al., 2001; Palmer et al., 2000; Tulina and Matunis, 2001; Yoshida et al., 2007; Zhang et al., 2003). It has been found that niche derived signals, which include signaling molecules, adhesive signals, and matrix proteins, are essential for the stem cell/progenitor cell maintenance, self-renewal, proliferation, and cell fate determination (Mandal et al., 2007; Nilsson et al., 2001; Van Mater et al., 2003)

The growing body of research suggests that a niche is a complex structure made up of cellular and extracellular components. Various cellular components that include mesenchymal, neuronal, glial, blood cells, vascular and physical parameters such as oxygen tension, extracellular matrix (ECM) rigidity, and shear stress have been identified in different niches (Morrison and Spradling, 2008; Ohlstein and Spradling, 2007; Song and Xie, 2002; Song et al., 2002). These components can be broadly categorized into two major classes; first, the physical niche that includes ECM and their associated structural protein, which can act as a scaffold to maintain the stem cells. The second class of cellular niche is defined by stromal cells, mesenchymal, vascular, neuronal, and glia, that secretes signaling molecules and ECM for the maintenance of the stem cells/progenitor cells. Interestingly, a niche can further be classified as static in terms of their location like the GSCs niche in *Drosophila* and or can be dynamic like the HSCs niches in the vertebrates. The niche provides location wherein a functional interaction between niche with stem cells and progeny occurs to control tissue homeostasis.

It has been observed that alterations in the niche components or signaling molecules are associated with many pathologies, including tissue degeneration, aging, and tumorigenesis (Carlson and Conboy, 2007; Carlson et al., 2009; Woolthuis et al., 2011)). Studies have elucidated that modifying the stem cells niche can reverse the effect of aging on stem cells and progenitor cells. In

recent times, several reports also point to the existence of modified microenvironments termed as a cancer stem cell niche (CSC niche), which via secreting various growth factors is capable of initiation and growth of the tumour (Plaks et al., 2015) . These aberrant cancer stem cells niches not only regulates cancer stem cells growth and proliferation but are also involved in transforming non-tumorigenic cells into tumorigenic cells through a process like Epithelial to Mesenchymal transition (EMT) (Erez et al., 2010) (Mao et al., 2013). Houses within the cancer stem cell niche, the cancer stem cells are protected from radiation, and chemical-induced cell death, explaining the therapy resistance found in some cancer patients (Chan et al., 2018; Huelsken and Hanahan, 2018). In such scenarios, targeting strategies that disturb the modified niche or alter the ability of the niche to produce growth factors may provide effective strategies for inhibiting tumour cell growth and malignant progression.

Apart from signalling molecules, intrinsic mechanical tensile generated by actomyosin complexes can also regulate stem cell niche. Recently, it has been shown that increased contractility mediated by actomyosin in hair follicle stem cell niche non-autonomous regulates maintenance and proliferation of stem cell (Ning W et al., 2020). The understanding of signaling molecules and mechanical properties that control the development of the stem cell niche will facilitate new and improved stem-cell-based therapies to revere the stem cell defect associated with aging and cancer.

5.1.1 *Drosophila* third-instar Lymph Gland as a model for progenitor- niche interaction

Given the significant conservation of signals and transcription factors with mammalian hematopoiesis, the *Drosophila* Lymph gland (larval hematopoietic organ) holds a great promise to unravel the novel aspects of the precursor cells and cellular niche maintenance.

In the third instar LG, four types of the population are identifiable by their different gene expression: the niche, the progenitor cells, IP cells, and differentiated cells. It has been previously demonstrated that Antp expressing niche serves as an essential regulator of *Drosophila* hematopoietic progenitor cells (Mandal et al., 2007; Mondal et al., 2011). Niche act as a source of Hh and Pvf1 molecules, which regulates Ci-155 in the progenitor cells, essential for its maintenance (Banerjee et al., 2019; Mandal et al., 2007; Mondal et al., 2011). Hematopoietic progenitor cells respond to Ci-155 signals in a dosage-dependent manner (Banerjee et al., 2019; Mandal et al., 2007). The increase or decrease level of Ci-155 can affect the progenitor cells maintenance or differentiation (Mandal et al., 2007; Mondal et al., 2011; Sharma et al., 2019)

It has also been shown that a decrease in niche number drastically affects the progenitor cell maintenance while an increase in the niche number enhances the progenitor cell maintenance (Banerjee et al., 2019; Sinenko et al., 2009; Tokusumi et al., 2012). So, it can be concluded that

niche plays a crucial role in maintaining the homeostasis of the lymph gland and change in niche function and/or in the cell number affect the progenitor maintenance.

In a diverse model system, it has been shown that growth factor, extracellular matrix, and nutritional status regulate the maintenance of niche function and/or cell number. However, in the *Drosophila* lymph gland, the information regarding niche cell maintenance is still in infancy. Few studies in the recent past have demonstrated the importance of intrinsic and extrinsic factors on *Drosophila* hematopoietic niche cell proliferation. For example, it has been shown that Dpp signaling, slit-Robo pathway, septate junction protein; Cora or Neurexin IV(NrxIV), and Lar negatively regulates niche size while InR, Akt1, Pdk1, or phosphoinositide-3 kinase (PI3K), Wg, Jumeau (a member of winges-helix/forkhead transcription factor), dMyc signaling positive regulates niche cell proliferation (Banerjee et al., 2019; Benmimoun et al., 2012; Dragojlovic-Munther and Martinez-Agosto, 2012; Kaur et al., 2019; Sinenko et al., 2009; Tokusumi et al., 2012).

These findings indicate an essential role of the above signaling molecules in maintaining niche cells number in *Drosophila* LG. Nevertheless, it remains yet to be fully addressed how niche cell proliferation is regulated with development. Therefore, with an intention to unraveling the molecular signals, which control the niche cell proliferation, first, the cell cycle dynamicity of the hematopoietic niche was explored. This was followed by analysing the involvement of the cellular pathways, which might regulate this dynamicity. Understanding such molecular mechanisms will provide the platform to recapitulate the niche ex vivo or in vitro.

5.2 Results

5.2.1 Revisiting the role of niche derived Hh in progenitor cell maintenance.

Several studies endorse the definite requirement of PSC in the maintenance of the hematopoietic progenitor cells (Baldeosingh et al., 2018; Benmimoun et al., 2012; Hao and Jin, 2017; Khadilkar et al., 2017; Mandal et al., 2007; Mondal et al., 2011; Tokusumi et al., 2012). However, the role of PSC as a niche in progenitor maintenance has been challenged by a study (Benmimoun et al., 2015). It was claimed that the genetic ablation of the niche via expressing apoptotic gene did not affect the progenitor cells. To resolve the above issue, the role of Hh in the hematopoietic niche was reinvestigated.

It has been shown that Hh is expressed in the niche while downstream transcriptional activator Ci-155 express in the progenitor cells (Mandal et al., 2007) (Figure 5.1 A). Thereafter, hematopoietic niche-specific Hh enhancer has been cloned and validated (Tokusumi et al., 2010) in the lymph gland. To reinvestigate the involvement of niche derived Hh signaling in the LG progenitor cell maintenance, the source of Hh and receiving cells of the Hh signaling were identified using Hh

antibody labeling. Two different protocols were adopted to detect the source of Hh (using detergent: which enables to visualize intracellular Hh) and the responder cells of the Hh (without detergent: helps in detecting extracellular Hh) in the lymph gland respectively (Figure 5.1B). Similar to the previous finding, the current study detects Hh in the niche while the extracellular Hh was evident in the progenitor cells, which also express the downstream transcriptional activator Ci-155. These results endorsed the previous finding that niche serves as a source of Hh while the MZ progenitors sense the signal.

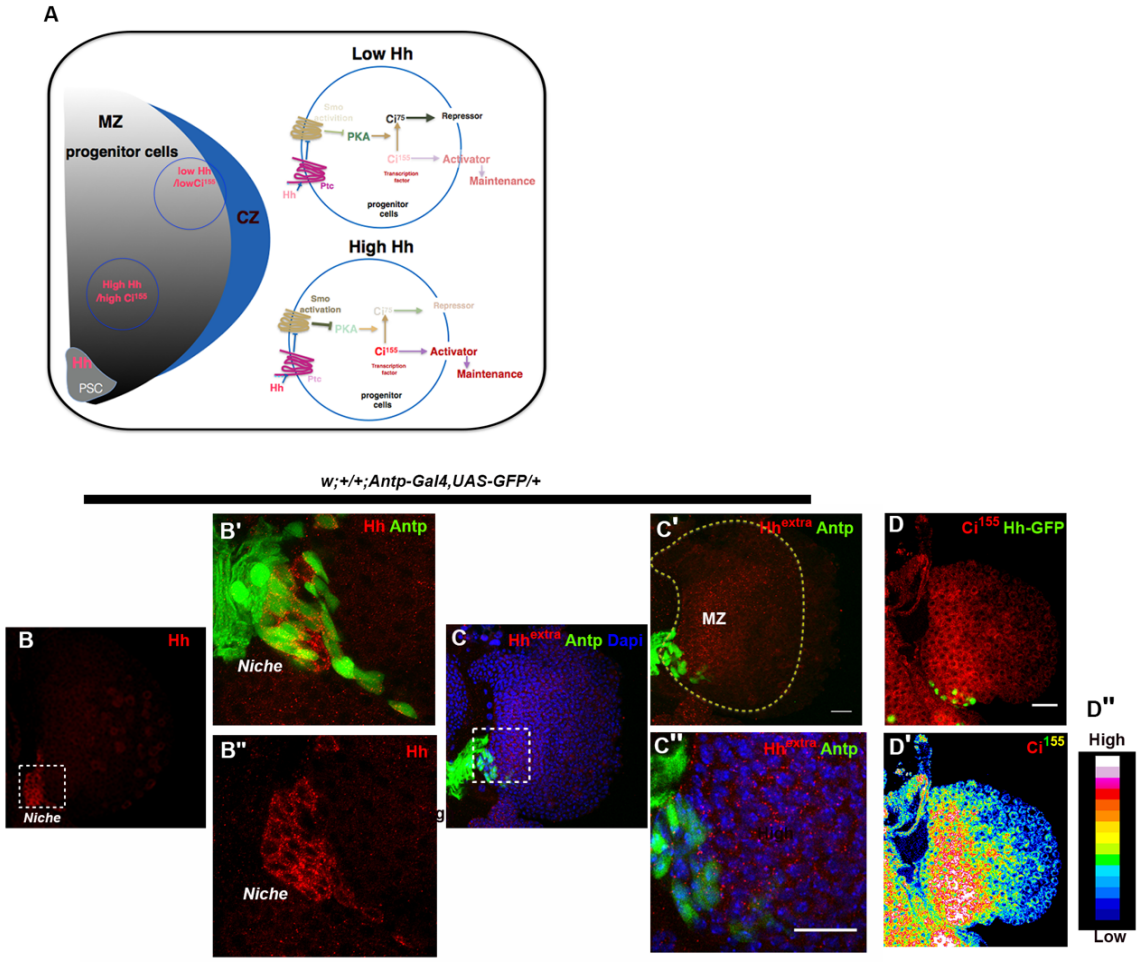

Figure 5.1 PSC serves as the source of Hh signaling. (A) Hh signaling in the lymph gland. The source of Hedgehog is the hematopoietic niche, and the Reception of Hh signaling in the progenitor cells requires the activity of two transmembrane proteins, Patched (the receptor) and Smoothened (transducer of Hh signaling). In the absence of Hh/very low Hh, Ptc inhibits the Smo activation, thereby prevents the activation of the intracellular effectors, while in high Hh condition, Binding of Hh with Ptc triggers their internalization, thereby allowing Smo to accumulate and signal the downstream effectors. The signaling thus initiated the activation of full-length Cubitus interuptus. The full-length protein accumulated acts as a transcriptional activator of Hh target genes. (B-B") Hh (Antibody, red) expresses in the PSC. B' and B" are the higher magnification of the PSC shown in B. (C-C') Upon detergent free permeabilization during antibody labeling, extracellular Hh (red) can be visualized in the progenitors cells (MZ). (C") Higher

magnification of a selected area in C (Antp-GFP marks the PSC). (D-D') Hematopoietic precursors cells near the source of Hh are higher in Ci-155, while those that are farther away have down-regulated it. Scale bar: 20 μm

Next, the functional requirement of the niche in progenitor cell maintenance was analyzed using niche-specific down-regulation of Hh signaling. The downregulation of Hh was performed through UAS-Hh RNAi using the niche-specific drivers like Antp-Gal4 and pCol-Gal4. To exclude the progenitor cell's proliferation phase and target the maintenance phase, the Hh inhibition in the niche was performed after the late second instar (Figure 5.2A).

Lowering the Hh activity in niche cells by expressing RNAi against Hh under either pCol-Gal4 (Figure 5.2C) or Antp GAL4 (Figure 5.2D) driver significantly reduced the progenitor cells (visualized by Ance) compared to the wild type lymph gland (Figure 5.2B). Analysing the progenitor cells via another progenitor specific marker (Shg) (Figure 5.2E-F) or differentiated cells marker (P1; Figure 5.2G-H) also revealed that lowering the Hh signals from the niche cells drastically affect the progenitor cells maintenance and increase their differentiation. Similarly, loss of Smo (a positive regulator of Ci) from the progenitor cells also resulted in reduction of progenitor cell number (Figure 5.2J). The above results suggest that reducing the Hh from the niche or its downstream signaling components in the progenitor cells severely affects the maintenance of the blood progenitors. Conversely, overexpression of Hh in the niche leads to the increment of the progenitor cells and a halt in their differentiation. (Figure 5.2I). Moreover, the progenitor's specific over-expression of the Smo also increases the progenitor number and reduces differentiation ((Figure 5.2K).

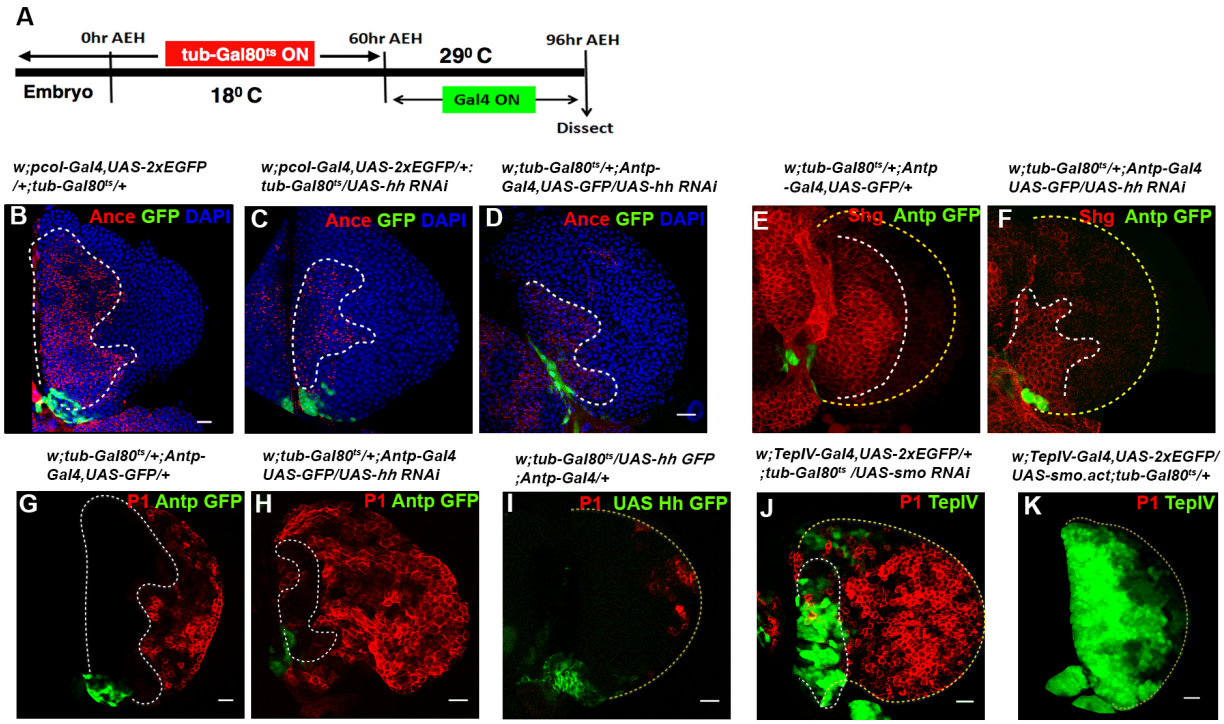

Figure 5.2 The level of Hh at the source downstream components activation in the MZ is critical for progenitor maintenance. (A) Scheme illustrating the timeline adopted for these studies. The synchronized larvae collected from the different crosses were kept at 18oC to keep the gal80 repression on till 7days AEL (equivalent to 60hrs at 25ºC). Following this, the vials were shifted to 29oC to express the RNAi to downregulate the desired gene function. (B-D Compare to control, loss of Hh from the niche post 60 hrs AEL by Col-Gal4 (C) or Antp-Gal4 (D) causes a decline in progenitor number (Ance) compared to control (A). Reduced progenitor cells number are seen upon using Shg as another independent marker for progenitors (E-F). (G-H) An increment in differentiation is seen upon loss of Hh signaling from the niche (P1 red) compared to control (I). Overexpression of Hh in the niche results in the expansion of progenitors and concomitant loss of differentiated cells (P1). (J-K) Down-regulation of Smo function in the progenitors (green) affects their maintenance while activation of Smo sustains their maintenance at the cost of differentiation (P1, red).

This targeted loss and gain of function data and expression analysis establishes the fact that niche-derived Hh indeed plays an essential role in progenitor cell maintenance and re-endorses the role of the niche in progenitor cell maintenance (Baldeosingh et al., 2018; Mandal et al., 2007).

5.2.2 Reduction in Hematopoietic niche cell number affects progenitor cell maintenance

To further probe into the role of Hh from the niche, the next approach was to reduce the number of niche cells by blocking their mitosis. The rationale was to test whether the reduction in the niche number and consequent decline in the strength of Hh signaling can affect the maintenance of the blood progenitors.

To block the cell cycle, the String knockdown was performed with two niche-specific gal4, Antp-Gal4, UAS-GFP (Mandal et al., 2007), and pCol-Gal4, UAS-GFP (Krzemien et al., 2007) in conjunction with gal80ts following the timeline is shown in Figure 5.2A. Since String is required for the G2-M transition (Edgar and O'Farrell, 1990), the down-regulation of Stg in the niche drastically reduced the niche number (Figure 5.2A-E). The depletion of Stg using Antp-Gal4, UAS-GFP, or pCol-Gal4, UAS-GFP resulted in an increment in the differentiation of the hematopoietic progenitor cells (E-G).

The above results demonstrate that the reduction in niche numbers directly affects the maintenance of the lymph gland progenitors. Since a similar response is evident with the loss of hh or its downstream players, it can be concluded that Hh from the niche is a crucial player for progenitor maintenance.

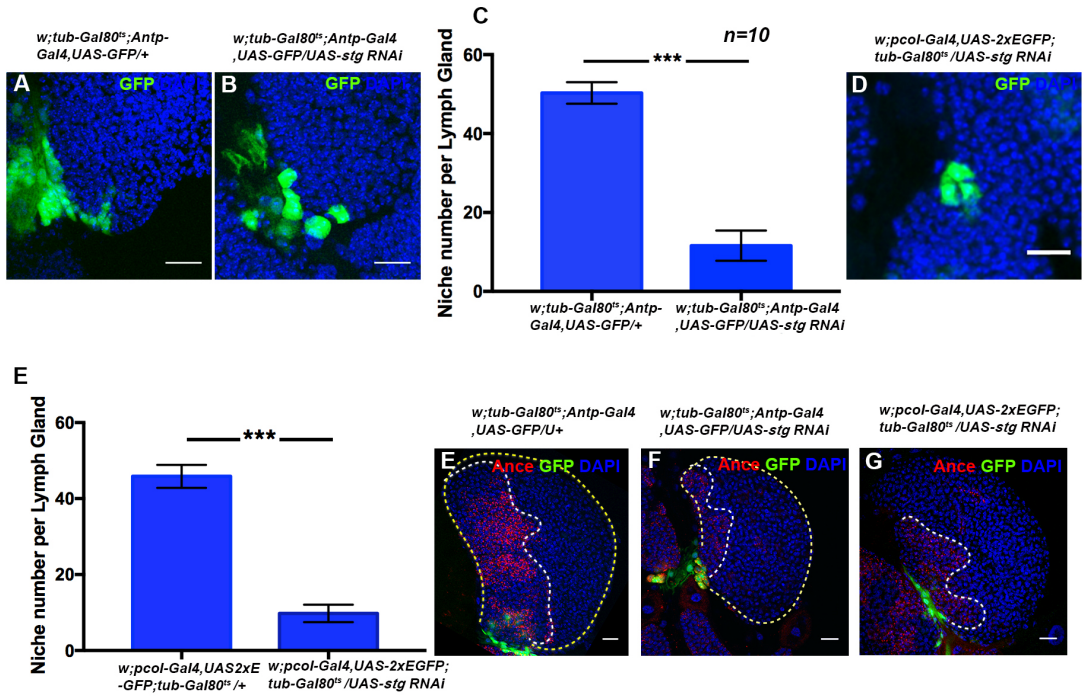

Figure 5.3 Reduction in niche cell number affects the progenitor cell number in the LG. (A-E) Down-regulation of string function in the niche either antp-Gal4 (A-C) or col-Gal4 (D-E) affects the niche cell number. P-value for tubgal80ts;antp-Gal4UASGFP>UAS-StgRNAi:4.349x10-7, Col-Gal4UASGFP;tubgal80ts>UAS-StgRNAi:7.163x10-6. Reduction in the Hh positive niche cell via string RNAi by Antp-Gal4(F) or Col-Gal4(G) causes a decline in progenitor number (Ance) compared to control (E).

5.2.3 Rate of proliferation of the niche cells declines with development

At the late phase of embryogenesis, niche cells account for ~15% of LG's total size (Jung et al., 2005; Pennetier et al., 2012). In comparison, the third instar stage only accounts for ~1% of LG's

total size, indicating a differential proliferation rate of a niche compared to other zones of the developing LG. Niche-specific loss and gain of function of Wg, Dpp, InR, Lar, PI3K, and dMyc experiments have established that niche cell number is tightly controlled in developing LG (Banerjee et al., 2019; Benmimoun et al., 2012; Dragojlovic-Munther and Martinez-Agosto, 2012; Kaur et al., 2019; Sinenko et al., 2009). It has been shown that the regulation of niche proliferation via the above pathways is essential for the maintenance of LG homeostasis. However, temporal profiling of niche proliferation with age in developing lymph glands is not worked out.

To determine the dynamics of proliferation, the niche cell number was quantified in the different developmental timeline (48 AEH, 72 AEH, 96 AEH, and 106 AEH). Interestingly, it was found that the niche number increased with age (Figure 5.3A), but its rate of increment was different in various developmental stages (Figure 5.3B). Quantitation of the above results suggests that increment at 48-60 hrs AEH and 60-72 hrs AEH was higher than increment at 96 hrs. In contrast, after 96 hrs AEH, the increment of the niche cell number was reduced (Figure 5.3B).

Next, the rate of proliferation was analyzed in different larvae stages. EdU labeling (green), which marks cells in the S phase, was used to detect the cell cycle progression in the niche (RFP).

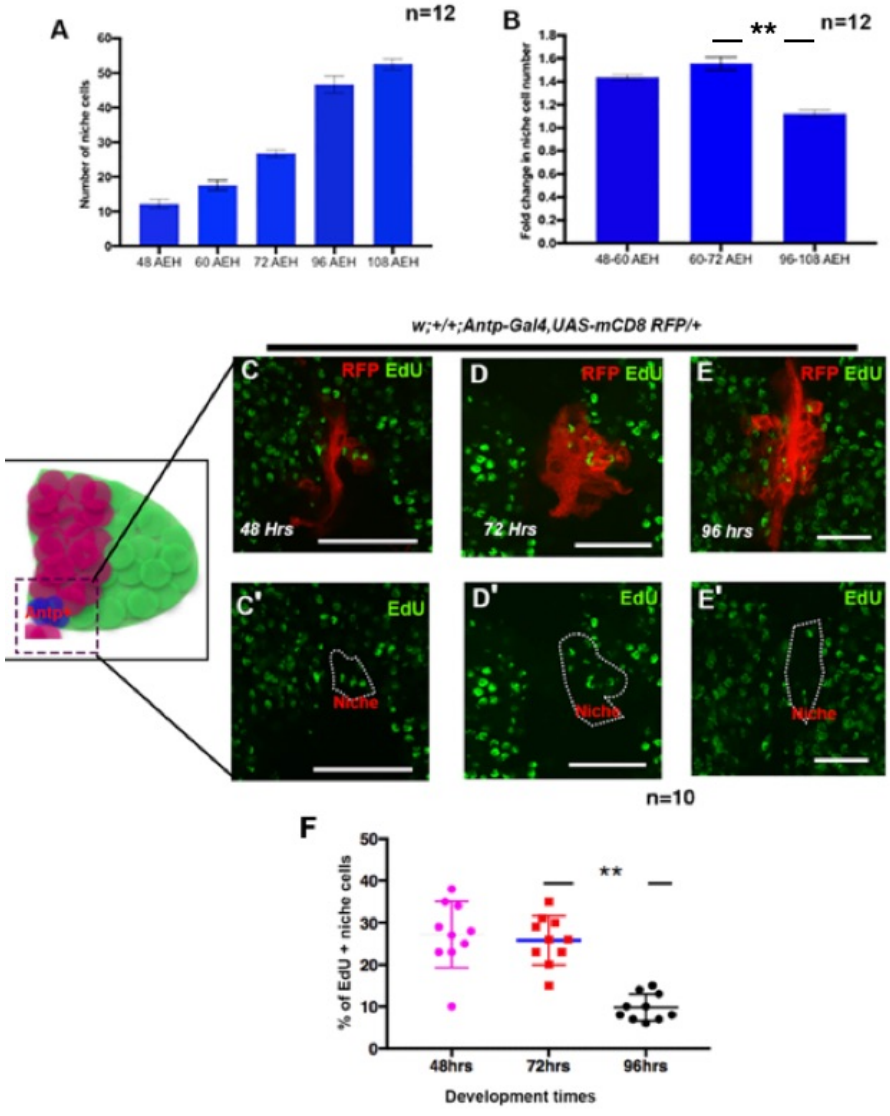

Figure 5.4 Niche cell proliferation reduced with age in developing the lymph gland(LG). (A) Quantification of Niche cell number with age in the developing LG. (B) Quantification of change in the niche cell number with age in the developing LG. (C-E') EdU incorporation at 48 AEH (C-C'), 72 AEH(D-D'), and 96 AEH (E-E') in the LG. A substantial reduction in EdU incorporation was seen in 96 AEH compared to 72 AEH and 48 AEH. (F) Quantification of EdU incorporation in the niche in 48hous, 72 hours, and 96 hours (P=1.678x10-6). Genotypes are shown on top of the corresponding panels scale bar=20μm. DAPI marks the nucleus.

The analysis of EdU suggests that there was a high proliferation of niche cells (Antp-Gal4, UAS-RFP) at 48 AEH (Figure 5.3C-C'), which persisted until 72 AEH (Figure 5.3D-D'). However, at 96 AEH, the EdU incorporation was drastically reduced, suggesting the decline in the proliferation of niche cells by the late third instar (Figure 5.3E-E'). Quantitation of the above results suggests that at 48 AEH and 72AEH, an average of 30% niche cells (Antp-Gal4, UAS-RFP) are dividing in the LG. In contrast, an average of 9% cells of the niche divides at 96 AEH (Figure 5.3F).
These results suggest that the rate of proliferation of the hematopoietic niche declines with age in developing lymph glands. Next, it was analyzed that how this niche proliferation is regulated in the developing LG with age.

5.2.4 Yorkie activity can be correlated with pattern of proliferation observed in hematopoietic niche

In the process of investigation, it was found that the activity of Yorkie (Yki), a transcriptional co-activator of the hippo pathway, was altered in the hematopoietic niche with age. Hippo is a well-conserved signaling pathway composed of two major kinases—Hippo (Hpo; Mst in the vertebrates) and Warts (Wts; Lats in the vertebrates) (Snigdha et al., 2019). Hpo activates Wts, which phosphorylates the transcriptional co-activator Yorkie (Yki, Yap, and Taz in the vertebrates). Phosphorylation of Yki leads to inhibition of its function by preventing it from accumulating in the nucleus (Staley and Irvine, 2012). Accumulation of Yki in the nucleus activates transcription of genes involved in promoting cell proliferation (Figure 5.5A).
To investigate the Yki activity in the developing niche, the expression of Diap1, a well-established downstream target of Yki, was examined (Figure 5.5B). The Diap1-GFP (Diap1-4.3-GFP) reporter lines harbor the GFP under the promoter that contains Yki binding sites (Zhang et al., 2008). Importantly, Diap1-GFP responds to alteration in Hippo signaling in the niche, as inhibition of Wts in the late third instar can increase its expression (Figure 5.5D-D') compared with wild-type niches (Figure 5.5C-C').

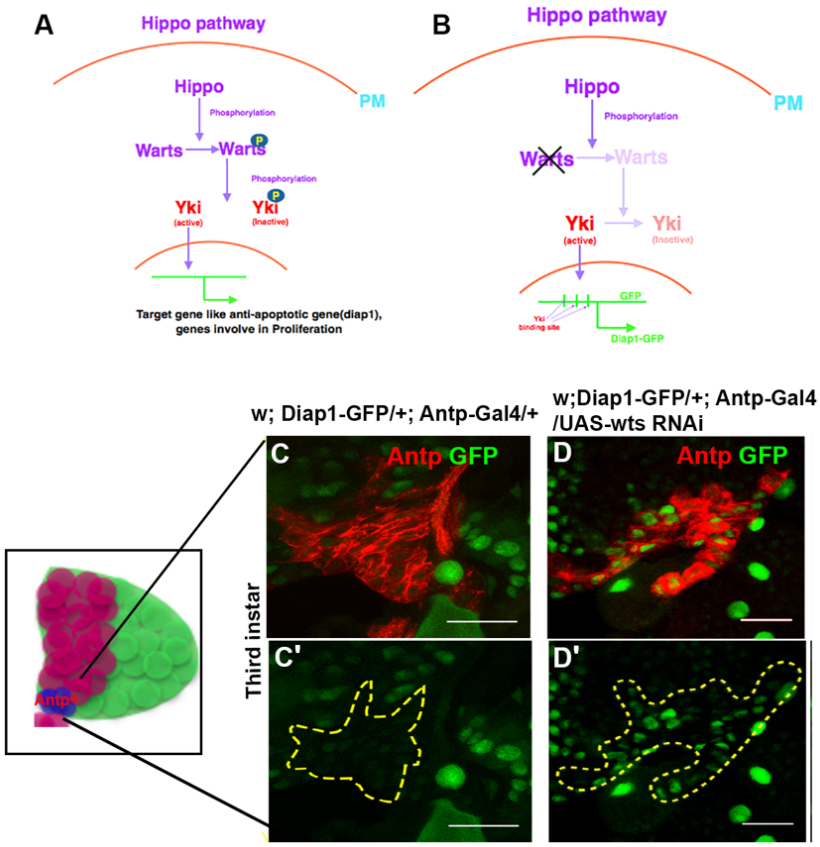

Figure 5.5 Yorkie activates Diap1-GFP in the niche. (A-B) Schematic representation of Hippo signaling. (A) Hippo pathway is composed of two major kinases—Hippo and Warts. Hpo activates Wts, which phosphorylates the transcriptional coactivator Yorkie and preventing it from accumulating in the nucleus. Accumulation of Yki in the nucleus activates transcription of genes involved in promoting cell proliferation. (B) Upon inhibition of Wts activates Yorkie, which can activate the target gene, including Diap1-GFP. (C-D') Basal level Yorkie activation in the wild type niche (red) indicated by a low level of Diap1-GFP expression (C-C'), which enhances upon inhibition of Wts in the niche (D-D').

Upon visualizing the Diap1-GFP expression in the hematopoietic niche at the different developmental timeline (48 AEH, 72 AEH, and 96 AEH), it was found that Yki activity was higher in early hours (48AEH and 72AEH), and its expression drastically reduced with age in developing hematopoietic niche (Figure 5.6A-C'). Quantitation of the above results suggests that the expression of Diap1-GFP at 48 AEH and 72 AEH was significantly higher than its expression in 96 AEH (Figure 5.6D).

The above results suggest a higher requirement of Yki activity in the early instar niche compare to the late third instar stage. Coincidently, the hematopoietic niche's proliferation pattern also seems to follow the same timeline (Figure 5.4C-F).

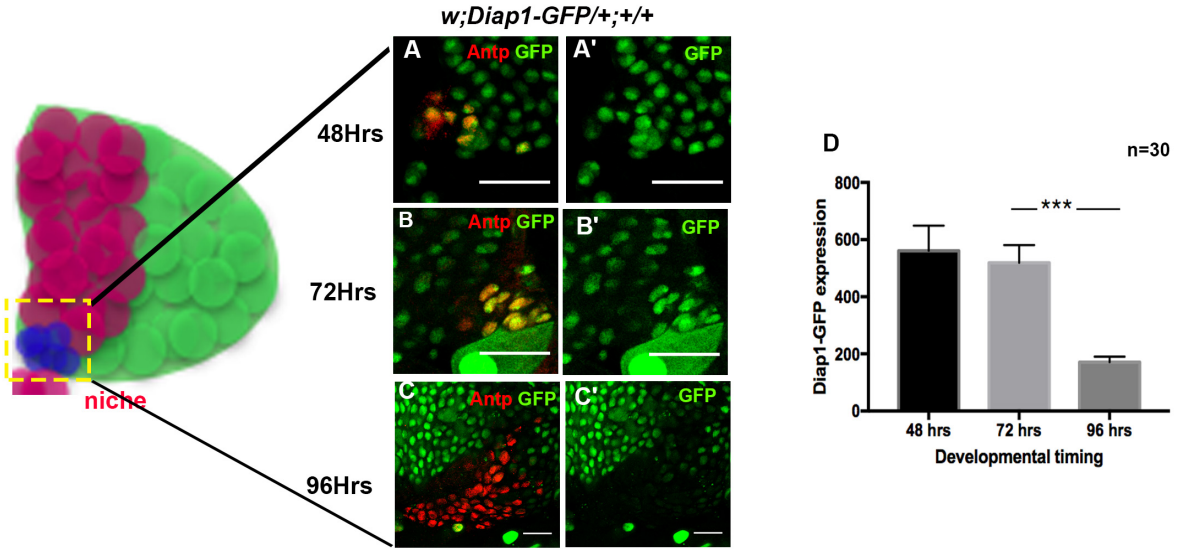

Figure 5.6 Yorkie activity decrease with age in the Hematopoietic niche. (A-C) Schematic representation of LG. Niche(Blue), Progenitor (Red), and Differentiated cell(green). Diap1-GFP(green), a reporter for Yorkie activity expression in the niche (red) at 48 hrs (A-A), 72 Hrs(B-B), and 96 Hrs (C-C). (D) Quantification Diap1-GFP expression reveals a stark decrease in Yorkie activity at 96 Hrs compared to 48 Hrs and 72 Hrs. (P-value= 3.29x10-5). Genotypes are shown on top of corresponding panels. Scale bar=20µm. DAPI marks the nucleus.

Next, the Yki activity and proliferation status were analyzed simultaneously in the hematopoietic niche to correlate the Yki activity with hematopoietic niche proliferation. In this experiment, EdU labeling detects the proliferation (shown in magenta), and Diap1-GFP (green) was analyzed in the different developmental timelines (48 AEH, 72 AEH, and 96 AEH). In the early instar (48 and 72 AEH), all the EdU positive cells in the niche were positive for Diap1-GFP (Figure 5.7A-B'). However, this was not the case in 96 AEH (Figure 5.7C-C''). Quantitation of the above results suggests that in early instar (48 AEH and 72 AEH), an average 80% dividing cell of the niche (EdU) was positive for Diap1. In sharp contrast, an average of 11% dividing niche cells was positive for Diap1-GFP at 96AEH (Figure 5.7D).

The above results suggest that the proliferation of the *Drosophila* hematopoietic niche cells declines with development and is in sync with the Yki activity in the developing niche cells.

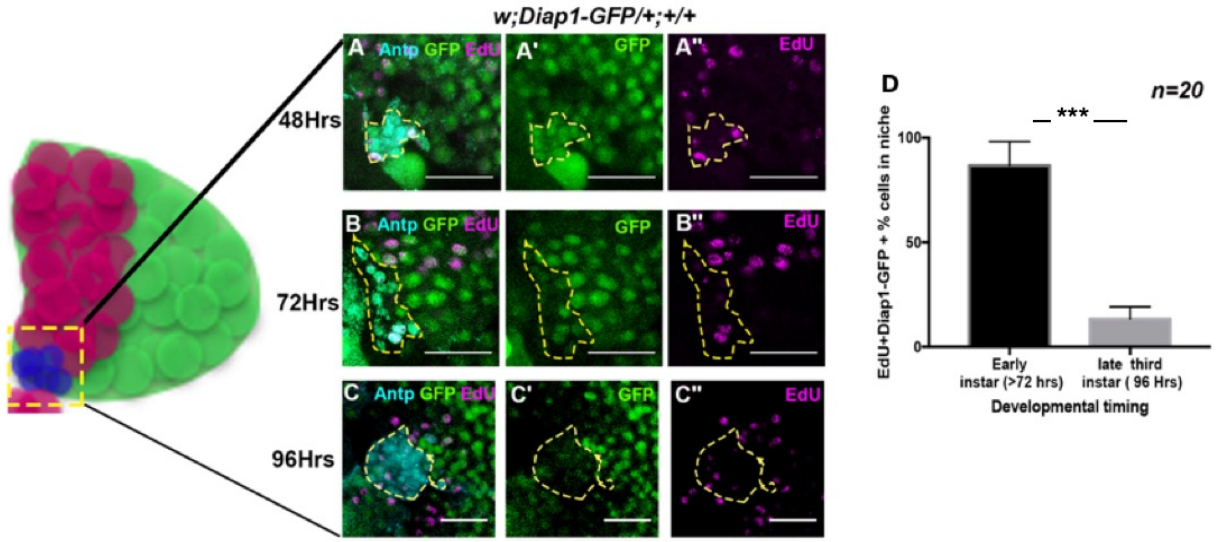

Figure 5.7 Diap1+EdU+ cells decline with ages in the hematopoietic niche. (A-B) Proliferative cells in S-phase (EdU+; Magenta) express Diap1 in the 48Hrs (A-A), 72 Hrs(B-B), and 96 Hrs. (D) Quantification of Diap1-GFP+ and EdU+ cells reveals a decline in the Diap1+EdU+ cells with age. The EdU+ niche, which is also positive for Diap1, decreased in 96 hours compared with the early hours (before 72 hrs), suggesting a relation between reduced Yorkie activity and decline in proliferation in late hours. (P-value= 9.070x10-6). Genotypes are shown on top of corresponding panels. Scale bar=20µm. DAPI marks the nucleus.

To determine the functional relationship between Yki activity and *Drosophila* hematopoietic niche proliferation, Yki was next down-regulated in the niche. The knockdown of Yki was performed using the expression of RNAi against the Yki in the niche. The effect of reduced Yki activity on hematopoietic niche cells resulted in a drastic reduction of the cell number, indicating that Yki activation is essential for niche cell proliferation (Figure 5.8D). The involvement of Yki in hematopoietic niche proliferation was further confirmed by increasing the Yki activation in the niche. Yki activation was performed in two ways: first, via inhibition of Warts (Wts), a negative regulator of Yki, and second by overexpression of Yki in the hematopoietic niche. Interestingly, activation of Yki via Wts RNAi (Figure 5.8C) or over-expression of Yki (Figure 5.8A-B) drastically increases the niche number. Quantitation of the above results suggests that the up-regulation of the activity of Yki leads to an increase in the niche cell number (Figure 5.8E).

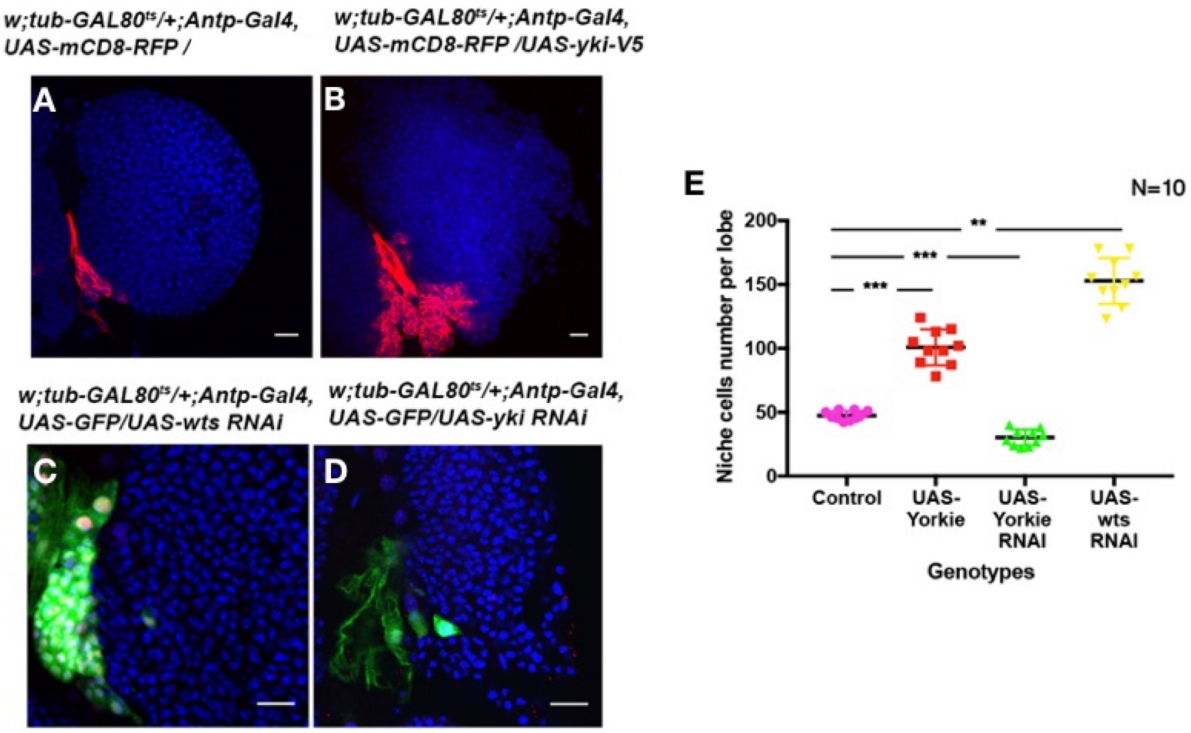

Figure 5.8 Yorkie activity regulates the maintenance of the hematopoietic niche. (A-B') Increment of Yki activity via overexpression of Yki (B-B') or inhibition of Wts (C-C') leads to increment in niche cell number. In contrast, down-regulation of Yki activity in the niche (red) leads to a strong reduction in the niche cell number compared to wild-type niches (A-A'). (C) Quantification of niche cell number of above genotype (P- value= UAS-yki; Antp-Gal4, UAS-mCD8RFP =3.490x10-6; Antp-Gal4,UAS-GFP/UAS-yki RNAi=1.763x10-6; Antp-Gal4,UAS-GFP/UAS-wts RNAi p=6.7041x10-7). Scale bar=20µm. DAPI marks the nucleus.

Next, it was analyzed whether knockdown of Wts, which leads to the increased activation of Yki, was sufficient to induce the niche cell proliferation. EdU incorporation was used to detect the proliferative cells in the third instar stage. In the 96 hours, the % of EdU positive niche cells in the Wts RNAi was drastically increased (Figure 5.9A-B') compared to control, suggesting that ectopic activation of Yki in the third instar is sufficient to enhance the proliferation within the niche. The Quantitation of the above results further endorses the high proliferation evidenced in the late third instar niche (Figure 5.9C).

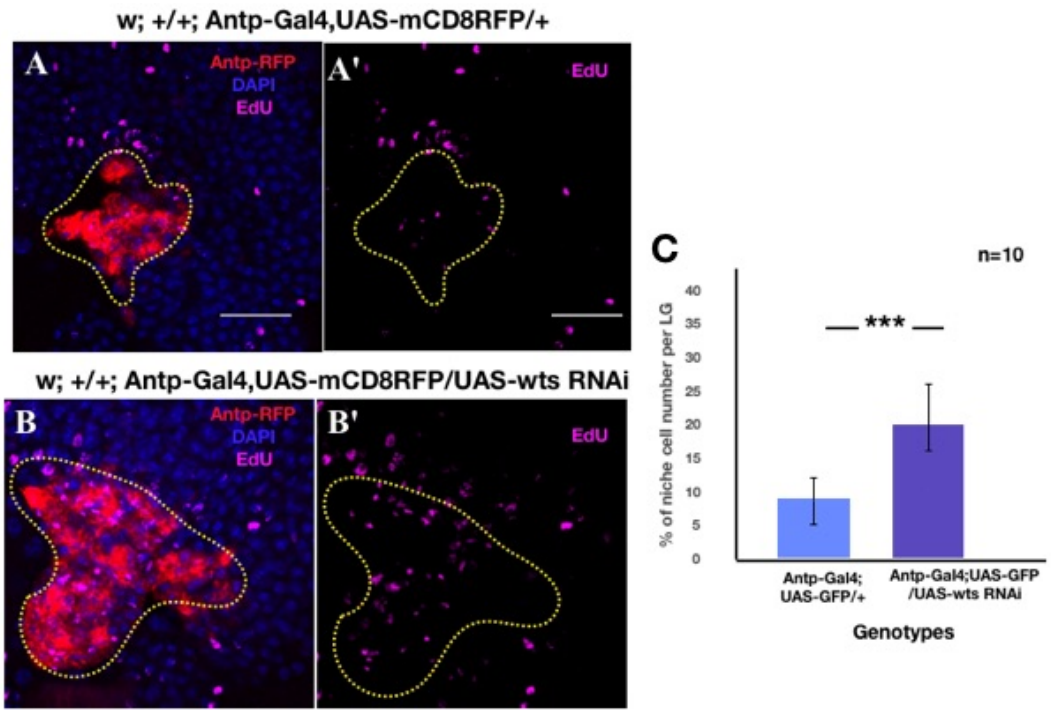

Figure 5.9 Ectopic activation of Yorkie activity via inhibition Wts leads to increase proliferation in the niche. (A-B') Down-regulation of Wts (B-B') in the niche (red) leads to a substantial increment in EdU incorporation (magenta) in the niche compare to wild type niche (A-A'). (C) Quantification of EdU incorporation in wild type niche and Wts RNAi ($p=6.7041x10^{-6}$). Scale bar=20µm. DAPI marks the nucleus. Yellow dotted line marks the niche.

Taken together, these results suggest that Yki activation positively regulates hematopoietic niche cell proliferation, and likewise, decreases the activity of Yki in the late third instar larvae was responsible for the reduced proliferation of hematopoietic niche.

5.2.5 Actomyosin regulates Yki activation in the hematopoietic niche.

Next, it was analyzed how Yki activity regulates proliferation in the developing hematopoietic niche. One likely candidate to drive the Yki activation would be enhanced actomyosin cytoskeleton (Benham-Pyle et al., 2015; Rauskolb et al., 2014; Sun and Irvine, 2011). It has been previously shown that the substantial accumulation of F-actin can be associated with the activation of Yki. Intriguingly, actin expression also shows a similar pattern to Yki activation and proliferation in the *Drosophila* hematopoietic niche. The F-actin was visualized in this study by using the actin-binding protein: Moesin expression via life-actin GFP. Indeed, the actin-binding protein was high in the early instar (48 AEH and 72 AEH) (Figure 5.10A-B) while its levels significantly declined in the late third instar niche (96AEH) (Figure 5.10C-C'). The above results suggest that developing hematopoietic niche expressed different level of actin at different development stages (Figure 5.10D).

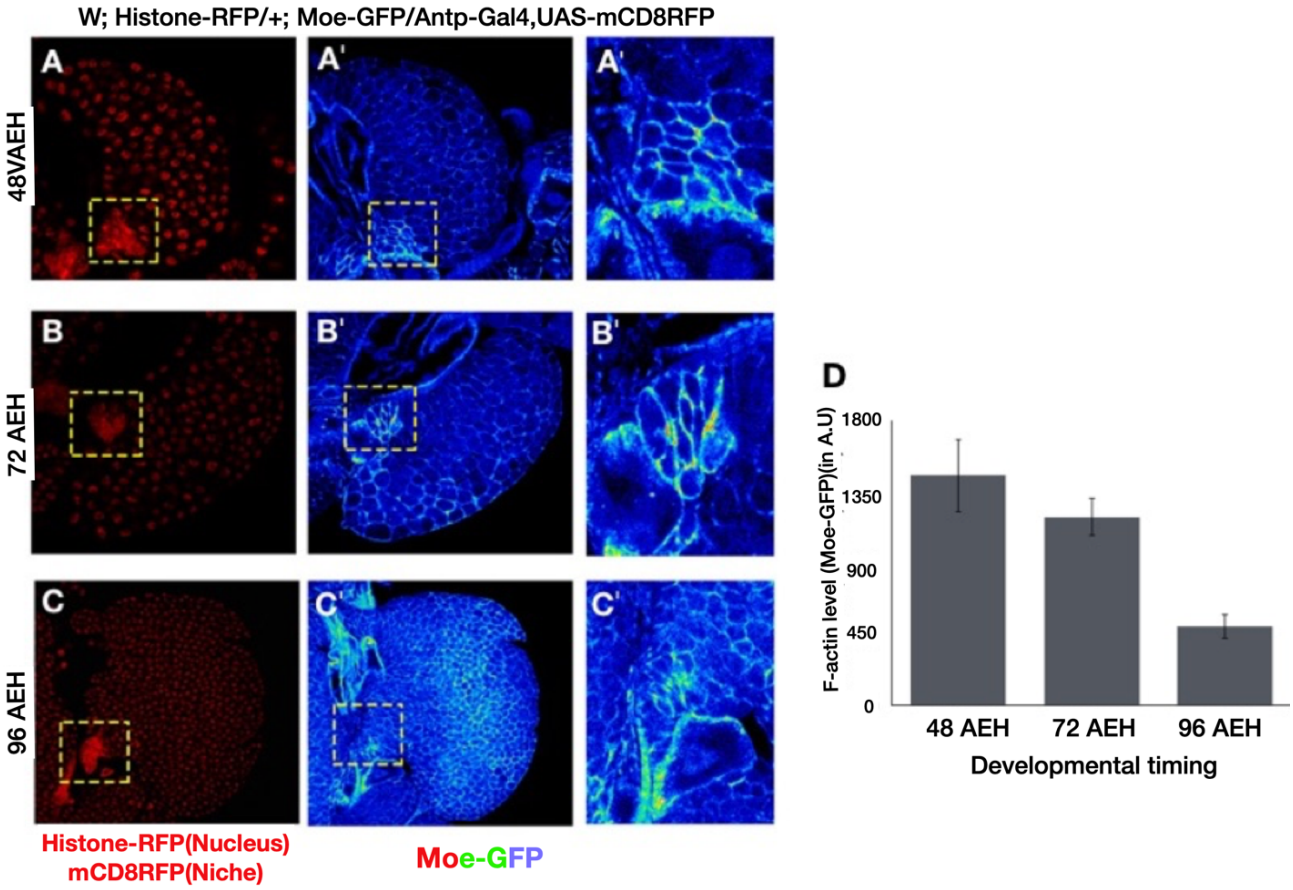

Figure 5.10 Actomyosin assembly decrease with age in the hematopoietic niche. (A-A") Actin expression visualised via moe-GFP is highly enriched in the early hematopoietic niche (48 hrs AEH and 72 hrsAEH), which down-regulated in the late third instar (96 hrs AEH). Zoom image of the niche (red; box) from A', B,' C' indicates a decline in actin expression in 96 hrs AEH (C') compared with 48 AEH (A') and 72 hrs AEH (B'). (D) Quantification of Moe-GFP in niche cells reveals a decline in the F-actin expression with age in the developing hematopoietic niche.

In order to compare actin expression directly with Diap1-GFP expression, Phalloidin, which has a strong binding capacity with F-actin (Warn and Robert-Nicoud, 1990) was employed to monitor actin levels in the niche at different development times along with Diap-GFP expression. Similar to Yki activity, the level of actin in the developing niche in different developmental time point was not uniform ((Figure 5.11A-C). Instead, it was highly enriched in the Diap1-GFP enriched early instar niche cells (Figure 5.11A-B"). Quite notably, levels of GFP in niche cells of late third instar is drastically reduced (Figure 5.11C-C"). The similarity in the dynamicity of the expression pattern of F-actin and Yki activation during proliferative state of niche cells strongly strongly implicate that actomyosin might niche cells proliferation via Yki activity.

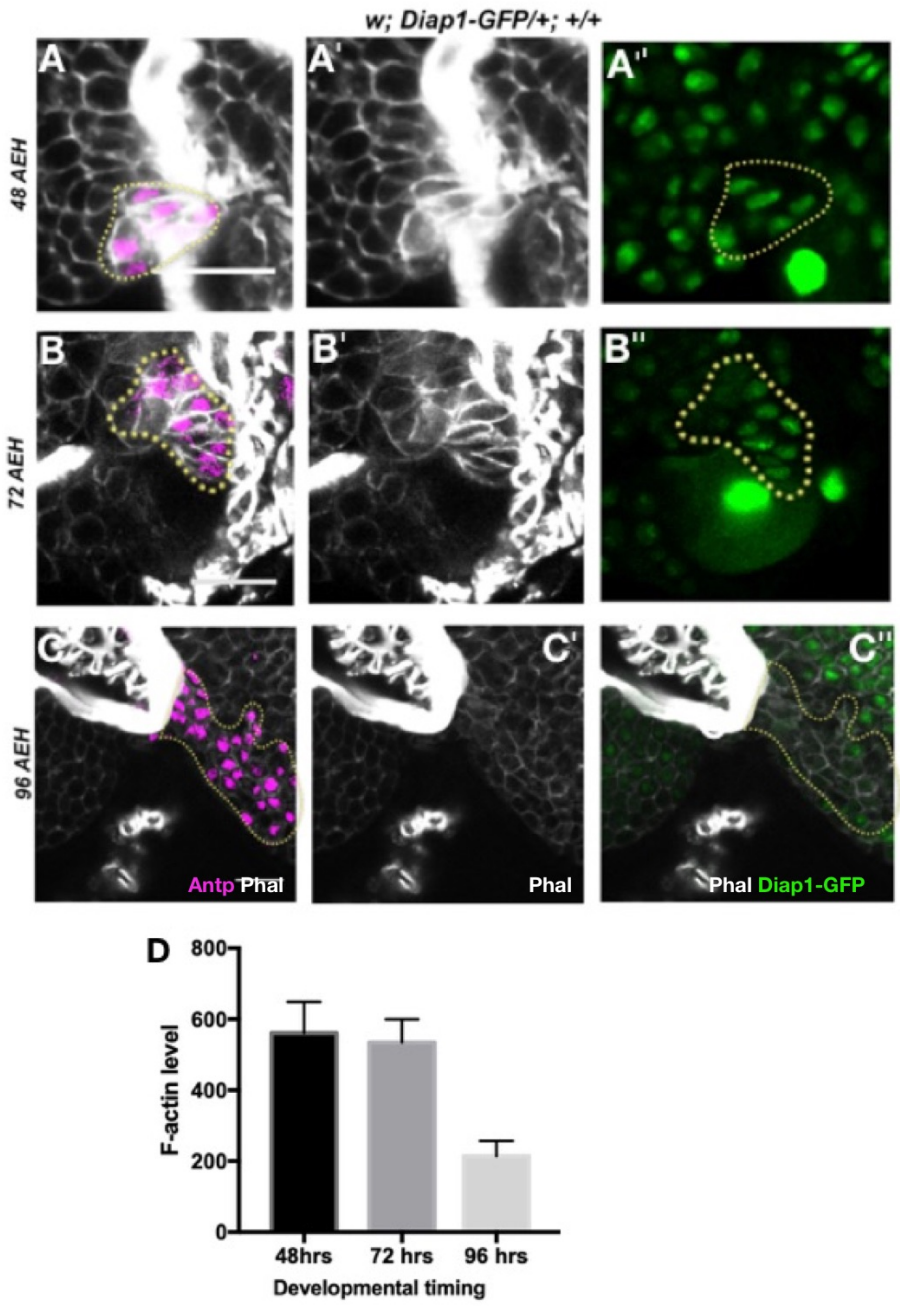

Figure 5.11 Reduction in Actomyosin assembly correlated with decrease Yki activity with age in the hematopoietic niche. (A-B) Early hematopoietic niche (Antp; Magenta) (48AEH) expresses a high level of actin (Phal; gray) and Diap1-GFP (green), which maintain till 72 AEH. In the late third instar, the expression of both actin and Diap1-GFP decreases. (D) Quantification of Actin in niche cells reveals a decline in the actin expression with age in the developing hematopoietic niche. Genotypes are shown on top of corresponding panels. Scale bar=20µm. DAPI marks the nucleus.

To investigate the influence of altered actomyosin activity on niche cell maintenance, the actomyosin activity was enhanced in the hematopoietic niche, and niche cell numbers were examined. ROCK was overexpressed in the niche using pCol-Gal4 to increase the actomyosin

activity in the niche. Similar to Yki over-activation, the expression of constitutively active forms of ROCK (UAS.ROCK) in the niche drastically increases the hematopoietic niche cell number (Figure 5.12A-C). This result was further endorsed by analyzing the Sqh-E21, a mutant that mimics the active form of sqh (Rauskolb et al., 2014) . Similar to ROCK over-activation, Sqh-E21 also shows an increment in the niche cell number (Figure 5.12D-E). Furthermore, the inhibition of Sqh drastically reduced the niche cell number (Figure 5.12F-H), suggesting that actomyosin positively regulates hematopoietic niche maintenance.

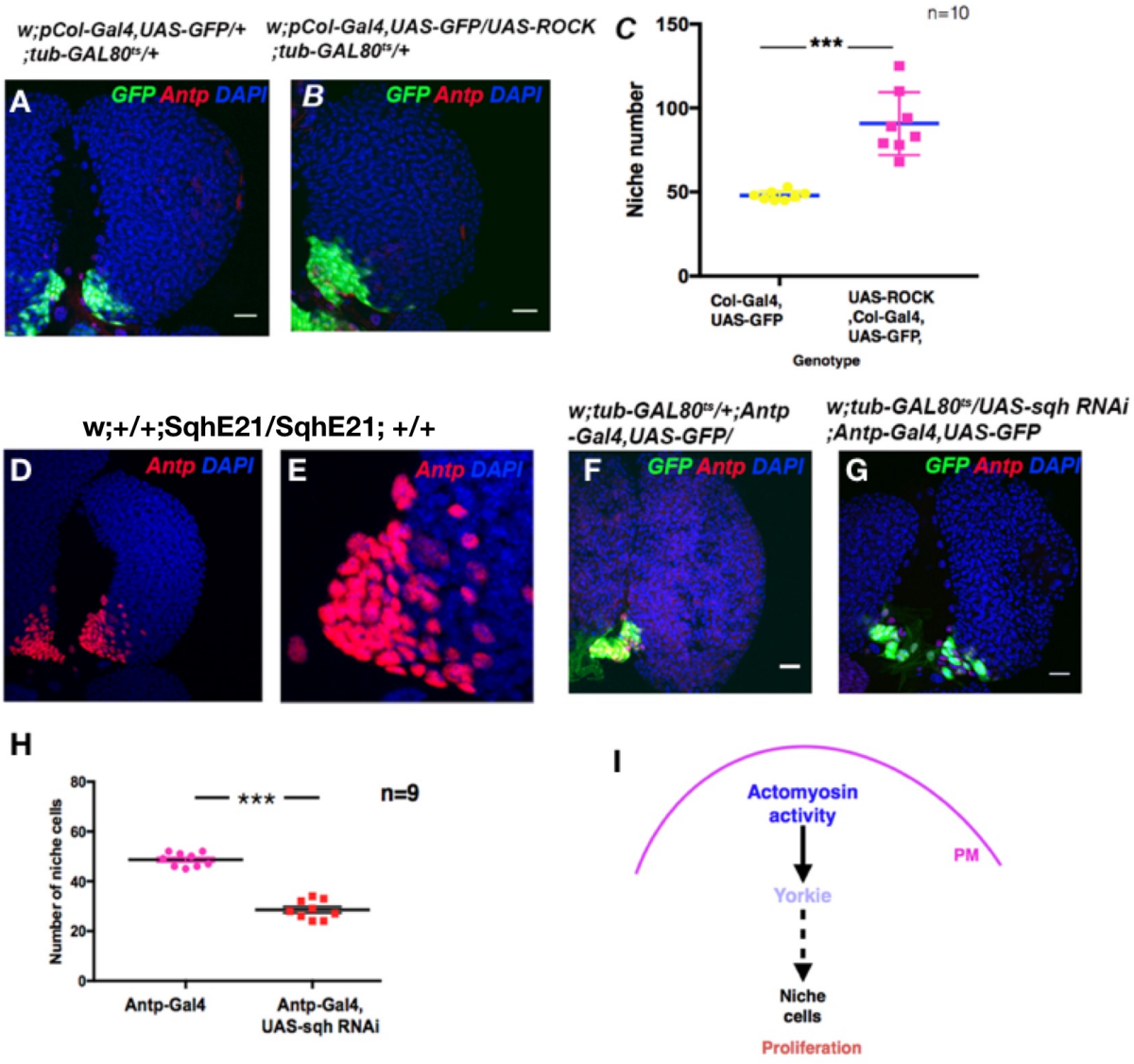

Figure 5.12 Actomyosin assembly regulates the maintenance of the Drosophila hematopoietic niche. (A-B') Overexpression of ROCK in the hematopoietic niche resulted in an increment of niche cell number compared with the wild type niche. (H) Quantification of niche cell number upon overexpression of ROCK (P= 8.620x10-87.(D-E) Niche cell number also increases in SqhA2021, a mutant form of sqh, mimicking the active form of sqh. (F-G) The downregulation of Sqh in niche leads to a drastic reduction in niche cell numbers compare with the wild type niche. (H) Quantification of niche cell number in sqh RNAi (P=

5.63x10-8). (I) A model describing the involvement of actomyosin assemble in the regulation of Drosophila hematopoietic niche cell maintenance.

Next, the effect of actomyosin knockdown on Yki activation in the hematopoietic niche was analyzed. The perterbation of actomyosin activity via inhibition of sqh or zip activity in the niche cells in early instar reduced the diap1 expression (Figure 5.13A-B"), suggesting that actomyosin regulates Yki in the hematopoietic niche. Furthermore, the activation of Yki can be maintained in the late third instar niche by expressing constitutively active forms of ROCK (UAS.ROCK) in the niche (Figure 5.13C-D').

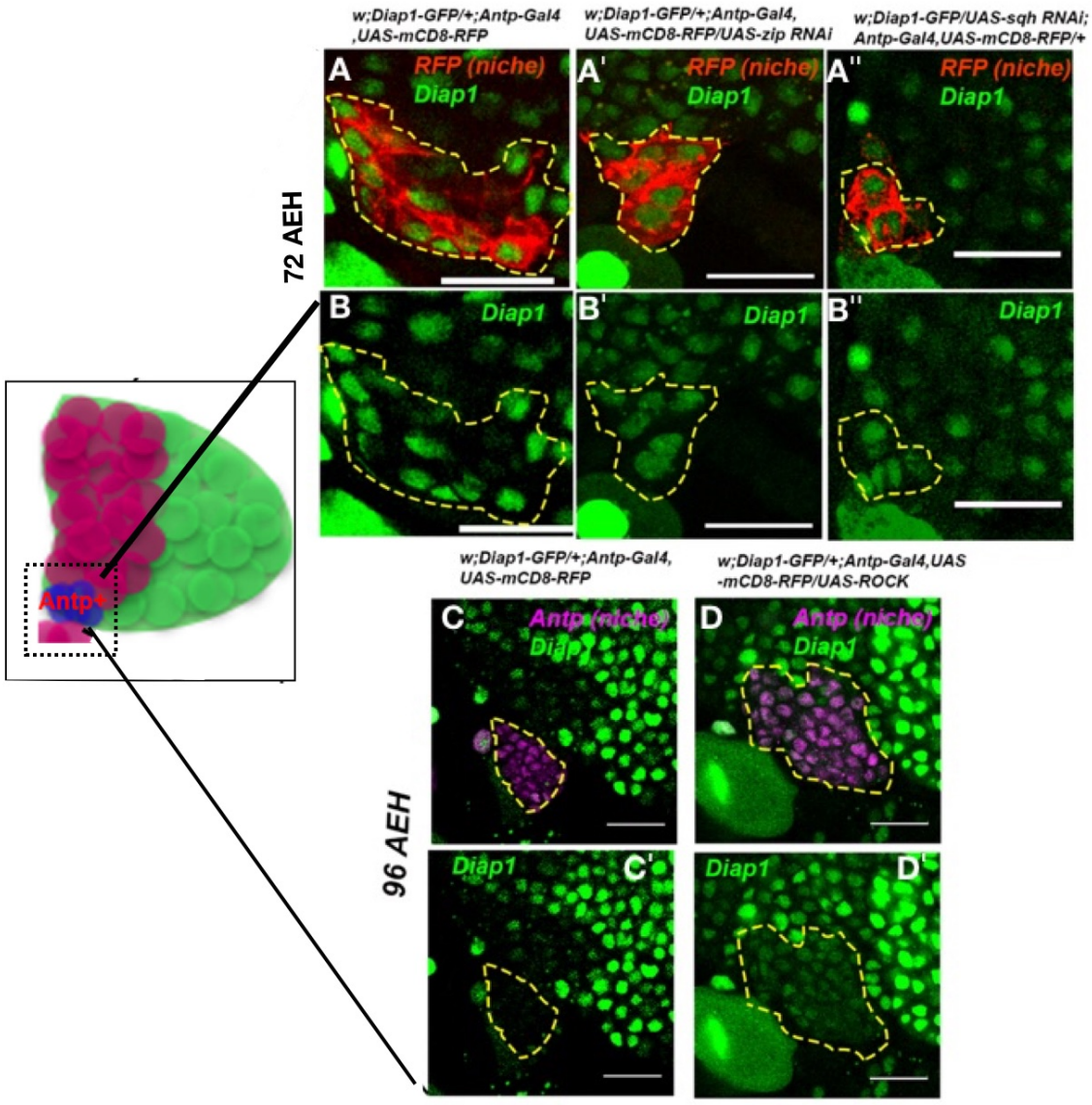

Figure 5.13 Actomyosin assembly regulates Yorkie activity in Drosophila hematopoietic niche. (A-C'') Downregulation of Sqh or Zip in the niche, which expresses high level of Diap1-GFP (72AEH), shows a reduction in Diap1-GFP expression compare with the niche with the same age. (D-E') Overexpression of ROCK in late third instar hematopoietic niche leads up-regulation of Diap1-GFP expression compared with the wild type niche.

Taken together, these results confirm that the actomyosin–Yki pathway is required for regulation of the hematopoietic niche cell proliferation in the *Drosophila* hematopoietic niche. These data also indicated that Yki acts downstream of actomyosin to regulate the proliferation of the niche cells.

5.2.6 Neighbouring myoepithelial cardioblast regulates Actomyosin-Yki cascade in *Drosophila* Hematopoietic Niche

The above results collectively suggest a model in which actomyosin induces the Yki activation, which is essential to regulate the hematopoietic niche cell proliferation. The temporal expression pattern of F-actin and Yki revealed the differential expression of these molecules in the developing niche. Thus, it raises how the actomyosin-Yki cascade can regulate the cells of the hematopoietic niche. As a part of the team engaged in the genome-wide screen to find regulators of niche maintenance, I had picked up Zasp, whose downregulation by Antp-Gal4, UAS-GFP leads to increased niche cell numbers (Figure 5.14D-F) and non-autonomously activate Yki in the niche. Zasp/cypher is a member of the PDZ and LIM domains containing Alp/Enigma protein (Liao et al.,2016). It is predominately expressed in muscle cells and works as an adaptor protein to stabilize Z-lines in striated and cardiac muscle. Next, the Zasp is downregulated by a pCol85-Gal4, which is also known to express in the niche. Surprisingly, the depletion of Zasp using pCol85-Gal4 did not increase the niche cell number (Figure 5.14A-C), which in contrast to the outcome seen upon using Antp Gal4 (Figure 5.14E).

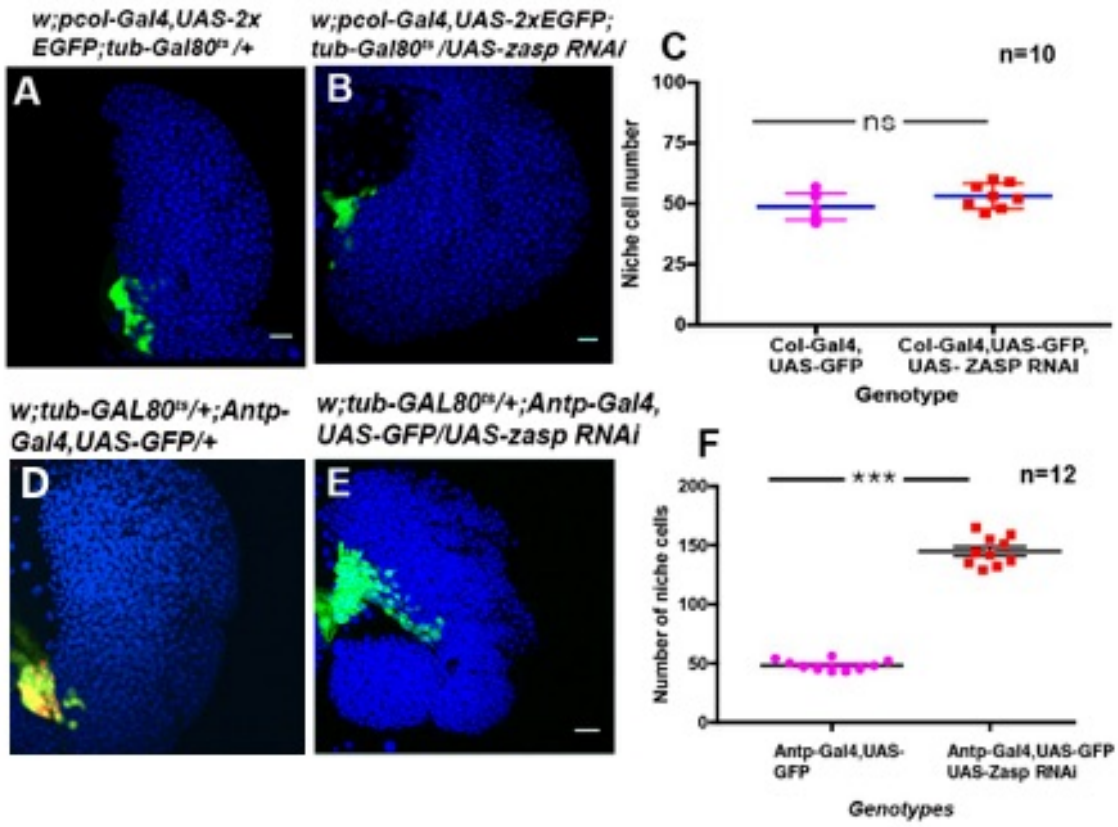

Figure 5.14 Knockdown of Zasp affects the maintenance of the Drosophila hematopoietic niche. (A-B) Expression of Zasp RNAi using Antp-Gal4 leads to increment in niche cells compared to wild type niche. (C) Quantification of niche cell number upon expression of Zasp RNAi (P= 4.760x10-6.). (D-E) The knockdown of Zasp using Col-Gal4, which also express in the niche, does not increase the niche cell number. Quantification of niche cell number upon expression of Zasp RNAi using Col-Gal4. Scale bar=20μm. DAPI marks the nucleus.

It was puzzling because both are validated drivers for the *Drosophila* hematopoietic niche. Through a closer examination of Antp-Gal4, UAS-GFP expression, it was noticed that apart from expression in the niche, Antp-Gal4 also expressed in four nearby cells (white arrow Figure 5.15B-B'), which lack pCol85-Gal4, UAS-GFP expression (Figure 5.15A-A'). Detailed characterization of these cells revealed that these Antp+Col- cells are the cardioblast as indicated by Z-band organization via F-actin (shown in magenta dotted line; Figure 5.15C-C"). These Antp + Col- myocardial cells can be visualized by Mef2-Gal4, UAS-mCD8-RFP, a pan muscle-specific driver (Cevik et al., 2019), while the Antp+ Col+ niche cells lack the expression of Mef2-Gal4, UAS-mCD8-RFP (Figure 5.15D-D"). Interestingly, this Antp+ cardioblast expressed a low level of Hand, a basic helix-loop-helix (bHLH) (Han et al., 2006) in comparison to the neighboring cardioblasts of the larval heart or dorsal vessel, which are enriched with Hand (Figure 5.15E-E"). The hand works as a transcription activator and plays evolutionarily conserved roles in cardiogenesis. It has been shown that it is involved in regulating the expression of genes encoding proteins essential to sarcomere formation (Hallier et al., 2015). The possible differential expression

of the Hand in the cardioblast regulates the differential ability of contraction in these cardioblasts. The above observation indicates that these Antp+Hand^{low} cardioblasts might differ from the rest of the cardioblast present in the anterior part of the dorsal vessel or the aorta. The above data also suggest that the downregulation of Zasp from just these four Antp positive cardioblasts is sufficient to cause an increment in the niche cell number.

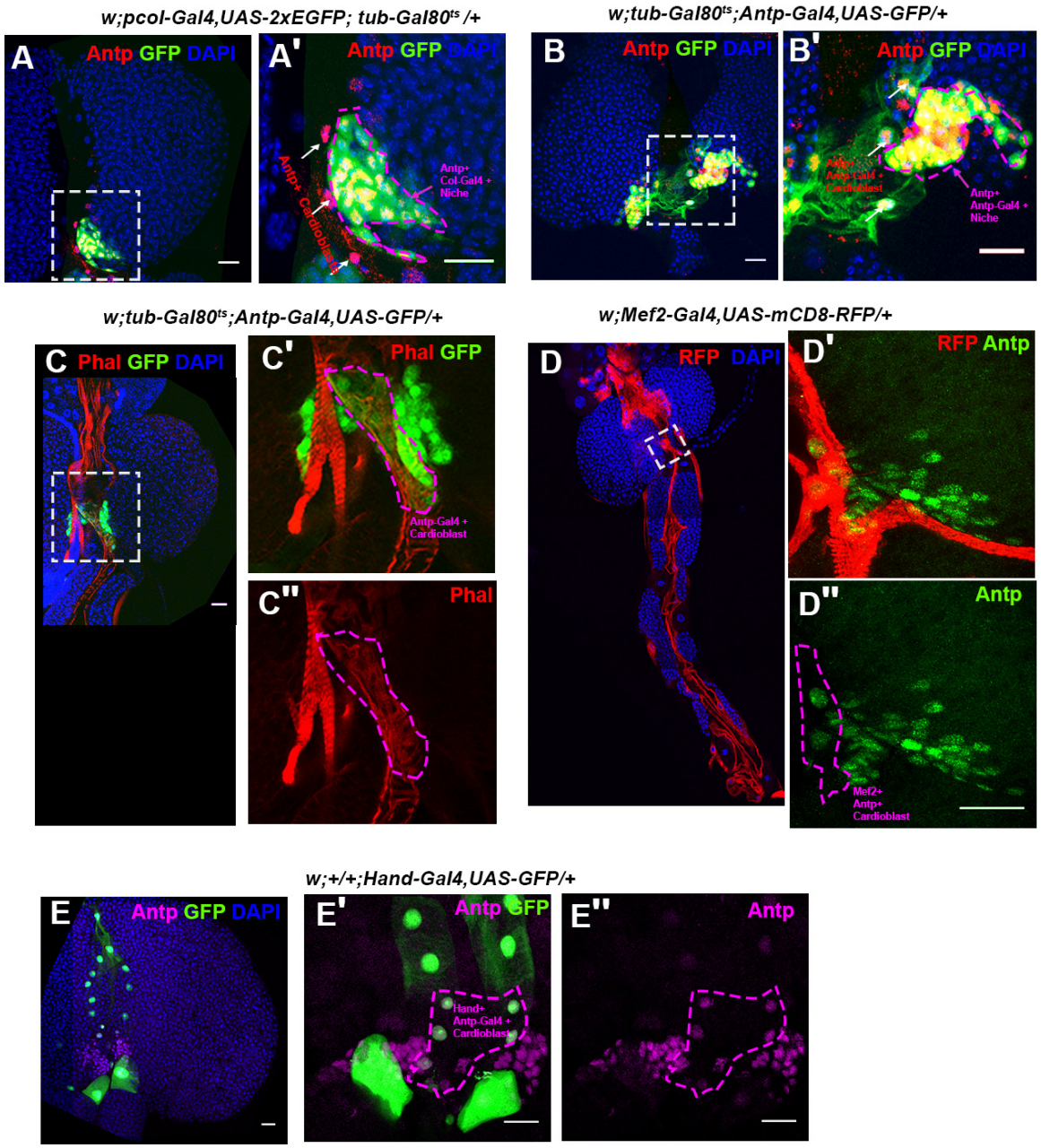

Figure 5.15 Loss Zasp in Antp positive cardioblast affects the maintenance of the Drosophila hematopoietic niche. (A-A) Co-expression of of Col-Gal4 (green) and Antp (red) reveals that Col-Gal4 expression only in the hematopoietic niche (magenta arrow). At the same time, Antp expresses in the niche and nearby cardioblast (white arrow). (B-B) Antp-Gal4 (Green) expression analysis reveals that Antp-Gal4 also express in nearby cardioblast (White Arrow). (C-C) These Antp positive cell are cardioblast (muscle)

evident by Z-band (Phal; red) pattern. (D-D) These Antp positive cardioblasts also express Mef2, pan muscle-specific protein. Above Gal4 analysis indicates that expression of Antp-Gal4 in the cardioblast might account for increase niche number phenotype when Zasp is knockdown using Antp-Gal4. (E-E) Analysis of Hand-Gal4 expression reveals that these Antp positive cardioblasts express low levels of Hand, suggesting the difference in the specification of these Antp positive cardioblasts from the rest of the Antp-Hand+ cardioblast.

The function of Zasp in the maintenance of muscle architecture and contractile machinery has been reported in C.elegans, *Drosophila*, zebrafish, and mouse system. In humans, mutations in ZASP are associated with cardiomyopathies and myofibrillar myopathies (Sheikh et al., 2007). Inhibition of Zasp in other *Drosophila* muscles, including associated ovary muscle, intestinal muscle, and IFM, is also associated with altered muscle integrity. Interestingly, knockdown of Zasp in these Antp positive cardioblast (green; white arrow) also disrupts the normal myofibril structure visualized by phalloidin staining (Red, compare Figure 5.16 A-A" with B-B").

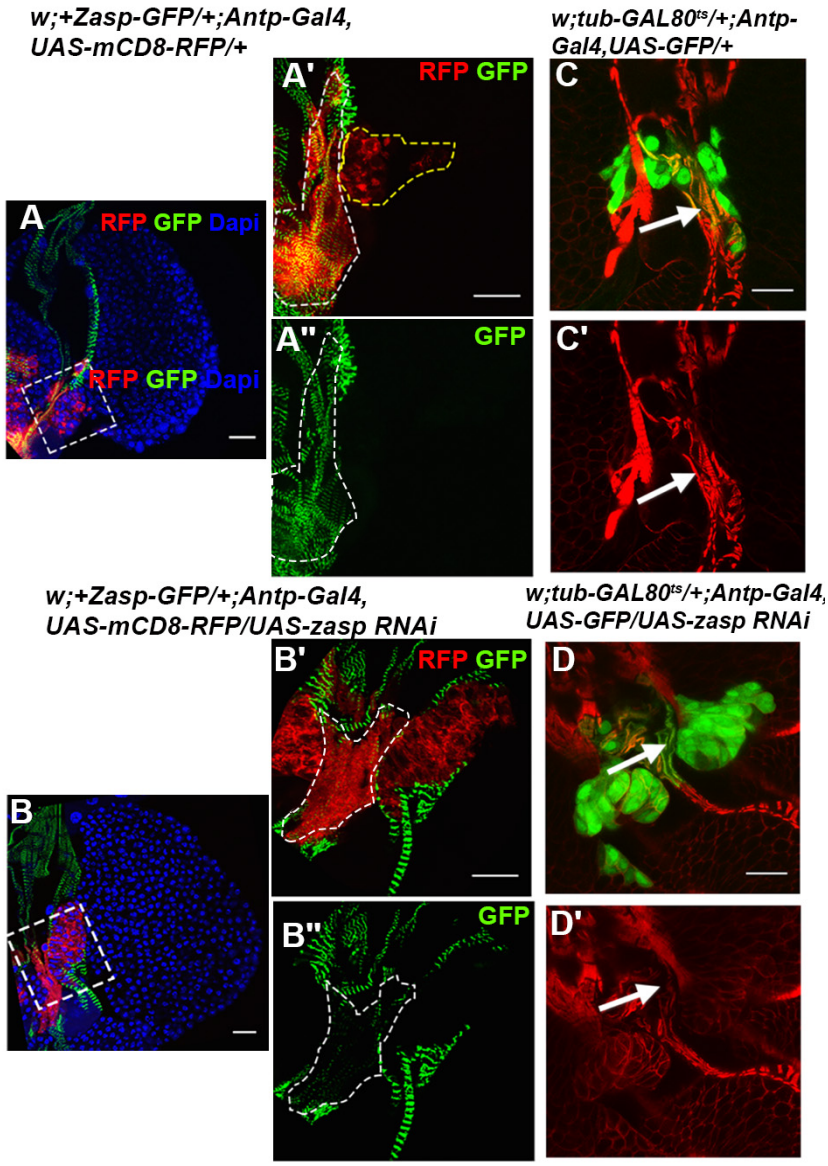

Figure 5.16 Loss Zasp in Antp positive cardioblast disturb muscle architecture. (A-A) Zasp-GFP expression in the nearby Antp positive cardioblast. (B-B) Expressing UAS-zaspRNAi using Antp-Gal4 disturbs Zasp-GFP expression in the Antp positive cardioblast (White dotted lines). Analysis of Sarcomere organization (phal; red) in a 3rd-instar larval muscle reveals that knockdown of Zasp disturbs muscle architecture in the Antp positive cardioblast.

Based on these data, it can be hypothesized that knockdown of Zasp in this Antp+ cardioblast severely affects the sarcomere structure, which non-autonomously affects the hematopoietic niche cell maintenance.

To test the above hypothesis, the knockdown of Zasp was performed using cardioblast-specific Antp-Gal4 and niche-specific Antp-Gal4. To create cardioblast-specific Antp-Gal4, the Antp-Gal4 was suppressed in the niche by using hematopoietic specific Gal80, which was recombined in the genotype by using the LexA-LexAop system (del Valle Rodriguez et al., 2011). In the lexA-Aop

system, LexA binds to and activates the lexA operator (LexAop). The lexA system is most often used in combination with GAL4, for instance, when one requires expressing any gene independent of GAL4. For this study, we expressed GAL80 in the lymph gland using hematopoietic specific LexA, which can suppress GAL4 expression in the hematopoietic niche (Figure 5.17A) and keep it on in the neighboring cardioblast of interest.

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Next, the different lexA enhancers from the Bloomington stock center were screened to search for the enhancer expressed in the lymph gland. Interestingly, Trio-lexA was exclusively expressed in the lymph gland. Expressing lexAOP-GAL80 under this enhancer was sufficient to suppress Antp-Gal4 in the hematopoietic niche (Figure 5.17B-C'), keeping it on only in cardioblasts (Figure 5.17C-C'; arrow in C'). A lineage tracing using the GTRACE technique (Evans et al., 2009) revealed that Antp-Gal4, Trio-Gal80 is only expressed in Antp positive cardioblast cells (Figure 5.17D-D'; yellow dotted line) and was off in the Antp positive niche cells (only red). Similar to Zasp knockdown using Antp-Gal4 (Figure 5.17E), the depletion of Zasp using Antp-Gal4, Trio-Gal80 drastically increased hematopoietic niche cell number (Figure 5.17E'). The quantitation of the above results also suggests that knockdown of Zasp by Antp-Gal4, Trio-Gal80 leads to increment in niche cell number (Figure 5.17F). Additionally, an increment in the number of niche cells is noticeable upon downregulation of Zasp by Mef2-Gal4, a well-known driver for cardioblasts (Figure 5.17G-I) express throughout development.

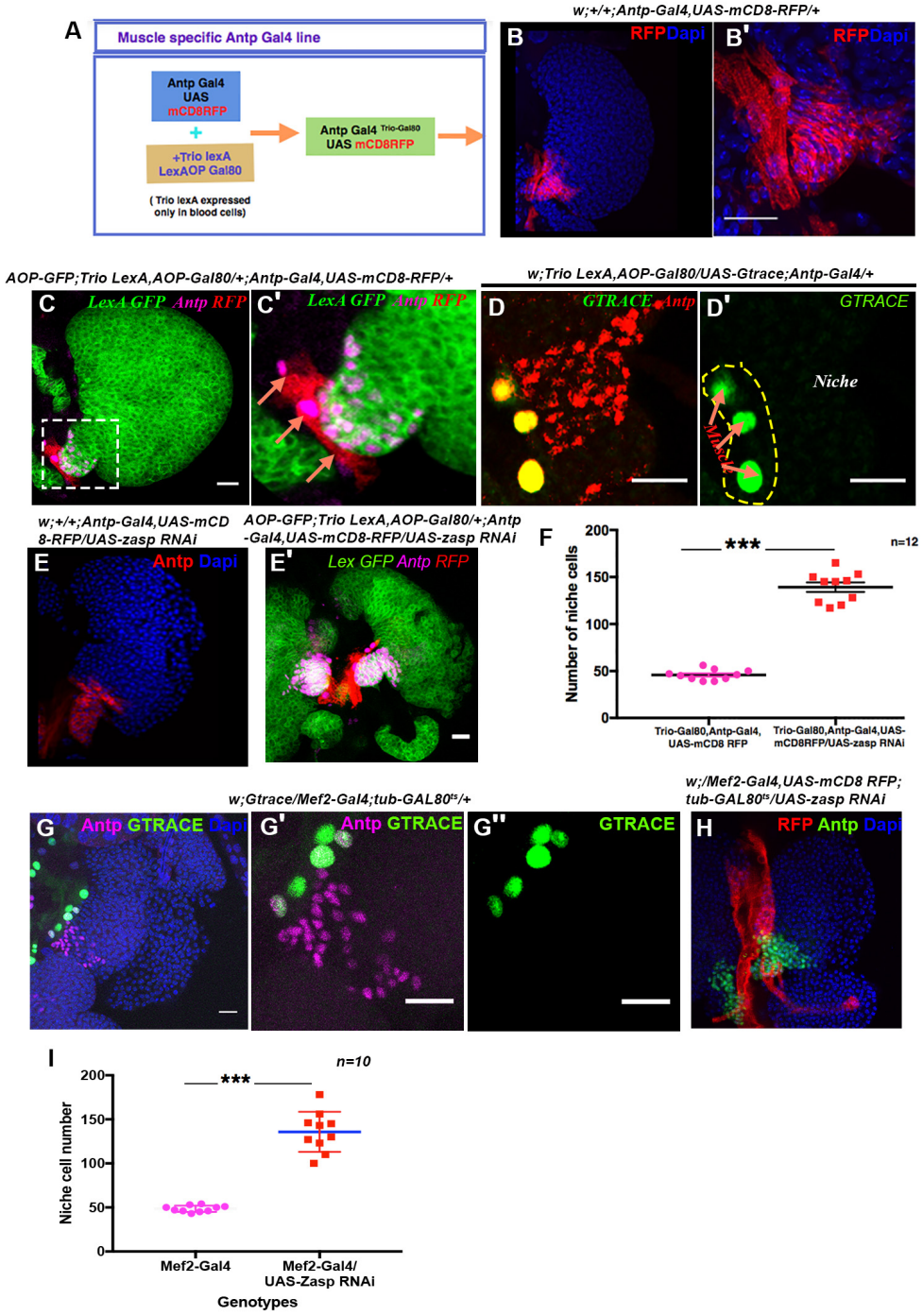

Figure 5.17 Knockdown of Zasp in the Antp positive cardioblast increases niche cell number. (A) Scheme representing the generation of cardioblast specific Antp-Gal4. (B-C) Antp-Gal4, UAS-mCD8-RFP expression (B-B) was suppressed in the hematopoietic niche using Trio-Gal80 (C-C), which specifically expressed in the hematopoietic system, including niche (green). (D-D) Antp-Gal4,Trio-Gal80 expresses only in cardioblast (arrow), indicated by Lineage tracing (green) of its expression from embryo to the third instar. It does not express in the niche (red) in all the developmental stages. (E-E) Knockdown of Zasp in the Antp positive cardioblast (red) using Antp-Gal4, Trio-Gal80 UAS-mCD8-RFP increases hematopoietic niche (magenta) cell number. (F) Quantification of niche cell number upon expression of Zasp RNAi using Antp-Gal4,Trio-Gal80, suggesting that loss of Zasp in the cardioblast affects hematopoietic niche cell maintenance. (G-H) The knockdown of Zasp in the cardioblast (red) using Mef2-Gal4, which also express in the cardioblast (red), increases the hematopoietic niche (magenta) cell number. (G-G) Lineage tracing of

Mef2-Gal4 (green) expression suggests its expression is only restricted to cardioblasts. (I) F) Quantification of niche cell number upon expression of Zasp RNAi using Mef2-Gal4.

Next, a niche-specific Antp-Gal4 was developed using Mhc-Gal80, a Muscle-specific Gal80 (Owusu-Ansah et al., 2013), which was also expressed in the cardioblast (Figure 5.18A). Expression of Mhc-Gal80 with Antp-Gal4 inhibited Gal4 expression in the cardioblast (red in C-C') and thereby generated niche-specific Antp-gal4 (Figure 5.18B-C'; green). Lineage tracing of Antp-Gal4, Mhc-Gal80 using GTRACE suggests that Antp-Gal4, Mhc-Gal80 is only expressed in the niche and not in the adjacent Antp+ cardioblast (red) (arrow; green) expression throughout development (Figure 5.18D-D'). The knockdown of Zasp using Antp-Gal4, Mhc-Gal80 did not increase hematopoietic niche cells (Figure 5.18E-F). Collectively, these results support the hypothesis that Antp+ cardioblast regulates the proliferation of hematopoietic niche cells.

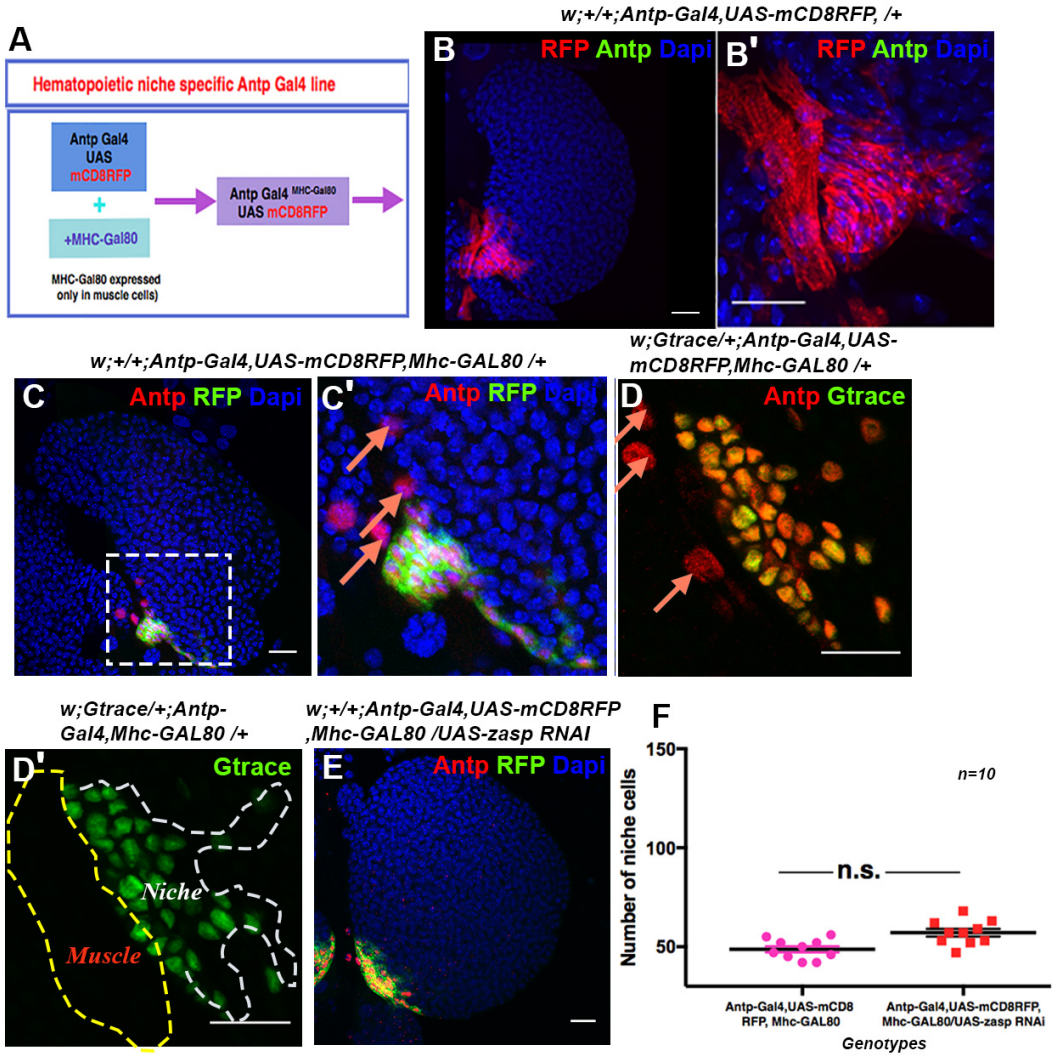

Figure 5.18 Knockdown of Zasp in niche cells does not recapitulate the increase niche phenotypes seen upon Zasp loss from the cardioblast. (A) Scheme representing the generation of niche specific Antp-Gal4. Suppression of Antp-Gal4 in the cardioblast using Mhc-Gal80, which specifically express in the cardioblast. (B) Antp-Gal4, UAS-mCD8-RFP express in the niche and cardioblast cells. (C-C) Suppression of Gal4 activity in the cardioblast (Arrow) using Mhc-Gal80 create Antp-Gal4, Mhc-Gal80, UAS-mCD8-RFP which only express in the niche (green). (E-F) Knockdown of Zasp using Antp-Gal4, Mhc-Gal80 does not increase the niche cell number, and neither phenocopies the phenotype seen in Zasp knockdown using Antp-Gal4. (C) Quantification of niche cell number upon expression of Zasp RNAi using Antp-Gal4, Mhc-Gal80, suggest that Zasp has no role in the niche cells.

Based on the above results, it can be inferred that being a structural protein, inhibition of Zasp disturbs the cardioblast integrity and function, which affects the hematopoietic niche cell proliferation.

5.2.7 Impairment of muscle contraction affect hematopoietic niche maintenance-

Next, it was assayed whether inhibition of Zasp function in the Antp positive cardioblast affects their contractility and increases the proliferation in hematopoietic niche cell number. To assay, the contraction of cardioblast in control and upon Zasp loss, two contractile points opposite to each other (Figure 5.19A-B; red lines) were assigned in the cardioblast from the genotype Antp-GalTrio-Gal80, UAS-mCD8-RFP (see the details in the method section), and their distance at different time point was noted. The live imaging of the cardioblast's pulsation reveals that loss of Zasp in this cardioblast severely reduced the contraction of these cells (Figure 5.19B) compared to wild-type Antp positive cardioblast (Figure 5.19A). This claim further clear from the quantitation of the above data (Figure 5.19C).

The above results suggest that knockdown of Zasp affects the structure and contractile function of the myoepithelial cardioblast, which in turn might results in the non-autonomous increment in the hematopoietic niche cell number.

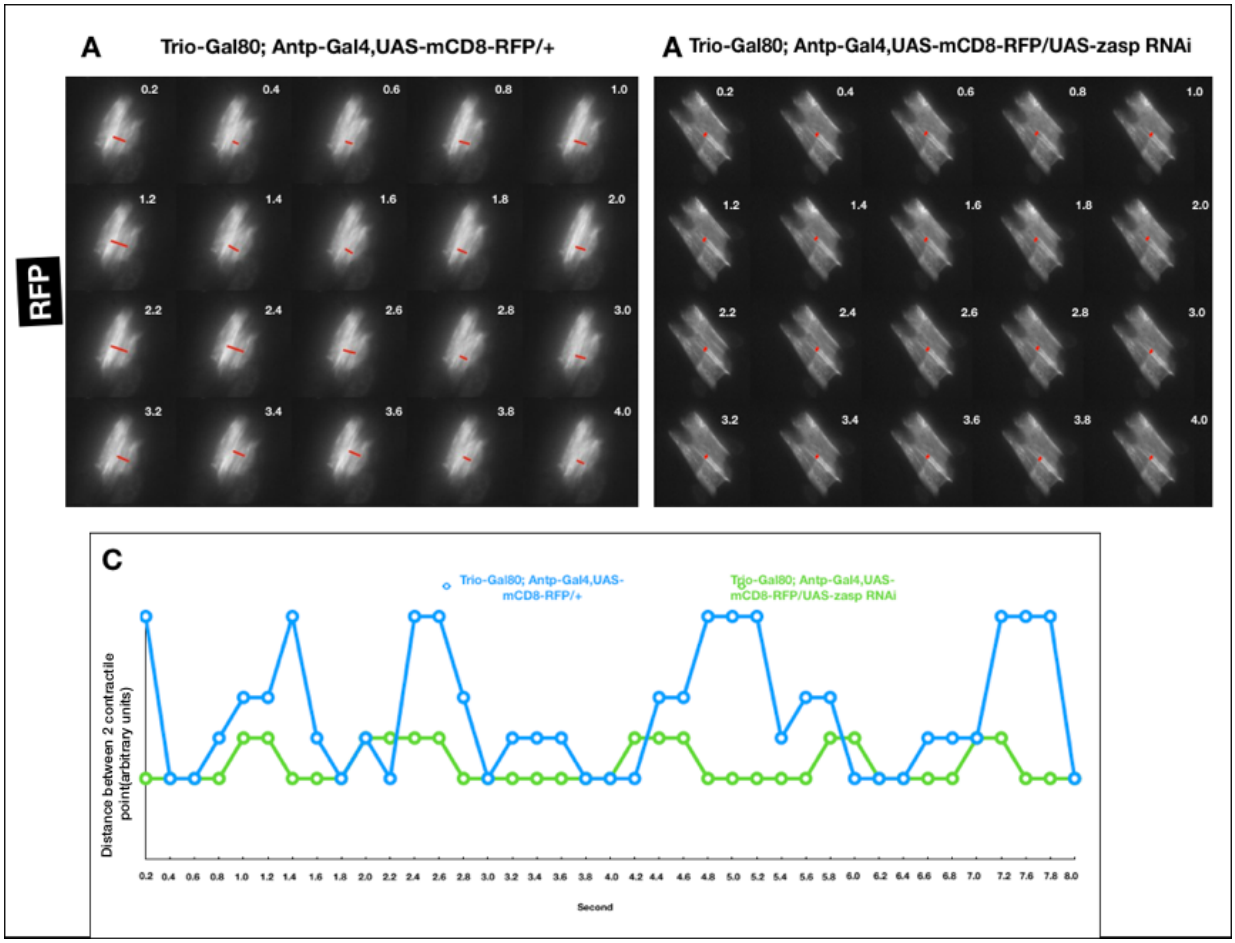

Figure 5.19. The knockdown of Zasp affects the contraction of Antp positive cardioblast. (A-C) Montages from the live imaging of the Antp-GalTrio-Gal80, UAS-mCD8-RFP cardioblast (A). and (B) upon knockdown of Zasp reduced contraction in Antp positive cardioblast. (C) Quantitation of the contraction in the above genotypes.

Next, it was analyzed whether directly inhibiting the cardioblast's contractility can affect the hematopoietic niche cell number. The calcium and ATP levels are known to be involved in cardiomyocyte function (Sullivan et al., 2000). The inhibition of RyR (Ryanodine receptor encodes an intracellular calcium channel) and bellwether (Blw, encodes the α subunit of mitochondrial ATP synthase) reduced the contractility of the cardiomyocytes. Interestingly, inhibition of RyR or Blw in the Antp positive cardioblast via Antp-GalTrio-Gal80, UAS-mCD8-RFP, increased the hematopoietic niche cell number (Figure 5.20A-C and E). Thus, it can be inferred that Antp positive cardioblast's contractility is essential for the maintenance of the hematopoietic niche. Moreover, analyzing the niche cell number in the lonely heart mutant (Loh, receptor for Pericardin, which regulates the heart integrity) also exhibit an increase in hematopoietic niche cells number (Figure 5.20D-E).

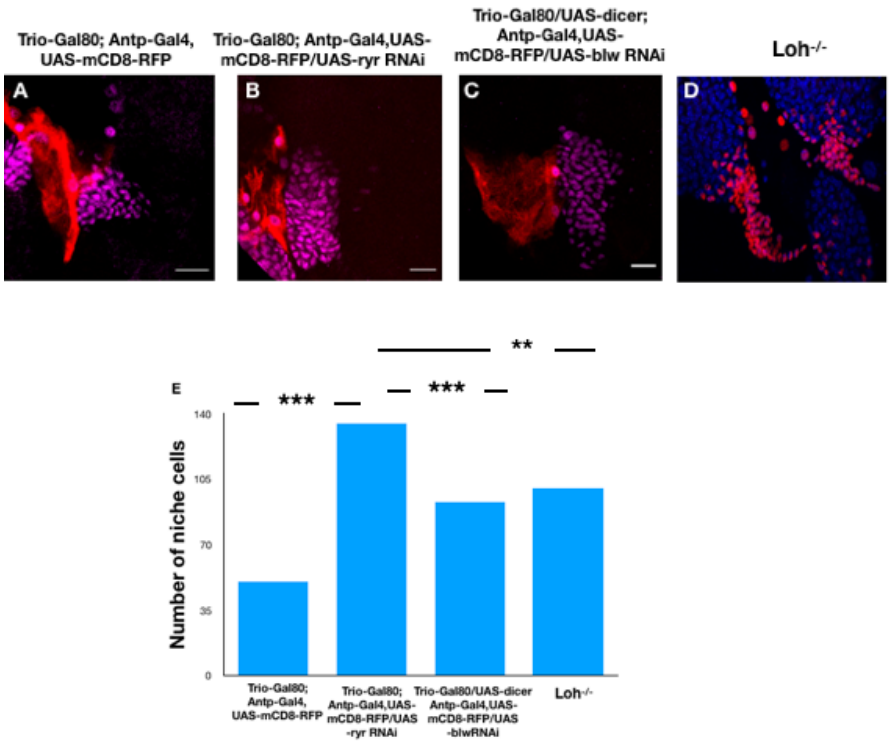

Figure 5.20 Inhibition of contraction in Antp positive cardioblast affects hematopoietic niche maintenance. Reduction in cytoplasmic calcium via knockdown of RyR receptor (B) or lowering down the energy level via knockdown of Blw (C) increases the hematopoietic niche number compared to control (A). (D) Niche cell number also increases in Loh-/- mutant. (E) Quantitation of the niche cell number in the above genotypes.

The above genetic data and the contraction assay suggest that loss of contractility in the cardioblast increases hematopoietic niche cell numbers. Thus, it can be concluded that the adjacent Antp positive cardioblasts' contraction is essential for maintaining the hematopoietic niche cells. It can also be thought that contraction in the myoepithelial cardioblast can generate a biophysical force, crucial for the *Drosophila* hematopoietic niche maintenance.

5.2.8 Cellular protrusion from the cardioblast is essential to maintain cardioblast-hematopoietic niche interaction

The next step was to understand the mechanism by which the hematopoietic niche senses myoepithelial cardioblast's contractility. At this time point, it was logical to speculate that there might exist a physical connection between the *Drosophila* hematopoietic niche and Antp positive cardioblast. This idea of a possible local physical contact between the cardioblast and the hematopoietic niche was probed into by the GRASP system (GFP reconstitution across synaptic partners). This genetic cassette can provide evidence of close contact between two diverse cells type (Feinberg et al., 2008).

In this GRASP system, GFP is split into two membrane-bound GFP fragments expressed in the two different cells. When these two cells are in close contact, the two membranes of the cells bind non-fluorescent fragments to generate fluorescent GFP (Figure 5.21A). For the current study, the membrane-bound GFP fragment 1 (CD GFP1-10) was expressed in the cardioblast using Mef2-Gal4. Fragment 2 (CD GFP11) was expressed in the hematopoietic system using Trio-LexA. Expression of these non-fluorescent GFP along with lymph gland-specific mcherry expression revealed that these non-fluorescent GFP fragments generated fluorescence along the boundary between muscle and lymph gland (Figure 5.21B).

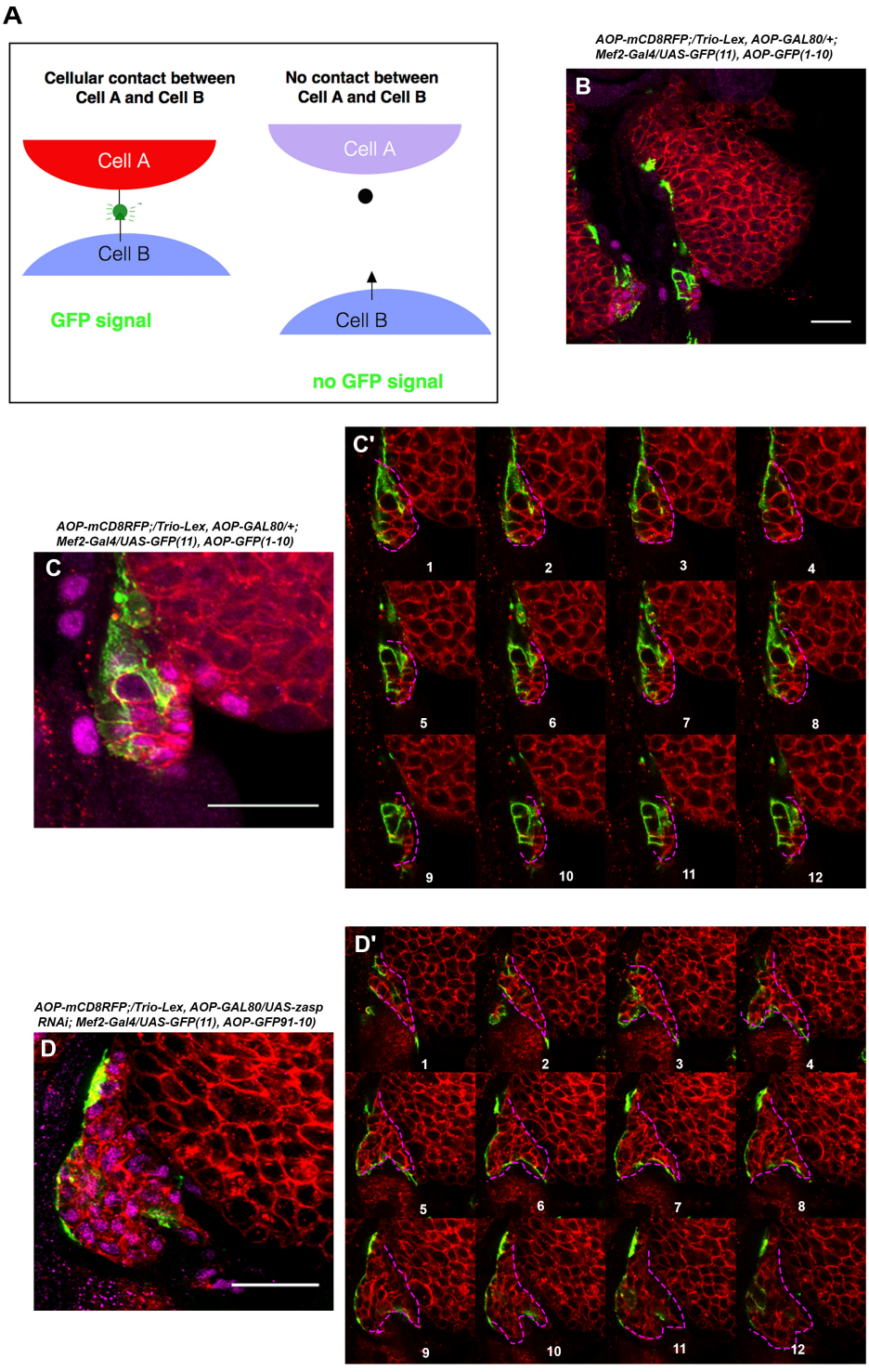

Figure 5.21 Knockdown of Zasp in cardioblast disturb physical interaction between cardioblast and hematopoietic niche. (A) Scheme representing the GRASP technique. The expression of one fragment of GFP (11) in the cardioblast and other fragments of GFP(1-10) expressed in the hematopoietic system where GFP is reconstituted at the point of contact between cardioblast and hematopoietic system. (C) GRASP (GFP) expression can be seen near the niche (magenta). (C) Z-stack reveals various contact points in the niche, which was lost upon expression of Zasp in the cardioblast(D-D), suggesting a close association between cardioblast and niche that might be essential for hematopoietic niche maintenance.

Interestingly, marking the niche with Antp revealed that apart from GRASP fluorescence in muscle boundary, the GRASP fluorescence signal can be seen in the hematopoietic niche cells (Figure 5.21C'). More importantly, these GRASP signal in the niche is severely affected in the Zasp RNAi condition (Figure 5.21D'), suggesting that cardioblast impairment using Zasp knockdown affects the cardioblast-niche local contact. The GFP reconstitution and protrusion pattern suggest that cardioblast-derived protrusion extends in the niche and makes a stable communication with niche cells essential for proper crosstalk between cardioblast and niche cells.

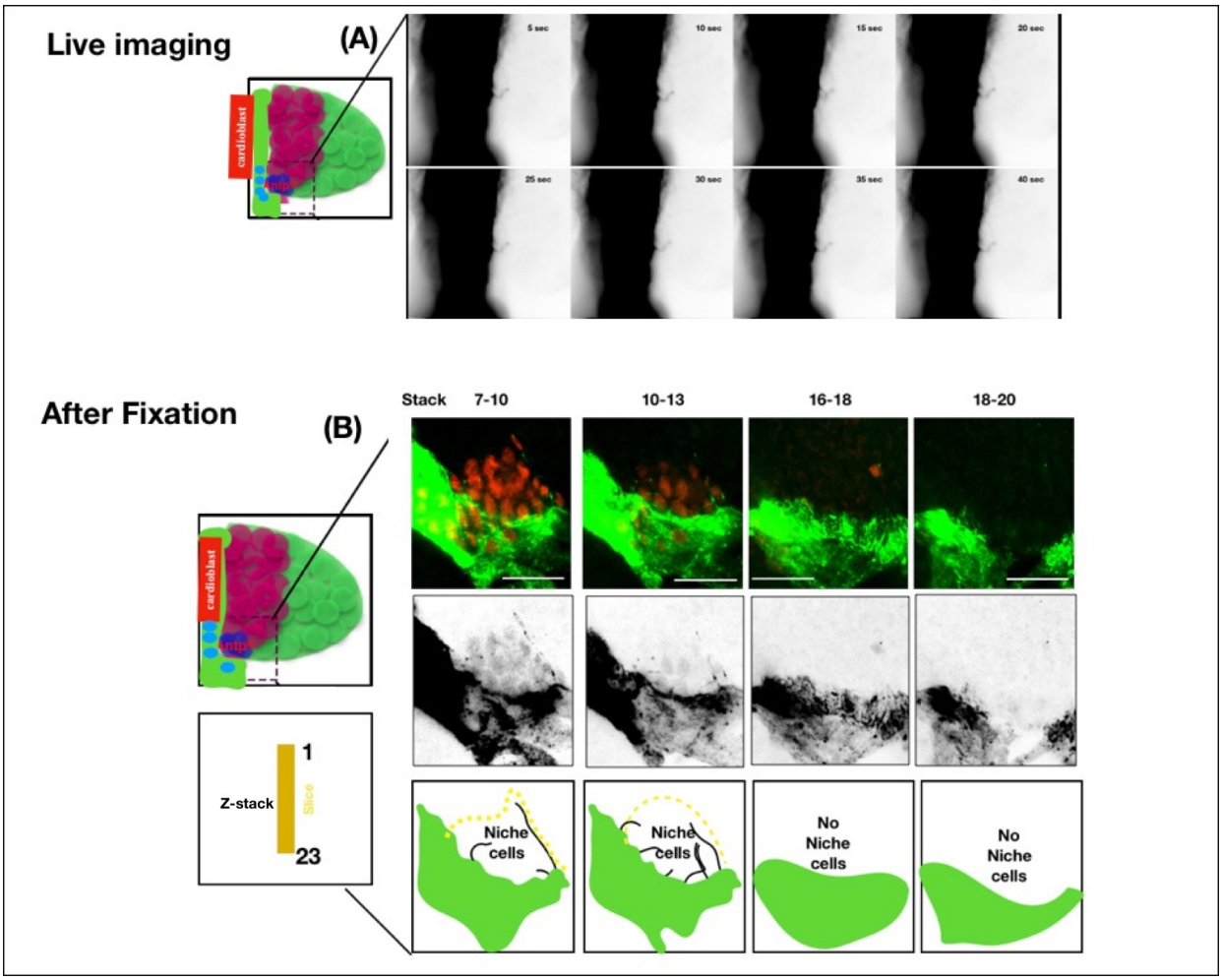

Figure 5.22 Cardioblast and hematopoietic niche interact via protrusion. (A) Live imaging near the niche found the existence of cardioblast derived protrusion. (B) Imaging of protrusion in a fixed sample revealed more than one cardioblast derived protrusion, which only found in the niche—image repressing the different focal plane (stack 1-23) of the sample.

In *Drosophila*, it has been shown that in the indirect flight muscles (IFM), the myotube physically attaches to the nearby myoblast cells via protrusion (Segal et al., 2016). To examine the muscle derived protrusion, CD4 tomato mcherry (tdTomato), which has been previously used to visualize protrusion/filopodia (Segal et al., 2016), was expressed in the cardioblast using Mef2-GAL4. Live imaging of the cardioblast and LG revealed the existence of thin protrusions proximal to the niche (Figure 5.22A), which can also be seen after the fixation. Interestingly, labeling the post-fixation, thin protrusions originating from the cardioblast cells directed towards the hematopoietic niche. Further, cardioblast originating protrusions can be seen in the Z section, where the niche is visible, suggesting that protrusions are specific for the niche.

To determine the functional importance of these protrusions originating from the cardioblast targeted towards the hematopoietic niche, the I-BAR domain protein IRSp53 was downregulated in the cardioblast, which is involved in the protrusion formation (Ahmed et al., 2010; Segal et al., 2016). IRSp53 acts as a mediator between curved sites of the plasma membrane and Ena recruitment, essential for filopodia formation. Knockdown of IRSp53 in *Drosophila* pupal IFM drastically affects filopodia formation (Segal et al., 2016). Interestingly, knockdown of IRSp53 in cardioblast using Antp-Gal4TrioGal80 (green) leads to increment in hematopoietic niche cells (magenta) number (Figure 5.21A-C). Moreover, knockdown of IRSp53 using Mef2-Gal4 also increase the hematopoietic niche cells number (Figure 5.21C), supporting the conclusion that physical interaction between the cardioblast and hematopoietic is essential for the regulation of niche cell proliferation.

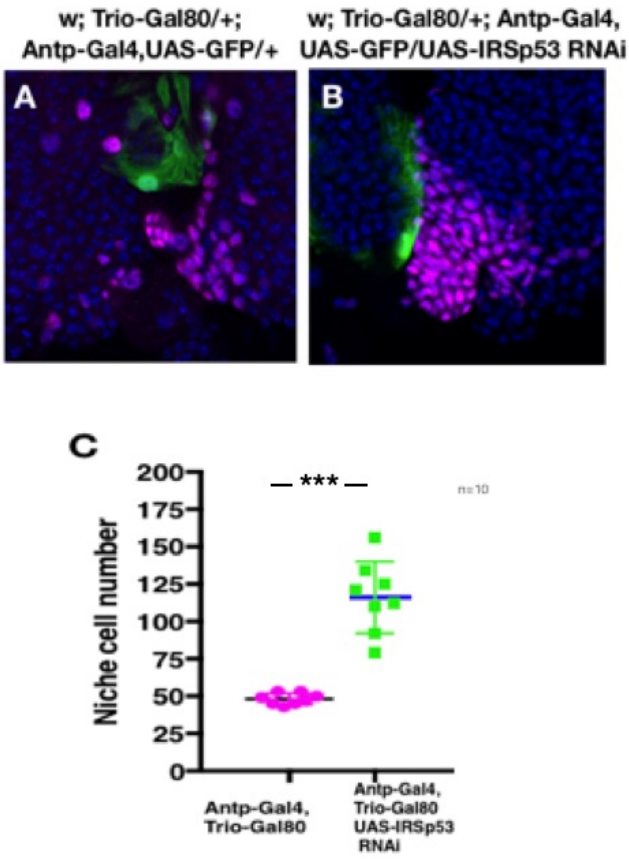

Figure 5.20 Knockdown of IRSp53 affects a hematopoietic niche cell number. Loss of IRSp53 (B), which involves protrusion formation, increases hematopoietic niche cell number compared to wild-type niches (A). (C) Quantitation of the niche cell number in the above genotype.

5.2.9 Impaired myoepithelial cardioblast increases actomyosin-Yki activation in hematopoietic niche

Next, the molecular mechanism by which cardioblast inhibits the hematopoietic niche cell proliferation was investigated. The clue came from the levels of phalloidin in the Zasp knockdown. During analysis, it was observed that although the F-actin was seen severely reduced in the Antp+ cardioblast, a significant increment of actin could also be seen in the adjacent niche (Figure 5.16C-D). Phal was rechecked in the niche at higher magnification to investigate whether cardioblast impairment could modulate actomyosin activity. Upon careful analysis of the high magnification image of the niche, it was noted that inhibiting Zasp up-regulated F-actin (Figure 5.23B-B"; red) in the niche (green) compared to the control (Figure 5.23A-A").

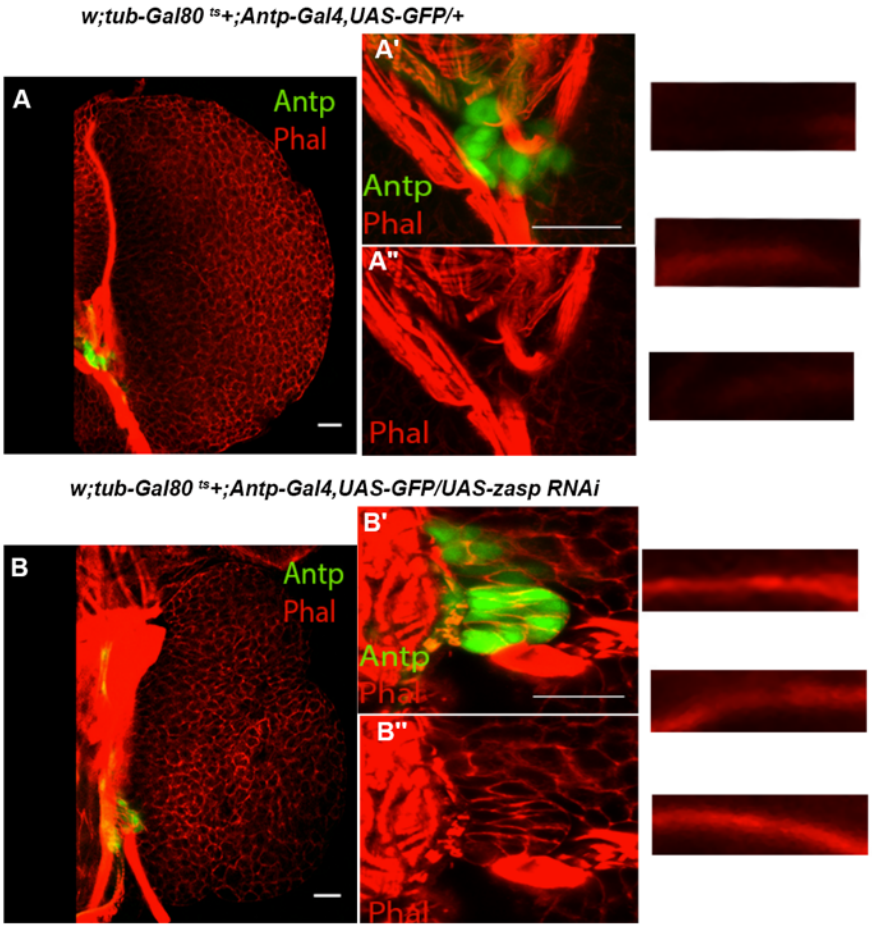

Figure 5.23 Impairment of cardioblast increase F-actin in the hematopoietic niche. (A-B") The basal level of F-actin (Phal; red) can be seen in the niche upon Zasp knockdown using Antp-Gal4. Zoom image of niche indicates the elevated F-actin expression (red) in the niche (B'-B'') (green) upon Zasp knockdown compare to wild type niche (A'-A"). Scale bar=20µm.

To examine the activity of myosin in impaired myepithial cardioblast condition, pMRLC, an indicator of the activated sqh were used to visualize phosphorylated myosin. Expression of Zasp RNAi driven by Antp-Gal4 resulted in a significant elevation of pMRLC (red) in the niche (green) (Figure 5.24B-B") compared with the wild type niche Figure 5.24A-A"). This result was confirmed by the knockdown of Zasp in the cardioblast using Antp-Gal4 Trio-Gal80 (Figure 5.24C-C') or Mef2-Gal4 (Figure 5.24D-E'). Knockdown of Zasp specifically in the cardioblast also significantly up-regulated pMRLC in the niche, suggesting that the structural impairment of cardioblast can affect actomyosin activity in the niche.

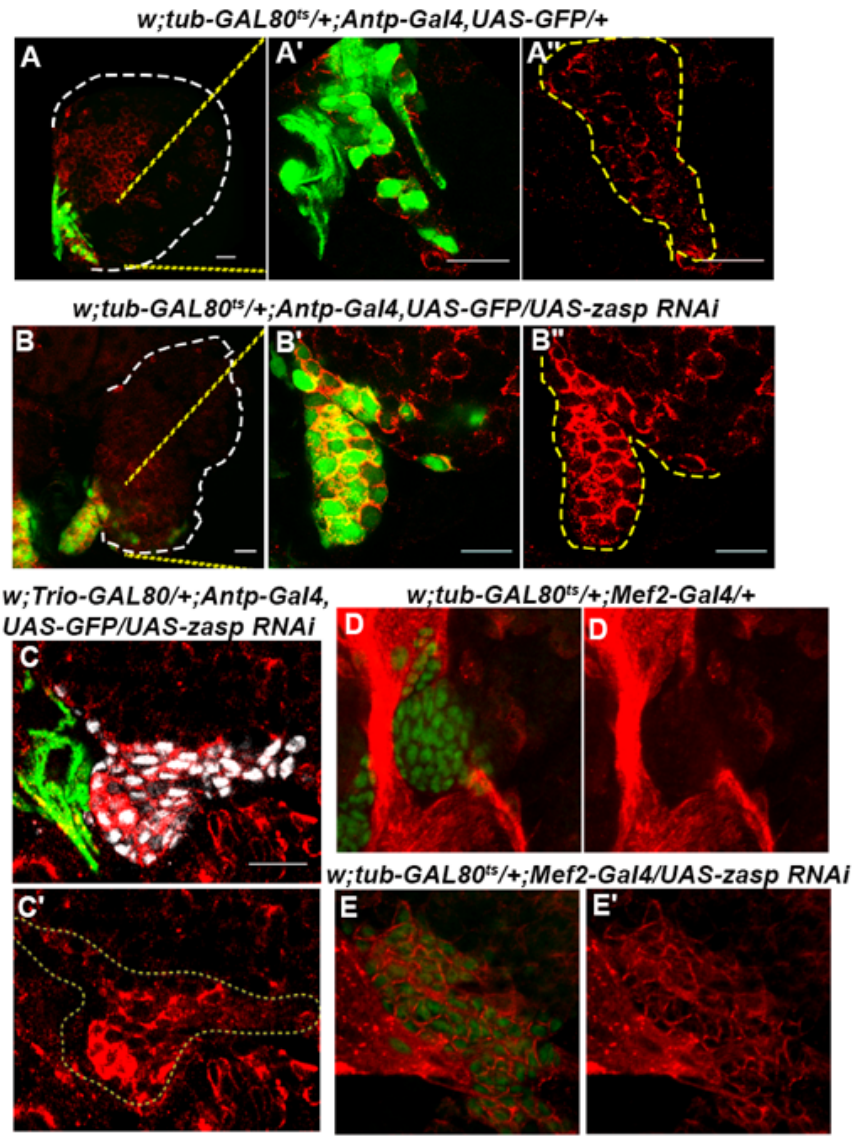

Figure 5.24 Impairment of cardioblast increase actomyosin assembly in the hematopoietic niche. (A-A") The basal level of actomyosin assembly (pMRLC) can be seen in the niche, which increases upon Zasp knockdown using Antp-Gal4 ("-'"). Zoom image of niche indicates the elevated pMRLC expression in the niche (arrows, "-B") upon Zasp knockdown. Knockdown of Zasp using Trio-Gal80; Antp-Gal4, UAS-GFP (antp+ cardioblast driver)(C-'') OR Mef2-Gal4 (pan muscle driver) (D-E') also increase the pMRLC in the niche, suggesting that impairment of cardioblast can cause non- autonomous activation of actomyosin in the niche. Scale bar=20μm.

It would be intriguing to see whether the autonomous activation of actomyosin assembly in the niche using Antp-Gal4, Mhc-Gal80 was sufficient to increase the proliferation in the niche. The niche-specific overexpression of ROCK using Antp-Gal4, Mhc-Gal80 does increase the niche number (Figure 5.25A-B and E) while niche-specific down-regulation of Sqh (Figure 5.25C) or Zip (Figure 5.25D-E) reduces the cell number. The above results suggest that indeed the actomyosin in the niche autonomously affects the hematopoietic niche proliferation. To unveil the genetic relationship between cardioblast impairment and actomyosin-mediated proliferation, ShqA20A21 has two mutated regulatory phosphorylation sites and behaves as a null allele (Rauskolb et al., 2014) was over-expressed in the Zasp RNAi condition. Interestingly, the expression of SqhA2021

in the Zasp knockdown condition decreases the niche cell number compared to the Zasp RNAi. Thus, lowering down the Myo II activity in the niche in impaired cardioblast condition is sufficient to rescue the hyperproliferation of niche cells seen in Zasp RNAi, suggesting that muscle impairment causes ectopic proliferation through the increase of actomyosin activity.

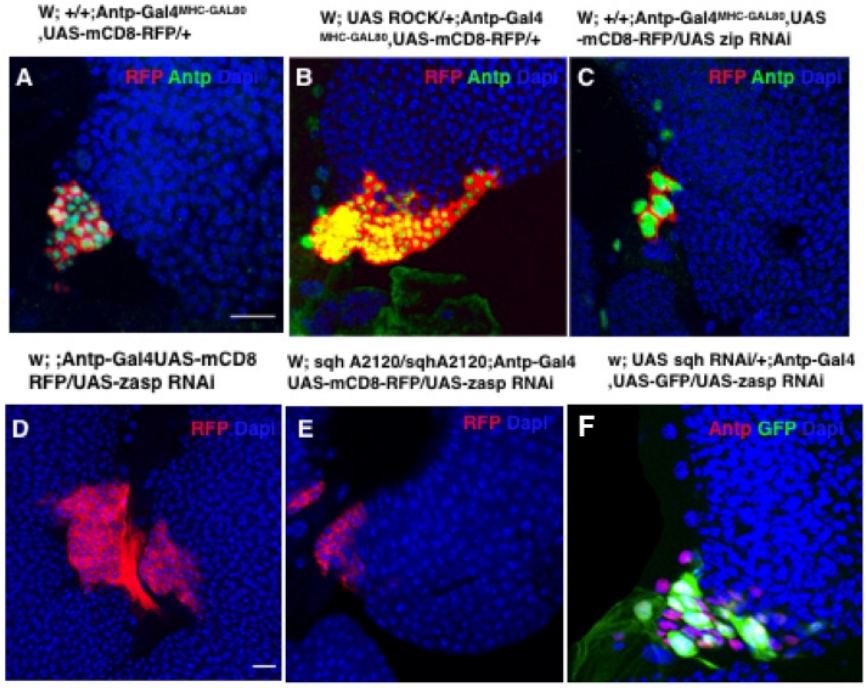

Figure 5.28 Over-activation of actomyosin assembly in niche increase the niche cell number. (A-C') Overexpression of ROCK (B) in niche via Antp-Gal4,Mhc-Gal80 increases niche cell number compared to wild-type niches (A). (C) The knockdown of Zip–in niche via Antp-Gal4,Mhc-Gal80 leads to a reduction in niche cell number. (D-F) Knockdown of Zasp in the cardioblast via Antp-Gal4 (D) leads to a strong increment in the niche cell number compare to wild type niche (A), which was rescued by the inhibition of sqh activity via sqhA2021(E) or Sqh RNAi(F). Scale bar=20μm. DAPI marks the nucleus.

Next, it was asked whether cardioblast impairment could affect the Yki activation similar to altered actomyosin assembly. A basal level of Diap1-GFP expression can be detected in the niche in wild type, indicating a low Yorkie activity (Figure 5.26 A-A'). Interestingly, knockdown of the Zasp in the cardioblast using Antp-Gal4 results in the up-regulation of Diap1-GFP (Figure 5.26 C-C') expression, mimicking the removal of Warts (a negative regulator of Yki activity) in the niche (Figure 5.26 B-B'). Moreover, inhibition of Zasp using Mef2-Gal4 also increased the hematopoietic niche cells number and Yki activation(Figure 5.26 D-D'). The above results suggest that cardioblast negatively regulates Yki activation in the hematopoietic and niche cells number.

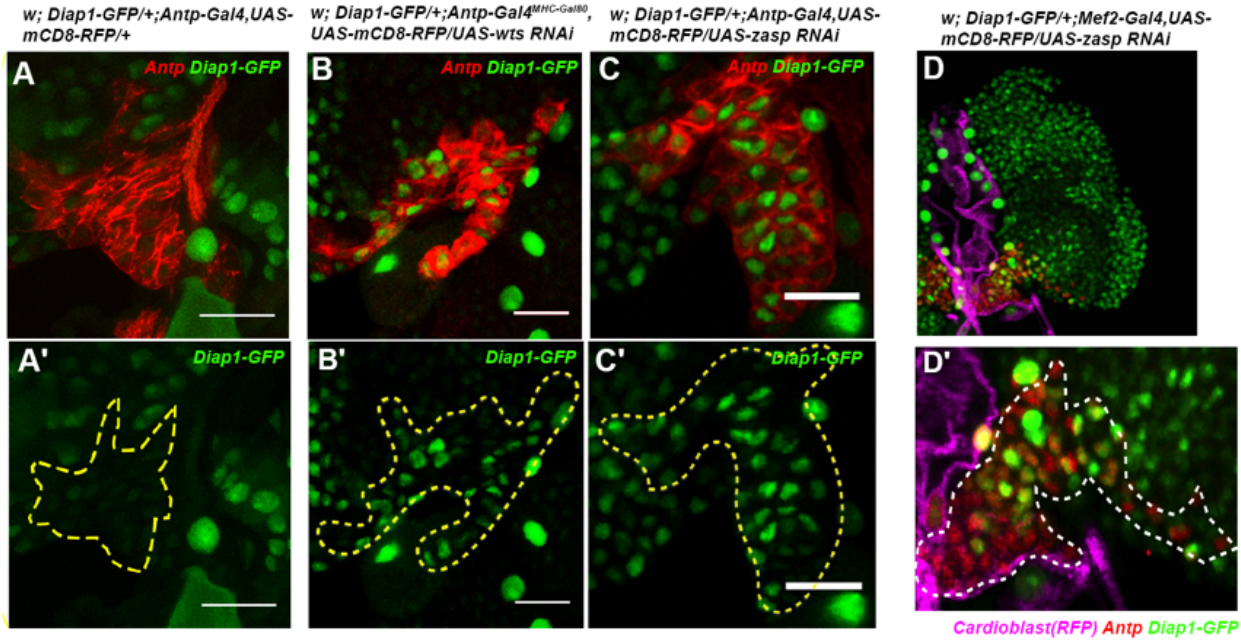

Figure 5.26 Cardioblast impairment causes the up-regulation of Yki activity in the niche. (A-B') Loss of Wts in the niche (red) leads to the up-regulation of Yki activity (Diap1-GFP; green). Knockdown of Zasp using Antp-Gal4 (C-C') or Mef2 Gal4 (magenta) (D-D') also increase Yki activity (GFP) in the niche (red) and phenocopy Wts phenotype. Genotypes are shown on top of corresponding panels. Scale bar=20µm. DAPI marks the nucleus.

This result was further endorsed by the direct visualization of the localization of Yki in niche using Yki-GFP in Zasp knockdown. In wild type niche, Yki (green) is predominantly expressed in the cytoplasm (Figure 5.27A'; arrow in A'), but upon inhibition of Zasp in the cardioblasts a partial translocation of Yki into the nucleus is evident as co-localization of Yki with DAPI is seen(Figure 5.27 B-B'; arrow in B').

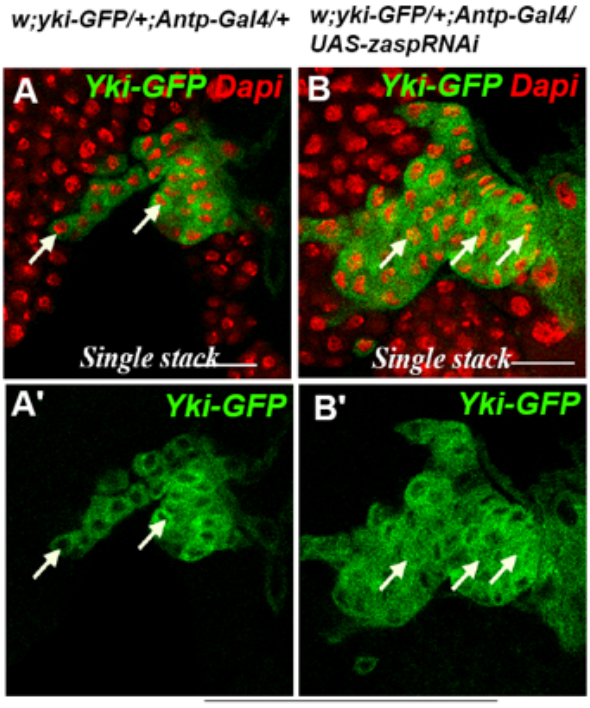

Figure 5.27 Cardioblast impairment causes the up-regulation of Yki activity in the niche. (A-B') Loss of Zasp using Antp-Gal4 (B- B') leads to the increment of enrichment of Yki-GFP (Diap1-GFP; green) in the nucleus compared with the wild type niche (arrow A'). Genotypes are shown on top of corresponding panels. Scale bar=20μm. DAPI marks the nucleus.

These results suggest that cardioblast can regulate the activation of Yki in the niche, which is essential for the proper proliferation of the cells constituting the hematopoietic niche. To understand the autonomous requirement of Yki in the *Drosophila* hematopoietic niche, both knockdowns of Yki and Yki activation were performed using niche-specific Antp-Gal4, Mhc-Gal80. The reduction of Yki levels in the niche drastically reduced the niche cell number (Figure 5.27C), whereas the autonomous activation of Yki in the niche increases the niche cell number (Figure 5.27A-B). Consistent with this, knockdown of Warts in the niche using Antp-Gal4, Mhc-Gal80 mCD8-RFP also increases niche cell numbers. (Figure 5.27D). These results suggest that the autonomous activation of Yki is sufficient to induce proliferation in the *Drosophila* hematopoietic niche.

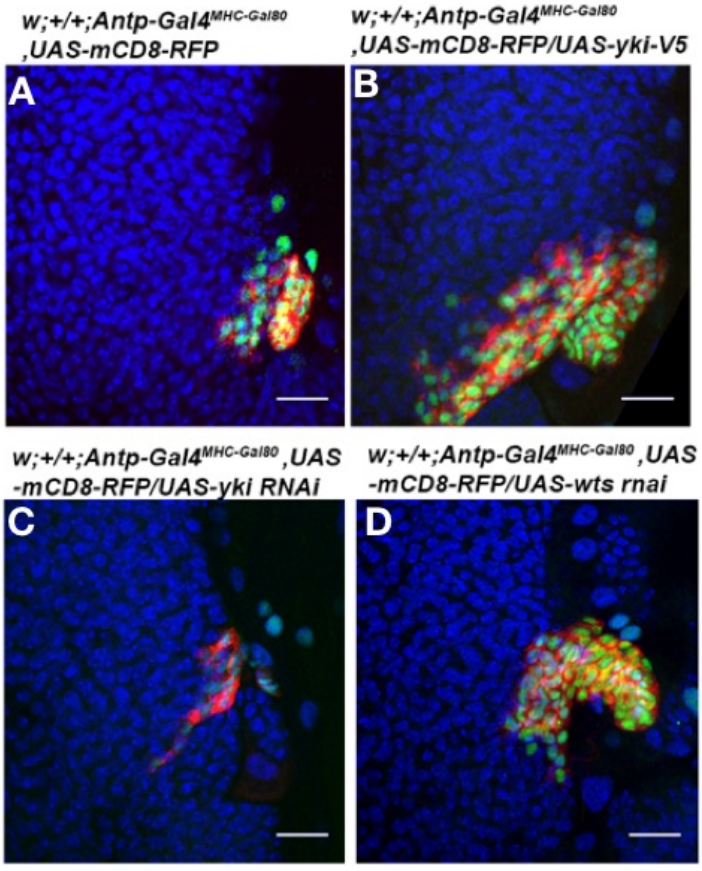

Figure 5.28 Autonomous activation of Yorkie in the niche is sufficient to maintain the niche cell number. (A-C') Overexpression of Yki(B) or inhibition of Wts (D) in niche via Antp-Gal4,Mhc-Gal80 increase niche cell number compared to wild type niche (A), suggesting that Yki activity autonomously regulates niche cell maintenance. (C) Expression of Yki RNAi in the niche (via Antp-Gal4,Mhc-Gal80 (red) leads to a strong reduction in the niche cell number compare to wild type niche (A). (Scale bar=20μm. DAPI marks the nucleus.

To genetically test whether the effects of the increase in niche number in impaired cardioblast condition is a direct consequence of Yki activation, Yki function was inhibited in zasp loss condition via a null allele, and niche number was assayed. Interestingly, the increased niche number is seen in zasp loss (Figure 5.29A-B) was drastically reduced upon inhibition of yki via the YkiB5 mutant allele (Figure 5.29C) or expressing Yki RNAi (Figure 5.29D). Moreover, an increase in expression of diap1-GFP and increased niche cell number seen in Zasp loss from the cardioblast (Figure 5.29F-F') were also rescued by RNAi-mediated downregulation of Yki (Figure 5.29G-G'). The above results indicate that increase cell proliferation in the niche is an outcome of increase Yki activation. Interestingly, inactivation of Myo II activity by expression of SqhA2021 under the Sqh promoter in impaired cardioblast also decrease the Diap1-expression seen in the Zasp loss from the cardioblast (Figure 5.29H-H'), attesting to the fact that impairment in cardioblast cause increment in the hematopoietic niche cells via actomyosin-Yki activation cascade.

Thus, cardiomyocytes act on the hematopoietic niche to orchestrate the actomyosin and Yki activation in the niche critical for hematopoietic niche maintenance.

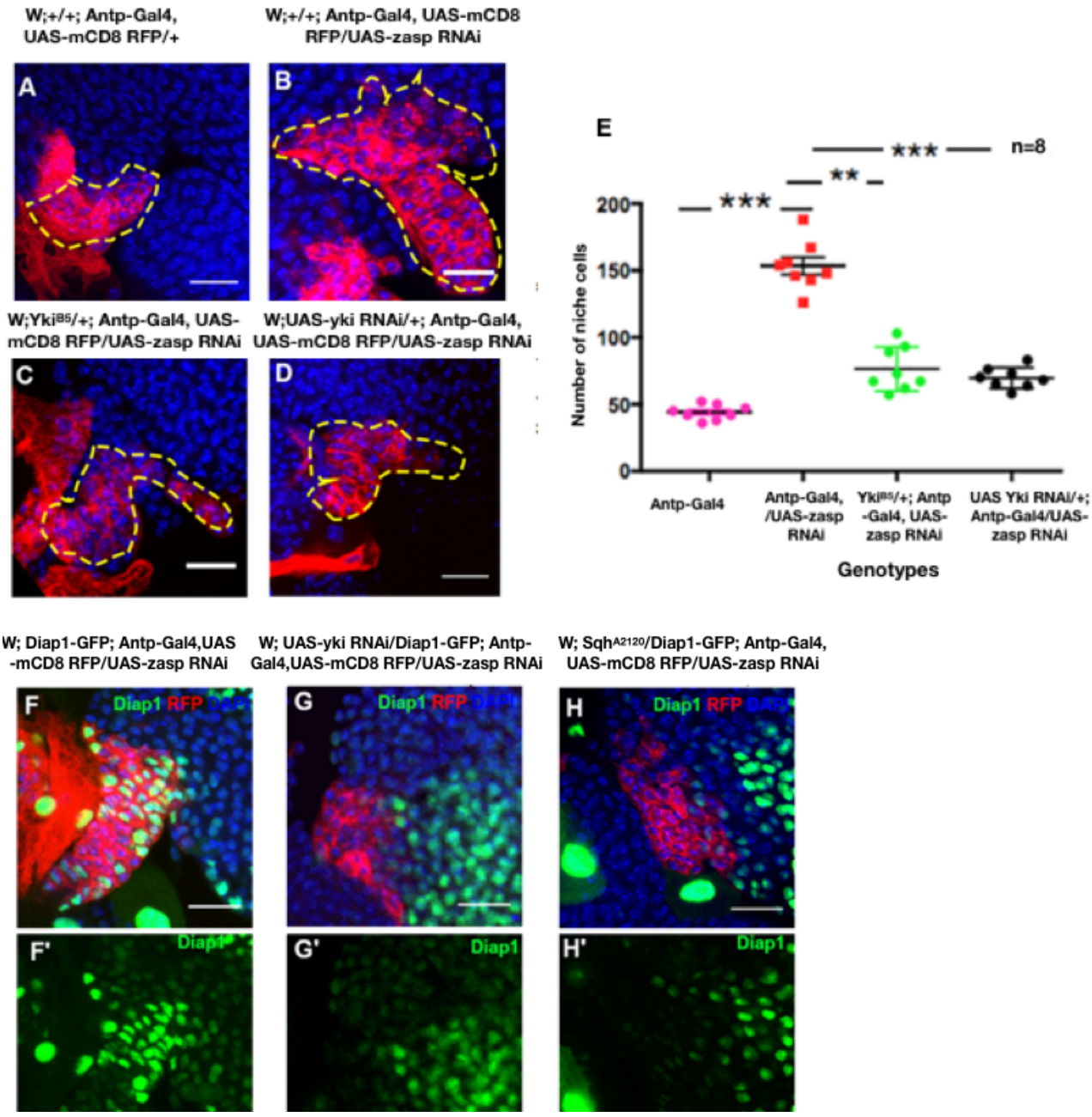

Figure 5.29 Down-regulation of Yki rescue the increase niche number seen in Zasp knockdown. (A-E) Inhibition of Zasp leads to increase niche number compare to control. Upon inhibition of Yki via Yki mutant (C) or expression of Yki RNAi (D)in the above background reascue the niche cells number. Quantification of niche cell number in the above genoptypes(P=UAS zasp RNAi; $1.402x10^{-7}$; Yki ^{B5} UAS zasp RNAi= $5.189x10^{-4}$. UAS-yki RNAi; UAS-zasp RNAi=$1.742x10^{-6}$. Genoptyes are shown on top of corresponding panels.

It is essential to understand how the Hippo pathway can interact with actomyosin to appreciate the network essential for the niche cells' proliferation. Hippo pathway interacts with actomyosin through Ajuba is very well worked out in *Drosophila* wing disc and mammalian cell lines (Rauskolb et al., 2014)}(Das Thakur et al., 2010). It is known that increase actomyosin activity

leads to an increased Ajuba-Warts complex at the plasma membrane. The reduction of the cytoplasmic pool of Wts leads to activation of Yki, resulting in an increase in proliferation. If the Ajuba-Warts axis is involved in the Niche proliferation, RNAi mediated down-regulation of Ajuba should suppress the increased cell number seen in the Zasp knockdown condition. Indeed, the expression of Ajuba RNAi in the Zasp knockdown condition decreases the niche cell number compared to the Zasp RNAi.

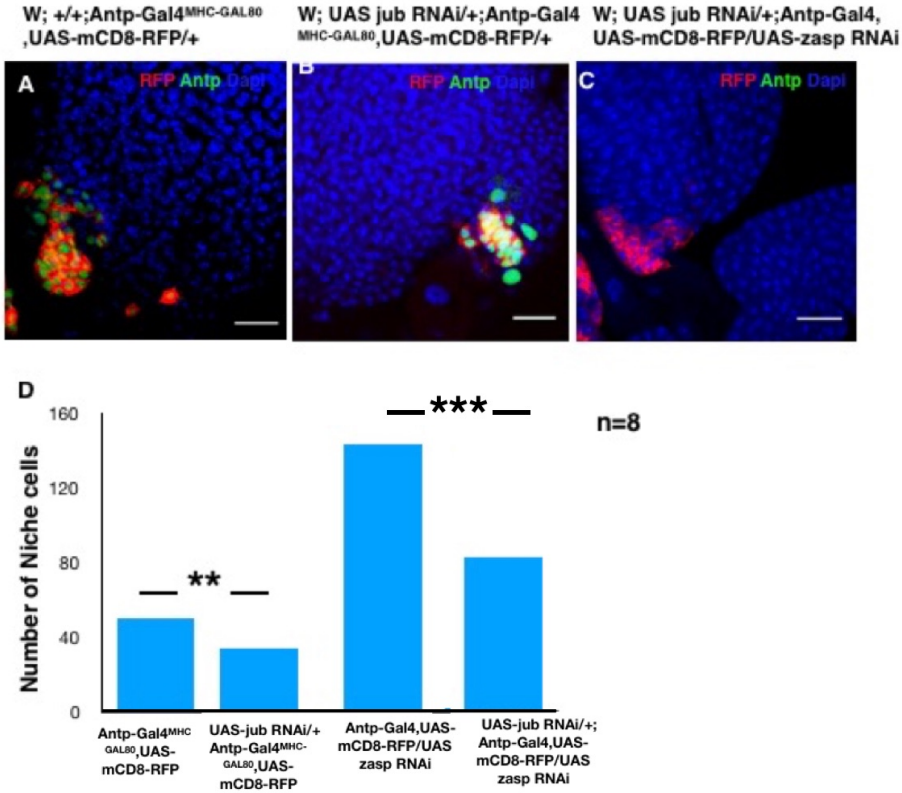

Figure 5.30 Inhibition of Ajuba rescue Zasp knockdown phenotypes (A-D) Down regulation of Ajuba (B)leads to reduction in niche cell numbers compare to control (A). Down-regulation of Ajuba in Zasp knockdown rescue the phenotypes. (D) Quantification of niche cell numbers in above genotypes.

Taken together, these data indicate that Ajuba acts downstream of actomyosin components to regulate the activation of Yki analogous to what has been reported earlier in the *Drosophila* wing disc (Das Thakur et al., 2010; Rauskolb et al., 2014).

5.2.10 Ectopic activation of Yki in niche disturbed lymph gland homeostasis

To further understand the phenotype upon Zasp downregulation from the cardioblasts, the differentiation status was assayed. The differentiated cells (P1; green) in the lymph gland (LG) were drastically increased upon knockdown of Zasp (Figure 5.31B) compare to the control LG

(Figure 5.31A). Although most of the LG exhibited enhanced differentiation, few cases of precocious rupture of the LG were noted (Figure 5.31C). Thus, although the impairment in cardioblast increased the niche cell number, it affected the primary function of the niche: progenitor maintenance. Therefore, the progenitors experienced a shift from maintenance to the differentiation program.

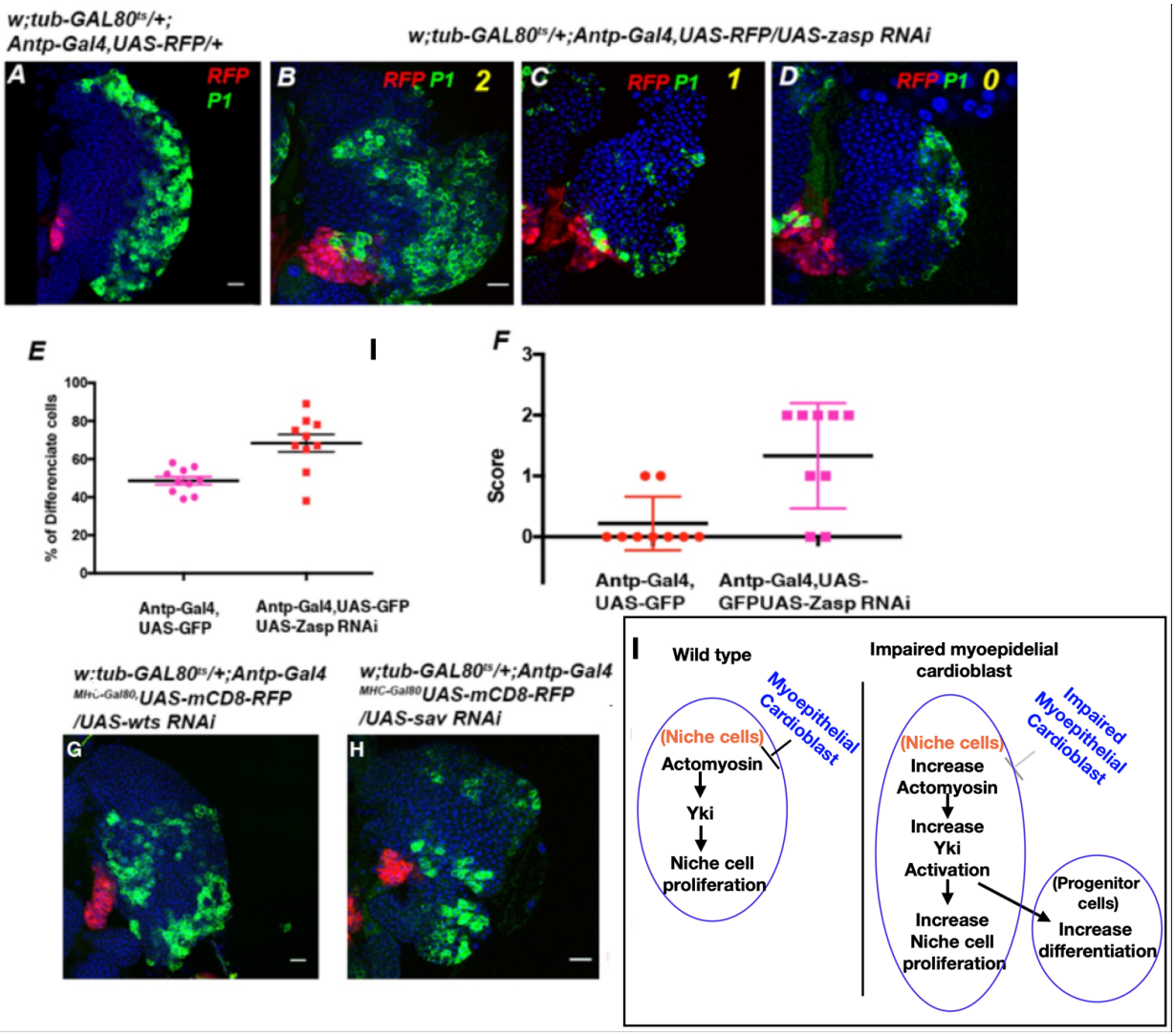

Figure 5.31 Ectopic activation of Yki in the niche increase lymph gland differentiation. (A-D) Downregulation of Zasp leads to ectopic differentiation in the lymph gland. (E) Quantification of differentiation index in Zasp RNAi suggests an increment of differentiation. (F) Score indicates the various phenotypes seen in Zasp RNAi. The majority of LG shows increased differentiation. (G-H) Autonomous activation of Yki in niche via down-regulation of Wts (G) or Sav (G) in the niche also phenocopy Zasp knockdown phenotype. (I) The model describes the involvement of the Actomyoisn-Yki cascade in the maintenance of niche cells as well as LG homeostasis and their regulation via nearby cardioblast.

Given that knockdown of Zasp in cardioblast ectopically activate Yki in the niche, it was next asked whether to increase Yki in niche sufficient to induce the ectopic differentiation in the lymph gland. Indeed, the inhibition of Wts (Figure 5.31G) or Sav (Figure 5.31H) in the niche was

sufficient to induce ectopic differentiation in the lymph gland. Thus, the impairment of myoepithelial cardioblast increased the Yki activation, which accounts for ectopic differentiation of progenitor cells and increment in niche cell number (Figure 5.31I).

The precocious rupture of the lymph gland seen might be due to a loss of the surrounding basement membrane (BM) of the lymph gland, as has been previously reported (Grigorian et al., 2013). To visualize the BM of lymph gland in Zasp RNAi or Wts RNAi, the expression of an extracellular matrix (ECM) protein, heparan sulfate proteoglycan Perlecan/Trol (Terribly Reduced Optic Lobes) was analyzed (Dragojlovic-Munther and Martinez-Agosto, 2013; Grigorian et al., 2013). In wild type lymph gland, expression of Trol is continuous around the lymph gland, indicative of the presence of a continuous BM (Figure 5.32A). However, the localisation of ECM was drastically disturbed upon Zasp knockdown in the cardioblast (Figure 5.32B-D), which reflects the disruption of BM in the mutant background. Moreover, the ectopic activation of Yki in the niche disrupts the ECM localisation in the LG. The effect of the Yki activation (due to impaired muscle-niche interaction or Wts inhibition) on the disruption of BM could be divided into three broad categories; normal, moderate, and severe types (A-D). Quantitative analysis suggests that most of the phenotypes belong to the "severe" types (Figure 5.32E). These results suggest that Yki activation in the niche disturbs the homeostasis of the lymph gland.

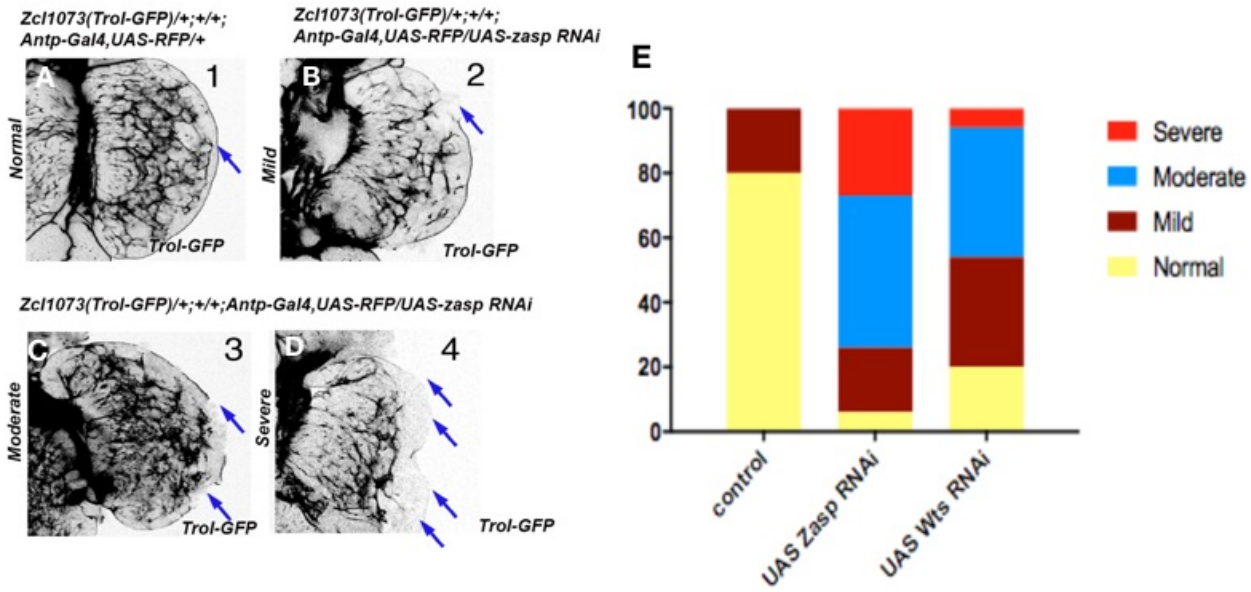

Figure 5.32 Ectopic activation of Yki in the niche disturbs lymph gland (LG) homeostasis. (A-E) The extracellular matrix analysis via Trol-GFP reveals the loss of the surrounding basement membrane (BM) (arrow) of the lymph gland upon Zasp knockdown. Autonomous activation of Yki via Wts RNAi in the niche also phenocopy zasp RNAi phenotype. In the wild-type lymph gland, expression of Trol is continuous around the lymph gland(A) indicating the presence of a continuous BM (A; arrow), while activation of Yki

leads to loss of BM (B-D) The phenotype of Yki activation can be divided into three broad categories; normal (A), mild moderate (B), and severe types (C and D). (E) Quantification of the above phenotype suggests that upon Yki activation in the niche (due to impaired muscle-niche interaction or loss of Wts), the basement membrane and LG homeostasis are drastically disturbed.

To further explore the mechanism by which the activation of Yki in niche leads to ectopic differentiation in the LG, activation of JNK signaling was examined. The JNK pathway has been implicated in the *Drosophila* hematopoietic progenitor differentiation (Owusu-Ansah and Banerjee, 2009). Recently, it has been shown that JNK signaling regulates fatty acid oxidation, which involves epigenetic modification required for progenitor cell differentiation (Tiwari et al., 2020).
To investigate whether Yki could activate JNK activity in the LG, TRE-GFP (Chatterjee and Bohmann, 2012; Shklover et al., 2015): the reporter of JNK signaling was examined. TRE-GFP has GFP under the control of AP-1 binding sites for JNK transcriptional effectors Jun/Fos. Up-regulation of Yki via expression of Wts RNAi in the niche driven by Antp–Gal4 resulted in a significant elevation of JNK signaling (green) in the niche (red) as well non-autonomous activation in the nearby progenitor cells (Figure 5.32C-C").

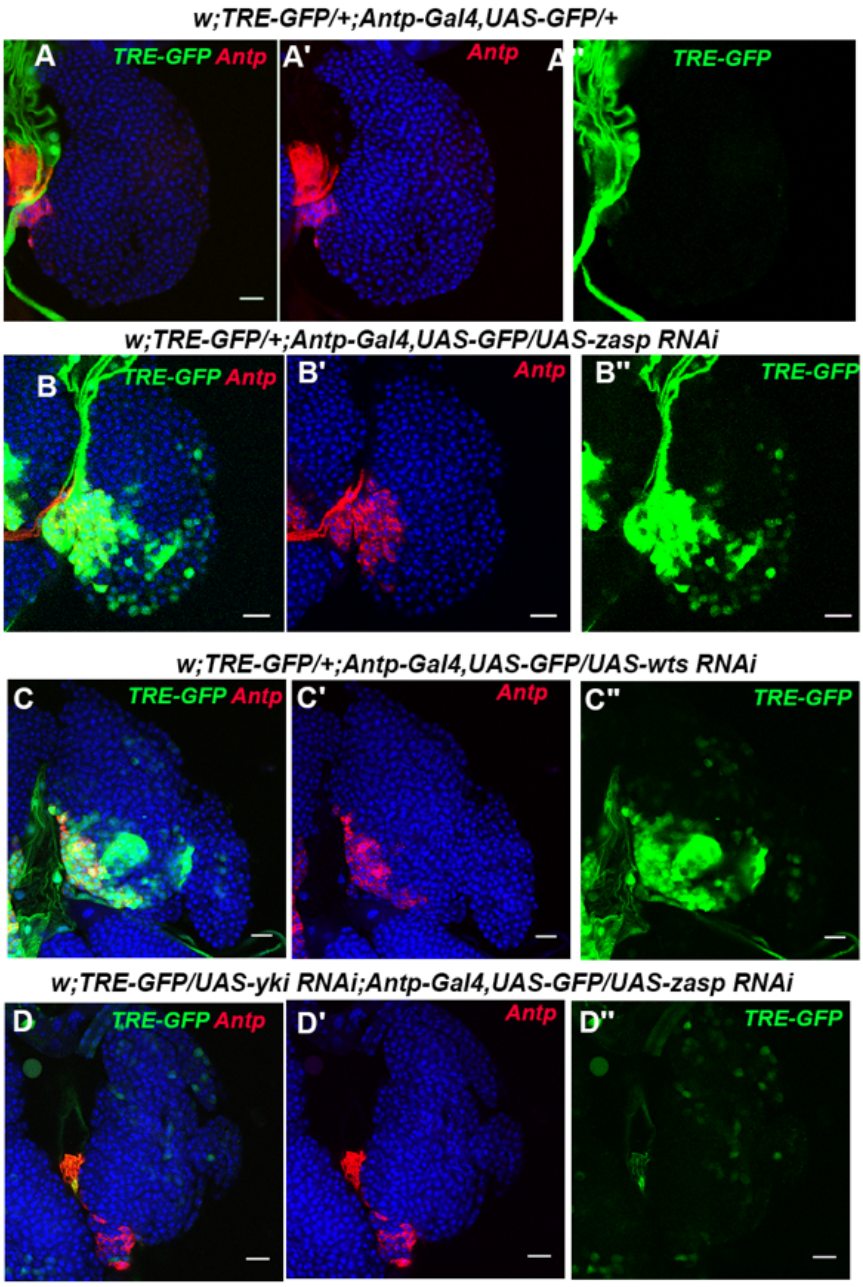

Figure 5.32 Ectopic activation of Yki up-regulates JNK activity in the LG. (A) TRE-GFP (green), a reporter for JNK signaling is expressed in basal level in the LG (A-A"). JNK signaling essential for the regulation of differentiation in the Lymph gland. Loss of Zasp (B-B") or Wts (C-C") increases JNK signaling reporter (TRE-GFP) in the niche (red) and in LG (blue). This increment was rescued upon the downregulation of Yki in the niche (D-D"), suggesting that activation of Yki in niche leads to the up-regulation of JNK signaling in the LG. Genotypes are shown on top of corresponding panels. Scale bar=20μm. DAPI marks the nucleus.

This non-autonomous activation of JNK in the progenitor cells can account for ectopic differentiation in the LG. Similar activation of JNK signaling was also found when Zasp was knockdown in the cardioblast (Figure 5.32B-B"), suggesting that ectopic activation of Yki signaling in the niche is sufficient to induce autonomous and non-autonomous JNK signaling in the

LG. Interestingly, inactivation of the Yki in this genetic background rescues JNK activation in the niche and progenitor cells (Figure 5.3D-D"), clearly suggesting that up-regulation of Yki in the niche (red) can activate autonomous and non-autonomous JNK signaling in the LG.

Together, these data suggest that the over-expression of Yki in the niche can activate non-autonomous JNK signaling in the LG, which induces ectopic differentiation. These data also explain the importance of regulation of Yki in the hematopoietic niche as hyper-activation of Yki in the niche can induce ectopic differentiation.

5.3 Discussion

The proper regulation of stem cells or progenitor cells is critical to maintaining normal tissue homeostasis (Biteau et al., 2011; Nakada et al., 2011). Stem cell niche is an entity that is essential to regulating the proliferation, maintenance, and differentiation of stem cells and their progeny (Morrison and Spradling, 2008) . It has been shown that stem cells lost their maintenance when the stem cell niche was altered, suggesting that the niche's homeostasis is essential. Therefore, alteration in hematopoietic niche numbers can affect progenitor cell maintenance. Regulation of niche is a complex process and need a tight regulation of complex signaling pathway. Various research in the diverse model organism has identified several molecular players secreted from niche essential to maintain homeostasis of the stem cell and progenitor cells ((Gattazzo et al., 2014; Zhang et al., 2003) but very little is known about the how cellular niche proliferate, and their proliferation rate is influenced by the cross talk of the diverse signaling pathway.

This study demonstrates the novel interaction between myoepithelial cardioblast and hematopoietic niche essential for *Drosophila* lymph gland (LG) homeostasis. This study also reveals a differential proliferation rate of a niche in developing the lymph gland at different development times. Furthermore, it has also been demonstrated that developmentally regulated actomyosin-Yki cascade in niche controls the proliferation of the hematopoietic niche. Interestingly, the hyper-activation of Yki in the niche non-autonomously activates JNK signaling, which elicits the differentiation signal in LG. Thus, the regulation of Yki in a niche in normal development is essential to maintain the lymph gland homeostasis.

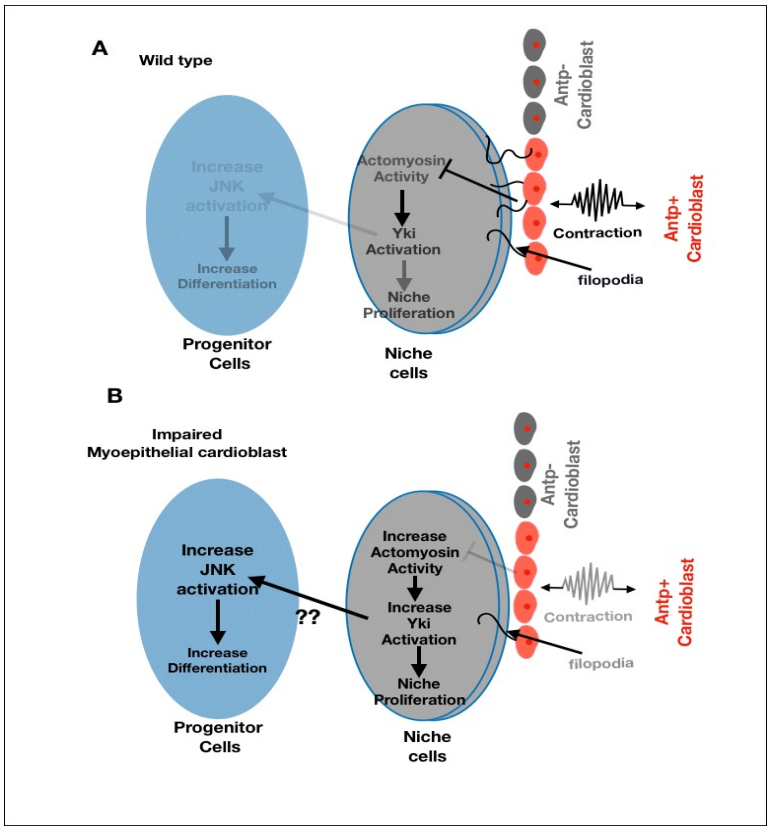

Figure 5.33 Myoepithelial cardioblast regulates niche proliferation and LG homeostasis (A) In wild type larvae, myoepithelial cardioblast regulates actomyosin activity and Yki activation in the hematopoietic niche. (B) Impairment of myoepithelial cardioblast contraction and filopodia formation increases actomyosin activity and Yki activation in the niche. Ectopic activation of Yki in niche up-regulates JNK activity in the niche and progenitor cells. Upregulation of JNK signaling in the progenitor cells causes ectopic differentiation in the LG. Thus, the regulation of Actomyosin-Yki activity in niche in normal development is essential to maintain the LG homeostasis.

Model describing the novel interaction between myoepithelial cardioblast and hematopoietic niche essential for *Drosophila* lymph gland (LG) homeostasis. This study demonstrated that actomyosin-Yki cascade in niche proliferation of the hematopoietic niche. Interestingly, the hyper-activation of Yki in the niche non-autonomously activates JNK signaling, which elicits the differentiation signal in LG. Thus, the regulation of Yki in the niche in normal development is essential to maintain the lymph gland homeostasis.

Yki and Mechanical intracellular tension modulate proliferation in the niche

Recent studies have revealed various signaling pathways, including Dpp, Wg, InR, and Lar involve in hematopoietic niche maintenance (Benmimoun et al., 2012; Kaur et al., 2019; Pennetier et al., 2012; Stofanko et al., 2010) whereas the involvement of Hippo pathway in hematopoietic niche maintenance remain elusive. The Hippo pathway is shown to play an evolutionarily conserved role in regulating cell growth, proliferation, and survival in tumorigenesis (Halder and Johnson, 2011; Irvine and Harvey, 2015; Kim et al., 2019; Snigdha et al., 2019; Zheng and Pan, 2019). This study suggests that Yki positively regulates the proliferation of the *Drosophila* hematopoietic niche. Similar to Yki, overexpression of ROCK, a positive regulator of actomyosin, also leads to increment in the niche cell number and Yki activation drastically. Thus, Yki activation could be considered one of the critical players of niche proliferation, along with previously reported players like insulin pathway, Dpp, and Wg signaling.

The increasing evidence suggests that Yki or Yap/Taz activity can be regulated by forces originating from the extracellular matrix, from shearing, stretching, and compression of cells (Elbediwy et al., 2016a; Elbediwy et al., 2016b; Sun and Irvine, 2016) . Interestingly, it has been shown that the contractility of muscle cells activates Yap, which activates Notch in neighbouring cells inhibiting their differentiation (Esteves de Lima et al., 2016). The link between mechanical forces and canonical Yki activity has been demonstrated in contexts of different tissue ((Isomursu et al., 2019; Moroishi et al., 2015; Panciera et al., 2017; Varelas, 2014) but niche specific role of actomyosin-Yki is not clear. It has been shown that apart from actomyosin-Jub (Rauskolb et al.,

2014), Wts can also be regulated through other upstream molecules like canonical hippo pathway and Msn (Jiang, 2018). It will be interesting in future studies to identify the other upstream regulators of Wts and actomyosin in the hematopoietic niche and their role in hematopoietic niche maintenance.

Myoepithelial cardioblast is a niche modulator in the Drosophila hematopoietic system

The results reported in this study establish the Antp+ myocardial cell (cardioblast) of *Drosophila* larval heart as an essential modulator of the hematopoietic niche. The reciprocal association between muscle function and immune response has been reported in *Drosophila* adult, zebrafish, and mammalian system (Chatterjee et al., 2016; Simpson et al., 2015; Yang et al., 2015). It has been demonstrated that impairment of muscle can affect the immune response (Chatterjee et al., 2016; Wu et al., 2020). However, direct evidence of muscle contraction and its effect on the hematopoietic system has been lacking. This study identifies the connection between hematopoiesis and myoepithelial cardioblast function. It has been found that impairment in myoepithelial cardioblast causes a drastic increment in niche cell proliferation through up-regulating the actomyosin mediated Yki activity. Additional work will be required to establish how impairment in myoepithelial cardioblast can up-regulate the actomyosin-Yki cascade in the niche. One possible explanation may be that muscle provides biophysical force or physical support to the niche, regulating the niche intercellular tension essential for niche homeostasis. Here, it might be possible that muscle-derived myopodia is involved in the muscle-hematopoietic niche interactions. It has been demonstrated that muscle-derived myopodia involve in muscle-myotube progenitor interaction and defect in muscle myopodia leads to a defect in muscle-myotube adhesion (Segal et al., 2016). Whether a similar mechanism is applicable here requires investigation. It is known in C.elegans, muscle regulates actomyosin activity in the epidermis through (Gillard et al., 2019; Zhang et al., 2011). It is a fascinating question, and future research in this direction will provide more informations about the importance of this mechanical support for maintenance of a stem cell niche.

Conclusion and Future Prospective -

Mechanical forces are important cues that dictate cellular fate via regulating maintenance or differentiation during tissue development (Hernandez-Hernandez et al., 2014; Vining and Mooney, 2017). In particular, it was demonstrated that actomyosin derived tension/force plays significant roles in various cellular processes such as size, shape, adhesion, migration, position, specification, and proliferation via modulating the gene expression within the cells (Munjal and Lecuit, 2014; Murrell et al., 2015; Rauskolb et al., 2014; Vicente-Manzanares et al., 2009). The coordination and integration of intracellular force generated by the actomyosin and gene expression within the cells control tissue patterning.

The *Drosophila* lymph gland has been widely used to study the basic biology of hematopoiesis, which opens the route to resolved the complex problem of vertebrate's hematopoietic system. Emerging evidences from different model system suggests that the precursor cells could encounter distinct mechanical tension in the tissue, regulating cell fate and function (Chaudhuri et al., 2016; Pathak et al., 2014; Shin et al., 2013). While much is known about crucial signaling molecules, which can regulate proliferation, maintenance, differentiation, and specification in the lymph gland (Banerjee et al., 2019; Benmimoun et al., 2012; Milton et al., 2014; Shim et al., 2012; Tiwari et al., 2020) the effects of mechanical cues/tension on lymph gland development is not worked out. This thesis describes the diverse role of actomyosin in lymph gland pattering. Here, using RNAi and Gal4-UAS system, we have identified the diverse role of actomyosin in the different cell type of lymph gland. Our data reveal a previously unknown role for actomyosin-mediated mechanotransduction in *Drosophila* hematopoiesis, whereby actomyosin regulates the proliferation and maintenance of the lymph gland cells.

The significant finding of this study is the discovery of the distinct role of actomyosin in LG morphogenesis. In progenitor cells, it plays dual roles. Early on, it is critical for progenitor cell proliferation through regulating G2 to M transition. Later on, in the quiescent progenitors, actomyosin activity regulates progenitor cells' maintenance. In the hematopoietic niche, the actomyosin activity is involved in the proliferation of niche cells.

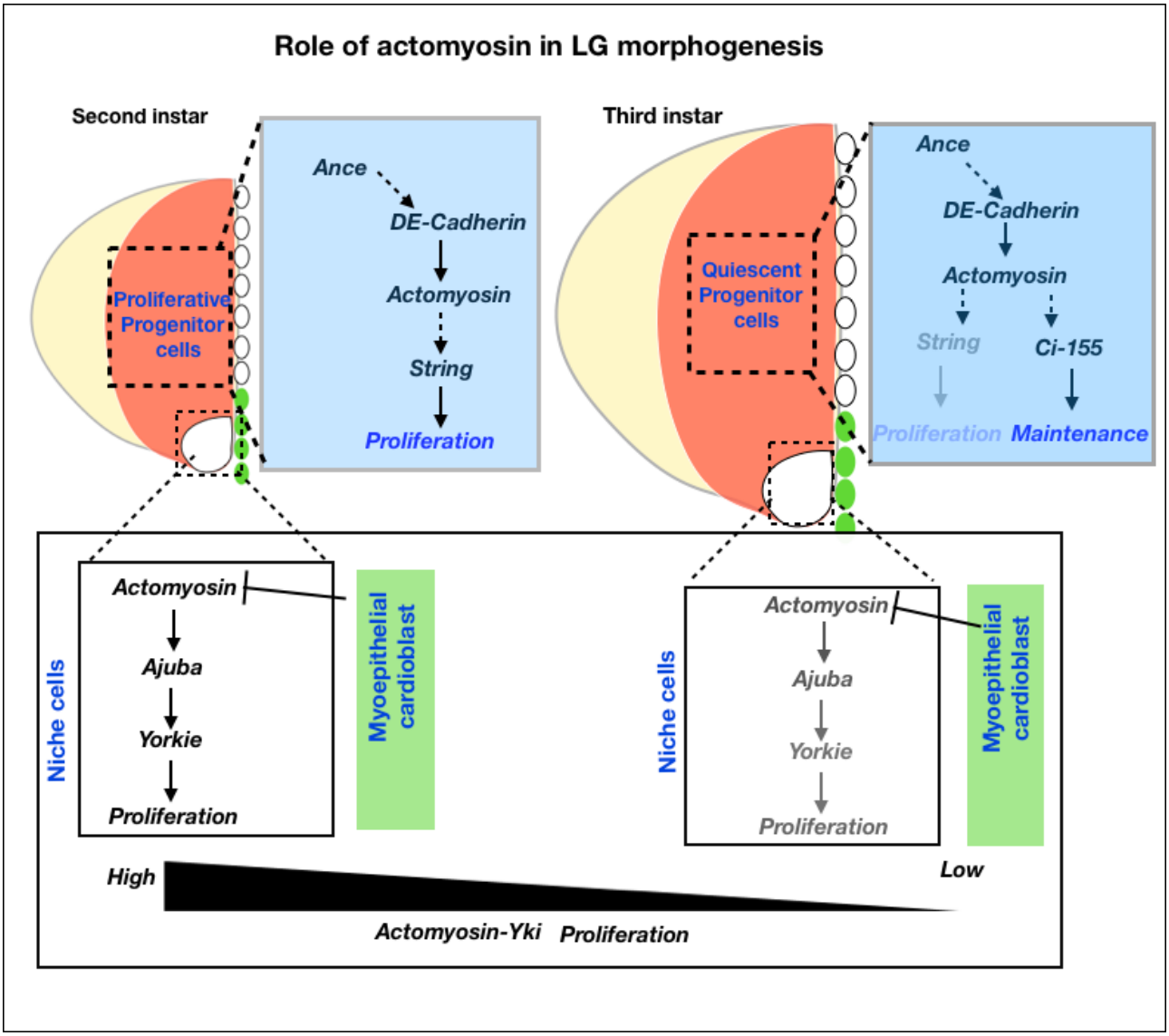

Figure 6.1 Diverse role of actomyosin complex in the developing *Drosophila* lymph gland.

In early instar, actomyosin-mediated intercellular tension regulates the proliferation of hematopoietic progenitor cells. The actomyosin genetically interacts with string, which is an essential regulator of G2 to M progression (Edgar, 1994). It will be interesting to test how G2 to M regulators like string physically interact with actomyosin in *Drosophila* hematopoietic progenitor cells. Although actomyosin involvement in proliferation has been reported in different model (Mammoto and Ingber, 2009; Mih et al., 2012; Rauskolb et al., 2014)) but its interaction with G2 specific regulator is not very well worked out.

Apart from the regulation of proliferation, actomyosin also involves in *Drosophila* hematopoietic progenitor cell maintenance. Previous studies have implicated Hh signaling as a positive regulator of progenitor cell maintenance (Mandal et al., 2007). This study has revealed that *Drosophila* hematopoietic progenitor is heterogeneous; Ance+ and Ance- progenitor cells. The existence of different cell types in LG opens a fundamental question of how this heterogeneity is established

and how different cell types in LG respond to Hh singling. Our results resolve these issues by demonstrating that level of Ci-155 is also controlled by actomyosin activity. In the model presented here, Hh secreted by niche regulates Ci-155 and generating a gradient of Ci-155 in the progenitor. Our findings indicate that actomyosin-mediated intracellular tension regulates the stability of Ci-155 that results in a sharp boundary within the MZ. During the transition from progenitor cells to IP cells, loss of Cell-cell adhesion and actomyosin assembly results in the desensitization of the blood progenitors to Hh signaling. Thus, the integration of mechanical force with signaling systems results in the pattering of LG progenitors. Previously, it has been demonstrated that activation of Ci-155 is regulated through a scaffold complex consisting of Cos2-Fu-Ci that must be translocated near the plasma membrane in the *Drosophila* wing disc (Ruel et al., 2003). We propose that the Cos2-Fu-Ci complex near the plasma membrane is supported by the actomyosin meshwork essential for Ci stabilization and activation.

Given their heterogeneous nature, additional genetic tools, and cell-specific actomyosin binding protein profiling will be needed to evaluate further the physical interaction between Ci-155 and actomyosin in Drosophila hematopoietic progenitor cells.

In niche, actomyosin is a decline with development and active at low levels in the wild-type third instar niche. It can be seen that early on, high actomyosin is essential for niche cell proliferation. In contrast, its repression in the late third instar niche is essential to maintain LG homeostasis, suggesting that aging can affect the actomyosin activity. Future studies will elucidate how aging regulates the actomyosin activation in the hematopoietic niche. It is also demonstrated that actomyosin regulates Yki through ajuba in the hematopoietic niche, and a high level of actomyosin can induce Yki activation in the late third instar niche. Furthermore, it is also shown that a high level of Yki induced the JNK activation in the LG and can lead to ectopic differentiation. It has been shown that Yki can induce JNK activity in the Drosophila wing disc, but the linear mechanism is not established (Ma et al., 2015). In the future, it will be worth to investigate the cross-talk between Yki and JNK in Drosophila hematopoietic niche.

The diverse responses of different cells type in LG to actomyosin require further investigation. However, it is plausible that these observed cell type-specific effects could be explained through the availability of specific proteins like Ajuba in the niche cells or Ci-155 in the progenitor cells. Beyond these signaling effectors, how actomyosin cross talks to other singling pathways, which are involved in *Drosophila* hematopoiesis, will be an exciting avenue for further study.

The actomyosin cytoskeleton is essential for various cellular processes, such as shape maintenance, migration, and proliferation. However, the basic assembly of actomyosin structure in every cell regulated through common protein such as myosin, actin, and a unique subset of accessory proteins

links it with other cellular structures, such as the plasma membrane (Zaidel-Bar et al., 2015). How actomyosin networks are enriched in specific cell types in the special-temporal manor is an area of intense research. In LG, actomyosin networks are also enriched differentially in niche and progenitor cells, as described in chapters 3, 4, and 5. The question then becomes what is regulating the differential enrichment of actomyosin in the lymph gland and why it is essential to maintain the lymph gland?

Our results suggest cardiomyocyte regulates actomyosin-Yki in the niche, which essential to maintain LG homeostasis. How could the impairment of cardiomyocyte trigger actomyosin-Yki activation in the *Drosophila* hematopoietic niche? Interestingly, YAP (Yki in Drosophila) was active in mammalian epithelial cells, fibroblasts, and endothelial cells, under stretch condition (Benham-Pyle et al., 2015; Li et al., 2018; Neto et al., 2018). Additionally, Yap is also active in endothelial cells during disturbed flow(Wang et al., 2016), suggesting the external input can also regulate Yap activity. Pathak et al., demonstrated that activation of Piezo1 channel through external traction force favours Yap activation through calcium signaling activation in human during human neural cells (Pathak et al., 2014). It is possible that the mechanosensitive receptors/ion channels that can mediate the Yki activation are localized at the niche. It will be fascinating to test this hypothesis by genetically knockdown the different mechanosensitive ion channels in the niche, which essential to the activation of Yki in the niche. Additionally, it is also possible that the mechanical activation channel triggered calcium-mediated signaling, which enhances actomyosin-Yki activation. Further experiments will be essential to determine the mechanical channel needed for the maintenance of the hematopoietic niche.

In sum, the current work contributes towards our understanding of *Drosophila* larval hematopoiesis, the heterogeneity in the progenitors, niche-progenitor cross-talk, progenitor maintenance through actomyosin activity, and cardiomyocytes-niche-progenitor interaction. Furthermore, we have characterized the cardiomyocytes-niche interaction, which is essential for lymph gland homeostasis. Overall, the study elucidates the role of actomyosin complex in *Drosophila* lymph gland morphogenesis and homeostasis.

Literature Cited

Adamo, L., Naveiras, O., Wenzel, P.L., McKinney-Freeman, S., Mack, P.J., Gracia-Sancho, J., Suchy-Dicey, A., Yoshimoto, M., Lensch, M.W., Yoder, M.C*., et al.* (2009). Biomechanical forces promote embryonic haematopoiesis. Nature *459*, 1131-1135.

Agaisse, H., Petersen, U.M., Boutros, M., Mathey-Prevot, B., and Perrimon, N. (2003). Signaling role of hemocytes in Drosophila JAK/STAT-dependent response to septic injury. Dev Cell *5*, 441-450.

Ahmed, S., Goh, W.I., and Bu, W. (2010). I-BAR domains, IRSp53 and filopodium formation. Semin Cell Dev Biol *21*, 350-356.

Amano, M., Mukai, H., Ono, Y., Chihara, K., Matsui, T., Hamajima, Y., Okawa, K., Iwamatsu, A., and Kaibuchi, K. (1996). Identification of a putative target for Rho as the serine-threonine kinase protein kinase N. Science *271*, 648-650.

Ammeux, N., Housden, B.E., Georgiadis, A., Hu, Y., and Perrimon, N. (2016). Mapping signaling pathway cross-talk in Drosophila cells. Proc Natl Acad Sci U S A *113*, 9940-9945.

Ando, Y., Yasuda, S., Oceguera-Yanez, F., and Narumiya, S. (2007). Inactivation of Rho GTPases with Clostridium difficile toxin B impairs centrosomal activation of Aurora-A in G2/M transition of HeLa cells. Mol Biol Cell *18*, 3752-3763.

Arakaki, Y., Kawai-Toyooka, H., Hamamura, Y., Higashiyama, T., Noga, A., Hirono, M., Olson, B.J., and Nozaki, H. (2013). The simplest integrated multicellular organism unveiled. PLoS One *8*, e81641.

Aranjuez, G., Burtscher, A., Sawant, K., Majumder, P., and McDonald, J.A. (2016). Dynamic myosin activation promotes collective morphology and migration by locally balancing oppositional forces from surrounding tissue. Mol Biol Cell *27*, 1898-1910.

Arber, S., Barbayannis, F.A., Hanser, H., Schneider, C., Stanyon, C.A., Bernard, O., and Caroni, P. (1998). Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. Nature *393*, 805-809.

Arnsdorf, E.J., Tummala, P., Kwon, R.Y., and Jacobs, C.R. (2009). Mechanically induced osteogenic differentiation--the role of RhoA, ROCKII and cytoskeletal dynamics. J Cell Sci *122*, 546-553.

Assoian, R.K., and Klein, E.A. (2008). Growth control by intracellular tension and extracellular stiffness. Trends Cell Biol *18*, 347-352.

Baldeosingh, R., Gao, H., Wu, X., and Fossett, N. (2018). Hedgehog signaling from the Posterior Signaling Center maintains U-shaped expression and a prohemocyte population in Drosophila. Dev Biol *441*, 132-145.

Bandura, J.L., Jiang, H., Nickerson, D.W., and Edgar, B.A. (2013). The molecular chaperone Hsp90 is required for cell cycle exit in Drosophila melanogaster. PLoS Genet *9*, e1003835.

Banerjee, U., Girard, J.R., Goins, L.M., and Spratford, C.M. (2019). Drosophila as a Genetic Model for Hematopoiesis. Genetics *211*, 367-417.

Barral, Y., Parra, M., Bidlingmaier, S., and Snyder, M. (1999). Nim1-related kinases coordinate cell cycle progression with the organization of the peripheral cytoskeleton in yeast. Genes Dev *13*, 176-187.

Behrndt, M., Salbreux, G., Campinho, P., Hauschild, R., Oswald, F., Roensch, J., Grill, S.W., and Heisenberg, C.P. (2012). Forces driving epithelial spreading in zebrafish gastrulation. Science *338*, 257-260.

Benham-Pyle, B.W., Pruitt, B.L., and Nelson, W.J. (2015). Cell adhesion. Mechanical strain induces E-cadherin-dependent Yap1 and beta-catenin activation to drive cell cycle entry. Science *348*, 1024-1027.

Benjamin, J.M., Kwiatkowski, A.V., Yang, C., Korobova, F., Pokutta, S., Svitkina, T., Weis, W.I., and Nelson, W.J. (2010). AlphaE-catenin regulates actin dynamics independently of cadherin-mediated cell-cell adhesion. J Cell Biol *189*, 339-352.

Benmimoun, B., Polesello, C., Haenlin, M., and Waltzer, L. (2015). The EBF transcription factor Collier directly promotes Drosophila blood cell progenitor maintenance independently of the niche. Proc Natl Acad Sci U S A *112*, 9052-9057.

Benmimoun, B., Polesello, C., Waltzer, L., and Haenlin, M. (2012). Dual role for Insulin/TOR signaling in the control of hematopoietic progenitor maintenance in Drosophila. Development *139*, 1713-1717.
Benson, K.R. (2001). T. H. Morgan's resistance to the chromosome theory. Nat Rev Genet *2*, 469-474.
Bernardoni, R., Vivancos, V., and Giangrande, A. (1997). glide/gcm is expressed and required in the scavenger cell lineage. Dev Biol *191*, 118-130.
Bernstein, K.E., Ong, F.S., Blackwell, W.L., Shah, K.H., Giani, J.F., Gonzalez-Villalobos, R.A., Shen, X.Z., Fuchs, S., and Touyz, R.M. (2013). A modern understanding of the traditional and nontraditional biological functions of angiotensin-converting enzyme. Pharmacol Rev *65*, 1-46.
Binggeli, O., Neyen, C., Poidevin, M., and Lemaitre, B. (2014). Prophenoloxidase activation is required for survival to microbial infections in Drosophila. PLoS Pathog *10*, e1004067.
Biteau, B., Hochmuth, C.E., and Jasper, H. (2011). Maintaining tissue homeostasis: dynamic control of somatic stem cell activity. Cell Stem Cell *9*, 402-411.
Blank, U., Karlsson, G., and Karlsson, S. (2008). Signaling pathways governing stem-cell fate. Blood *111*, 492-503.
Blanpain, C., and Fuchs, E. (2009). Epidermal homeostasis: a balancing act of stem cells in the skin. Nat Rev Mol Cell Biol *10*, 207-217.
Bouldin, C.M., Snelson, C.D., Farr, G.H., 3rd, and Kimelman, D. (2014). Restricted expression of cdc25a in the tailbud is essential for formation of the zebrafish posterior body. Genes Dev *28*, 384-395.
Bour, B.A., O'Brien, M.A., Lockwood, W.L., Goldstein, E.S., Bodmer, R., Taghert, P.H., Abmayr, S.M., and Nguyen, H.T. (1995). Drosophila MEF2, a transcription factor that is essential for myogenesis. Genes Dev *9*, 730-741.
Boutros, R., Lobjois, V., and Ducommun, B. (2007). CDC25 phosphatases in cancer cells: key players? Good targets? Nat Rev Cancer *7*, 495-507.
Brand, A.H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*, 401-415.
Brembeck, F.H., Rosario, M., and Birchmeier, W. (2006). Balancing cell adhesion and Wnt signaling, the key role of beta-catenin. Curr Opin Genet Dev *16*, 51-59.
Brown, S., Hu, N., and Hombria, J.C. (2001). Identification of the first invertebrate interleukin JAK/STAT receptor, the Drosophila gene domeless. Curr Biol *11*, 1700-1705.
Bryan, B.A., Dennstedt, E., Mitchell, D.C., Walshe, T.E., Noma, K., Loureiro, R., Saint-Geniez, M., Campaigniac, J.P., Liao, J.K., and D'Amore, P.A. (2010). RhoA/ROCK signaling is essential for multiple aspects of VEGF-mediated angiogenesis. FASEB J *24*, 3186-3195.
Bunt, S., Hooley, C., Hu, N., Scahill, C., Weavers, H., and Skaer, H. (2010). Hemocyte-secreted type IV collagen enhances BMP signaling to guide renal tubule morphogenesis in Drosophila. Dev Cell *19*, 296-306.
Bylund, J.B., Trinh, L.T., Awgulewitsch, C.P., Paik, D.T., Jetter, C., Jha, R., Zhang, J., Nolan, K., Xu, C., Thompson, T.B.*, et al.* (2017). Coordinated Proliferation and Differentiation of Human-Induced Pluripotent Stem Cell-Derived Cardiac Progenitor Cells Depend on Bone Morphogenetic Protein Signaling Regulation by GREMLIN 2. Stem Cells Dev *26*, 678-693.
Calabrese, E.J. (2018). Muller's nobel prize research and peer review. Philos Ethics Humanit Med *13*, 15.
Carlson, M.E., and Conboy, I.M. (2007). Loss of stem cell regenerative capacity within aged niches. Aging Cell *6*, 371-382.
Carlson, M.E., Suetta, C., Conboy, M.J., Aagaard, P., Mackey, A., Kjaer, M., and Conboy, I. (2009). Molecular aging and rejuvenation of human muscle stem cells. EMBO Mol Med *1*, 381-391.
Cartagena-Rivera, A.X., Logue, J.S., Waterman, C.M., and Chadwick, R.S. (2016). Actomyosin Cortical Mechanical Properties in Nonadherent Cells Determined by Atomic Force Microscopy. Biophys J *110*, 2528-2539.
Cerenius, L., Lee, B.L., and Soderhall, K. (2008). The proPO-system: pros and cons for its role in invertebrate immunity. Trends Immunol *29*, 263-271.

Cevik, D., Acker, M., Michalski, C., and Jacobs, J.R. (2019). Pericardin, a Drosophila collagen, facilitates accumulation of hemocytes at the heart. Dev Biol *454*, 52-65.
Chakrabarti, S., Dudzic, J.P., Li, X., Collas, E.J., Boquete, J.P., and Lemaitre, B. (2016). Remote Control of Intestinal Stem Cell Activity by Haemocytes in Drosophila. PLoS Genet *12*, e1006089.
Chan, C.J., Heisenberg, C.P., and Hiiragi, T. (2017). Coordination of Morphogenesis and Cell-Fate Specification in Development. Curr Biol *27*, R1024-R1035.
Chan, J.S.K., Sng, M.K., Teo, Z.Q., Chong, H.C., Twang, J.S., and Tan, N.S. (2018). Targeting nuclear receptors in cancer-associated fibroblasts as concurrent therapy to inhibit development of chemoresistant tumors. Oncogene *37*, 160-173.
Chatterjee, A., Roy, D., Patnaik, E., and Nongthomba, U. (2016). Muscles provide protection during microbial infection by activating innate immune response pathways in Drosophila and zebrafish. Dis Model Mech *9*, 697-705.
Chatterjee, D., Katewa, S.D., Qi, Y., Jackson, S.A., Kapahi, P., and Jasper, H. (2014). Control of metabolic adaptation to fasting by dILP6-induced insulin signaling in Drosophila oenocytes. Proc Natl Acad Sci U S A *111*, 17959-17964.
Chatterjee, N., and Bohmann, D. (2012). A versatile PhiC31 based reporter system for measuring AP-1 and Nrf2 signaling in Drosophila and in tissue culture. PLoS One *7*, e34063.
Chaudhuri, O., Gu, L., Klumpers, D., Darnell, M., Bencherif, S.A., Weaver, J.C., Huebsch, N., Lee, H.P., Lippens, E., Duda, G.N.*, et al.* (2016). Hydrogels with tunable stress relaxation regulate stem cell fate and activity. Nat Mater *15*, 326-334.
Chen, C.S., Hong, S., Indra, I., Sergeeva, A.P., Troyanovsky, R.B., Shapiro, L., Honig, B., and Troyanovsky, S.M. (2015). alpha-Catenin-mediated cadherin clustering couples cadherin and actin dynamics. J Cell Biol *210*, 647-661.
Chen, Y., Cardinaux, J.R., Goodman, R.H., and Smolik, S.M. (1999). Mutants of cubitus interruptus that are independent of PKA regulation are independent of hedgehog signaling. Development *126*, 3607-3616.
Chen, Y., and Jiang, J. (2013). Decoding the phosphorylation code in Hedgehog signal transduction. Cell Res *23*, 186-200.
Choi, N.H., Kim, J.G., Yang, D.J., Kim, Y.S., and Yoo, M.A. (2008). Age-related changes in Drosophila midgut are associated with PVF2, a PDGF/VEGF-like growth factor. Aging Cell *7*, 318-334.
Chugh, P., and Paluch, E.K. (2018). The actin cortex at a glance. J Cell Sci *131*.
Croft, D.R., and Olson, M.F. (2006). The Rho GTPase effector ROCK regulates cyclin A, cyclin D1, and p27Kip1 levels by distinct mechanisms. Mol Cell Biol *26*, 4612-4627.
Crozatier, M., Ubeda, J.M., Vincent, A., and Meister, M. (2004). Cellular immune response to parasitization in Drosophila requires the EBF orthologue collier. PLoS Biol *2*, E196.
Das Thakur, M., Feng, Y., Jagannathan, R., Seppa, M.J., Skeath, J.B., and Longmore, G.D. (2010). Ajuba LIM proteins are negative regulators of the Hippo signaling pathway. Curr Biol *20*, 657-662.
Decotto, E., and Spradling, A.C. (2005). The Drosophila ovarian and testis stem cell niches: similar somatic stem cells and signals. Dev Cell *9*, 501-510.
del Valle Rodriguez, A., Didiano, D., and Desplan, C. (2011). Power tools for gene expression and clonal analysis in Drosophila. Nat Methods *9*, 47-55.
Dey, N.S., Ramesh, P., Chugh, M., Mandal, S., and Mandal, L. (2016). Dpp dependent Hematopoietic stem cells give rise to Hh dependent blood progenitors in larval lymph gland of Drosophila. Elife *5*.
Di Talia, S., and Wieschaus, E.F. (2012). Short-term integration of Cdc25 dynamics controls mitotic entry during Drosophila gastrulation. Dev Cell *22*, 763-774.
Domnina, L.V., Ivanova, O.Y., Pletjushkina, O.Y., Fetisova, E.K., Chernyak, B.V., Skulachev, V.P., and Vasiliev, J.M. (2004). Marginal blebbing during the early stages of TNF-induced apoptosis indicates alteration in actomyosin contractility. Cell Biol Int *28*, 471-475.
Dragojlovic-Munther, M., and Martinez-Agosto, J.A. (2012). Multifaceted roles of PTEN and TSC orchestrate growth and differentiation of Drosophila blood progenitors. Development *139*, 3752-3763.

Dragojlovic-Munther, M., and Martinez-Agosto, J.A. (2013). Extracellular matrix-modulated Heartless signaling in Drosophila blood progenitors regulates their differentiation via a Ras/ETS/FOG pathway and target of rapamycin function. Dev Biol *384*, 313-330.
Drechsler, M., Schmidt, A.C., Meyer, H., and Paululat, A. (2013). The conserved ADAMTS-like protein lonely heart mediates matrix formation and cardiac tissue integrity. PLoS Genet *9*, e1003616.
Dumstrei, K., Wang, F., and Hartenstein, V. (2003). Role of DE-cadherin in neuroblast proliferation, neural morphogenesis, and axon tract formation in Drosophila larval brain development. J Neurosci *23*, 3325-3335.
Dupont, S., Morsut, L., Aragona, M., Enzo, E., Giulitti, S., Cordenonsi, M., Zanconato, F., Le Digabel, J., Forcato, M., Bicciato, S*., et al.* (2011). Role of YAP/TAZ in mechanotransduction. Nature *474*, 179-183.
Duvic, B., Hoffmann, J.A., Meister, M., and Royet, J. (2002). Notch signaling controls lineage specification during Drosophila larval hematopoiesis. Curr Biol *12*, 1923-1927.
Dzierzak, E., and Bigas, A. (2018). Blood Development: Hematopoietic Stem Cell Dependence and Independence. Cell Stem Cell *22*, 639-651.
Edgar, B.A. (1994). Cell cycle. Cell-cycle control in a developmental context. Curr Biol *4*, 522-524.
Edgar, B.A., Lehman, D.A., and O'Farrell, P.H. (1994). Transcriptional regulation of string (cdc25): a link between developmental programming and the cell cycle. Development *120*, 3131-3143.
Edgar, B.A., and O'Farrell, P.H. (1990). The three postblastoderm cell cycles of Drosophila embryogenesis are regulated in G2 by string. Cell *62*, 469-480.
Edwards, K.A., Demsky, M., Montague, R.A., Weymouth, N., and Kiehart, D.P. (1997). GFP-moesin illuminates actin cytoskeleton dynamics in living tissue and demonstrates cell shape changes during morphogenesis in Drosophila. Dev Biol *191*, 103-117.
Edwards, K.A., and Kiehart, D.P. (1996). Drosophila nonmuscle myosin II has multiple essential roles in imaginal disc and egg chamber morphogenesis. Development *122*, 1499-1511.
El-Amraoui, A., and Petit, C. (2010). Cadherins as targets for genetic diseases. Cold Spring Harb Perspect Biol *2*, a003095.
Elbediwy, A., Vincent-Mistiaen, Z.I., Spencer-Dene, B., Stone, R.K., Boeing, S., Wculek, S.K., Cordero, J., Tan, E.H., Ridgway, R., Brunton, V.G*., et al.* (2016a). Integrin signalling regulates YAP and TAZ to control skin homeostasis. Development *143*, 1674-1687.
Elbediwy, A., Vincent-Mistiaen, Z.I., and Thompson, B.J. (2016b). YAP and TAZ in epithelial stem cells: A sensor for cell polarity, mechanical forces and tissue damage. Bioessays *38*, 644-653.
Erez, N., Truitt, M., Olson, P., Arron, S.T., and Hanahan, D. (2010). Cancer-Associated Fibroblasts Are Activated in Incipient Neoplasia to Orchestrate Tumor-Promoting Inflammation in an NF-kappaB-Dependent Manner. Cancer Cell *17*, 135-147.
Esteves de Lima, J., Bonnin, M.A., Birchmeier, C., and Duprez, D. (2016). Muscle contraction is required to maintain the pool of muscle progenitors via YAP and NOTCH during fetal myogenesis. Elife *5*.
Evans, C.J., Hartenstein, V., and Banerjee, U. (2003). Thicker than blood: conserved mechanisms in Drosophila and vertebrate hematopoiesis. Dev Cell *5*, 673-690.
Evans, C.J., Liu, T., and Banerjee, U. (2014). Drosophila hematopoiesis: Markers and methods for molecular genetic analysis. Methods *68*, 242-251.
Evans, C.J., Olson, J.M., Ngo, K.T., Kim, E., Lee, N.E., Kuoy, E., Patananan, A.N., Sitz, D., Tran, P., Do, M.T*., et al.* (2009). G-TRACE: rapid Gal4-based cell lineage analysis in Drosophila. Nat Methods *6*, 603-605.
Ewen-Campen, B., Yang-Zhou, D., Fernandes, V.R., Gonzalez, D.P., Liu, L.P., Tao, R., Ren, X., Sun, J., Hu, Y., Zirin, J*., et al.* (2017). Optimized strategy for in vivo Cas9-activation in Drosophila. Proc Natl Acad Sci U S A *114*, 9409-9414.
Feinberg, E.H., Vanhoven, M.K., Bendesky, A., Wang, G., Fetter, R.D., Shen, K., and Bargmann, C.I. (2008). GFP Reconstitution Across Synaptic Partners (GRASP) defines cell contacts and synapses in living nervous systems. Neuron *57*, 353-363.

Ferguson, G.B., and Martinez-Agosto, J.A. (2014). Yorkie and Scalloped signaling regulates Notch-dependent lineage specification during Drosophila hematopoiesis. Curr Biol *24*, 2665-2672.
Ferraro, F., Lymperi, S., Mendez-Ferrer, S., Saez, B., Spencer, J.A., Yeap, B.Y., Masselli, E., Graiani, G., Prezioso, L., Rizzini, E.L.*, et al.* (2011). Diabetes impairs hematopoietic stem cell mobilization by altering niche function. Sci Transl Med *3*, 104ra101.
Fessler, J.H., Nelson, R.E., and Fessler, L.I. (1994). Preparation of extracellular matrix. Methods Cell Biol *44*, 303-328.
Flores-Benitez, D., and Knust, E. (2015). Crumbs is an essential regulator of cytoskeletal dynamics and cell-cell adhesion during dorsal closure in Drosophila. Elife *4*.
Fogarty, C.E., Diwanji, N., Lindblad, J.L., Tare, M., Amcheslavsky, A., Makhijani, K., Bruckner, K., Fan, Y., and Bergmann, A. (2016). Extracellular Reactive Oxygen Species Drive Apoptosis-Induced Proliferation via Drosophila Macrophages. Curr Biol *26*, 575-584.
Fossett, N., Hyman, K., Gajewski, K., Orkin, S.H., and Schulz, R.A. (2003). Combinatorial interactions of serpent, lozenge, and U-shaped regulate crystal cell lineage commitment during Drosophila hematopoiesis. Proc Natl Acad Sci U S A *100*, 11451-11456.
Fossett, N., and Schulz, R.A. (2001). Functional conservation of hematopoietic factors in Drosophila and vertebrates. Differentiation *69*, 83-90.
Fossett, N., Tevosian, S.G., Gajewski, K., Zhang, Q., Orkin, S.H., and Schulz, R.A. (2001). The Friend of GATA proteins U-shaped, FOG-1, and FOG-2 function as negative regulators of blood, heart, and eye development in Drosophila. Proc Natl Acad Sci U S A *98*, 7342-7347.
Fournier, D., Luft, F.C., Bader, M., Ganten, D., and Andrade-Navarro, M.A. (2012). Emergence and evolution of the renin-angiotensin-aldosterone system. J Mol Med (Berl) *90*, 495-508.
Franc, N.C., Dimarcq, J.L., Lagueux, M., Hoffmann, J., and Ezekowitz, R.A. (1996). Croquemort, a novel Drosophila hemocyte/macrophage receptor that recognizes apoptotic cells. Immunity *4*, 431-443.
Francis, D., Chanana, B., Fernandez, B., Gordon, B., Mak, T., and Palacios, I.M. (2019). YAP/Yorkie in the germline modulates the age-related decline of germline stem cells and niche cells. PLoS One *14*, e0213327.
Fuchs, S., Frenzel, K., Hubert, C., Lyng, R., Muller, L., Michaud, A., Xiao, H.D., Adams, J.W., Capecchi, M.R., Corvol, P.*, et al.* (2005). Male fertility is dependent on dipeptidase activity of testis ACE. Nat Med *11*, 1140-1142; author reply 1142-1143.
Fuchs, S., Xiao, H.D., Cole, J.M., Adams, J.W., Frenzel, K., Michaud, A., Zhao, H., Keshelava, G., Capecchi, M.R., Corvol, P.*, et al.* (2004). Role of the N-terminal catalytic domain of angiotensin-converting enzyme investigated by targeted inactivation in mice. J Biol Chem *279*, 15946-15953.
Fujihara, Y., Tokuhiro, K., Muro, Y., Kondoh, G., Araki, Y., Ikawa, M., and Okabe, M. (2013). Expression of TEX101, regulated by ACE, is essential for the production of fertile mouse spermatozoa. Proc Natl Acad Sci U S A *110*, 8111-8116.
Furusawa, C., and Kaneko, K. (2012). A dynamical-systems view of stem cell biology. Science *338*, 215-217.
Gao, H., Wu, X., and Fossett, N. (2009). Upregulation of the Drosophila Friend of GATA gene U-shaped by JAK/STAT signaling maintains lymph gland prohemocyte potency. Mol Cell Biol *29*, 6086-6096.
Gao, H., Wu, X., and Fossett, N. (2013). Drosophila E-cadherin functions in hematopoietic progenitors to maintain multipotency and block differentiation. PLoS One *8*, e74684.
Gattazzo, F., Urciuolo, A., and Bonaldo, P. (2014). Extracellular matrix: a dynamic microenvironment for stem cell niche. Biochim Biophys Acta *1840*, 2506-2519.
Ge, Y., Miao, Y., Gur-Cohen, S., Gomez, N., Yang, H., Nikolova, M., Polak, L., Hu, Y., Verma, A., Elemento, O.*, et al.* (2020). The aging skin microenvironment dictates stem cell behavior. Proc Natl Acad Sci U S A *117*, 5339-5350.
Ghosh, S., Singh, A., Mandal, S., and Mandal, L. (2015). Active hematopoietic hubs in Drosophila adults generate hemocytes and contribute to immune response. Dev Cell *33*, 478-488.
Gillard, G., Nicolle, O., Brugiere, T., Prigent, S., Pinot, M., and Michaux, G. (2019). Force Transmission between Three Tissues Controls Bipolar Planar Polarity Establishment and Morphogenesis. Curr Biol *29*, 1360-1368 e1364.

Giordani, G., Barraco, M., Giangrande, A., Martinelli, G., Guadagnuolo, V., Simonetti, G., Perini, G., and Bernardoni, R. (2016). The human Smoothened inhibitor PF-04449913 induces exit from quiescence and loss of multipotent Drosophila hematopoietic progenitor cells. Oncotarget *7*, 55313-55327.
Gkretsi, V., and Stylianopoulos, T. (2018). Cell Adhesion and Matrix Stiffness: Coordinating Cancer Cell Invasion and Metastasis. Front Oncol *8*, 145.
Goodwin, K., Lostchuck, E.E., Cramb, K.M.L., Zulueta-Coarasa, T., Fernandez-Gonzalez, R., and Tanentzapf, G. (2017). Cell-cell and cell-extracellular matrix adhesions cooperate to organize actomyosin networks and maintain force transmission during dorsal closure. Mol Biol Cell *28*, 1301-1310.
Goto, A., Kadowaki, T., and Kitagawa, Y. (2003). Drosophila hemolectin gene is expressed in embryonic and larval hemocytes and its knock down causes bleeding defects. Dev Biol *264*, 582-591.
Goupil, E., Amini, R., Hall, D.H., and Labbe, J.C. (2017). Actomyosin contractility regulators stabilize the cytoplasmic bridge between the two primordial germ cells during Caenorhabditis elegans embryogenesis. Mol Biol Cell *28*, 3789-3800.
Green, J.B., and Sharpe, J. (2015). Positional information and reaction-diffusion: two big ideas in developmental biology combine. Development *142*, 1203-1211.
Green, M.M. (2010). 2010: A century of Drosophila genetics through the prism of the white gene. Genetics *184*, 3-7.
Grigorian, M., Liu, T., Banerjee, U., and Hartenstein, V. (2013). The proteoglycan Trol controls the architecture of the extracellular matrix and balances proliferation and differentiation of blood progenitors in the Drosophila lymph gland. Dev Biol *384*, 301-312.
Grosshans, J., and Wieschaus, E. (2000). A genetic link between morphogenesis and cell division during formation of the ventral furrow in Drosophila. Cell *101*, 523-531.
Gupton, S.L., and Waterman-Storer, C.M. (2006). Spatiotemporal feedback between actomyosin and focal-adhesion systems optimizes rapid cell migration. Cell *125*, 1361-1374.
Halder, G., and Johnson, R.L. (2011). Hippo signaling: growth control and beyond. Development *138*, 9-22.
Hales, K.G., Korey, C.A., Larracuente, A.M., and Roberts, D.M. (2015). Genetics on the Fly: A Primer on the Drosophila Model System. Genetics *201*, 815-842.
Hall, E.T., Hoesing, E., Sinkovics, E., and Verheyen, E.M. (2019). Actomyosin contractility modulates Wnt signaling through adherens junction stability. Mol Biol Cell *30*, 411-426.
Hallier, B., Hoffmann, J., Roeder, T., Togel, M., Meyer, H., and Paululat, A. (2015). The bHLH Transcription Factor Hand Regulates the Expression of Genes Critical to Heart and Muscle Function in Drosophila melanogaster. PLoS One *10*, e0134204.
Han, Z., Yi, P., Li, X., and Olson, E.N. (2006). Hand, an evolutionarily conserved bHLH transcription factor required for Drosophila cardiogenesis and hematopoiesis. Development *133*, 1175-1182.
Handorf, A.M., Zhou, Y., Halanski, M.A., and Li, W.J. (2015). Tissue stiffness dictates development, homeostasis, and disease progression. Organogenesis *11*, 1-15.
Hansson, E.M., Lendahl, U., and Chapman, G. (2004). Notch signaling in development and disease. Semin Cancer Biol *14*, 320-328.
Hao, Y., and Jin, L.H. (2017). Dual role for Jumu in the control of hematopoietic progenitors in the Drosophila lymph gland. Elife *6*.
Hartenstein, V. (2006). Blood cells and blood cell development in the animal kingdom. Annu Rev Cell Dev Biol *22*, 677-712.
Hashimoto, T., Perlot, T., Rehman, A., Trichereau, J., Ishiguro, H., Paolino, M., Sigl, V., Hanada, T., Hanada, R., Lipinski, S.*, et al.* (2012). ACE2 links amino acid malnutrition to microbial ecology and intestinal inflammation. Nature *487*, 477-481.
Heer, N.C., and Martin, A.C. (2017). Tension, contraction and tissue morphogenesis. Development *144*, 4249-4260.
Heer, N.C., Miller, P.W., Chanet, S., Stoop, N., Dunkel, J., and Martin, A.C. (2017). Actomyosin-based tissue folding requires a multicellular myosin gradient. Development *144*, 1876-1886.

Heller, E., and Fuchs, E. (2015). Tissue patterning and cellular mechanics. J Cell Biol *211*, 219-231.
Hernandez-Hernandez, V., Rueda, D., Caballero, L., Alvarez-Buylla, E.R., and Benitez, M. (2014). Mechanical forces as information: an integrated approach to plant and animal development. Front Plant Sci *5*, 265.
Holz, A., Bossinger, B., Strasser, T., Janning, W., and Klapper, R. (2003). The two origins of hemocytes in Drosophila. Development *130*, 4955-4962.
Huelsken, J., and Hanahan, D. (2018). A Subset of Cancer-Associated Fibroblasts Determines Therapy Resistance. Cell *172*, 643-644.
Hunter, M.V., Lee, D.M., Harris, T.J., and Fernandez-Gonzalez, R. (2015). Polarized E-cadherin endocytosis directs actomyosin remodeling during embryonic wound repair. J Cell Biol *210*, 801-816.
Hurst, D., Rylett, C.M., Isaac, R.E., and Shirras, A.D. (2003). The drosophila angiotensin-converting enzyme homologue Ance is required for spermiogenesis. Dev Biol *254*, 238-247.
Hyvari, L., Ojansivu, M., Juntunen, M., Kartasalo, K., Miettinen, S., and Vanhatupa, S. (2018). Focal Adhesion Kinase and ROCK Signaling Are Switch-Like Regulators of Human Adipose Stem Cell Differentiation towards Osteogenic and Adipogenic Lineages. Stem Cells Int *2018*, 2190657.
Ikeya, T., Galic, M., Belawat, P., Nairz, K., and Hafen, E. (2002). Nutrient-dependent expression of insulin-like peptides from neuroendocrine cells in the CNS contributes to growth regulation in Drosophila. Curr Biol *12*, 1293-1300.
Inaba, M., Yuan, H., and Yamashita, Y.M. (2011). String (Cdc25) regulates stem cell maintenance, proliferation and aging in Drosophila testis. Development *138*, 5079-5086.
Ingham, P.W., and Placzek, M. (2006). Orchestrating ontogenesis: variations on a theme by sonic hedgehog. Nat Rev Genet *7*, 841-850.
Irvine, K.D., and Harvey, K.F. (2015). Control of organ growth by patterning and hippo signaling in Drosophila. Cold Spring Harb Perspect Biol *7*.
Irving, P., Ubeda, J.M., Doucet, D., Troxler, L., Lagueux, M., Zachary, D., Hoffmann, J.A., Hetru, C., and Meister, M. (2005). New insights into Drosophila larval haemocyte functions through genome-wide analysis. Cell Microbiol *7*, 335-350.
Isomursu, A., Lerche, M., Taskinen, M.E., Ivaska, J., and Peuhu, E. (2019). Integrin signaling and mechanotransduction in regulation of somatic stem cells. Exp Cell Res *378*, 217-225.
Ito, K., Awano, W., Suzuki, K., Hiromi, Y., and Yamamoto, D. (1997). The Drosophila mushroom body is a quadruple structure of clonal units each of which contains a virtually identical set of neurones and glial cells. Development *124*, 761-771.
Jarov, A., Williams, K.P., Ling, L.E., Koteliansky, V.E., Duband, J.L., and Fournier-Thibault, C. (2003). A dual role for Sonic hedgehog in regulating adhesion and differentiation of neuroepithelial cells. Dev Biol *261*, 520-536.
Jeanes, A., Gottardi, C.J., and Yap, A.S. (2008). Cadherins and cancer: how does cadherin dysfunction promote tumor progression? Oncogene *27*, 6920-6929.
Jia, D., Soylemez, M., Calvin, G., Bornmann, R., Bryant, J., Hanna, C., Huang, Y.C., and Deng, W.M. (2015). A large-scale in vivo RNAi screen to identify genes involved in Notch-mediated follicle cell differentiation and cell cycle switches. Sci Rep *5*, 12328.
Jiang, J. (2018). Misshapen Connects Food, Mechanosensing, and Intestinal Growth. Dev Cell *45*, 417-418.
Jodoin, J.N., Coravos, J.S., Chanet, S., Vasquez, C.G., Tworoger, M., Kingston, E.R., Perkins, L.A., Perrimon, N., and Martin, A.C. (2015). Stable Force Balance between Epithelial Cells Arises from F-Actin Turnover. Dev Cell *35*, 685-697.
Johnston, L.A., and Edgar, B.A. (1998). Wingless and Notch regulate cell-cycle arrest in the developing Drosophila wing. Nature *394*, 82-84.
Jokubaitis, V.J., Sinka, L., Driessen, R., Whitty, G., Haylock, D.N., Bertoncello, I., Smith, I., Peault, B., Tavian, M., and Simmons, P.J. (2008). Angiotensin-converting enzyme (CD143) marks hematopoietic stem cells in human embryonic, fetal, and adult hematopoietic tissues. Blood *111*, 4055-4063.
Jordan, P., and Karess, R. (1997). Myosin light chain-activating phosphorylation sites are required for oogenesis in Drosophila. J Cell Biol *139*, 1805-1819.

Jung, S.H., Evans, C.J., Uemura, C., and Banerjee, U. (2005). The Drosophila lymph gland as a developmental model of hematopoiesis. Development *132*, 2521-2533.
Jurado, J., de Navascues, J., and Gorfinkiel, N. (2016). alpha-Catenin stabilises Cadherin-Catenin complexes and modulates actomyosin dynamics to allow pulsatile apical contraction. J Cell Sci *129*, 4496-4508.
Kasza, K.E., and Zallen, J.A. (2011). Dynamics and regulation of contractile actin-myosin networks in morphogenesis. Curr Opin Cell Biol *23*, 30-38.
Kaur, H., Sharma, S.K., Mandal, S., and Mandal, L. (2019). Lar maintains the homeostasis of the hematopoietic organ in Drosophila by regulating insulin signaling in the niche. Development *146*.
Kawano, R., Ohta, K., and Lupo, G. (2017). Cadherin-7 enhances Sonic Hedgehog signalling by preventing Gli3 repressor formation during neural tube patterning. Open Biol *7*.
Khadilkar, R.J., and Tanentzapf, G. (2019). Septate junction components control Drosophila hematopoiesis through the Hippo pathway. Development *146*.
Khadilkar, R.J., Vogl, W., Goodwin, K., and Tanentzapf, G. (2017). Modulation of occluding junctions alters the hematopoietic niche to trigger immune activation. Elife *6*.
Kiger, J.A., Jr., Natzle, J.E., and Green, M.M. (2001). Hemocytes are essential for wing maturation in Drosophila melanogaster. Proc Natl Acad Sci U S A *98*, 10190-10195.
Kim, A.R., Choi, E.B., Kim, M.Y., and Choi, K.W. (2017). Angiotensin-converting enzyme Ance is cooperatively regulated by Mad and Pannier in Drosophila imaginal discs. Sci Rep *7*, 13174.
Kim, C.L., Choi, S.H., and Mo, J.S. (2019). Role of the Hippo Pathway in Fibrosis and Cancer. Cells *8*.
Kimble, J.E., and White, J.G. (1981). On the control of germ cell development in Caenorhabditis elegans. Dev Biol *81*, 208-219.
Klinedinst, S.L., and Bodmer, R. (2003). Gata factor Pannier is required to establish competence for heart progenitor formation. Development *130*, 3027-3038.
Kocks, C., Cho, J.H., Nehme, N., Ulvila, J., Pearson, A.M., Meister, M., Strom, C., Conto, S.L., Hetru, C., Stuart, L.M*., et al.* (2005). Eater, a transmembrane protein mediating phagocytosis of bacterial pathogens in Drosophila. Cell *123*, 335-346.
Kohlstedt, K., Kellner, R., Busse, R., and Fleming, I. (2006). Signaling via the angiotensin-converting enzyme results in the phosphorylation of the nonmuscle myosin heavy chain IIA. Mol Pharmacol *69*, 19-26.
Komaki, G., Tamai, H., Mori, T., Nakagawa, T., and Mori, S. (1988). Changes in serum angiotensin-converting enzyme in acutely starved non-obese patients. A possible dissociation between angiotensin-converting enzyme and the thyroid state. Acta Endocrinol (Copenh) *118*, 45-50.
Krege, J.H., John, S.W., Langenbach, L.L., Hodgin, J.B., Hagaman, J.R., Bachman, E.S., Jennette, J.C., O'Brien, D.A., and Smithies, O. (1995). Male-female differences in fertility and blood pressure in ACE-deficient mice. Nature *375*, 146-148.
Krzemien, J., Dubois, L., Makki, R., Meister, M., Vincent, A., and Crozatier, M. (2007). Control of blood cell homeostasis in Drosophila larvae by the posterior signalling centre. Nature *446*, 325-328.
Krzemien, J., Oyallon, J., Crozatier, M., and Vincent, A. (2010). Hematopoietic progenitors and hemocyte lineages in the Drosophila lymph gland. Dev Biol *346*, 310-319.
Kulkarni, V., Khadilkar, R.J., Magadi, S.S., and Inamdar, M.S. (2011). Asrij maintains the stem cell niche and controls differentiation during Drosophila lymph gland hematopoiesis. PLoS One *6*, e27667.
Kumper, S., Mardakheh, F.K., McCarthy, A., Yeo, M., Stamp, G.W., Paul, A., Worboys, J., Sadok, A., Jorgensen, C., Guichard, S*., et al.* (2016). Rho-associated kinase (ROCK) function is essential for cell cycle progression, senescence and tumorigenesis. Elife *5*, e12994.
Kurucz, E., Vaczi, B., Markus, R., Laurinyecz, B., Vilmos, P., Zsamboki, J., Csorba, K., Gateff, E., Hultmark, D., and Ando, I. (2007). Definition of Drosophila hemocyte subsets by cell-type specific antigens. Acta Biol Hung *58 Suppl*, 95-111.
Kyba, M., and Daley, G.Q. (2003). Hematopoiesis from embryonic stem cells: lessons from and for ontogeny. Exp Hematol *31*, 994-1006.

Lai, C.M., Lin, K.Y., Kao, S.H., Chen, Y.N., Huang, F., and Hsu, H.J. (2017). Hedgehog signaling establishes precursors for germline stem cell niches by regulating cell adhesion. J Cell Biol *216*, 1439-1453.
Lambert, D.W., Clarke, N.E., and Turner, A.J. (2010). Not just angiotensinases: new roles for the angiotensin-converting enzymes. Cell Mol Life Sci *67*, 89-98.
Langevin, J., Morgan, M.J., Sibarita, J.B., Aresta, S., Murthy, M., Schwarz, T., Camonis, J., and Bellaiche, Y. (2005). Drosophila exocyst components Sec5, Sec6, and Sec15 regulate DE-Cadherin trafficking from recycling endosomes to the plasma membrane. Dev Cell *9*, 365-376.
Lanot, R., Zachary, D., Holder, F., and Meister, M. (2001). Postembryonic hematopoiesis in Drosophila. Dev Biol *230*, 243-257.
Lebestky, T., Chang, T., Hartenstein, V., and Banerjee, U. (2000). Specification of Drosophila hematopoietic lineage by conserved transcription factors. Science *288*, 146-149.
Lebestky, T., Jung, S.H., and Banerjee, U. (2003). A Serrate-expressing signaling center controls Drosophila hematopoiesis. Genes Dev *17*, 348-353.
Lecuit, T., Lenne, P.F., and Munro, E. (2011). Force generation, transmission, and integration during cell and tissue morphogenesis. Annu Rev Cell Dev Biol *27*, 157-184.
Lee, R.T., Zhao, Z., and Ingham, P.W. (2016). Hedgehog signalling. Development *143*, 367-372.
Leitao, A.B., and Sucena, E. (2015). Drosophila sessile hemocyte clusters are true hematopoietic tissues that regulate larval blood cell differentiation. Elife *4*.
Lemaitre, B., Reichhart, J.M., and Hoffmann, J.A. (1997). Drosophila host defense: differential induction of antimicrobial peptide genes after infection by various classes of microorganisms. Proc Natl Acad Sci U S A *94*, 14614-14619.
Li, Q., Nirala, N.K., Nie, Y., Chen, H.J., Ostroff, G., Mao, J., Wang, Q., Xu, L., and Ip, Y.T. (2018). Ingestion of Food Particles Regulates the Mechanosensing Misshapen-Yorkie Pathway in Drosophila Intestinal Growth. Dev Cell *45*, 433-449 e436.
Liao, K.A., Gonzalez-Morales, N., and Schock, F. (2016). Zasp52, a Core Z-disc Protein in Drosophila Indirect Flight Muscles, Interacts with alpha-Actinin via an Extended PDZ Domain. PLoS Genet *12*, e1006400.
Lin, C., Datta, V., Okwan-Duodu, D., Chen, X., Fuchs, S., Alsabeh, R., Billet, S., Bernstein, K.E., and Shen, X.Z. (2011). Angiotensin-converting enzyme is required for normal myelopoiesis. FASEB J *25*, 1145-1155.
Lin, X., Ruiz, J., Bajraktari, I., Ohman, R., Banerjee, S., Gribble, K., Kaufman, J.D., Wingfield, P.T., Griggs, R.C., Fischbeck, K.H.*, et al.* (2014). Z-disc-associated, alternatively spliced, PDZ motif-containing protein (ZASP) mutations in the actin-binding domain cause disruption of skeletal muscle actin filaments in myofibrillar myopathy. J Biol Chem *289*, 13615-13626.
Lindberg, H., Nielsen, D., Jensen, B.V., Eriksen, J., and Skovsgaard, T. (2004). Angiotensin converting enzyme inhibitors for cancer treatment? Acta Oncol *43*, 142-152.
Liu, C., Xiong, Y., Feng, J., Zhang, L., and Zhao, Y. (2015a). Sqh is involved in the regulation of Ci stability in Hh signaling pathway. J Mol Cell Biol *7*, 584-587.
Liu, Z., Zhong, G., Chai, P.C., Luo, L., Liu, S., Yang, Y., Baeg, G.H., and Cai, Y. (2015b). Coordinated niche-associated signals promote germline homeostasis in the Drosophila ovary. J Cell Biol *211*, 469-484.
Liu, Z.C., Odell, N., and Geisbrecht, E.R. (2013). Drosophila importin-7 functions upstream of the Elmo signaling module to mediate the formation and stability of muscle attachments. J Cell Sci *126*, 5210-5223.
Louradour, I., Sharma, A., Morin-Poulard, I., Letourneau, M., Vincent, A., Crozatier, M., and Vanzo, N. (2017). Reactive oxygen species-dependent Toll/NF-kappaB activation in the Drosophila hematopoietic niche confers resistance to wasp parasitism. Elife *6*.
Ma, X., Chen, Y., Xu, W., Wu, N., Li, M., Cao, Y., Wu, S., Li, Q., and Xue, L. (2015). Impaired Hippo signaling promotes Rho1-JNK-dependent growth. Proc Natl Acad Sci U S A *112*, 1065-1070.
Makhijani, K., Alexander, B., Tanaka, T., Rulifson, E., and Bruckner, K. (2011). The peripheral nervous system supports blood cell homing and survival in the Drosophila larva. Development *138*, 5379-5391.

Makki, R., Meister, M., Pennetier, D., Ubeda, J.M., Braun, A., Daburon, V., Krzemien, J., Bourbon, H.M., Zhou, R., Vincent, A.*, et al.* (2010). A short receptor downregulates JAK/STAT signalling to control the Drosophila cellular immune response. PLoS Biol *8*, e1000441.
Mammoto, A., Huang, S., Moore, K., Oh, P., and Ingber, D.E. (2004). Role of RhoA, mDia, and ROCK in cell shape-dependent control of the Skp2-p27kip1 pathway and the G1/S transition. J Biol Chem *279*, 26323-26330.
Mammoto, A., and Ingber, D.E. (2009). Cytoskeletal control of growth and cell fate switching. Curr Opin Cell Biol *21*, 864-870.
Mammoto, T., and Ingber, D.E. (2010). Mechanical control of tissue and organ development. Development *137*, 1407-1420.
Manaka, J., Kuraishi, T., Shiratsuchi, A., Nakai, Y., Higashida, H., Henson, P., and Nakanishi, Y. (2004). Draper-mediated and phosphatidylserine-independent phagocytosis of apoptotic cells by Drosophila hemocytes/macrophages. J Biol Chem *279*, 48466-48476.
Mandal, L., Banerjee, U., and Hartenstein, V. (2004). Evidence for a fruit fly hemangioblast and similarities between lymph-gland hematopoiesis in fruit fly and mammal aorta-gonadal-mesonephros mesoderm. Nat Genet *36*, 1019-1023.
Mandal, L., Martinez-Agosto, J.A., Evans, C.J., Hartenstein, V., and Banerjee, U. (2007). A Hedgehog- and Antennapedia-dependent niche maintains Drosophila haematopoietic precursors. Nature *446*, 320-324.
Mao, Y., Keller, E.T., Garfield, D.H., Shen, K., and Wang, J. (2013). Stromal cells in tumor microenvironment and breast cancer. Cancer Metastasis Rev *32*, 303-315.
Martin, A.C., Gelbart, M., Fernandez-Gonzalez, R., Kaschube, M., and Wieschaus, E.F. (2010). Integration of contractile forces during tissue invagination. J Cell Biol *188*, 735-749.
Martin, A.C., Kaschube, M., and Wieschaus, E.F. (2009). Pulsed contractions of an actin-myosin network drive apical constriction. Nature *457*, 495-499.
Mata, J., Curado, S., Ephrussi, A., and Rorth, P. (2000). Tribbles coordinates mitosis and morphogenesis in Drosophila by regulating string/CDC25 proteolysis. Cell *101*, 511-522.
McGuire, S.E., Le, P.T., Osborn, A.J., Matsumoto, K., and Davis, R.L. (2003). Spatiotemporal rescue of memory dysfunction in Drosophila. Science *302*, 1765-1768.
Medvinsky, A., and Dzierzak, E. (1996). Definitive hematopoiesis is autonomously initiated by the AGM region. Cell *86*, 897-906.
Meilhac, S.M., Adams, R.J., Morris, S.A., Danckaert, A., Le Garrec, J.F., and Zernicka-Goetz, M. (2009). Active cell movements coupled to positional induction are involved in lineage segregation in the mouse blastocyst. Dev Biol *331*, 210-221.
Meitinger, F., and Palani, S. (2016). Actomyosin ring driven cytokinesis in budding yeast. Semin Cell Dev Biol *53*, 19-27.
Mendes Pinto, I., Rubinstein, B., Kucharavy, A., Unruh, J.R., and Li, R. (2012). Actin depolymerization drives actomyosin ring contraction during budding yeast cytokinesis. Dev Cell *22*, 1247-1260.
Meserve, J.H., and Duronio, R.J. (2017). A population of G2-arrested cells are selected as sensory organ precursors for the interommatidial bristles of the Drosophila eye. Dev Biol *430*, 374-384.
Mih, J.D., Marinkovic, A., Liu, F., Sharif, A.S., and Tschumperlin, D.J. (2012). Matrix stiffness reverses the effect of actomyosin tension on cell proliferation. J Cell Sci *125*, 5974-5983.
Millan, J., Cain, R.J., Reglero-Real, N., Bigarella, C., Marcos-Ramiro, B., Fernandez-Martin, L., Correas, I., and Ridley, A.J. (2010). Adherens junctions connect stress fibres between adjacent endothelial cells. BMC Biol *8*, 11.
Milton, C.C., Grusche, F.A., Degoutin, J.L., Yu, E., Dai, Q., Lai, E.C., and Harvey, K.F. (2014). The Hippo pathway regulates hematopoiesis in Drosophila melanogaster. Curr Biol *24*, 2673-2680.
Mondal, B.C., Mukherjee, T., Mandal, L., Evans, C.J., Sinenko, S.A., Martinez-Agosto, J.A., and Banerjee, U. (2011). Interaction between differentiating cell- and niche-derived signals in hematopoietic progenitor maintenance. Cell *147*, 1589-1600.
Moroishi, T., Park, H.W., Qin, B., Chen, Q., Meng, Z., Plouffe, S.W., Taniguchi, K., Yu, F.X., Karin, M., Pan, D.*, et al.* (2015). A YAP/TAZ-induced feedback mechanism regulates Hippo pathway homeostasis. Genes Dev *29*, 1271-1284.

Morris, L.X., and Spradling, A.C. (2011). Long-term live imaging provides new insight into stem cell regulation and germline-soma coordination in the Drosophila ovary. Development *138*, 2207-2215.
Morrison, S.J., and Spradling, A.C. (2008). Stem cells and niches: mechanisms that promote stem cell maintenance throughout life. Cell *132*, 598-611.
Moujaber, O., and Stochaj, U. (2020). The Cytoskeleton as Regulator of Cell Signaling Pathways. Trends Biochem Sci *45*, 96-107.
Mui, K.L., Chen, C.S., and Assoian, R.K. (2016). The mechanical regulation of integrin-cadherin crosstalk organizes cells, signaling and forces. J Cell Sci *129*, 1093-1100.
Mukherjee, T., Kim, W.S., Mandal, L., and Banerjee, U. (2011). Interaction between Notch and Hif-alpha in development and survival of Drosophila blood cells. Science *332*, 1210-1213.
Munjal, A., and Lecuit, T. (2014). Actomyosin networks and tissue morphogenesis. Development *141*, 1789-1793.
Munjal, A., Philippe, J.M., Munro, E., and Lecuit, T. (2015). A self-organized biomechanical network drives shape changes during tissue morphogenesis. Nature *524*, 351-355.
Muratoglu, S., Garratt, B., Hyman, K., Gajewski, K., Schulz, R.A., and Fossett, N. (2006). Regulation of Drosophila friend of GATA gene, u-shaped, during hematopoiesis: a direct role for serpent and lozenge. Dev Biol *296*, 561-579.
Murrell, M., Oakes, P.W., Lenz, M., and Gardel, M.L. (2015). Forcing cells into shape: the mechanics of actomyosin contractility. Nat Rev Mol Cell Biol *16*, 486-498.
Nakada, D., Levi, B.P., and Morrison, S.J. (2011). Integrating physiological regulation with stem cell and tissue homeostasis. Neuron *70*, 703-718.
Nappi, A.J. (1975). Inhibition by parasites of melanotic tumour formation in Drosophila melanogaster. Nature *255*, 402-404.
Negre, N., Ghysen, A., and Martinez, A.M. (2003). Mitotic G2-arrest is required for neural cell fate determination in Drosophila. Mech Dev *120*, 253-265.
Nelson, R.E., Fessler, L.I., Takagi, Y., Blumberg, B., Keene, D.R., Olson, P.F., Parker, C.G., and Fessler, J.H. (1994). Peroxidasin: a novel enzyme-matrix protein of Drosophila development. EMBO J *13*, 3438-3447.
Nelson, W.J., and Weis, W.I. (2016). 25 Years of Tension over Actin Binding to the Cadherin Cell Adhesion Complex: The Devil is in the Details. Trends Cell Biol *26*, 471-473.
Neto, F., Klaus-Bergmann, A., Ong, Y.T., Alt, S., Vion, A.C., Szymborska, A., Carvalho, J.R., Hollfinger, I., Bartels-Klein, E., Franco, C.A.*, et al.* (2018). YAP and TAZ regulate adherens junction dynamics and endothelial cell distribution during vascular development. Elife *7*.
Nie, J., Mahato, S., and Zelhof, A.C. (2014). The actomyosin machinery is required for Drosophila retinal lumen formation. PLoS Genet *10*, e1004608.
Nilsson, S.K., Johnston, H.M., and Coverdale, J.A. (2001). Spatial localization of transplanted hemopoietic stem cells: inferences for the localization of stem cell niches. Blood *97*, 2293-2299.
Noma, K., Kihara, Y., and Higashi, Y. (2012). Striking crosstalk of ROCK signaling with endothelial function. J Cardiol *60*, 1-6.
Ohlstein, B., and Spradling, A. (2007). Multipotent Drosophila intestinal stem cells specify daughter cell fates by differential notch signaling. Science *315*, 988-992.
Olofsson, B., and Page, D.T. (2005). Condensation of the central nervous system in embryonic Drosophila is inhibited by blocking hemocyte migration or neural activity. Dev Biol *279*, 233-243.
Olson, M.F., and Sahai, E. (2009). The actin cytoskeleton in cancer cell motility. Clin Exp Metastasis *26*, 273-287.
Orkin, S.H., and Zon, L.I. (2008). Hematopoiesis: an evolving paradigm for stem cell biology. Cell *132*, 631-644.
Otsuki, L., and Brand, A.H. (2018). Cell cycle heterogeneity directs the timing of neural stem cell activation from quiescence. Science *360*, 99-102.
Owusu-Ansah, E., and Banerjee, U. (2009). Reactive oxygen species prime Drosophila haematopoietic progenitors for differentiation. Nature *461*, 537-541.
Owusu-Ansah, E., Song, W., and Perrimon, N. (2013). Muscle mitohormesis promotes longevity via systemic repression of insulin signaling. Cell *155*, 699-712.

Palmer, T.D., Willhoite, A.R., and Gage, F.H. (2000). Vascular niche for adult hippocampal neurogenesis. J Comp Neurol *425*, 479-494.
Pan, D. (2010). The hippo signaling pathway in development and cancer. Dev Cell *19*, 491-505.
Panciera, T., Azzolin, L., Cordenonsi, M., and Piccolo, S. (2017). Mechanobiology of YAP and TAZ in physiology and disease. Nat Rev Mol Cell Biol *18*, 758-770.
Pandya, P., Orgaz, J.L., and Sanz-Moreno, V. (2017). Actomyosin contractility and collective migration: may the force be with you. Curr Opin Cell Biol *48*, 87-96.
Paszek, M.J., Zahir, N., Johnson, K.R., Lakins, J.N., Rozenberg, G.I., Gefen, A., Reinhart-King, C.A., Margulies, S.S., Dembo, M., Boettiger, D*., et al.* (2005). Tensional homeostasis and the malignant phenotype. Cancer Cell *8*, 241-254.
Pathak, M.M., Nourse, J.L., Tran, T., Hwe, J., Arulmoli, J., Le, D.T., Bernardis, E., Flanagan, L.A., and Tombola, F. (2014). Stretch-activated ion channel Piezo1 directs lineage choice in human neural stem cells. Proc Natl Acad Sci U S A *111*, 16148-16153.
Pauli, A., Althoff, F., Oliveira, R.A., Heidmann, S., Schuldiner, O., Lehner, C.F., Dickson, B.J., and Nasmyth, K. (2008). Cell-type-specific TEV protease cleavage reveals cohesin functions in Drosophila neurons. Dev Cell *14*, 239-251.
Pennetier, D., Oyallon, J., Morin-Poulard, I., Dejean, S., Vincent, A., and Crozatier, M. (2012). Size control of the Drosophila hematopoietic niche by bone morphogenetic protein signaling reveals parallels with mammals. Proc Natl Acad Sci U S A *109*, 3389-3394.
Perrimon, N., Ni, J.Q., and Perkins, L. (2010). In vivo RNAi: today and tomorrow. Cold Spring Harb Perspect Biol *2*, a003640.
Pines, J. (1999). Four-dimensional control of the cell cycle. Nat Cell Biol *1*, E73-79.
Plaks, V., Kong, N., and Werb, Z. (2015). The cancer stem cell niche: how essential is the niche in regulating stemness of tumor cells? Cell Stem Cell *16*, 225-238.
Priti, A., Ong, H.T., Toyama, Y., Padmanabhan, A., Dasgupta, S., Krajnc, M., and Zaidel-Bar, R. (2018). Syncytial germline architecture is actively maintained by contraction of an internal actomyosin corset. Nat Commun *9*, 4694.
Rabinovitch, P.S. (1994). DNA content histogram and cell-cycle analysis. Methods Cell Biol *41*, 263-296.
Rahman, M.S., Akhtar, N., Jamil, H.M., Banik, R.S., and Asaduzzaman, S.M. (2015). TGF-beta/BMP signaling and other molecular events: regulation of osteoblastogenesis and bone formation. Bone Res *3*, 15005.
Ranieri, N., Therond, P.P., and Ruel, L. (2014). Switch of PKA substrates from Cubitus interruptus to Smoothened in the Hedgehog signalosome complex. Nat Commun *5*, 5034.
Ratheesh, A., Biebl, J., Vesela, J., Smutny, M., Papusheva, E., Krens, S.F.G., Kaufmann, W., Gyoergy, A., Casano, A.M., and Siekhaus, D.E. (2018). Drosophila TNF Modulates Tissue Tension in the Embryo to Facilitate Macrophage Invasive Migration. Dev Cell *45*, 331-346 e337.
Rauskolb, C., Pan, G., Reddy, B.V., Oh, H., and Irvine, K.D. (2011). Zyxin links fat signaling to the hippo pathway. PLoS Biol *9*, e1000624.
Rauskolb, C., Sun, S., Sun, G., Pan, Y., and Irvine, K.D. (2014). Cytoskeletal tension inhibits Hippo signaling through an Ajuba-Warts complex. Cell *158*, 143-156.
Rehorn, K.P., Thelen, H., Michelson, A.M., and Reuter, R. (1996). A molecular aspect of hematopoiesis and endoderm development common to vertebrates and Drosophila. Development *122*, 4023-4031.
Riether, C., Schurch, C.M., and Ochsenbein, A.F. (2015). Regulation of hematopoietic and leukemic stem cells by the immune system. Cell Death Differ *22*, 187-198.
Rizki, T.M., Rizki, R.M., and Carton, Y. (1990). Leptopilina heterotoma and L. boulardi: strategies to avoid cellular defense responses of Drosophila melanogaster. Exp Parasitol *70*, 466-475.
Rosenthal, T., and Gavras, I. (2009). Angiotensin inhibition and malignancies: a review. J Hum Hypertens *23*, 623-635.
Rothenbusch-Fender, S., Fritzen, K., Bischoff, M.C., Buttgereit, D., Oenel, S.F., and Renkawitz-Pohl, R. (2017). Myotube migration to cover and shape the testis of Drosophila depends on Heartless, Cadherin/Catenin, and myosin II. Biol Open *6*, 1876-1888.

Roubinet, C., Tsankova, A., Pham, T.T., Monnard, A., Caussinus, E., Affolter, M., and Cabernard, C. (2017). Spatio-temporally separated cortical flows and spindle geometry establish physical asymmetry in fly neural stem cells. Nat Commun *8*, 1383.
Rousseau-Plasse, A., Wdzieczak-Bakala, J., Lenfant, M., Ezan, E., Genet, R., Robinson, S., Briscoe, T., Melville, J., and Riches, A. (1998). Lisinopril, an angiotensin I-converting enzyme inhibitor, prevents entry of murine hematopoietic stem cells into the cell cycle after irradiation in vivo. Exp Hematol *26*, 1074-1079.
Royou, A., Sullivan, W., and Karess, R. (2002). Cortical recruitment of nonmuscle myosin II in early syncytial Drosophila embryos: its role in nuclear axial expansion and its regulation by Cdc2 activity. J Cell Biol *158*, 127-137.
Ruel, L., Rodriguez, R., Gallet, A., Lavenant-Staccini, L., and Therond, P.P. (2003). Stability and association of Smoothened, Costal2 and Fused with Cubitus interruptus are regulated by Hedgehog. Nat Cell Biol *5*, 907-913.
Rugendorff, A., Younossi-Hartenstein, A., and Hartenstein, V. (1994). Embryonic origin and differentiation of the Drosophila heart. Rouxs Arch Dev Biol *203*, 266-280.
Rupes, I., Webb, B.A., Mak, A., and Young, P.G. (2001). G2/M arrest caused by actin disruption is a manifestation of the cell size checkpoint in fission yeast. Mol Biol Cell *12*, 3892-3903.
Samakovlis, C., Kimbrell, D.A., Kylsten, P., Engstrom, A., and Hultmark, D. (1990). The immune response in Drosophila: pattern of cecropin expression and biological activity. EMBO J *9*, 2969-2976.
Samuel, M.S., Lopez, J.I., McGhee, E.J., Croft, D.R., Strachan, D., Timpson, P., Munro, J., Schroder, E., Zhou, J., Brunton, V.G*., et al.* (2011). Actomyosin-mediated cellular tension drives increased tissue stiffness and beta-catenin activation to induce epidermal hyperplasia and tumor growth. Cancer Cell *19*, 776-791.
Schlichting, K., Demontis, F., and Dahmann, C. (2005). Cadherin Cad99C is regulated by Hedgehog signaling in Drosophila. Dev Biol *279*, 142-154.
Schmidt, A., Durgan, J., Magalhaes, A., and Hall, A. (2007). Rho GTPases regulate PRK2/PKN2 to control entry into mitosis and exit from cytokinesis. EMBO J *26*, 1624-1636.
Scott, J.A., Shewan, A.M., den Elzen, N.R., Loureiro, J.J., Gertler, F.B., and Yap, A.S. (2006). Ena/VASP proteins can regulate distinct modes of actin organization at cadherin-adhesive contacts. Mol Biol Cell *17*, 1085-1095.
Segal, D., Dhanyasi, N., Schejter, E.D., and Shilo, B.Z. (2016). Adhesion and Fusion of Muscle Cells Are Promoted by Filopodia. Dev Cell *38*, 291-304.
Seidel, H.S., and Kimble, J. (2015). Cell-cycle quiescence maintains Caenorhabditis elegans germline stem cells independent of GLP-1/Notch. Elife *4*.
Seita, J., and Weissman, I.L. (2010). Hematopoietic stem cell: self-renewal versus differentiation. Wiley Interdiscip Rev Syst Biol Med *2*, 640-653.
Sever, R., and Brugge, J.S. (2015). Signal transduction in cancer. Cold Spring Harb Perspect Med *5*.
Sharma, S.K., Ghosh, S., Geetha, A.R., Mandal, S., and Mandal, L. (2019). Cell Adhesion-Mediated Actomyosin Assembly Regulates the Activity of Cubitus Interruptus for Hematopoietic Progenitor Maintenance in Drosophila. Genetics *212*, 1279-1300.
Sheikh, F., Bang, M.L., Lange, S., and Chen, J. (2007). "Z"eroing in on the role of Cypher in striated muscle function, signaling, and human disease. Trends Cardiovasc Med *17*, 258-262.
Shim, J., Gururaja-Rao, S., and Banerjee, U. (2013a). Nutritional regulation of stem and progenitor cells in Drosophila. Development *140*, 4647-4656.
Shim, J., Mukherjee, T., and Banerjee, U. (2012). Direct sensing of systemic and nutritional signals by haematopoietic progenitors in Drosophila. Nat Cell Biol *14*, 394-400.
Shim, J., Mukherjee, T., Mondal, B.C., Liu, T., Young, G.C., Wijewarnasuriya, D.P., and Banerjee, U. (2013b). Olfactory control of blood progenitor maintenance. Cell *155*, 1141-1153.
Shin, J.W., Buxboim, A., Spinler, K.R., Swift, J., Christian, D.A., Hunter, C.A., Leon, C., Gachet, C., Dingal, P.C., Ivanovska, I.L*., et al.* (2014). Contractile forces sustain and polarize hematopoiesis from stem and progenitor cells. Cell Stem Cell *14*, 81-93.

Shin, J.W., Swift, J., Ivanovska, I., Spinler, K.R., Buxboim, A., and Discher, D.E. (2013). Mechanobiology of bone marrow stem cells: from myosin-II forces to compliance of matrix and nucleus in cell forms and fates. Differentiation *86*, 77-86.
Shklover, J., Levy-Adam, F., and Kurant, E. (2015). The role of Drosophila TNF Eiger in developmental and damage-induced neuronal apoptosis. FEBS Lett *589*, 871-879.
Simpson, R.J., Kunz, H., Agha, N., and Graff, R. (2015). Exercise and the Regulation of Immune Functions. Prog Mol Biol Transl Sci *135*, 355-380.
Sinenko, S.A., Mandal, L., Martinez-Agosto, J.A., and Banerjee, U. (2009). Dual role of wingless signaling in stem-like hematopoietic precursor maintenance in Drosophila. Dev Cell *16*, 756-763.
Sinka, L., Biasch, K., Khazaal, I., Peault, B., and Tavian, M. (2012). Angiotensin-converting enzyme (CD143) specifies emerging lympho-hematopoietic progenitors in the human embryo. Blood *119*, 3712-3723.
Snigdha, K., Gangwani, K.S., Lapalikar, G.V., Singh, A., and Kango-Singh, M. (2019). Hippo Signaling in Cancer: Lessons From Drosophila Models. Front Cell Dev Biol *7*, 85.
Song, X., and Xie, T. (2002). DE-cadherin-mediated cell adhesion is essential for maintaining somatic stem cells in the Drosophila ovary. Proc Natl Acad Sci U S A *99*, 14813-14818.
Song, X., Zhu, C.H., Doan, C., and Xie, T. (2002). Germline stem cells anchored by adherens junctions in the Drosophila ovary niches. Science *296*, 1855-1857.
Sorrentino, R.P., Carton, Y., and Govind, S. (2002). Cellular immune response to parasite infection in the Drosophila lymph gland is developmentally regulated. Dev Biol *243*, 65-80.
St Johnston, D. (2015). The renaissance of developmental biology. PLoS Biol *13*, e1002149.
Staley, B.K., and Irvine, K.D. (2012). Hippo signaling in Drosophila: recent advances and insights. Dev Dyn *241*, 3-15.
Steck, K., Walker, S.J., Itskov, P.M., Baltazar, C., Moreira, J.M., and Ribeiro, C. (2018). Internal amino acid state modulates yeast taste neurons to support protein homeostasis in Drosophila. Elife *7*.
Steinhart, Z., and Angers, S. (2018). Wnt signaling in development and tissue homeostasis. Development *145*.
Stepniak, E., Radice, G.L., and Vasioukhin, V. (2009). Adhesive and signaling functions of cadherins and catenins in vertebrate development. Cold Spring Harb Perspect Biol *1*, a002949.
Stofanko, M., Kwon, S.Y., and Badenhorst, P. (2008). A misexpression screen to identify regulators of Drosophila larval hemocyte development. Genetics *180*, 253-267.
Stofanko, M., Kwon, S.Y., and Badenhorst, P. (2010). Lineage tracing of lamellocytes demonstrates Drosophila macrophage plasticity. PLoS One *5*, e14051.
Sullivan, K.M., Scott, K., Zuker, C.S., and Rubin, G.M. (2000). The ryanodine receptor is essential for larval development in Drosophila melanogaster. Proc Natl Acad Sci U S A *97*, 5942-5947.
Sumi, A., Hayes, P., D'Angelo, A., Colombelli, J., Salbreux, G., Dierkes, K., and Solon, J. (2018). Adherens Junction Length during Tissue Contraction Is Controlled by the Mechanosensitive Activity of Actomyosin and Junctional Recycling. Dev Cell *47*, 453-463 e453.
Sun, G., and Irvine, K.D. (2011). Regulation of Hippo signaling by Jun kinase signaling during compensatory cell proliferation and regeneration, and in neoplastic tumors. Dev Biol *350*, 139-151.
Sun, S., and Irvine, K.D. (2016). Cellular Organization and Cytoskeletal Regulation of the Hippo Signaling Network. Trends Cell Biol *26*, 694-704.
Sun, S., Reddy, B.V., and Irvine, K.D. (2015). Localization of Hippo signalling complexes and Warts activation in vivo. Nat Commun *6*, 8402.
Suzuki, A., Scruggs, A., and Iwata, J. (2015). The temporal specific role of WNT/beta-catenin signaling during myogenesis. J Nat Sci *1*, e143.
Swaminathan, A., and Pile, L.A. (2010). Regulation of cell proliferation and wing development by Drosophila SIN3 and String. Mech Dev *127*, 96-106.
Sykiotis, G.P., and Bohmann, D. (2008). Keap1/Nrf2 signaling regulates oxidative stress tolerance and lifespan in Drosophila. Dev Cell *14*, 76-85.
Tepass, U., Fessler, L.I., Aziz, A., and Hartenstein, V. (1994). Embryonic origin of hemocytes and their relationship to cell death in Drosophila. Development *120*, 1829-1837.

Terriente-Felix, A., Li, J., Collins, S., Mulligan, A., Reekie, I., Bernard, F., Krejci, A., and Bray, S. (2013). Notch cooperates with Lozenge/Runx to lock haemocytes into a differentiation programme. Development *140*, 926-937.
Tian, A., Shi, Q., Jiang, A., Li, S., Wang, B., and Jiang, J. (2015). Injury-stimulated Hedgehog signaling promotes regenerative proliferation of Drosophila intestinal stem cells. J Cell Biol *208*, 807-819.
Tiwari, S.K., Toshniwal, A.G., Mandal, S., and Mandal, L. (2020). Fatty acid beta-oxidation is required for the differentiation of larval hematopoietic progenitors in Drosophila. Elife *9*.
Tokusumi, Y., Tokusumi, T., Shoue, D.A., and Schulz, R.A. (2012). Gene regulatory networks controlling hematopoietic progenitor niche cell production and differentiation in the Drosophila lymph gland. PLoS One *7*, e41604.
Tokusumi, Y., Tokusumi, T., Stoller-Conrad, J., and Schulz, R.A. (2010). Serpent, suppressor of hairless and U-shaped are crucial regulators of hedgehog niche expression and prohemocyte maintenance during Drosophila larval hematopoiesis. Development *137*, 3561-3568.
Trappmann, B., Gautrot, J.E., Connelly, J.T., Strange, D.G., Li, Y., Oyen, M.L., Cohen Stuart, M.A., Boehm, H., Li, B., Vogel, V*., et al.* (2012). Extracellular-matrix tethering regulates stem-cell fate. Nat Mater *11*, 642-649.
Tulina, N., and Matunis, E. (2001). Control of stem cell self-renewal in Drosophila spermatogenesis by JAK-STAT signaling. Science *294*, 2546-2549.
van den Ameele, J., and Brand, A.H. (2019). Neural stem cell temporal patterning and brain tumour growth rely on oxidative phosphorylation. Elife *8*.
Van Mater, D., Kolligs, F.T., Dlugosz, A.A., and Fearon, E.R. (2003). Transient activation of beta -catenin signaling in cutaneous keratinocytes is sufficient to trigger the active growth phase of the hair cycle in mice. Genes Dev *17*, 1219-1224.
Varelas, X. (2014). The Hippo pathway effectors TAZ and YAP in development, homeostasis and disease. Development *141*, 1614-1626.
Vicente-Manzanares, M., Ma, X., Adelstein, R.S., and Horwitz, A.R. (2009). Non-muscle myosin II takes centre stage in cell adhesion and migration. Nat Rev Mol Cell Biol *10*, 778-790.
Vining, K.H., and Mooney, D.J. (2017). Mechanical forces direct stem cell behaviour in development and regeneration. Nat Rev Mol Cell Biol *18*, 728-742.
Vishnubhotla, R., Bharadwaj, S., Sun, S., Metlushko, V., and Glover, S.C. (2012). Treatment with Y-27632, a ROCK Inhibitor, Increases the Proinvasive Nature of SW620 Cells on 3D Collagen Type 1 Matrix. Int J Cell Biol *2012*, 259142.
Vissers, J.H., Manning, S.A., Kulkarni, A., and Harvey, K.F. (2016). A Drosophila RNAi library modulates Hippo pathway-dependent tissue growth. Nat Commun *7*, 10368.
Vlisidou, I., and Wood, W. (2015). Drosophila blood cells and their role in immune responses. FEBS J *282*, 1368-1382.
Vyas, N., Goswami, D., Manonmani, A., Sharma, P., Ranganath, H.A., VijayRaghavan, K., Shashidhara, L.S., Sowdhamini, R., and Mayor, S. (2008). Nanoscale organization of hedgehog is essential for long-range signaling. Cell *133*, 1214-1227.
Wang, K.C., Yeh, Y.T., Nguyen, P., Limqueco, E., Lopez, J., Thorossian, S., Guan, K.L., Li, Y.J., and Chien, S. (2016). Flow-dependent YAP/TAZ activities regulate endothelial phenotypes and atherosclerosis. Proc Natl Acad Sci U S A *113*, 11525-11530.
Wangler, M.F., Yamamoto, S., and Bellen, H.J. (2015). Fruit flies in biomedical research. Genetics *199*, 639-653.
Wangler, M.F., Yamamoto, S., Chao, H.T., Posey, J.E., Westerfield, M., Postlethwait, J., Members of the Undiagnosed Diseases, N., Hieter, P., Boycott, K.M., Campeau, P.M*., et al.* (2017). Model Organisms Facilitate Rare Disease Diagnosis and Therapeutic Research. Genetics *207*, 9-27.
Warn, R.M., and Robert-Nicoud, M. (1990). F-actin organization during the cellularization of the Drosophila embryo as revealed with a confocal laser scanning microscope. J Cell Sci *96 (Pt 1)*, 35-42.
Weaver, V.M. (2017). Cell and tissue mechanics: the new cell biology frontier. Mol Biol Cell *28*, 1815-1818.
Wee, P., and Wang, Z. (2017). Epidermal Growth Factor Receptor Cell Proliferation Signaling Pathways. Cancers (Basel) *9*.

Wilkinson, S., and Frame, M.C. (2016). The ROCKs on which tumour cells thrive. Elife *5*, e14511.
Winter, C.G., Wang, B., Ballew, A., Royou, A., Karess, R., Axelrod, J.D., and Luo, L. (2001). Drosophila Rho-associated kinase (Drok) links Frizzled-mediated planar cell polarity signaling to the actin cytoskeleton. Cell *105*, 81-91.
Wolpert, L. (2016). Positional Information and Pattern Formation. Curr Top Dev Biol *117*, 597-608.
Woolthuis, C.M., de Haan, G., and Huls, G. (2011). Aging of hematopoietic stem cells: Intrinsic changes or micro-environmental effects? Curr Opin Immunol *23*, 512-517.
Wu, J., Weisshaar, N., Hotz-Wagenblatt, A., Madi, A., Ma, S., Mieg, A., Hering, M., Mohr, K., Schlimbach, T., Borgers, H.*, et al.* (2020). Skeletal muscle antagonizes antiviral CD8(+) T cell exhaustion. Sci Adv *6*, eaba3458.
Xie, T., and Spradling, A.C. (1998). decapentaplegic is essential for the maintenance and division of germline stem cells in the Drosophila ovary. Cell *94*, 251-260.
Xiong, F., Ma, W., Hiscock, T.W., Mosaliganti, K.R., Tentner, A.R., Brakke, K.A., Rannou, N., Gelas, A., Souhait, L., Swinburne, I.A.*, et al.* (2014). Interplay of cell shape and division orientation promotes robust morphogenesis of developing epithelia. Cell *159*, 415-427.
Xu, C., Luo, J., He, L., Montell, C., and Perrimon, N. (2017). Oxidative stress induces stem cell proliferation via TRPA1/RyR-mediated Ca(2+) signaling in the Drosophila midgut. Elife *6*.
Yang, H., Kronhamn, J., Ekstrom, J.O., Korkut, G.G., and Hultmark, D. (2015). JAK/STAT signaling in Drosophila muscles controls the cellular immune response against parasitoid infection. EMBO Rep *16*, 1664-1672.
Yasothornsrikul, S., Davis, W.J., Cramer, G., Kimbrell, D.A., and Dearolf, C.R. (1997). viking: identification and characterization of a second type IV collagen in Drosophila. Gene *198*, 17-25.
Yi, W., Zhang, Y., Tian, Y., Guo, J., Li, Y., and Guo, A. (2013). A subset of cholinergic mushroom body neurons requires Go signaling to regulate sleep in Drosophila. Sleep *36*, 1809-1821.
Yim, E.K., and Sheetz, M.P. (2012). Force-dependent cell signaling in stem cell differentiation. Stem Cell Res Ther *3*, 41.
Yoon, C., Yang, H.S., Jeon, I., Chang, Y., and Park, S.M. (2011). Use of angiotensin-converting-enzyme inhibitors or angiotensin-receptor blockers and cancer risk: a meta-analysis of observational studies. CMAJ *183*, E1073-1084.
Yoshida, S., Sukeno, M., and Nabeshima, Y. (2007). A vasculature-associated niche for undifferentiated spermatogonia in the mouse testis. Science *317*, 1722-1726.
Zaidel-Bar, R., Zhenhuan, G., and Luxenburg, C. (2015). The contractome--a systems view of actomyosin contractility in non-muscle cells. J Cell Sci *128*, 2209-2217.
Zhan, Y.P., Liu, L., and Zhu, Y. (2016). Taotie neurons regulate appetite in Drosophila. Nat Commun *7*, 13633.
Zhang, H., Landmann, F., Zahreddine, H., Rodriguez, D., Koch, M., and Labouesse, M. (2011). A tension-induced mechanotransduction pathway promotes epithelial morphogenesis. Nature *471*, 99-103.
Zhang, H., Li, C., Chen, H., Wei, C., Dai, F., Wu, H., Dui, W., Deng, W.M., and Jiao, R. (2015). SCF(Slmb) E3 ligase-mediated degradation of Expanded is inhibited by the Hippo pathway in Drosophila. Cell Res *25*, 93-109.
Zhang, J., Niu, C., Ye, L., Huang, H., He, X., Tong, W.G., Ross, J., Haug, J., Johnson, T., Feng, J.Q.*, et al.* (2003). Identification of the haematopoietic stem cell niche and control of the niche size. Nature *425*, 836-841.
Zhang, L., Ren, F., Zhang, Q., Chen, Y., Wang, B., and Jiang, J. (2008). The TEAD/TEF family of transcription factor Scalloped mediates Hippo signaling in organ size control. Dev Cell *14*, 377-387.
Zhang, X., Li, Q., Wang, L., Liu, Z.J., and Zhong, Y. (2018). Active Protection: Learning-Activated Raf/MAPK Activity Protects Labile Memory from Rac1-Independent Forgetting. Neuron *98*, 142-155 e144.
Zheng, Y., and Pan, D. (2019). The Hippo Signaling Pathway in Development and Disease. Dev Cell *50*, 264-282.
Zhou, S., Chen, S., Jiang, Q., and Pei, M. (2019). Determinants of stem cell lineage differentiation toward chondrogenesis versus adipogenesis. Cell Mol Life Sci *76*, 1653-1680.

Zielke, N., and Edgar, B.A. (2015). FUCCI sensors: powerful new tools for analysis of cell proliferation. Wiley Interdiscip Rev Dev Biol *4*, 469-487.
Zielke, N., Korzelius, J., van Straaten, M., Bender, K., Schuhknecht, G.F.P., Dutta, D., Xiang, J., and Edgar, B.A. (2014). Fly-FUCCI: A versatile tool for studying cell proliferation in complex tissues. Cell Rep *7*, 588-598.

Research publications

1. Sharma, S. K., Ghosh, S., Geetha, A. R., Mandal, S. & Mandal, L. Cell Adhesion- Mediated Actomyosin Assembly Regulates the Activity of Cubitus Interruptus for Hematopoietic Progenitor Maintenance in Drosophila. *Genetics* **212**, 1279-1300, doi:10.1534/genetics.119.302209 (2019).

2. Kaur, H., Sharma, S. K., Mandal, S. & Mandal, L. Lar maintains the homeostasis of the hematopoietic organ in Drosophila by regulating insulin signaling in the niche. *Development* **146**, doi:10.1242/dev.178202 (2019).

3. Sharma S.K., Mandal S & Mandal L. DE-cadherin mediated actomyosin assembly controls G2/M progression in the *Drosophila* hematopoietic progenitor cells (Manuscript under preparation)

4. Sharma S.K., Kaur, H., Mandal S & Mandal L. Maintenance of the Hematopoietic niche in *Drosophila* larva depends on the neighbouring cardioblasts (Manuscript under preparation)