HEMOCYTE-PERICARDIAL CELL INTERACTION

HEMOCYTE-PERICARDIAL CELL INTERACTION DURING THE GROWTH OF THE DORSAL VESSEL

BY

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Abstract

Drosophila melanogaster has a tubular heart called the dorsal vessel, which is composed of contractile cardiomyocytes and hemolymph filtering pericardial cells. During larval development the dorsal vessel (heart) grows in size, and the luminal space inside the heart expands, however it has not been clear which cells are responsible for laying the extracellular matrix (ECM) during this expansion. Hemocytes (white blood cells), pericardial cells and cells of the fat body are candidate cell types that may secrete ECM for assembly during the growth of the heart lumen. With gene knock-down techniques we are exploring whether hemocytes participate in assembly of the heart ECM at this location. Additionally, studies of fluorescently tagged hemocytes in intact larvae reveal that hemocytes aggregate around pericardial cells of the dorsal vessel in 3rd instars. Confocal studies of dissected larval hearts indicate that hemocytes aggregate within infoldings of basement membrane associated with pericardial cells. Hemocyte-pericardial cell association could indicate that hemocytes take up proteins that are produced by pericardial cells and deliver them to other locations or that there might be a previously unidentified hematopoietic site at the *Drosophila* larval heart.

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ABBREVIATIONS and SYMBOLS

+	Wild type chromosome
αPS5	Integrin subunit
BMP	Bone Morphogenic Protein
βPS	Integrin subunit
CyO	"Curly O" balancer chromosome
da	"daughterless" ubiquitous gene
DV	Dorsal vessel
ECM	Extracellular Matrix
FGF	Fibroblast Growth Factor
hl	Heart lumen
Hml	"hemolectin" hemocyte specific gene
HPs	Hematopoietic Pockets
Lan A	Laminin A
lz	"lozenge" crystal cell specific gene
PBS	Phosphate buffered saline
PBT	PBS + 0.1% Triton
prc	Pericardin mutant
PVF	PDGF (Platelet Derived Growth Factor)/VEGF (Vascular Endothelial Growth Factor) factor
PVR	PDGF/VEGF receptor
Pxn	"Peroxidasin" hemocyte specific gene
Shi	"shibire" gene that encodes Dynamin
SJ	Septate junction
TM6	"Third multiple six" balancer
Vkg	"Viking" Collagen IV gene

Chapter 1.0: Introduction

This thesis explores the intersection of 3 topics of interest: the heart, blood cells and extracellular matrix. One of our primary aims was to characterize the association of the *Drosophila melanogaster* heart (dorsal vessel-DV) with clusters of white blood cells (hemocytes). We sought to gain knowledge of new cell adhesions, signaling cues and proliferative niches by exploring if blood cells associate with the heart cells. Our second aim was to test whether hemocytes are responsible for secreting or assembling extracellular matrix proteins (ECM) of the heart during larval stages. ECM is fundamental to all multicellular organisms, so by identifying types of cells that contribute to ECM remodeling throughout development, we can learn about how this universal and dynamic intercellular space is regulated. ECM proteins are also important to study because cell-ECM interactions are essential to tissue integrity and function. Sessile hemocytes of *Drosophila* larvae are important to study because their capacity for homing and proliferation is similar to vertebrate tissue-resident macrophages that are capable of self-renewal (Sieweke & Allen 2013; Davies et al. 2013). Likewise, the composition and function of *Drosophila* ECM and DV bear many similarities with their vertebrate counterparts, as reviewed below. *Drosophila* model offers us a possibility to study the behavior of heart and blood cells in vivo or in vitro with organisms that can be easily manipulated genetically.

This introduction touches upon many aspects of these 3 intersecting topics. The first two chapters introduce the reader to the structure of the DV. The details on these chapters are important to interpret why it may be significant for *Drosophila* hemocytes to cluster in certain positions in the heart. This information also gives landmarks that are useful when trying to discern the exact location of hemocyte

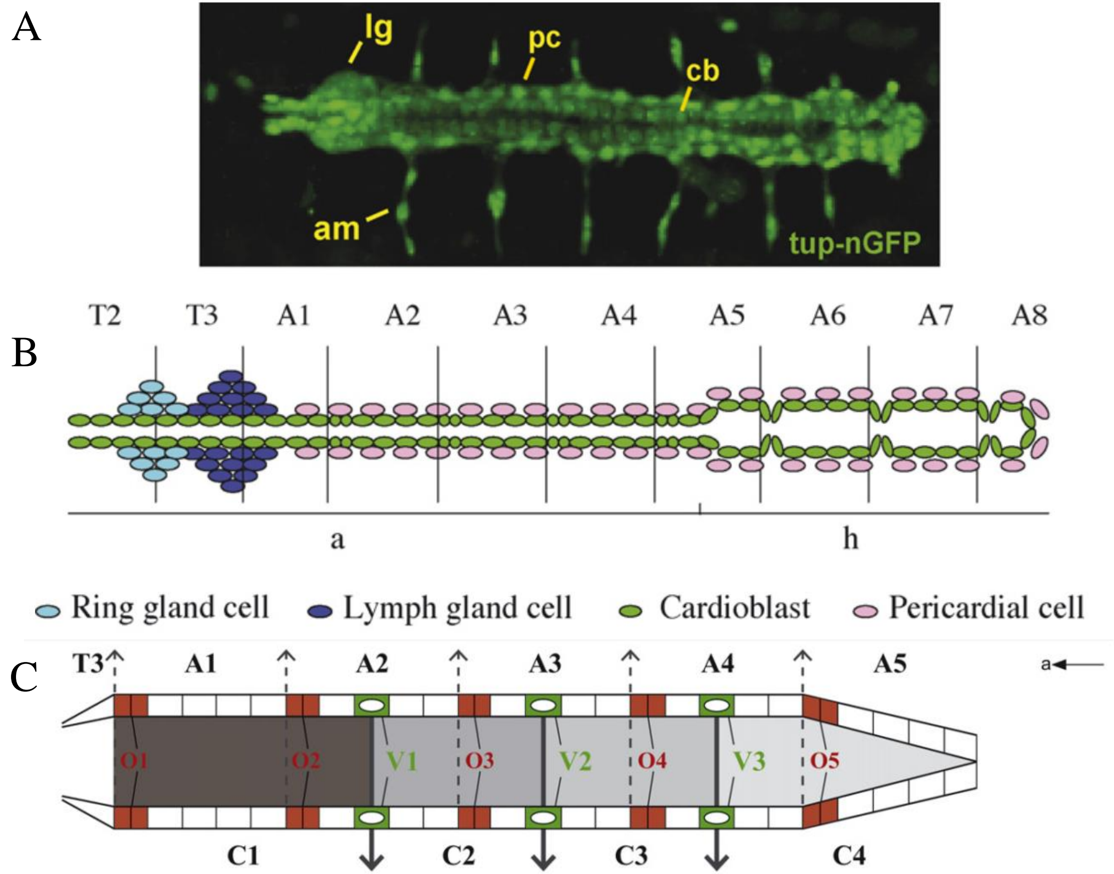
clusters. However, the structure of the DV changes with each stage, so the next section focuses on the DV development, in order to review the origins of the DV cells and the structural differences that arise within these cells throughout the lifespan. The section on ECM describes what kind of structural meshwork hemocytes have to pass through in order to associate with the heart. Additionally, this section gives an idea of which ECM should be secreted while heart is growing and changing. The last section describes various behaviors of hemocytes in different stages so that we can compare the unexplored DV associated sessile hemocytes to well-characterized kinds of blood cells of *Drosophila*.

1.1 Structure of *Drosophila melanogaster* dorsal vessel

The dorsal vessel (DV) of *Drosophila melanogaster*, which is a contractile tubular organ located at the dorsal midline, shares similarities with the embryonic vertebrate heart in terms of structure, function and development. Both the DV and the vertebrate heart pumps hemolymph (blood) from a posterior to anterior direction (Zaffran 2002). Additionally, muscle filaments of cardiomyocytes of the DV form intercalated discs that resemble the cardiac muscle of vertebrates (Frasch 1999; Rugendorff et al. 1994). According to Tao & Schulz 2007, the mature DV extends from segments T2 to A8 (Figure 1.1). However Zeitouni et al. 2007 notes that the embryonic and larval DV is between T3-A7 and that the adult DV lacks the 2 posterior segments (A6 and A7). Rugendorff et al. 1994 describes the DV as follows: Cardiomyocytes and pericardial cells are the two cell types that the make up the DV. Contractile cardiomyocytes line the inside of the heart and form the heart tube, whereas excretory pericardial cells are found on the outer side and are not capable of

contraction. Segmental pairs of alary muscles attach the DV to the epidermis. The anterior part of the DV is called ‘aorta’ and it is anteriorly connected to the ring gland and lymph gland. The ‘heart’ of the fly is considered to be located at the posterior of DV. This antero-posterior polarity of the DV is another feature that is shared by vertebrate hearts (Zaffran 2002). As reviewed by Lehmacher et al. 2012, a subset of heart cells make up inflow tracks called ostia, whereas another subset forms intercardiac valves which separate the heart into chambers (Figure 1.1 D). There is thought to be 2 pacemaker regions in the DV (Ocorr et al. 2014).

Figure 1.1: Structure of the DV throughout different developmental stages. (A) View of the heart from embryonic stage 16. lg: lymph gland, am: alary muscle, pc: pericardial cell, cb: cardioblast. (B) General structure of the heart before metamorphosis. (C) Dorsal vessel of the adult fly. Red: ostia, Green: intercardiac valves. Adapted from (A,B) Tao & Schulz 2007 and (C) Lehmacher et al. 2012.


A
lg
pc
cb
am
tup-nGFP
B
T2
T3
A1
A2
A3
A4
A5
A6
A7
A8
a
h
Ring gland cell
Lymph gland cell
Cardioblast
Pericardial cell
C
T3
A1
A2
A3
A4
A5
a
O1
O2
V1
O3
V2
O4
V3
O5
C1
C2
C3
C4

1.2 Pericardial cells

I found that hemocytes are found in close proximity of the pericardial cells; therefore I will review the function and structure of pericardial cells. In *Drosophila*, There are 120 pericardial cells in late stage embryos (Ward & Skeath 2000; Denholm & Skaer 2009) and 40 in larvae and adults (Sellin et al. 2006; Das et al. 2008; Denholm & Skaer 2009). It is known that some of the embryonic pericardial cells are destined to make up a structure called the wing heart that is located in the fly wing (Tögel et al. 2008), however the reason for the loss of pericardial cells in the larvae and adults is unexplored (Denholm & Skaer 2009). On the other hand, there are no ultrastructural changes between larval and adult stage pericardial cells, which suggest that pericardial cell physiology is constant throughout different developmental stages (Lehmacher et al. 2012).

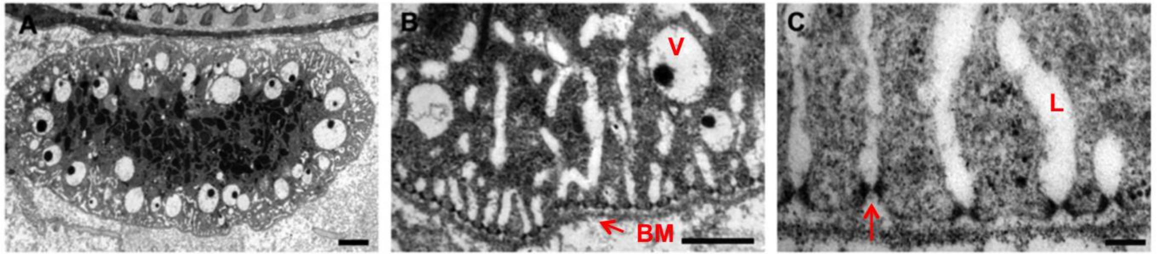
Drosophila has two kinds of nephron-like cells (nephrocytes) that filter and metabolize compounds found in the hemolymph: pericardial cells of the DV and garland cells that are connected to the oesophagus. (Crossley 1985; Kowalevsky 1889; Locke & Russell 1998; Weavers et al. 2009) Pericardial cells are common to most insects (Mills & King 1965). A study by Crossley 1972 showed that both pericardial cells and white blood cells (hemocytes) of the insect *Calliphora erythrocephala* have a function in cellular uptake of tracer compounds. Soluble particles with a diameter smaller than approximately 12 nm can be sequestered by pericardial cells, whereas bigger compounds or bacteria are taken up by the hemocytes. Zhang et al. 2013 used GFP-fused secretion peptide as a tracer to measure the hemocyte filtering ability of *Drosophila* pericardial cells. The secreted GFP-tagged peptide accumulated in pericardial cells regardless of which six tissue sources

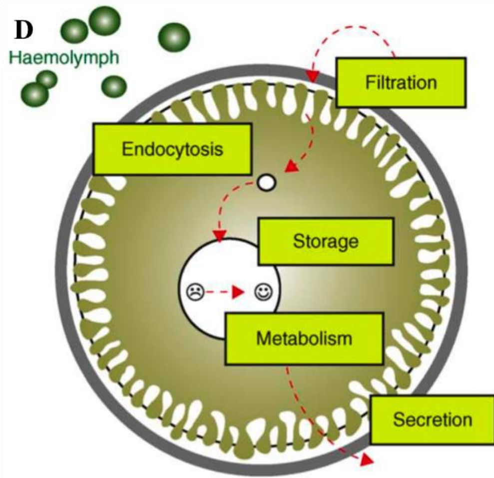
originally produced it, suggesting that pericardial cells can take up material from the hemolymph independent of the source of the material. Garland nephrocytes were also capable of taking in secreted peptides in embryos and larvae, however pericardial cells were the only ones to do so in the adults. The same study also used different sized Dextran dyes to show that pericardial cells can take up smaller compounds more efficiently. The sequestered compounds are thought to be digested inside the pericardial cell, sometimes stored long-term (Kowalevsky 1889), and eventually accumulated in a central vacuole, which releases its contents into the hemolymph (Mills & King 1965). The plasma membrane of nephrocytes invaginates into structures called lacunae that have 30nm entrances: the nephrocyte diaphragms (Figure 1.2; Weavers et al. 2009). Compounds that selectively pass through the negative charged basement membrane that covers the nephrocytes and get past nephrocyte diaphragm can be endocytosed off the walls of the lacunae (Crossley 1985; Locke & Russell 1998; Weavers et al. 2009). Endocytosis through foot processes is also possible, but is less common (Denholm & Skaer 2009).

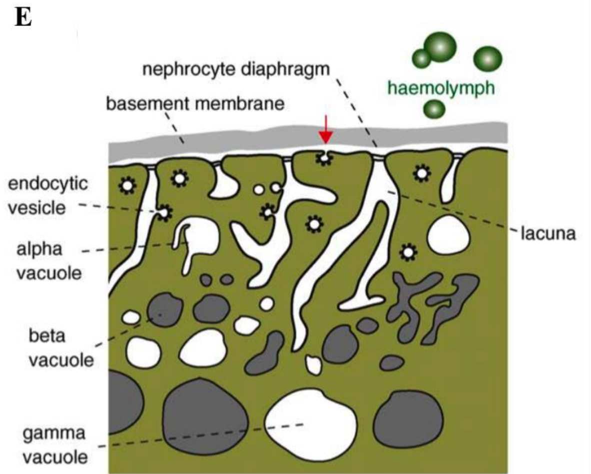
There is evidence to suggest that pericardial cells also have secretory functions: *Drosophila* pericardial cells secrete a collagen-like protein *Pericardin (prc)* in embryos after stage 13 (Chartier et al. 2002). Additionally, pericardial cells of *Calliphora* synthesize bactericidal lysozyme, and release it into the hemolymph (Crossley 1972). These findings suggest that pericardial cells might have functions in producing ECM molecules or fighting pathogenesis by secreting bactericidal elements.

Figure 1.2: Structure of *Drosophila* pericardial cells.

(A-C) Electron micrographs showing the ultrastructure of larval pericardial cells. (B) Various sizes of vacuoles are visible close to the plasma membrane. BM (basement membrane) is shown with the red arrow and it covers the plasma membrane. V: Vacuole. (C) Nephrocyte diaphragms (red arrow) form the entrances of lacunae. L: Lacunae. (D) Illustration shows the main steps of hemolymph filtration by nephrocytes. (E) Picture depicts common structures involved in endocytosis. Compounds can be taken up by pericardial cells via the endocytic vesicles of lacunae or foot processes (red arrow) in order to be delivered to different types of vacuoles that assist the breakdown of these compounds. Scale bars: 2µm in A, 500 nm in B, 100 nm in C. (A-C) Genotype: *Dot-Gal4/MHC-ANF-RFP/Hand-GFP*. This is a control line that is used to represent a wild type phenotype. (A-C) Modified from Zhang et al. 2013. (D, E) Adapted from Denholm & Skaer 2009.


A
B
V
BM
C
L


D
Haemolymph
Filtration
Endocytosis
Storage
Metabolism
Secretion


E
nephrocyte diaphragm
haemolymph
basement membrane
endocytic vesicle
alpha vacuole
lacuna
beta vacuole
gamma vacuole

1.3 Development of the dorsal vessel

It was previously noted that DV of *Drosophila* is similar to an embryonic vertebrate heart before it goes through cardiac looping (Frasch 1999). Moreover the two systems share common pathways that are active during heart development. For example, Bone Morphogenic Protein (BMP), Wnt, Fibroblast Growth Factor (FGF) and Notch pathways are each critical in DV development and vertebrate heart development (Zaffran 2002). Moreover, the vertebrate *Nkx2* transcriptional regulation network is expressed in precardiac cells and is essential to cardiac fate specification similar to their *Drosophila* orthologs in the *tinman* network (Zaffran 2002; Cripps & Olson 2002).

1.3.1 Embryonic stage

Drosophila embryogenesis lasts approximately a day at around room temperature and is divided into 17 stages. Previous reviews have described DV embryonic development in great detail (Zaffran 2002; Cripps & Olson 2002; Sorrentino et al. 2005; Tao & Schulz 2007; Lehmacher et al. 2012). During early stages of embryonic development, mesoderm on the ventral side spreads laterally and eventually gets specified as dorsal mesoderm. A subset of the dorsal mesoderm gets further specified as cardiogenic mesoderm. Later, the cardiogenic mesoderm gives rise to heart progenitors. These progenitors give rise to either pericardial cells or cardioblasts. The cardioblasts give rise to cardiomyocytes or specialized cells such as valve or ostial cells. At stage 11, bilateral rows of cardioblasts appear. Starting from stage 13, cardioblasts on both sides of the embryo continue migrating to the dorsal

side until they meet in the middle at stage 16 to form the dorsal vessel lumen. DV starts contracting and pumping hemolymph by stage 17.

Various studies have also focused on the role of cell-cell and cell-ECM adhesions in DV organogenesis and maintenance. For example, the production of *Drosophila* homolog of cell adhesion transmembrane protein E-Cadherin in cardioblasts during embryogenesis is essential for proper lumen formation (Haag et al. 1999). Additionally, Pericardin is a key ECM protein that is vital for the formation of an organized DV with properly aligned cells (Chartier et al. 2002). ECM proteins of the DV will be covered below in detail.

1.3.2 Larval stage

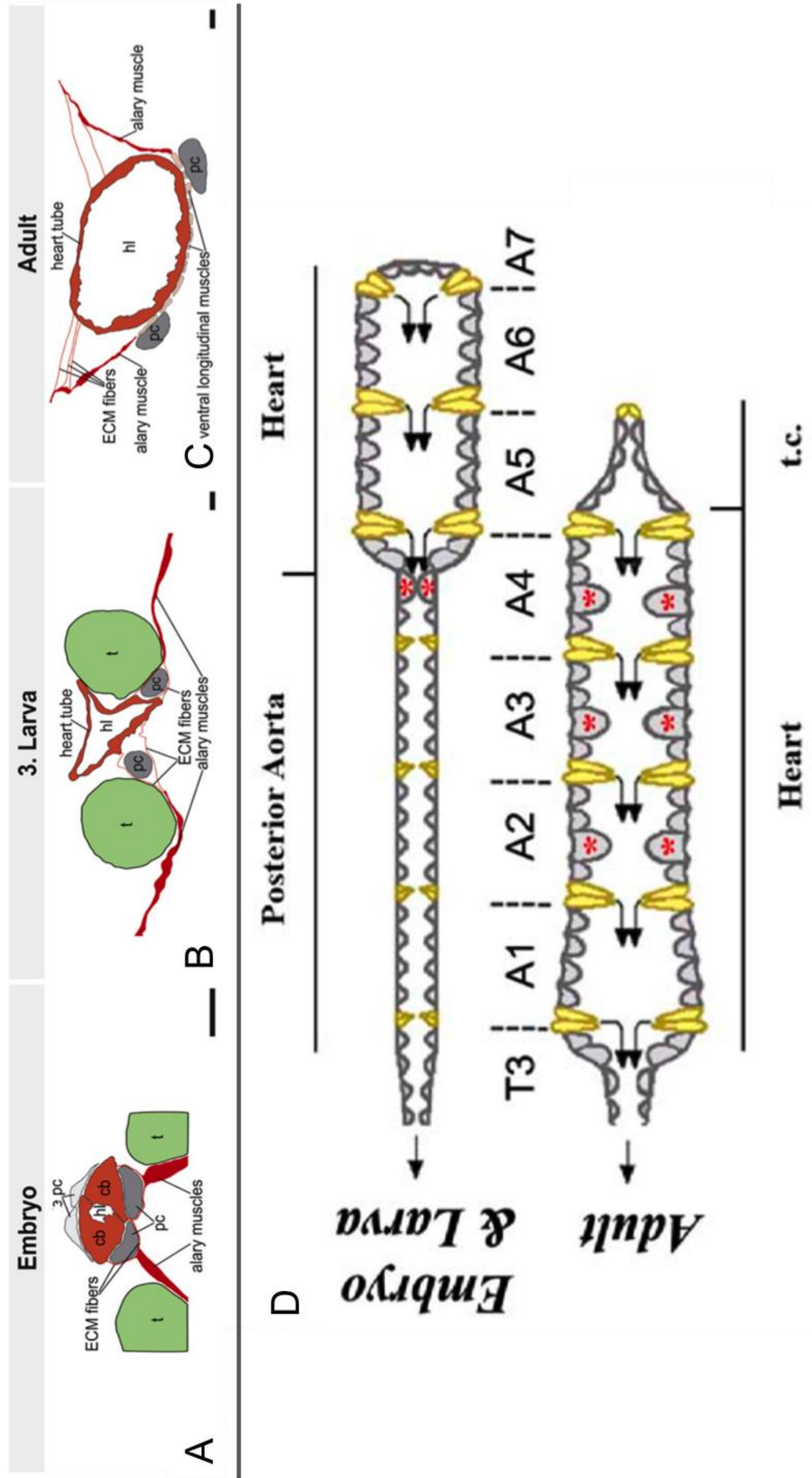
There are 3 stages to larval development: 1st instar (L1), 2nd instar (L2) and 3rd instar (L3). During larval development there are no new cardiac cells born (Lehmacher et al. 2012), however the DV expands in length and diameter (Bogatan et al. 2015). The growth of the DV is possible because cardiomyocytes grow and elongate as they build more myofilaments (Lehmacher et al. 2012). As the DV grows in size, the luminal space inside the heart also expands (Figure 1.3). The ECM network of the DV must be remodeled in order to sustain the growth of DV. One of the main objectives of this thesis is identifying the cells responsible for the DV ECM production.

1.3.3 Adult stage

Lehmacher et al. 2012 describe the changes that take place during pupation, as flies go through metamorphosis and the heart is remodeled: The function and structure of pericardial cells stay same, but there are differences that take place in the cardiomyocytes. In the adult stage, cardiomyocytes have more myofilaments, and 12 bundles of new longitudinal muscles derived from larval lymph gland like cells (Shah et al. 2011) are present on the ventral side of the DV. The 3rd instar larval heart contains one valve in segment A4, which is located between the heart and aorta. On the other hand, the adult fly DV harbors 3 valves in segments A2, A3 and A4. In the adults, ostial progenitors located in the larval aorta are differentiated, and the larval aorta gets included in the adult heart (Figure 1.3 D; Zeitouni et al. 2007). The posterior part of the adult heart makes up the terminal chamber, a structure that is not contractile (Monier et al. 2005).

Figure 1.3: Changes in DV between different developmental stages.

(A-C) Transverse cross sections of the heart from 3 developmental stages, showing the progressive growth of the heart lumen and changes in cardiomyocytes. cb: cardioblast, hl: heart lumen, pc: pericardial cell, t: trachea. (D) Adult DV is structurally different than embryonic & larval DV. The heart tube is shown without pericardial cells. tc: terminal chamber, yellow cells: ostial progenitors, yellow cells with arrows: ostia, grey cells: cardiomyocytes, grey cells with asterisk: intercardiac valves. (A-C) Modified from Lehmacher et al. 2012. (D) Modified from Zeitouni et al. 2007.


A
Embryo
ECM fibers
3 pc
cb
hl
pc
alary muscles
B
3. Larva
heart tube
hl
pc
ECM fibers
alary muscles
C
Adult
heart tube
hl
ECM fibers
alary muscle
pc
ventral longitudinal muscles
D
Embryo & Larva
Adult
Posterior Aorta
Heart
T3
A1
A2
A3
A4
A5
A6
A7
t.c.

1.4 Key structures of the DV ECM network and other ECM related proteins

ECM is the extracellular dynamic medium that provides the tissue with support as well as enabling cells to communicate with each other and sense the environment (Badylak et al. 2009). The structural integrity of the DV depends on ECM as well as ECM-cell adhesions. For example; ECM networks are important for linking the alary muscles to the DV, because the alary muscles are connected to the ECM, which encloses the pericardial cells (Lehmacher et al. 2012; Hollfelder et al. 2014). There are two questions regarding ECM that are important to this project: 1) Which cells produce the ECM proteins of the DV? 2) How do hemocytes interact with the DV ECM? In order to answer these questions, first we will review the type of ECM and ECM-cell adhesions of the DV.

1.4.1 Basement membrane of the DV

Basement membranes are protective ECM sheaths that adhere to animal cells and convey signals between the environment and the cells (Yurchenco 2011). Close interactions of hemocytes with the DV cells mean that hemocytes might be be interacting with the basement as well. Previous electron microscopy (EM) data suggests that the basement membrane covers the luminal and abluminal sides of both cardiomyocytes and pericardial cells (Figure 1.2 B) in embryos, larvae and adults (Lehmacher et al. 2012; Rugendorff et al. 1994; Tepass & Hartenstein 1994; Hollfelder et al. 2014; Zhang et al. 2013). This finding is supported with the expression pattern of a main basement membrane component, Laminin (Figure 1.4 A; Yarnitzky & Volk 1996; this thesis). Laminin function and expression in the DV will be discussed in detail below.

Drosophila contains homologs of all major vertebrate BM proteins such as polymerizing macromolecules Laminin and Collagen IV; heparan sulfate proteoglycan Perlecan; glycoprotein Nidogen; and ECM connecting cell receptors like Integrin and Dystroglycan (Hynes & Zhao 2000; Yurchenco 2011). Laminins are possibly the most important basement membrane protein. They anchor cells through sulfated glycolipids or proteins such as integrins, dystroglycan, and heparan sulfates; therefore forming a scaffold for other ECM components to assemble on (Yurchenco 2011; Hollfelder et al. 2014). For example collagen IV binds to the scaffold of laminin, either directly or through nidogen (McKee et al. 2007). Other ECM components besides laminin can directly adhere to cells as well, however research on vertebrates showed that laminins are required to bind to cells to initiate the polymerization and accumulation of rest of the ECM proteins in order to form a proper basement membrane (Smyth et al. 1998; Li et al. 2005; McKee et al. 2007; Yurchenco 2011).

We studied the larval expression of some core basement membrane related proteins of the DV such as Laminin A, Collagen IV, Intergrin and a novel Drosphila DV protein Pericardin in order to understand the hemocyte-pericardial cell interactions in regards to ECM network. To test the idea of hemocytes contributing to ECM of the DV, we knocked down transcripts for ECM related components in hemocytes and looked for DV morphology and survival defects.

1.4.2 Cell-ECM adhesions

Hemocytes that adhere to the DV must overcome the adhesions that DV cells make with their surroundings. The type of adhesion can be determined according to electron microscopy (EM) data or by identifying the proteins in that adhesion complex. For example, adherens junctions connect cells to other cells or to the ECM by making use of adhesion proteins such as integrins and cadherins (Tepass & Hartenstein 1994). Homologs of vertebrate integrins and cadherins are found in *Drosophila* (Hynes & Zhao 2000). Hemi-adherens junctions are a type of cell-substrate adherens junction that mainly connects cells to the basement membrane (Tepass & Hartenstein 1994). According to the EM data, hemi-adherens junctions connect cardiomyocytes to the basement membrane during embryonic, larval and adult stages (Lehmacher et al. 2012; Rugendorff et al. 1994; Tepass & Hartenstein 1994).

Less is known about the possible pericardial cell-ECM adherens interactions. The only junctions on pericardial cell membrane that were observed with EM are the nephrocyte diaphragms (Figure 1.2). These structures are thought to be made of adherens junctions and are referred to as nephrocyte junctions as well (Rugendorff et al. 1994). Even though these structures show us that the adhesion molecules on the same plasma membrane can come together to form an invagination, it is still a mystery what kinds of junctions attach pericardial cells to the basement membrane during larval and adult stages. However, it is very likely that the attachment of pericardial cells to the ECM involves integrins, because it is known that integrin subunits are required for proper localization of the pericardial cells during DV morphogenesis in embryos (Stark et al. 1997) and the immunolabelling of subunit

βPS integrin antibody is strong in larval and adult pericardial cell membranes (antibody used in this project).

Besides comprehensive research on adherens junctions, there is also interesting data on septate junctions of the DV. Septate junctions connect two cells at about a 15 nm distance with particles that include claudin-like transmembrane proteins, which are usually positioned in a ladder-like fashion (Furuse & Tsukita 2006; Tepass et al. 2001). There are no septate junctions in the DV (Tepass & Hartenstein 1994; Rugendorff et al. 1994), however eight septate junction proteins are required for the pericardial cell-cabioblast adhesion after the DV organogenesis (Yi et al. 2008). Moreover, it was observed that some septate junction proteins are localized at the cell membrane of pericardial cells and cardioblasts in the late stage embryos (Yi et al. 2008). Interestingly, septate junctions are found on *Drosophila* epithelia and glia (Tepass et al. 2001), which suggests that the same septate junction proteins have a different role in the DV (Nelson & Beitel 2009; Yi et al. 2008). One of the current models suggested involves septate junction proteins on pericardial cells and cardioblasts to interact with each other directly or through ECM proteins such as Pericardin (Figure 1.4B; Yi et al. 2008). The nature of this interaction is unknown and could explain how cells attach to the basement membrane in the DV.

1.4.3 Laminin

In both mammals and *Drosophila*, laminins can form mesh-like structures that contribute to tissue integrity as well as having functions such as instructing other cells to differentiate and migrate (Yarnitzky & Volk 1996; Colognato & Yurchenco 2000). In mammals, they are composed of 3 disulfide-linked polypeptide chains called α, β

and γ (Colognato & Yurchenco 2000). Drosophila genome contains 1 β gene called *lanB1*, one γ gene called *lanB2* and 2 laminin α genes called *laminin A* and *wing blister* (Brandt & Paululat 2013).

In *laminin A* loss of function mutants, pericardial cells detach from cardioblasts at an embryonic stage as early as stage 16 (Yarnitzky & Volk 1996). At stage 17, more severe defects are observed, such as twists and breaks in the heart (Haag et al. 1999). It was reported that Laminin is deposited along luminal and abluminal sides of cardioblasts in late stage embryos (Figure 1.4A Yarnitzky & Volk 1996). We have studied Laminin expression in the DV of 3rd instars in relation to the position of hemocytes, pericardial cells and the heart tube because of the importance of laminin as the main glycoprotein building block of the basement membrane and its widespread expression at the DV.

Previous research shows that laminins are deposited in hemocytes and fat body cells in *Drosophila* during embryonic stages (Kusche-Gullberg et al. 1992). It could be that the hemocytes or fat bodies continue to contain Laminin throughout larval development and contribute to the Laminin composition of the DV as the heart lumen grows. We did not directly test if DV Laminin is secreted by hemocytes or fat cells during the larval stages of development, but this is a possible topic of interest for future projects at our lab.

1.4.4 Pericardin

Unique to *Drosophila*, Pericardin (Prc) is a collegen-like ECM protein that is required for maintaining proper attachment between pericardial cells and cardioblasts (Chartier et al. 2002; Drechsler et al. 2013). First detectable at stage 13, Pericardin is

produced in pericardial cells, oenocytes and some cardioblasts of the embryo (Chartier et al. 2002). DV Pericardin is localized to the abluminal side of cardioblasts and around pericardial cells starting from embryonic stages (Chartier et al. 2002). After the 1st instar hatches as a larva, fat bodies start secreting most of the Pericardin that gets incorporated into the DV ECM (Drechsler et al. 2013). In this study, we have observed the Pericardin networks of the DV relative to the position of hemocytes and cardiac cells.

Pericardin homozygous mutants have pericardial cells that are detached from the dorsal vessel. (Drechsler et al. 2013) Using this phenotype to our advantage, we observed the behavior of hemocytes when pericardial cells are detached from the heart tube.

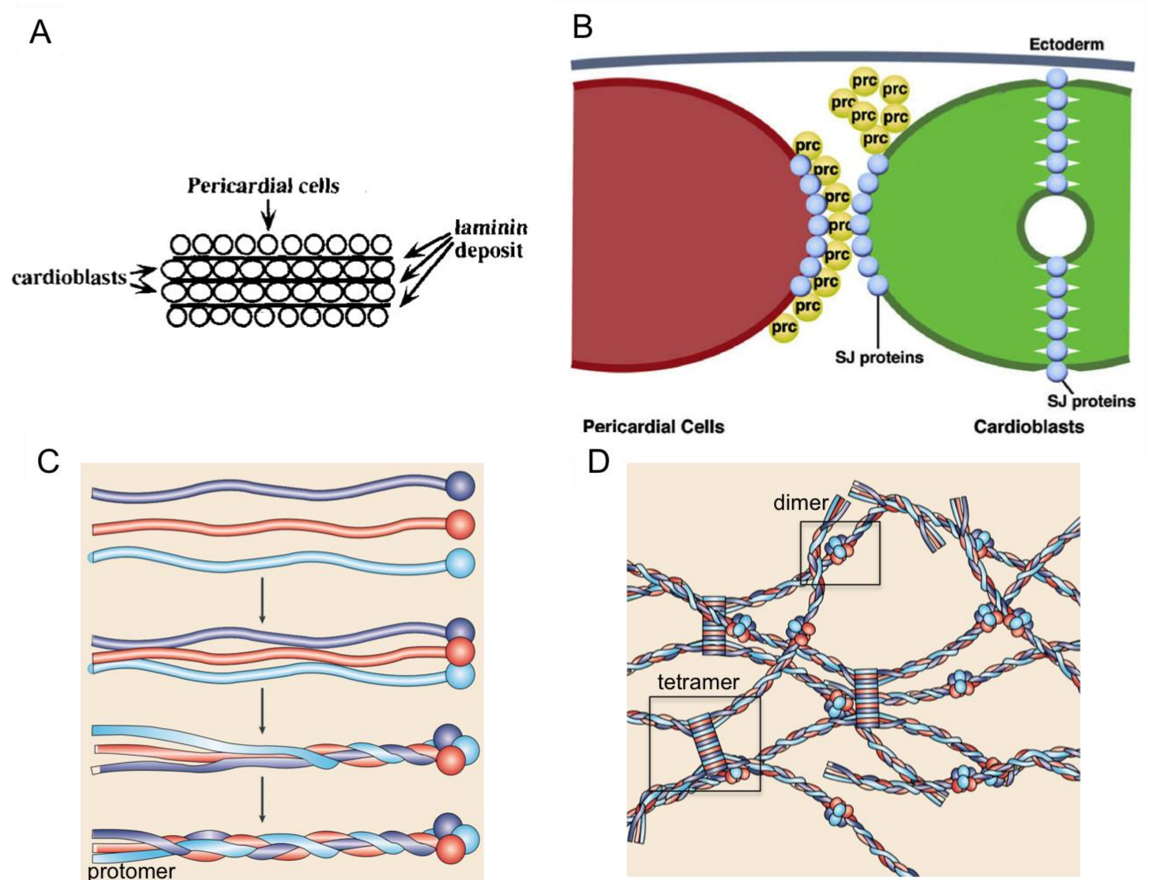
1.4.5 Type IV Collagen

In the animal kingdom, one of the major components of the basement membrane is Collagen IV, and is only localized to the basement membranes (Kalluri 2003; Hynes & Zhao 2000). Kalluri 2003 reviews the way in which collagen IV assembles into ECM networks: 3 α-chains trimerize to make a protomer, which can bind to another protomer to form a dimer. Additionally, 2 dimers can associate to form a tetramer (Figure 1.4C). Mammals have 6 different α-chains (Hudson et al. 1993), whereas *Drosophila* has 2 α-chain genes: *viking* (*vkg*) and *collagen at 25C* (*cg25C*) (Natzle et al. 1982; Yasothornsrikul et al. 1997). Collagen IV is an important component in DV structure, because in Cg25C mutants, alary muscles dissociate from the DV at late embryonic stages (Hollfelder et al. 2014).

In *Drosophila,* during embryonic stages, hemocytes are known to provide basement membranes with Collagen IV (Bunt et al. 2010; Olofsson & Page 2005). During larval stages, the fat body organ that is made up of adipocytes secretes Collagen IV into the hemolymph in its trimeric form (Pastor-Pareja & Xu 2011). The Fat body, but not hemocytes is the major source of Collagen IV that gets incorporated into overall basement membrane of the larvae (Pastor-Pareja & Xu 2011). However, it is not known if hemocytes or fat bodies are the ones responsible for secreting the most of Collagen IV that makes up the DV ECM. We knocked down Vkg that is expressed from hemocytes or fat bodies to find the cell type that contributes most to basement membrane of the DV up until third instar stage.

Figure 1.4: ECM proteins of the DV.

(A) Illustration showing location of laminin deposit along the DV in a stage 16 embryo. (B) Transverse cross section demonstrating the location of septate junction proteins along the cell membrane of pericardial cells and cardioblasts. SJ: septate junction, prc: Pericardin (C) α-chains of Collagen IV interact to make protomers. (D) Dimers and tetramers make up Collagen IV networks. (A) Adapted from Yarnitzky & Volk 1996. (B) Modified from Yi et al. 2008. (C, D) Modified from Kalluri 2003.


A
Pericardial cells
laminin deposit
cardioblasts
B
Ectoderm
prc
SJ proteins
SJ proteins
Pericardial Cells
Cardioblasts
C
protomer
D
dimer
tetramer

1.4.6 Integrin

Integrins are transmembrane receptors that are composed of β and α subunits (Gotwals et al. 1994). They connect cells to ECM and they also have functions in cell signaling (Vanderploeg et al. 2012; Gotwals et al. 1994). We used the expression pattern of an integrin subunit in 3rd instar DV to immunolabel different cell types, mainly pericardial cells. This helped us see relative positions of relevant cells in multiple experiments.

1.4.7 Talin (encoded by *rhea*)

Talin is one of the cytoplasmic proteins that link transmembrane protein integrin to the actin cytoskeleton (Vanderploeg et al. 2012). Extacellular domains of integrins bind to ECM, so Talin takes part in making connections between the ECM and the cytoplasmic domain (Gotwals et al. 1994). By knocking down Talin in hemocytes, we expected to sever the communication between hemocytes and their environment.

1.4.8 Dynamin (encoded by *shibire*)

Dynamin contributes to endocytosis by assisting in the production of clathrin coated vesicles (Masur et al. 1990; See Damke 1996 for review). Dynamin may also have a role in microtubule bundling (Shpetner & Vallee 1989). Impairing the ability of a cell to perform endocytosis or to form microtubules may have drastic effects on any cell, so we knocked down Dynamin in the hemocytes to see if we get a more disorganized DV ECM, showing hemocytes are critical for DV ECM growth and

remodeling. If hemocytes have a role in remodeling the DV, then they might endocytose previous ECM structures so that new networks can be rebuilt.

1.5 Hemocytes of *Drosophila*

Hemocytes are functionally similar to vertebrate white blood cells, because they can defend the organism against microbic infestation, engulf apoptotic cells and produce ECM (Hoffmann et al. 1999; Gold & Bruckner 2014). There are 3 main types of hemocytes: phagocytotic plasmatocytes that are the most common type; encapsulating lamellocytes, which are produced when there is an immune challenge; and crystal cells (<5%) that destroy pathogens through melanization (Evans et al. 2003; Meister & Lagueux 2003; Lanot et al. 2001).

Another function of hemocytes is to secrete basement proteins such as Laminin, Collagen IV and Papilin (Kusche-Gullberg et al. 1992; Knibiehler et al. 1987; Yasothornsrikul et al. 1997). Functionally impairing hemocytes can give rise to embryos with morphologically abnormal tissues that lack a robust ECM network. For example both hemocyte migration defects and *laminin A* mutations give rise to developmental defects of the gut, which suggests that hemocytes play an important role in producing Laminin A for the basement membrane of the gut (Yarnitzky & Volk 1996). Another study shows that preventing migration of hemocytes prevent ECM deposition at the embryonic ventral nerve cord, which gives rise to developmental defects (Olofsson & Page 2005). These examples show secretory functions of hemocytes and make them a good candidate for the cell type that may be producing the ECM of DV during larval growth.

1.5.1 Production of hemocytes (hematopoiesis) in embryo and larval lymph gland

Hematopoiesis in Drosophila starts with the head mesoderm that gives rise to hemocyte progenitors that go through four cell divisions starting from stage 8 until stage 11 (Tepass et al. 1994; Rehorn et al. 1996). Between stage 11-16, these cells differentiate into about 700 plasmatocytes and migrate to other tissues (Lebestky 2000; Stramer et al. 2005; Wood & Jacinto 2007; Siekhaus et al. 2010). There is also about 30 crystal cells born from the head mesoderm but they concentrate at the gut for unknown reasons instead of migrating like plasmatocytes (Lebestky 2000). Plasmatocytes that are born in the first wave can linger for a long time, even until the adulthood (Holz et al. 2003).

The other source of hematopoiesis is the lymph glad, which is attached to the anterior side of the dorsal vessel (Lebestky 2000; Mandal et al. 2004). Lymph gland is a hemocyte producing structure that grows throughout larval development and provides the most of hemocytes at the start of metamorphosis (Figure 1.5; Tepass et al. 1994; Lebestky 2000; Rizki 1978). The lymph gland is divided into 3 main sections according to their functions: medullary zone is where prohemocytes are stored, cortical zone contains differentiating hemocytes and the posterior signaling center controls the hemocyte differentiation rate. (Jung et al. 2005; Lebestky et al. 2003; Krzemień et al. 2007). The lymph gland is disintegrated in the adults (Lanot et al. 2001).

1.5.2 Structure of hematopoietic pockets

During larval development, the lymph gland hemocytes are not released into the hemolymph unless there is an immune response (Holz et al. 2003; Honti et al. 2010).

However, larvae have sessile hemocyte containing hematopoietic pockets (HPs), which repeat segmentally from A1-A7, in addition to circulating hemocytes (Figure 1.5A; Lanot et al. 2001; Makhijani et al. 2011; Leitã O & Sucena 2015). These hemocytes descend from the differentiated embryonic plasmatocytes (Makhijani & Brückner 2012; Makhijani et al. 2011; Honti et al. 2010). The hemocyte clusters within HPs occupy the space between the epidermis of the larval body wall and the muscle tissue (Makhijani et al. 2011). 1st instar HPs surround the liver-like organs called oenocytes (Gutierrez et al. 2007) which are located at the lateral midline (Makhijani et al. 2011). During 2nd and 3rd instar stages, the stripes with hematopoietic pockets extend further up to the dorsal midline (Makhijani et al. 2011). HP hemocytes are attracted to and associate with the peripheral nervous system (PNS) neurons as well as requiring their presence for survival-inducing signals (Figure 1.5B; Makhijani et al. 2011). The signals that attract HP hemocytes are very specific in location, because the pattern of HP bands is restored after distributing all sessile hemocytes by touching the larva with a paintbrush (Makhijani et al. 2011)

The adhesive properties of hemocytes in HPs can give us an idea on possible mechanisms that might trigger formation of hemocyte clusters associated with the DV. No desmosomes or septate junctions between the hemocytes were observed in HP clusters, however these hemocytes associate closely with each other, forming cellular interdigitations (Lanot et al. 2001). Despite the interdigitations, some of the HP hemocytes are in a dynamic state, capable of migrating into other clusters (Makhijani et al. 2011). Perhaps more research on hemocyte cell receptors is necessary to understand this dynamic, yet consistent pattern of HP clusters. One interesting finding in this avenue is that hemocytes require a Nimrod transmembrane

receptor called Eater in order to bind and phagocytose Gram positive bacteria or to form HPs (Bretscher et al. 2015; Kocks et al. 2005; Kurucz, Markus, et al. 2007). It is likely that Eater is a key protein in cell-cell adhesion in hemocytes (Bretscher et al. 2015). Additionaly, it was found that integrin subunit βPS is required for adhesion and migration of hemocytes in embryonic stage (Comber et al. 2013). It is likely that after embryogenesis, integrin continues the role of adhering hemocytes to their surroundings as well as assisting their migration through bridging intracellular actin cytosketon to the ECM network (Comber et al. 2013).

1.5.3 Function of hematopoietic pockets

It is likely that HPs function as a proliferative niche, because HP hemocytes proliferate significantly faster than circulating hemocytes (Makhijani et al. 2011). It was observed that HP plasmatocytes divide, giving rise other plasmatocytes (Leitã O & Sucena 2015). Moreover, some HP plasmatocytes transdifferentiate into crystal cells (Leitã O & Sucena 2015), which is a critical process because the crystal cells that originate from embryonic hemocytes do not multiply by themselves during larval stages (Krzemien et al. 2010; Lanot et al. 2001). The trasdifferentiation is triggered by a plasmatocyte that express the membrane bound ligand Serrate, which can activate the Notch receptors on the adjacent plasmatocyte (Leitã O & Sucena 2015). The activated Notch signaling causes the production of a crystal cell transcription factor called Lozenge, which commits the cell to become a crystal cell (Leitã O & Sucena 2015). Presence of HPs in the larva is extremely important to this process, because plasmatocytes need to be closely associated as they are in HPs in order for the Serrate-Notch communication to happen (Leitã O & Sucena 2015).

Proliferation of hemocytes in larval HPs is also significant for metamorphosis (Lanot et al. 2001). There is a greater number of circulating hemocytes observed as the larval development progresses (Lanot et al. 2001). It is likely that there are two contributors to this phenomenon: the expansion of hemocyte population, and having hemocytes readily available in circulation in late larva to prepare for metamorphosis in which the apoptotic tissue removal will take place (Lanot et al. 2001).

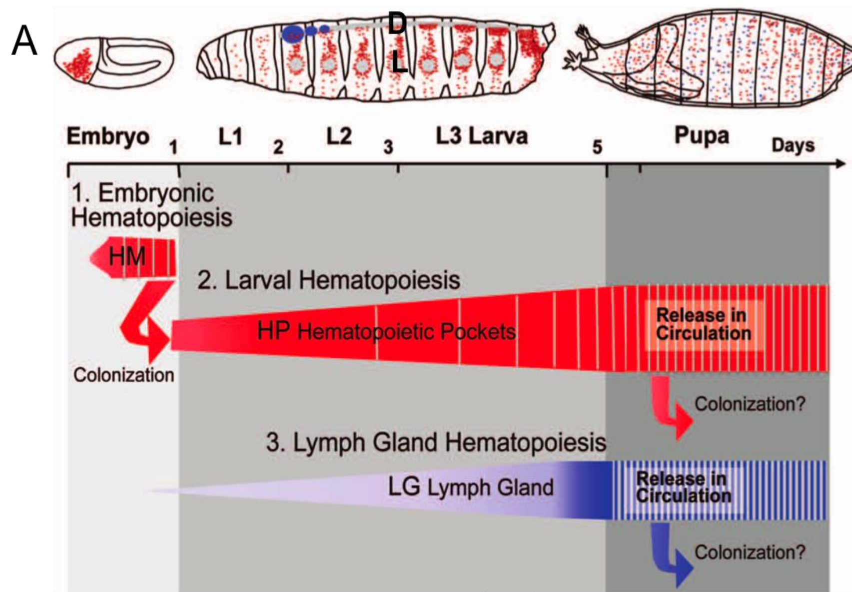
Another function of larval HPs is to respond to immune stimulation. HPs produce lamellocytes upon parasitic infection (Márkus et al. 2009). Moreover, following a parasitic infection, it was observed that the number of sessile HP hemocytes decreased (Márkus et al. 2009). This observation is explained with the data that there is no increase in the rate of mitosis in HPs in the inoculated larvae, yet the number of hemocytes circulating in hemolymph increase (Márkus et al. 2009). This shows that when HP hemocytes detect that there is a parasitic attack, they dissociate from HPs to migrate to other tissues and scan for foreign bodies. Conversely, it was previously reported that only the circulating hemocytes, but not the tissue bound hemocytes in close proximity of a pinched epidermis wound site got recruited to the site of injury (Babcock et al. 2008). According to authors, this data suggests that only circulating hemocytes respond to injury; while sessile tissue bound hemocytes remain unresponsive. However the total number of sessile vs. circulating cells were not quantified in this study, or the pattern of HPs were not observed before and after the injury, so the response of HPs to injury is not described in detail so far.

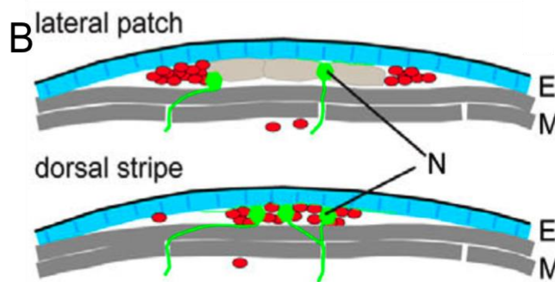
1.5.4 Hematopoietic hubs of the adults

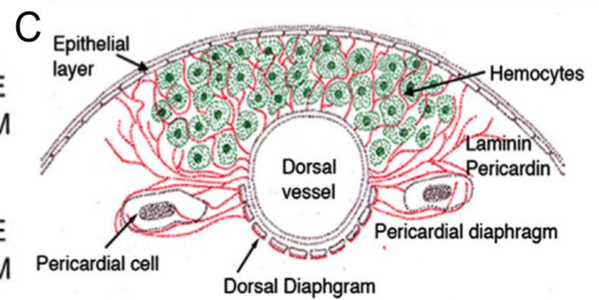
The adult flies lack a lymph gland or the HPs that are formed during larval stages, however hemocytes that are produced during embryonic and larval stages can persist into adulthood (Holz et al. 2003; Honti et al. 2014). Hemocytes of the adults reside in hematopoietic hubs that form between the epithelial layer and the dorsal vessel on segments A1-A4 (Fig.1.5C; Ghosh et al. 2015). These hubs are composed of hemocytes that are embedded in an ECM network of Laminin A and Pericardin (Ghosh et al. 2015). There are also hemocyte progenitors that descend from the lymph gland among these hubs, which differentiate into crystal cells and plasmatocytes (Ghosh et al. 2015). Even though differentiated hemocytes do not proliferate in the healthy adult, when there is a bacterial infection, plasmatocytes of the hematopoietic hubs are capable of proliferating (Ghosh et al. 2015).

Figure 1.5: ***Drosophila*** **hematopoietic sites**.

(A) Hematopoiesis throughout development. red: hemocytes, blue: lymph gland, D: dorsal side, L: lateral side. (B) Transverse cross sections of lateral and dorsal HPs. red: hemocytes, E: epidermis, M: muscle N: PNS neurons (C) Transverse cross section of the adult DV. Hematopoietic hub is intertwined with an ECM network of laminin A and Pericardin; and is located between the epithelial layer and the DV. (A) Modified from (Makhijani & Brückner 2012). (B) Adapted from Makhijani et al. 2011. (C) Adapted from (Ghosh et al. 2015).


A
D
L
Embryo
1
L1
2
L2
3
L3 Larva
5
Pupa
Days
1. Embryonic Hematopoiesis
HM
Colonization
2. Larval Hematopoiesis
HP Hematopoietic Pockets
Release in Circulation
Colonization?
3. Lymph Gland Hematopoiesis
LG Lymph Gland
Release in Circulation
Colonization?


B
lateral patch
E
M
N
dorsal stripe
E
M


C
Epithelial layer
Hemocytes
Laminin
Pericardin
Dorsal vessel
Pericardial diaphragm
Pericardial cell
Dorsal Diaphgram

1.5.5 Hemocytes that associate with the DV

It was reported that sometimes hemocytes are found in close proximity of the DV (Lehmacher et al. 2012) and are possibly associated with it (Makhijani et al. 2011). However it was not clear if these hemocytes are tethered to DV cells or accumulate in the heart lumen as a result of hemolymph circulation. Babcock et al. 2008 supported the latter idea and postulated that hemocytes that appear to be associated with the DV are in fact a result of a circulation build-up. It was also noted that DV clusters are different from HPs because they do not associate with PNS neurons (Makhijani et al. 2011). Even if the DV associated hemocytes are not a result of circulation build-up and there are unknown signals that attract hemocytes to the DV, these hemocytes must have a different microenvironment than HPs just based on the locational differences. There are a lot of unexplored genes that might affect the number of hemocytes that associate with the DV. Stofanko et al. 2008 identified the accumulation of hemocytes along the DV as an abnormal phenotype and listed the genes whose misexpression in hemocytes can trigger this phenotype. Most of these gain of function experiments involved genes whose functions are not known, however there are some interesting genes that might give insight into the nature of hemocyte adhesions. For example, the accumulation of hemocytes along the DV is observed when an unidentified gene CG32813 that leads to an increase in βPS integrin is overexpressed. In parallel to this finding, the overexpression of integrin subunit αPS3 is also associated with the same phenotype. Future research on the list of genes in this study can illuminate at what kind of adhesive properties are required for hemocytes to accumulate at the DV.

1.5.6 A key signal along hemocyte migration routes: PVF/PVR

It is interesting to know if there are any signals that may attract hemocytes into the heart, especially if these signals have homologs in vertebrates. *Drosophila* homologs of VEGF and VEGFR proteins are a candidate for such a signaling pathway. Vascular endothelial growth factor (VEGF) is a protein family that has important roles in angiogenesis and cell proliferation in vertebrates (Ferrara et al. 2003). Additionally, VEGFs promote chemotaxis of monocytes (Clauss et al. 1990). VEGF ligands secreted from various cells perform their activity by binding to VEGF receptors (VEGFRs) that are found on endothelial cells from blood vessels and lymphatic endothelium (Kukk et al. 1996; Jeltsch et al. 1997). These receptors are tyrosine kinases that have immunoglobulin like domains (Shibuya et al. 1990; Terman et al. 1991).

There is one *Drosophila* homolog of VEGFR called PVR (PDGF/VEGF receptor) and 3 homologs for VEGF called PVF1, PVF2 and PVF3 (PDGF/VEGF factor; Cho et al. 2002). Similar to its vertebrate homolog, *Drosophila* VEGF may have a role in cell proliferation. Ectopically expressed PVF2 triggers hemocyte proliferation in 3rd instar larvae (Munier et al. 2002). Another proposed role for PVF is that is a ligand produced by the cells that exist along hemocyte migration pathways and act as a chemoattractant for PVR which is a receptor that is produced by the hemocytes (Cho et al. 2002). PVR is expressed in hemocytes starting from stage 8 when they are born and throughout larval stages (Cho et al. 2002). In wild type embryos, hemocytes move into the tail region at stage 11, but in PVR mutants hemocytes fail to enter the tail (Cho et al. 2002). Hemocytes normally scatter inside the embryo at stage 13, but in PVR mutants they form aggregates at the anterior

region instead (Cho et al. 2002). There is further evidence for PVF being a chemoattractant for PVR, such as the expression pattern of PVF matching the hemocyte routes (Cho et al. 2002). For example it was observed that from stage 11 to stage 15, all three kinds of PVFs are expressed at tissues such as trachea, salivary glands and foregut at times when hemocytes are known to migrate to those areas (Cho et al. 2002). Moreover, ectopic expression of PVF in the salivary duct, foregut, trachea and midline glia of the embryo relocated hemocytes to these areas (Cho et al. 2002). It was also reported that PVF2 is expressed along dorsal vessel during embryonic stages to guide hemocytes (Wood et al. 2006). Considering that PVF is chemoattractant for hemocytes in the embryo, we think that it might have the same role in larvae, possibly attracting hemocytes to the heart. However this idea is beyond the scope of this project and can be tested in future experiments. We focused our attention on PVR in this project. PVR is an anti-apoptotic cell survival factor for hemocytes (Brückner et al. 2004). We used the dual function of PVR in migration and survival to our advantage in order to impair hemocytes as much as possible so that we can observe if hemocytes are necessary for proper DV formation.

1.6 Project outline: Hypothesis and thesis objectives

Hypothesis 1: Hemocytes adhere to the dorsal vessel in late third instar larvae in order to form cell clusters that responds to immune stimulation.

Rationale: We have noticed that sometimes hemocyte clusters can be found in close proximity of the heart. The structure and function of such clusters was to be explored, because hemocyte clustering may be an indication of hematopoietic sites.

Objectives:

1. Using a transgenic line that enables us to trace hemocytes and visualize the DV, I will determine whether hemocytes are found in close proximity of the DV in late stage embryos, L1, L2, L3 and adults.
2. Using the same transgenic line from objective 1 and antibodies that labeled basement membrane proteins and pericardial cells, I will determine whether there is a specific location along the L3 DV where hemocytes cluster. A microbial infection will be used to determine if an immune reaction will change the behaviour of DV associated hemocytes.
3. Using the same transgenic line from objective 1 again, the change in the amount of DV associated hemocytes of the L3 larvae will be quantified according to developmental time and in presence or absence of a microbial infection.
4. Using an additional transgenic marker to trace crystal cells in addition to a general hemocyte marker, the localization of crystal cells along the DV will be determined for larvae with or without microbial infection. Identifying different kinds of hemocytes within the DV associated clusters is important to understand the function of these clusters as a hematopoietic niche.
5. Larvae with a *pericardin* mutation in addition to the hemocyte tracking transgene will be created to observe if hemocytes cluster along the DV when a component of the heart ECM is missing and pericardial cells are detached from the DV. Changing the ECM networks and pericardial-cardiomyocyte adhesions of the DV, and observing the change in behavior of hemocytes in response to this change is critical to understand what component of the DV is required for hemocytes to associate with the DV.

Hypothesis 2: Hemocytes or fat bodies take part in the dorsal vessel growth during larval stages by secreting Collagen IV that is found at the DV.

Rationale: In order to identify the cells that produce a major ECM macromolecule for the DV, we tested the two cell types that are likely to produce Collagen IV up until 3rd instar stage.

Objectives:

1. Hemocytes will be impaired using a hemocyte driver and UAS lines for *shi*, *talin* and *laminin* RNAi, and PVR dominant negative. Lethality rates will be assessed by performing a survival count after pupal eclosure, because high lethality rates might point to an effect on ECM assembly, potentially including the heart ECM.
2. Fluorescence quantification of ubiquitous GFP tagged Collagen IV in the L3 DV will be done to identify if either hemocytes or fat cells are responsible for producing the Collagen IV that is associated with the DV. Each cell type is targeted using a *UAS GFP RNAi* line and a Gal4 with tissue specific promoter in order to knock down Collagen IV produced in that specific cell type.
3. Fluorescence quantification of ubiquitous GFP tagged Collagen IV in the L3 DV will be done to assess if hemocytes secrete Collagen IV associated with the DV. Hemocytes might take part in laying the ECM of the DV even if they only carry the protein products to the DV instead of producing it originally. Fluorescence intensity of the GFP in DV will be measured in larvae with hemocytes that are impaired by either using *UAS stroy* (apoptosis inducing) or UAS PVR^{DN} (apoptosis inducing with migration defects) together with a hemocyte Gal4 driver.

Chapter 2.0: Methods

Drosophila stocks: *w; Hml-Gal4* (Bloomington 6395), *w; Pxn-Gal4* (K. Bruckner), *w; da Gal4* (Bloomington 55851), *R4-Gal4* (33832), *UAS-shi RNAi* (Dynamin, Bloomington 36921), *UAS-rhea RNAi* (Talin, VDRC 40399), *UAS-lanA RNAi* (laminin, VDRC 18573), *UAS-wb RNAi* (laminin), *UAS-mys RNAi* (integrin, VDRC 103704), *UAS* PVR^{DN} (K. Bruckner), *UAS rpr.27 (*stroy, B. Reed*)*, *HmlΔ-DsRed* (Makhijani et al. 2011)*, UAS GFP RNAi* (Bloomington 9330)*, Lz Gal4; UAS GFP* (lozenge, Bloomington 6313), *prc* ^{MB03017}*/TM6C,Sb* (pericardin, Bloomington 23836), *vkg* ^{cc0079}*, yw* (Bloomington 6598), *D/TM6*, *ubi.GFP, D/TM3, Sco/Cyo*. The line that was used to observe the heart lumen and hemocytes at the same time (*HmldsRed, Vkg-GFP /Cyo; Pxn Gal4*) was generated by me using lines we have obtained from Bloomington Stock Centre and K. Bruckner. Promoter of gene *hemolectin (hml)* or *peroxidasin (pxn)* is used to target most of the hemocytes (Sinenko & Mathey-Prevot 2004; Clark et al. 2011), except mature crystal cells that have lost the *hml* expression and instead express *lozenge (lz)* (Mukherjee et al. 2011).

The line to knock down GFP (*vkg; UAS GFP RNAi*) was generated by me using *vkg*^{cc0079} GFP trap (1) and *UAS GFP RNAi* (Bloomington 9330).

Table 2.1: A list of Gal4 lines and their expression pattern throughout development

	Embryo	Larva	Pupa	Adult	Donor/Reference Paper
Hml Gal4	N/A	Expressed by hemocytes at low levels	N/A	N/A	Akira Goto (donor)
Pxn Gal4 (transcriptional activity of the gene and immuno-location of the polypeptide is described instead of the Gal4 expression)	Hemocyte, Garland cell, Posterior midgut primordium, Head sensory system, Ring gland	Hemocyte, Fat body, Gastric caecum	N/A	Spermatozoon	Katja Bruckner (donor), Berkeley Drosophila Genome Project, Nelson et al. 1994, Hammonds et al. 2013, Tomancak et al. 2002, Tomancak et al. 2007, Wasbrough et al. 2010
da Gal4	ubiquitous	N/A	N/A	N/A	Elisabeth Knust (donor), Wodarz et al. 1995
R4 Gal4	N/A	Fat body, Salivary glands	N/A	N/A	Jae Park (donor)
Lz Gal4	Hemocyte precursor, Crystal cell	Crystal cell	NA	Crystal cell	John Pollock (donor), Fossett et al. 2001, Leitã O & Sucena 2015, Ghosh et al. 2015

Immunolabeling: All steps were done at room temperature according to the protocol by Alayari et al. 2009. Samples were fixed in 1ml of 4% paraformaldehyde in PBS for 20 minutes in a 24 well plate, followed by a 10 minute PBT wash 3 times. Maximum of 6 samples were put on a shaker in 150 µlt of PBT solution with 5 µlt of primary antibody for 2 hours in a 96 well plate. Samples were washed 3 times with PBT for 10

minutes each. Samples were put on a shaker in 150 µlt of PBT solution with 1 µlt of secondary antibody for 1 hour, and then washed 3 times for 10 minutes each with PBT again. Finally, samples were washed with PBS for 10 minutes, then put in 50% glycerol in PBS and stored at 4ºC indefinitely until mounted with 70% glycerol in PBS for imaging.

Antibodies used: α-βPS (Integrin, DF.6g11-s) was mainly used to label pericardial cells, but when there is no Vkg-GFP it also helped see the general outline of the heart by also labelling cardiomyocytes. α-LanA (Laminin) and α-Prc (Pericardin EC11 anti-Pericardin) were used to visualize ECM components.

Live imaging of the embryo: Live imaging of late stage embryo (Fig. 3.1A) was performed by Dr. Qamber Syed, using the hanging drop method described in Reed et al. 2009.

Heat fix for 1st and 2nd instars: Larvae on petri dishes were soaked in bleach for 3 minutes, and then washed with ddH_2O into a nitex sieve. Nitex sieve was immersed into 10mL of wash solution (0.5%NaCl, 0.03% Triton-X) in a small glass vial. The vial was put into boiling water and agitated for 10 seconds before placing the tube on ice and adding cold wash solution into the vial. After the larvae sank to the bottom, wash solution was replaced with PBT (1xPBS and 0.3% Triton-X). Next, PBT was replaced with 70% glycerol and specimens were mounted on a slide. Experiment performed for Fig 3.1 B, C.

Ethanol treatment for 3rd instars: Larvae were dipped in 90-100% ethanol for approximately 5 minutes, then imaged with Leica M165 FC dissection microscope within 20 minutes (Fig 3.1 D, E).

Dissecting and imaging the adult: Adult female that hatched 2 days ago was dissected according to the protocol adapted from Vogler & Ocorr 2009 in order to view the DV. Vaseline was used to hold flies in place on the dissection plate. The sample was fixed in 4% paraformaldehyde in PBS for 15 minutes, then washed with PBT and PBS for 10 minutes each at room temperature. The sample was put in 50% glycerol at 4°C for at least 30 minutes before imaging the dorsal side of the fly with Leica M165 FC dissection microscope (Fig. 3.1 F). Then, the sample was transferred to 70% glycerol and mounted on a slide before imaging it at confocal.

Larval dissections: Larval dissections were done according to the protocol adapted from (Vogler & Ocorr 2009). Larvae were immobilized by pinning them on a dissection plate.

Confocal imaging: All the images were taken with Leica SP5 confocal microscope unless stated otherwise. Frontal stack images were taken 1.3 µm apart and projected. Transverse cross sections were not projected.

Collection of larvae that were dissected: Approximately 100 flies were put in houses made up of plastic beakers with pinholes for air exchange and apple juice agar plates (0.02 gr/ml Agar, 2.5% sucrose, 25% apple juice in water) with a streak of yeast paste in the middle. The flies laid eggs for 2 hours before I collected the agar plates and stored them at room temperature. The next day, 25 fluorescent 1st instars were transferred to a yeast plate with yeast fly food. For hemocyte quantification experiments I collected approximately 25 newly hatched 1st instars every 30 min and transferred them to a yeast plate so that the larvae were approximately the same age. I have recorded the time when larvae started having branched spiracles to mark the time they became 3rd instars.

Microbial infection: The yeast plates with microbial agents were not treated with antibiotic and developed the infection naturally because the infection was carried over with eggs. The fly eggs were bleached to kill the infection, however in time the infection spread again. Therefore, in order to obtain plates without the infection, antibiotic was used. Plates with no infection had chloramphenicol added in the fly food so that the final concentration is 25µg/ml. Additionally, 100 ml of 25µg/ml chloramphenicol solution in water was spread out on each plate. Stock solutions for chloramphenicol was prepared with ethanol, but diluted with water to make working solutions. The microbial agent will be sequenced in the future in order to identify it. The pharmacological effect of chloramphenicol on wild type larvae was not assessed.

Staining for hemocyte quantification: Fluorescent progeny of *HmldsRed, vkgGFP/Cyo* males and *yw* females was kept on microbial plates or antibiotic plates at room temperature. 10-11 larvae were dissected in 70 minutes every 4 hours starting from when larvae become 3rd instars and until most of the larvae on plates started pupating. Some of the larvae were not used in hemocyte quantification due to dissection or imaging problems, so the sample size for each group is approximately 7-10. During dissections the already dissected samples were kept on ice in 1ml fixative (4% paraformaldehyde in PBS) with at most 6 larvae in each well of a 24 well plate. The samples were fixed for 15 minutes at room temperature, followed by Hoechst 33348 staining (1µg/ml in 1ml water) for 15 min. Hoechst stain was used to visualize the pericardial cells because they take up the dye and the staining is stronger in pericardial cells than it is in other cells. Samples were washed with PBS for 10 minutes on shaker, then put in 50% glycerol for at least 30 minutes at 4°C before

being transferred into 70% glycerol in PBS. Samples were mounted on a slide and imaged within a week.

Volume measuring for hemocyte quantification: Confocal stacks were converted to RGB in ImageJ (Java 1.6.0_24 (64-bit)). The color was adjusted so that Vkg-GFP, HmldsRed and Hoechst are all visible. A scale was set using the scale bar on the imported stack, this is important to define each pixel in μmeters. The distance between each stack was set to 1.3 μm. A trapezoid was drawn boxing the area between anterior side of 1st and 7th pericardial cell pair starting counting from the posterior end of the DV. The image was cropped so that all the areas outside of this region were not considered in the quantification. Image was resliced at every μm to obtain computer constructed transverse cross sections. The ImageJ plugin Volumest was used to measure the total volume covered by hemocytes that are located underneath the ECM layers of the DV. Figure 3.4 explains further which hemocytes were considered for quantification. The areas of interest on each image was drawn by hand. This analysis was performed by Pouya Arefi, Ana Stosic, Mahan Ghodrati and me. Pouya Arefi and I adjusted and outlined the steps in this protocol to quantify hemocytes. Settings for Volumest: slice thickness: 1 μm, number of steps: 1, gridth width: 3 μm, object type: irregular. Mann-Whitney rank order test was performed to detect if there is a difference between developmental stages or between antibiotic and microbial infection treatments.

Virtually constructed transverse cross sections and 3D projections: Using ImageJ, a scale was set on the stack, using the scale bar on the imported stack. The distance between each stack was set to 1.3 μm. To obtain cross sections, image was resliced at every μm. Volume Viewer plugin was used to construct 3D projections.

Pupal Survival Test: UAS RNAi lines and *UAS PVR^{DN}* were crossed to a respective balancer stock (*Sco/Cyo* or *D/TM3*), which had the balancer on the chromosome that corresponded to the chromosome that had the RNAi or PVR^{DN} insertion. Heterozygous virgins with balancers were collected from this cross and crossed to males with Gal4 drivers or *yw* (no driver) for control. Progeny was raised at 25°C until pupation. Because 50% of flies are expected to have a balancer, this formula was used to calculate % lethality: 50%-[(Number of flies without balancer/Total number of flies that eclosed)*100]. If the lethality rate was less than 0 then it was represented as 0. Chi Square test was performed using the observed and expected numbers of flies with and without a balancer. I detected if there is any difference within each group (a group consisted flies that had a specific UAS line or balancer as well as either one of these: *Hml Gal4, Pxn Gal4, yw*).

GFP fluorescence intensity comparison: Gal4 or *yw* virgin females were crossed with appropriate UAS males in a house (Table 2.2). Mahan Ghodrati helped collect virgins and flip plates sometimes. 15 fluorescent 1st instars were collected on a yeast plate every 30 minutes and treated with antibiotic in the way described above. When some of the larvae on the plate started pupating (around 100-110 hours old since 1st instar collection), rest of the larvae were dissected immediately and immunostained with α-BPS. This staining allowed me to see the pericardial cells and avoid the regions with hemocytes when choosing areas to measure the GFP intensity. Larvae from GFP RNAi experiments in Fig. 3.8 were imaged between 35-38 hours and 20 minutes after immunostaining. Larvae from the experiment in Fig. 3.10 were imaged between 27-29 hours and 10 minutes after immunostaining. The images in the stacks were cropped so that I only measured the fluorescence of the ventral half of the heart (this was a single-

blinded study). A trapezoid between 2 parallel pericardial cells located on segment A5 was chosen to measure the average fluorescence intensity. The background average fluorescence intensity of the same area was subtracted from the DV GFP measurement. A two-tailed t-test was used to compare each treatment to the control.

Stock maintenance: Flies were kept at room temperature on yeast fly food and flipped every 2 weeks. Ana Stosic maintained the stocks and prepared the fly flood and apple juice agar plates.

Table 2.2: Explanation for knock down experiments

Aim	UAS lines crossed to Gal4 or control lines	Gal4 drivers used to perform the knockdown	Control line
Fluorescently tagged Collagen IV detection at the DV when hemocytes have migration or survival defects	vkg; UAS GFP RNAi	HmldsRed,vkg/Cyo; Pxn Gal4	HmldsRed,vkg/Cyo
Fluorescently tagged Collagen IV detection at the DV when Collagen IV secretion from targeted tissues is inhibited	vkg; UAS GFP RNAi	Pxn Gal4 R4 Gal4 da-Gal4	yw
Measuring lethality rates in flies with impaired hemocytes or hemocytes with ECM related protein production defects	UAS LanA RNAi UAS Shi RNAi UAS Talin RNAi UAS PVR^{DN} UAS wb RNAi	Hml Gal4 Pxn Gal4	yw

Chapter 3.0: Results

3.1 Hemocytes cluster at the DV in 3rd instars, but not in embryos, L1, L2 or adults.

Migratory hemocytes were visible in late stage embryos (Fig 3.1 A). Even though the *HmldsRed* expression was not too strong in embryonic stages, these hemocytes were easy to identify because they are strongly labeled with Vkg-GFP. Some hemocytes come in contact with the DV as they migrate in late embryos, however no hemocyte clustering at the DV was observed at this stage.

There were a few individual hemocytes found in close proximity of the DV in 1st and 2nd instar larvae, but no clusters of DV associated hemocytes (Fig. 3.1 B, C). The accumulation of sessile hemocytes at these stages pertains mostly to the segmental accumulation of epidermal Hemotopoietic Pockets (HPs) described by Makhijani et al. 2011.

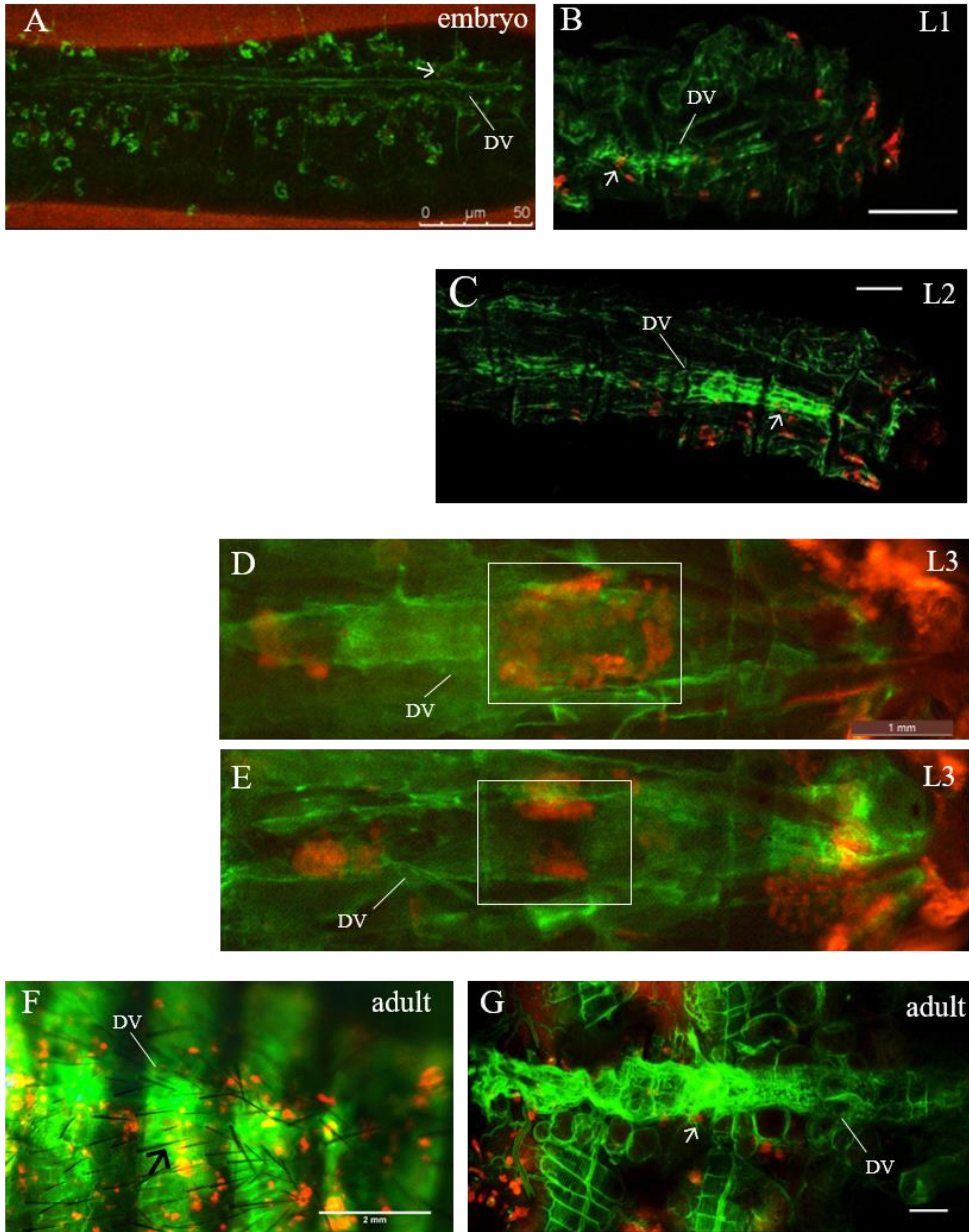
In some 3rd instars, there are no hemocytes associated with the DV, yet hemocytes reside on the segmental HPs of the body wall epidermis that is located on the dorsal side of the DV (Fig. 3.1 D). However, in others, clumps of hemocytes are associated with the DV (Fig. 3.1 E). It was hard to determine whether these clumps were inside the heart lumen or around the heart until the larva was dissected, but hemocytes are close enough to the heart that they move together with the heart when it pumps in intact larvae.

Figure 3.1 F and G show the dorsal and ventral view of a dissected adult dorsal vessel respectively. The hemocytes observed on the dorsal side of the heart are consistent with the findings of Ghosh et al. 2015, which showed that adults have hematopoietic hubs on the dorsal side of the DV. These hubs are only visible from the

dorsal side of the abdomen through the cuticle, and do not extend to the ventral side of the DV.

Figure 3.1: Hemocytes found in proximity of the *Drosophila* dorsal vessel throughout development.

(A, B, C, D, E, F) Dorsal and (G) ventral view of the DV obtained from *Drosophila* at different developmental stages. Subsequent figures of all frontal sections will be ventral views. Posterior is on the right in this figure and in all subsequent figures. (A, B, C, G) Few hemocytes (arrow) can be found in close proximity of the DV during embryonic, L1, L2 and adult stages. (D) Some L3s do not have any DV associated hemocytes, only the hematopoietic pockets (boxed area) located along the larval body wall. (E) Some L3s have hemocyte clusters (boxed area) associated with the DV. (F) Hemocytes (h) are located on the epidermis on dorsal side of the heart of the adult. (G) Ventral view of the same dissected fly from (F) does not show many DV associated hemocytes (arrow). DV: dorsal vessel. *HmldsRed* (red) is used to label most hemocytes, especially the plasmatocytes. *Vkg-GFP* (green) is used to visualize Collagen VI and it outlines the DV. *HmldsRed, Vkg-GFP* construct will be used in subsequent figures. n=20 for each observation. Scale bar: (A, B, C, G) 50 µm, (D, E) 1mm, (F) 2 mm.


A
embryo
DV
0 μm 50
B
L1
DV
C
L2
DV
D
L3
DV
1 mm
E
L3
DV
F
adult
DV
2 mm
G
adult
DV
Vkg HmldsRed

3.2 Hemocyte clusters accumulate inside the DV ECM pockets that ensheath pericardial cells in 3rd instars.

When the larvae with DV associated hemocyte clumps were dissected to expose the ventral surface of the DV, it was observed that the hemocytes appear around and in between pericardial cells of the heart proper (Fig. 3.2). The majority of the clusters are associated with the parts of the DV that reside on segments A5 and A6.

The epidermal HPs are located more dorsally compared to the DV hemocytes (Figure 3D). However, these HPs can appear to overlap with a DV hemocyte cluster when an intact larva is observed dorsally. The only way to distinguish these different hemocyte groups would be to observe if the cluster moves with the pumping heart or to dissect the larvae and image the DV together with the epidermis.

Basement membrane proteins such as Viking and Laminin A, and *Drosophila* DV protein Pericardin enclose the lateral compartments of the DV where pericardial cells and hemocytes reside together slightly ventrally to the heart lumen (Figure 3.2 and 3.3). Hemocytes appear to be in close contact with each other and with pericardial cells. There was comparatively little Viking-GFP, Laminin A or Pericardin immunolabeling observed in between the hemocyte clusters and pericardial cells, showing that pericardial cells are in close contact with hemocytes instead of indirectly contacting these clusters through a thick basement membrane (Figure 3.3 E^2, F^2).

It is possible that cardiomyocytes are connected to the DV hemocytes as well, however there is a basement membrane around the heart tube (the structure that immediately surrounds the heart lumen), which could make this association more

indirect than it is with pericardial cells. For example in Fig. 3.2 C, a layer of Viking is readily detectable around the heart tube.

Figure 3.2: Location of DV associated hemocyte clusters in third instars.

(A) Frontal sections showing the ventral view of a dissected DV from a migratory 3rd instar. s: segmental boundaries. Vkg (green) covers the DV(dv). Plenty of hemocytes appear between the pericardial cells (asterisk) and the heart lumen (hl). Boxed area is enlarged in B. (B) Hemocytes are found in clusters that appear to be in the space between pericardial cell and the heart lumen. Additionally, there are hemocytes in between two adjacent pericardial cells (arrow). The line stands for the position of the transverse cross section shown in C. (C) Hemocyte clusters (arrow) are found adjacent to the pericardial cells enclosed within pockets of Vkg networks. These networks cover the heart lumen as well. Dorsal is at top here and in subsequent cross sections. (D) A cut through sagital 3D construction of the same DV from A-C. Inset shows the location of the section. Dorsal side is up and posterior is on right. Arrowhead is pointing at the HP associated with the epidermis of the body wall. Scale bars: (A) 75 µm, (B) 25 µm, (C) 10 µm. Genotype: HmldsRed,Vkg-GFP/+. Magenta: HmldsRed expressing hemocytes in here and in subsequent figures. Blue: α-BPS integrin to see the pericardial cells.

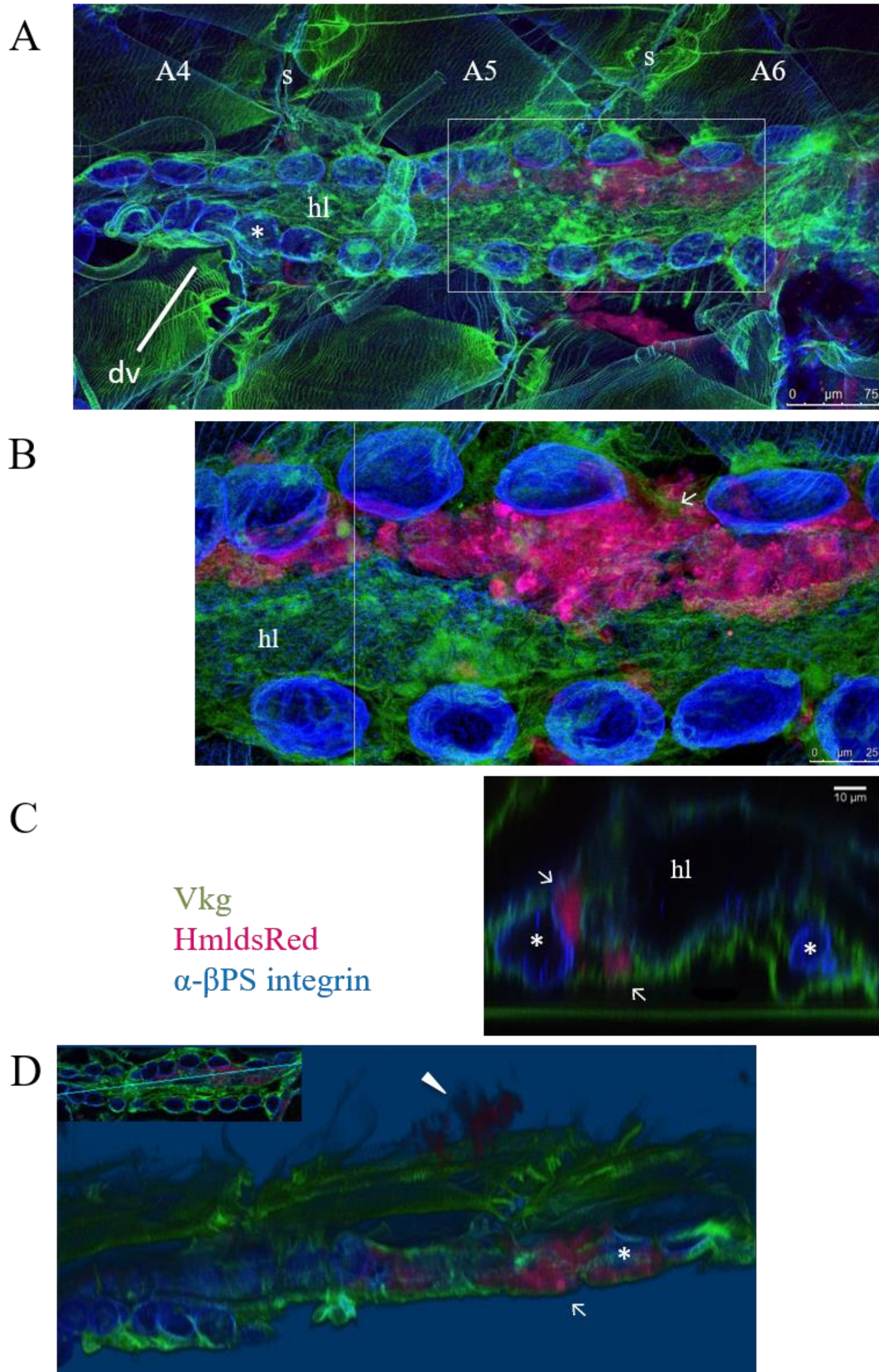
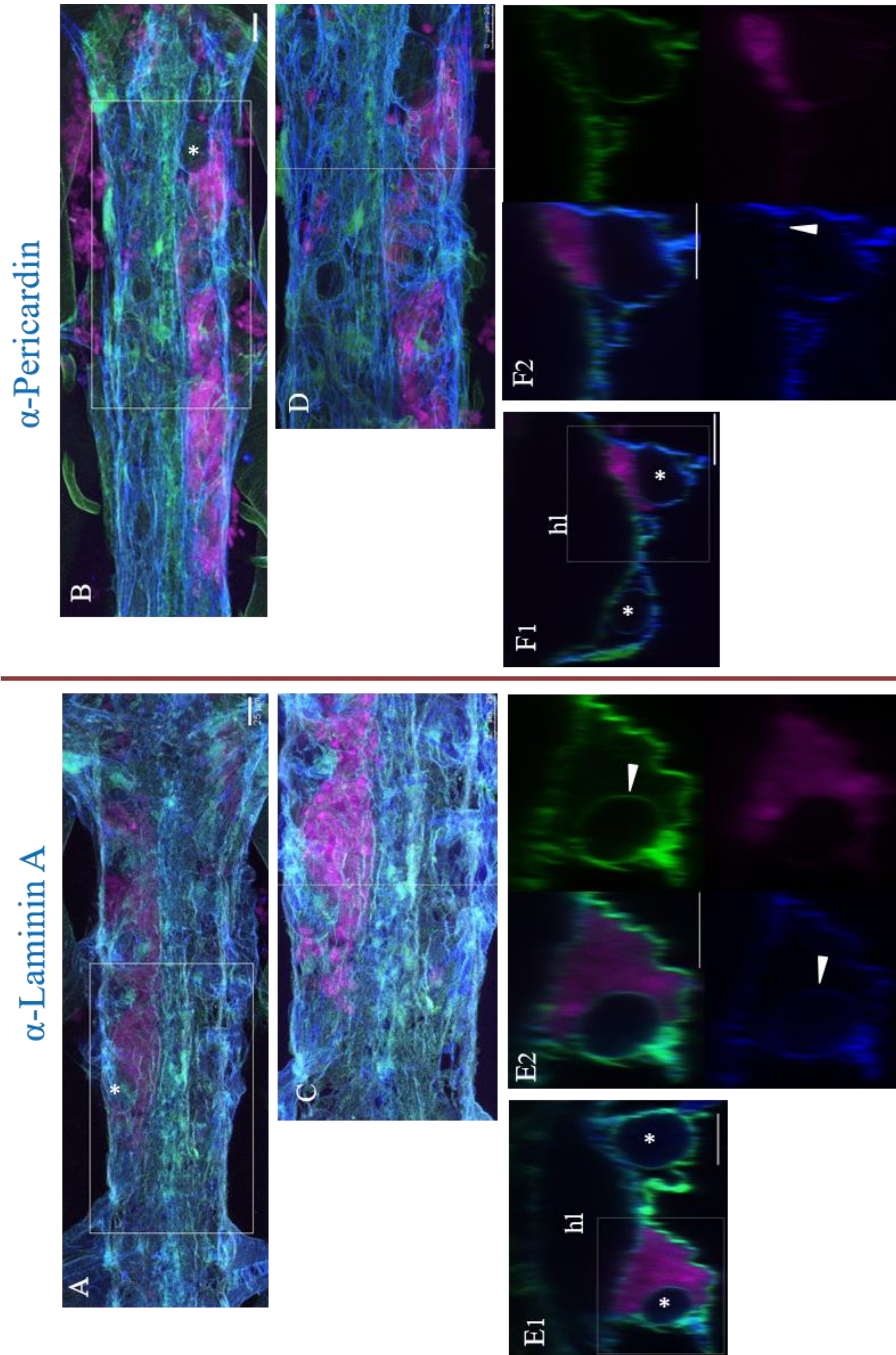

A
A4
s
A5
s
A6
hl
*
dv
0 µm 75
B
hl
0 µm 25
C
Vkg
HmldsRed
α-βPS integrin
10 µm
hl
*
*
D
*

Figure 3.3: Sheaths of Pericardin and Laminin envelope DV associated hemocyte clusters in third instars.

Localization of Laminin (A, C, E) and Pericardin (B, D, F) at the DV. Pericardial cells are not immunolabelled, but their positions are shown with asterisk. (A, B) Laminin and Pericardin cover the DV. Posterior on right. Boxed areas are enlarged in C and D. (C, D) Clusters of hemocytes are visible under Laminin and Pericardin layers. The lines stand for the positions of the transverse cross sections shown in E and F. (E^1, F^1) Both Laminin and Pericardin networks are detectable around the heart lumen as well as around pericardial cells. Hemocytes cluster between these networks and the pericardial cells. Boxed areas are enlarged in E^2 and F^2. Dorsal is on top. (E^2, F^2) Pericardin, Laminin and Viking (arrowheads) proteins are at threshold for detection in between hemocytes and pericardial cells. Green: Vkg-GFP, Magenta: hemocytes, Blue: Pericardin or Laminin. Scale bar: 25 μm. Genotype: HmldsRed, Vkg-GFP/+


α-Pericardin
B
D
F1
F2
hl
α-Laminin A
A
C
E1
E2
hl

3.3 The number of hemocytes at the DV is affected by the developmental time as well as the presence of a microbial immune reaction.

A large variation in the number of hemocytes is observed among 3rd instars that are approximately the same age. Even when flies are allowed to lay embryos for a very short amount of time, by the time most of the embryos become 3rd instars, others can be at different developmental stages. To address this issue, I collected newly hatched 1st instars on a plate (instead of embryos) and these larvae only differed by ±3 hours by the time they got to 3rd instar stage (Ashburner 1989). This experiment helped me observe the 3rd instars that are approximately at the same stage every four hours in order to determine if hemocyte clustering is affected by developmental time.

In previous studies, it was observed that hematopoietic sites in adults and larvae respond to immune activation (Márkus et al. 2009; Ghosh et al. 2015). We sought to determine if an immune reaction would cause more or less hemocytes to accumulate at the DV. I grew larvae on yeast plates infected with an unknown microbe (described in detail in the methods) to trigger an immune stimulus. This microbe grows on yeast and we have not yet identified the exact species, but the morphology and behavior of the microbe fits a gram negative bacteria (Sultan et al. 2001) that was previously shown to grow on *Drosophila* food. Because this microbe forms a biofilm on top of the yeast plates, the microbe is likely to be ingested by the larvae that eat this food. It is yet to be independently determined if this microbe causes an immune reaction in *Drosophila* larvae, however assuming that it would, I have quantified the changes in the number of DV associated hemocytes when larvae are exposed to this microbial agent. The control larvae had antibiotic on plates, which

prevented the growth of this microbial agent, however it was not assessed if there was a secondary effect of this antibiotic on larval hemocyte counts.

While quantifying DV associated hemocytes, we only considered the hemocytes that are found inside the ECM pockets where pericardial cells reside. Figure 3.4 A-B shows epidermal HPs and other locations where hemocytes can be found in close proximity of the DV. For quantification, we measured the volume of spaces occupied by hemocytes in transverse cross sections of confocal stacks, so we could easily identify the DV associated hemocytes enclosed within the ECM layers. Hemocyte clumps that are found inside the heart lumen are not likely to be true sessile hemocytes, because they would block the hemolymph flow. Such hemocyte clusters could be a result of individual hemocytes accumulating at the narrow parts of the heart lumen while dissecting the larvae, which will be discussed further below. Therefore, we did not consider these luminal hemocytes in the hemocyte quantifications (Fig. 3.4 B1). Furthermore, the HPs on epidermis can extend on the ventral side and contact the DV bilaterally (Fig. 3.4 B2). These hemocytes were still considered as a part of the epidermal HPs if Viking layers of the DV ECM did not enclose them. HP hemocytes were not included in DV hemocyte quantification. Additionally, if there were regions without the *Hmldsred* expression embedded within hemocyte clusters (Fig. 3.4 A), these regions were excluded from the measurements. Such gaps could be there because there are no hemocytes in that space or because there is a hemocyte such as a mature crystal cell that does not express *HmldsRed*. Because I could not distinguish between these possibilities, we excluded these dark regions all together. We limited our measurements to the region between the first and seventh pericardial cell pairs starting from the posterior side (usually A6), because this is the region where most

hemocytes accumulate. We did not count the hemocytes on the posterior most side of the heart proper because most of the time there are a lot of hemocytes here and the distinction between the epidermal hemocytes and DV-hemocytes is less clear.

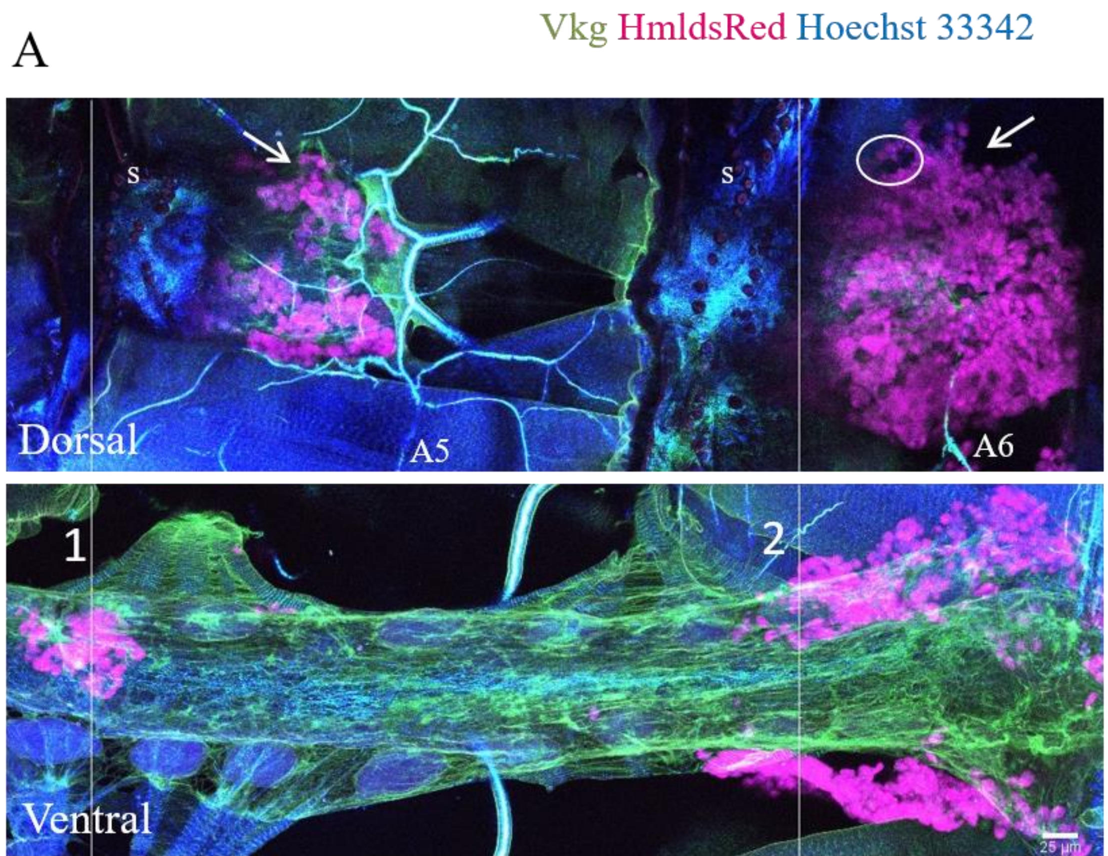
The median values of volume measurements from samples collected every 4 hours after becoming 3rd instar is shown in Fig 3.4 C. Larvae grown on antibiotic have an increase in DV hemocyte quantity at 32-36 hours after becoming 3rd instars. There is a decline in DV hemocyte volume after this stage until +48 hours. Shortly after 48 hours, most of the remaining larvae started pupating. Larvae grown with the microbial agent had an increase in hemocyte counts between +12-28 hours. After 28 hours there was a general trend showing a decline in DV hemocyte quantity until 44 hours. Shortly after 44 hours, most of the larvae pupated. The reason for larvae pupating faster on microbial plates could be an unpredicted temperature or humidity change even though the larvae were grown in a controlled incubator. Another reason for the difference in pupation time could be an unknown secondary effect of the immune reaction that triggered early pupation. Regardless of this difference, both treatments had the maximum amount of DV hemocytes 20 hours before pupation. In both treatments the second day of 3rd instar development had significantly more hemocytes than the first day.

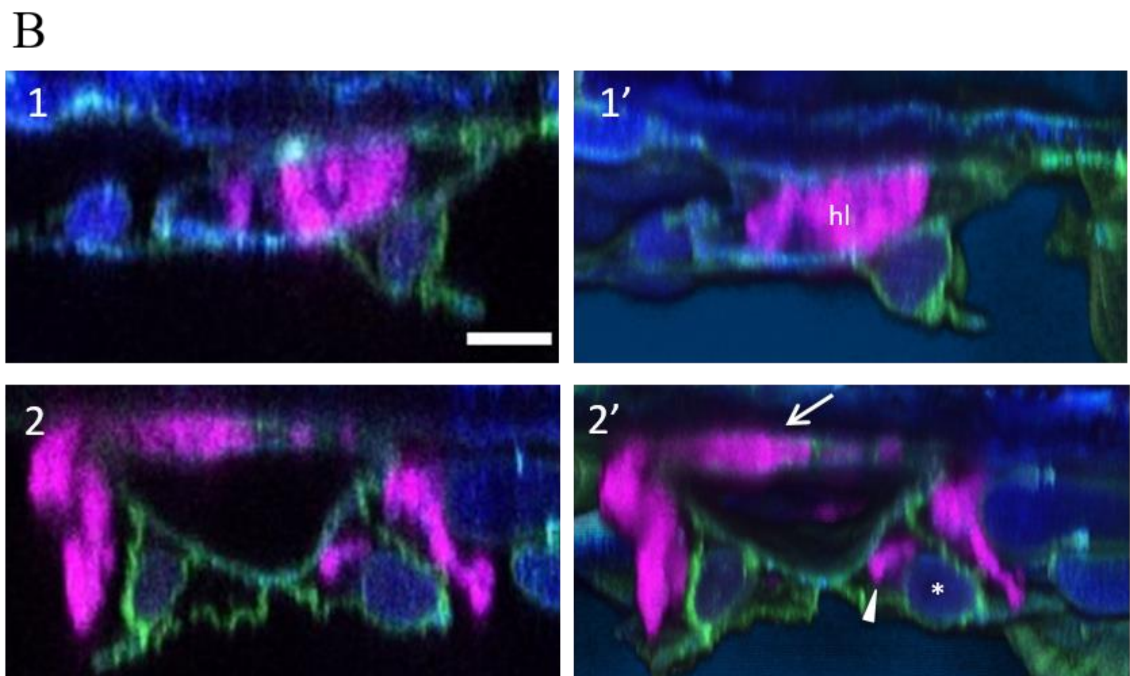
Between +12-32 hours of third instar development, larvae grown on plates with the microbial agent has significantly more DV hemocytes than the ones grown on plates with antibiotic. However, this comparison is done without considering the earlier pupation time for larvae grown on microbial plates. To overcome the bias that may have resulted from different pupation times, I have also compared the total data collected from two different treatment groups without considering developmental

hours. In total, larvae grown on bacteria have significantly more DV hemocytes than the larvae grown on antibiotic plates. All tests of significance were done according to one tailed Mann-Whitney rank order test ($P<0.05$).

Figure 3.4: Quantification and characterization of the DV associated hemocytes.
(A) Dorsal and ventral views of a representative 3rd instar DV dissection. Dorsal view shows the epidermis associated hematopoietic pockets (arrow) on segments A5 and A6. Segmental boundaries (s) can be used to identify the borders of A5 and A6. The epidermis associated HPs are found in clusters, however there are uniform circular gaps in between them, which are most likely occupied by mature crystal cells. Two of such gaps are encircled. The ventral view completely overlaps with the dorsal view and shows the DV that lays attached to the epidermis. There are hemocyte clusters found along the heart, but their exact position is not clear from the frontal view. The two lines (1,2) represent the location of two transverse cross sections in B including such hemocytes. (B) Virtually constructed transverse cross sections show different positions where hemocytes can be found. Primed (') numbers show the 3D constructed images of respective cross sections. Dorsal is up. (B1) Hemocytes can be found inside the heart lumen. Such hemocytes are not considered for DV associated hemocyte quantification (B2) On the dorsal side of the heart, the epidermis harbours HPs (arrow), which extend down towards the lateral sides of the heart. These HPs were not considered for DV hemocyte quantification either. Only the hemocytes (arrowhead) found adjacent to the pericardial cells (asterisk) wrapped in Vkg layers were quantified. Scale bar: 25 µm. (C) Quantification of DV hemocytes by measuring the volume covered by hemocytes expressing the HmldsRed marker. Median values for volume measurements are plotted on the graph. Larvae grown on yeast plates with microbial infection are compared to larvae grown on plates with antibiotic. Asterisk denotes the stages (12, 16, 20, 24, 28, 32 hours) where larvae from infected plates have significantly more hemocytes than the larvae grown on antibiotic plates. There

is an increase in the volume covered by DV hemocytes in both treatments until mid-late third instar stage (approximately 32nd hour after becoming 3rd instar). After that, there is a decline in the hemocyte count until pupation. Larvae grown on antibiotic plates pupated at 48 hours, whereas the larvae grown on infected plates pupated earlier at 44 hours. Larvae grown on microbial plates have significantly more DV associated hemocytes than the ones grown on antibiotic plates. The first day of 3rd instar (first +24 hours) is associated with significantly less DV hemocytes than the 2nd day of 3rd instar in both antibiotic and microbial treatments. All tests to detect significance was done according to one tailed Mann-Whitney rank order test ($P \leq 0.05$). Genotype: HmldsRed, Vkg-GFP/+.


A
Vkg HmldsRed Hoechst 33342
s
s
Dorsal
A5
A6
1
2
Ventral
25 µm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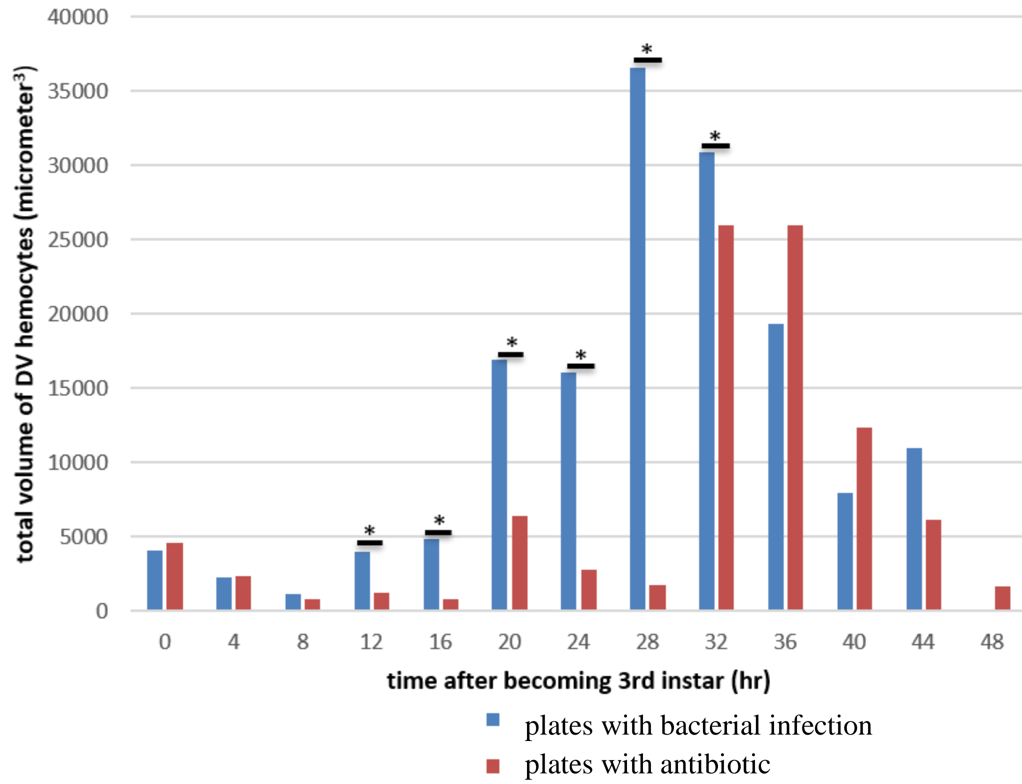


3.4 DV associated hemocyte clusters could form through the accumulation of hemocytes that enter the DV from the posterior side or through relocalization of HP hemocytes of the epidermis.

Many of the 3rd instars have hemocytes accumulated at the posterior side of the DV (Fig. 3.5 A). This includes the region posterior to pericardial cells and the heart lumen. An ECM network that contains Vkg covers the posterior tip of the heart. Figure 3.5 A shows two nearby sections from this region that contain a big hemocyte cluster. One part of the hemocyte clump is not fully covered by the Vkg layer, potentially allowing the entrance of hemocytes into this area (Fig. 3.5 A_1). Further down on the posterior, the clump is completely covered with a network of Viking (Fig. 3.5 A_2). This shows that even if some sections of the posterior heart appear to be fully covered by ECM proteins such as Vkg, others may contain openings that could allow an easy passage for many hemocytes to migrate into the heart. If this were the only mode through which all DV hemocytes entered the DV, then we would see the posterior most regions of the DV filling up with hemocytes before more anterior regions did so as well. However, this is not observed in many larvae that I have screened. I observed some larval hearts where clusters are found on the anterior side of the heart proper without any posterior clusters (results not shown).

Another way for hemocytes to accumulate at the DV ECM pockets could be from relocating hemocytes of the segmental HPs of the epidermis. In some larvae, especially during later stages of the instar development, the HPs are not confined to a planar band of hemocytes located on the epidermis. The sides of the band extend ventrally, down towards the sides of the DV (Fig 3.4 B_1, Fig. 3.5 B_2, C). These extensions are usually outside the Vkg layers of the DV, however sometimes they can

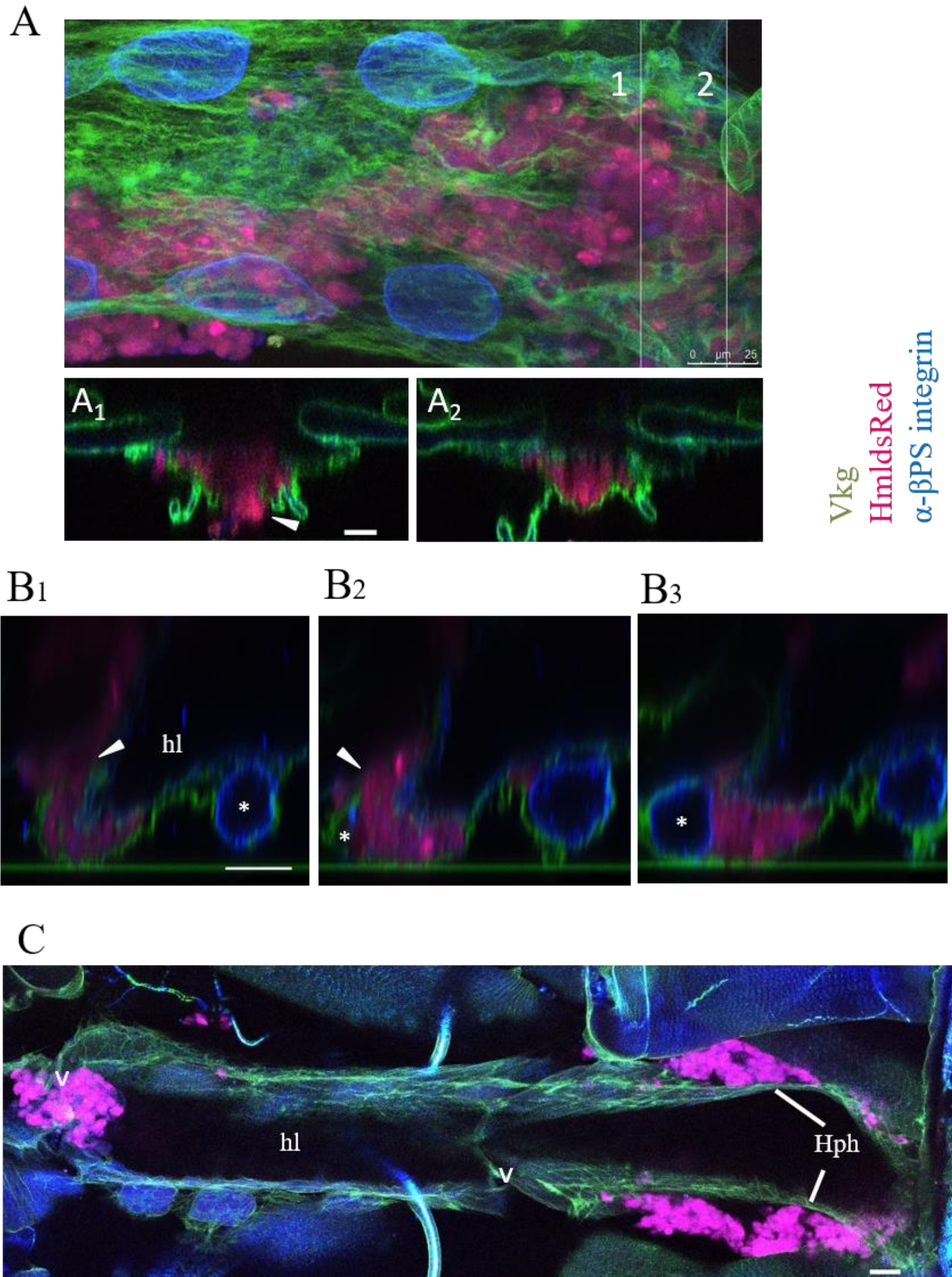
be observed to extend into the ECM pockets where pericardial cells are found (Fig. 3.5 B). The extension of these clumps into the heart is possible through openings in the Vkg network, which suggests that this opening could be a basement membrane gap that is utilized by hemocytes to migrate through ECM networks of the DV.

In dissected 3rd instars, clumps of hemocytes can be found inside the heart lumen (Fig. 3.5 C), however the only DV associated clumps in the living larvae are usually located at the sides of the DV where pericardial cells reside. It is likely that these clumps form when the larva is dissected but the heart is still pumping. Because the heart loses its shape and collapses during the dissection, hemocytes can get stuck in the heart lumen, especially where the valves are located. Indeed, the clusters that I see in the heart lumen are almost always located where the valves are.

Figure 3.5: Possible modes of hemocyte migration that result in hemocytes to associate with the DV.

(A) The posteriormost terminal part of this DV harbours hemocyte clusters. Lines denoted with 1, 2 show the locations of the transverse cross sections shown below. ($A_{1,2}$) Dorsal is up. (A_1) Hemocytes are covered with the Vkg layers of the terminal DV. Heart lumen is not visible here and possibly the tubular heart lumen does not extend this far. The Vkg layer that covers the hemocytes have an opening (arrowhead), which would allow hemocytes to easily enter this pocket. (A_2) Hemocyte cluster is completely covered with a Vkg layer. ($B_{1,2,3}$) 3 transverse cross sections of DV that are a few µm apart from each other. (B_1) Hemocytes from the HPs of the epidermis located dorsally extend down to the lateral side of the DV. Arrowhead shows the Vkg layer opening though which hemocytes could enter the ECM pockets that suspend the pericardial cells adjacent to the heart lumen. In this picture the pericardial cell on the left is not found in the same cross section with the hemocyte cluster, however the contralateral pericardial cell is denoted with asterisk. (B_2) The tip of the pericardial cell (asterisk) associated with hemocyte cluster is visible. Hemocyte cluster extends beyond the pericardial cell pockets covered with Vkg through an opening (arrowhead). (B_3) Pericardial cell (asterisk) associated with the hemocytes is visible and enclosed in a Vkg pocket. (C) This frontal section shows hemocytes that are clustered inside the heart. It is possible that hemocytes enter the heart through the heart lumen, however the clustering of the hemocytes is localized to the anterior heart valve (v). Posterior valve is also shown, but has no hemocytes. Clustering of the hemocytes at the heart valve can be a consequence of larval dissection. This image is

also significant to see how posterior side of the DV can be flanked by HPs that have extended ventrally, towards the DV. Hph: Hematopoietic pocket hemocytes. All images are from migratory 3rd instar larvae. Scale bar: 25 μm. Genotype: HmldsRed, Vkg-GFP/+.


A
1
2
0 μm 25
A1
A2
Vkg
HmldsRed
α-βPS integrin
B1
B2
B3
hl
*
C
hl
Hph
v

3.5 Similar to HPs of the epidermis, DV associated hemocyte clusters contain crystal cells.

DV associated hemocyte clusters contain crystal cells in addition to plasmatocytes in larvae that are grown on antibiotic plates or microbial plates (Fig. 3.6 A, B). The composition of DV associated hemocyte clusters is similar to HPs, which contain crystal cells as well as plasmatocytes (Fig. 3.6 C; Leitã O & Sucena 2015). Additionally, the posterior tip of the DV that extends beyond pericardial cells and cardiomyocytes harbors hemocyte clusters that contain crystal cells and plasmatocytes. It was observed that crystal cells are closely associated with each other, plasmatocytes and pericardial cells (Fig. 3.6 D).

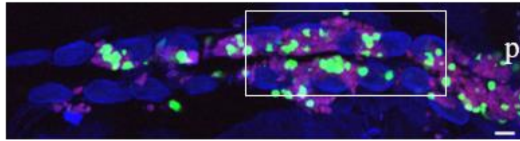
There is an unidentified cell type detected within the DV associated hemocyte clusters. There were many of the same kind of cells observed; one is shown in Fig. 3.6 B' as an example. This cell is not labeled by *HmldsRed* or *Lz-GFP*, meaning that it is not a mature crystal cell, a plasmatocyte or an old plasmatocyte in the process of transdifferentiating into a crystal cell. Unlike crystal cells, the unidentified cells contain a lot of Integrin on their plasma membranes. These cells are embedded within the hemocyte clusters, and they are found adjacent to plasmatocytes and crystal cells. This indicates that all three kinds of cells could be adhering to each other though cell-cell connections. The unidentified cell is bigger than a plasmatocyte, which suggests that it could be a lamellocyte.

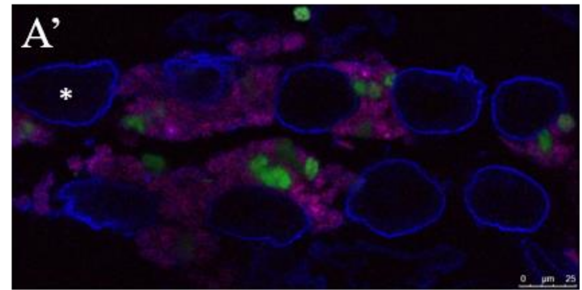
Figure 3.6: Dorsal vessel associated hemocyte clusters and the hematopoietic pockets of the epidermis both contain crystal cells in third instars.

(A, B) 3rd instars contain crystal cells (green) in the DV associated hemocyte clusters both when grown on antibiotic plates and on plates with microbial infection. Hemocyte clusters at the very posterior end of heart (p) also contain crystal cells. Rectangular region is enlarged in primed (') pictures. (B') DV associated hemocyte clusters contain few unlabeled cells (arrowhead) that express integrin on their cell membranes and are neither mature crystal cells nor plasmatocytes. (C) Dorsal view of a 3rd instar containing the HPs of the epidermis. The segmental boundary between A5 and A6 can be distinguished (s). The HPs on both segments contain crystal cells. (D) Some crystal cells are found adjacent to a pericardial cell. Asterisk: pericardial cell. HmldsRed: labels most hemocytes, Lz-GFP: labels crystal cells. Scale bar: 25 µm. Genotype: Lz Gal4/+(or Y); UAS GFP/HmldsRed.

A

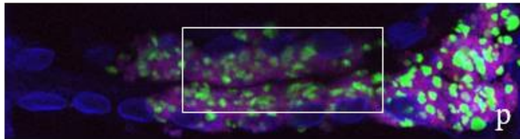
microbial infection

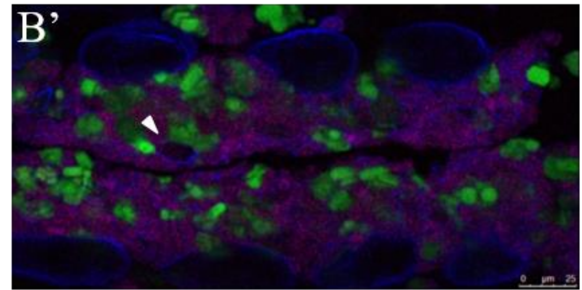




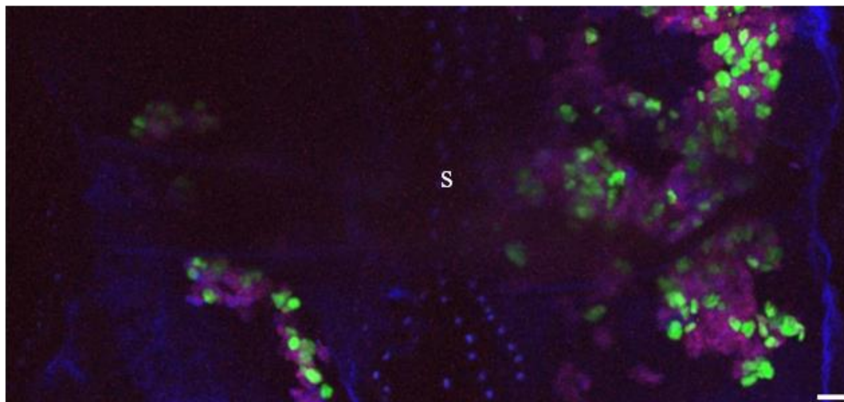
B

antibiotic

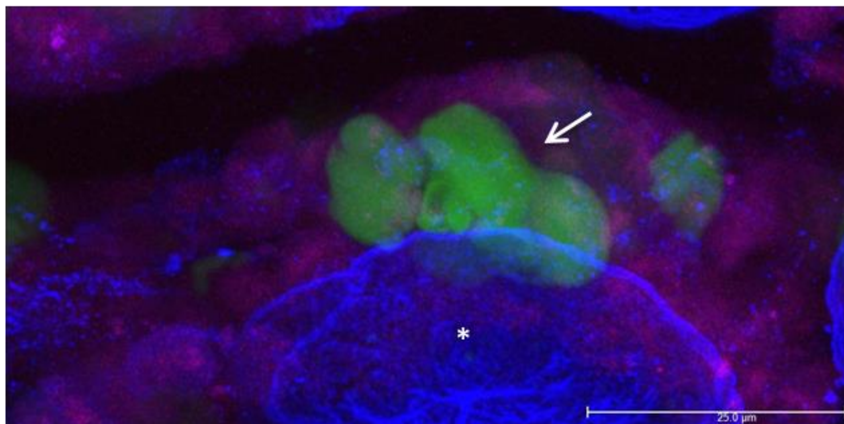




C



D



Lz-GFP
HmldsRed
α-βPS integrin

3.6 Hemocytes do not cluster at the DV when Pericardin network of the DV is disrupted.

Because Pericardin is a key ECM protein that forms networks and connects pericardial cells to the heart tube, we sought to examine the possible requirement of pericardial cells and Pericardin protein for hemocyte accumulation at the DV. This could reveal if cardiomyocytes or pericardial cells are responsible for attracting hemocytes into the DV via an unknown signal.

The morphology of the DV of heterozygous *prc/*+ or *prc/*TM6 mutants is close to the morphology of a wild type DV (Fig 3.7 A). The Pericardin networks still exist in the DVs of both mutants as revealed by the Pericardin antibody. However it was not quantified if pericardial cells are more detached from the heart tube then they are in the wild type. Additionally, DV associated hemocyte clusters were observed in these mutants that are grown with the microbial agent or the antibiotic. Substantial accumulations of hemocytes were seen in *prc/TM6* mutants, however this has not yet been quantified.

Homozygous *prc* mutant DVs are morphologically different than the wild type DVs (Figure 3.7 A, Drechsler et al. 2013). No Prc could be detected with the Prc antibody and pericardial cells have detached from the heart tube. The HPs still exist in *prc* homozygous mutants, but there are no hemocytes at the heart tube or associated with pericardial cells.

In most homozygous *prc* mutants grown with microbial agent or antibiotic, the heart tube is smaller in diameter than it is in the wild type. However, in some, the diameter of the heart tube is bigger. It is possible that there is a range in the severity of the phenotype and some larvae could have heart tubes that are more similar to the wild

type. In such a larva, I have found 2 small clusters of hemocytes at the DV (Fig. 3.7 B). One of the clusters is associated with the pericardial cell and the other is found adjacent to the heart tube. This suggests that both structures could have the potential to associate with hemocytes or attract them into the DV. Further screening of homozygous *prc* mutants and quantification of such observations are required to elucidate this idea.

It is also interesting to note that the cluster that was found near the pericardial cell was found in a position that is not commonly occupied by the hemocytes. In wild type larvae, hemocytes are often found on the dorsal, ventral or luminal side of the pericardial cell and rarely found on the abluminal side. This could be because there is more space between the pericardial cells and the basement membrane on these sides, giving hemocytes more space to occupy. In this case, the small cluster is found on the abluminal side of the pericardial cell. Perturbing the ECM through a *prc* mutation causes interesting changes in DV associated hemocyte clustering behavior and that needs further examination.

Figure 3.7: Hemocyte clustering at the DV in Pericardin mutants.

(A) Frontal stacks of migratory 3rd instars. Label on the left identifies the third chromosome. *prc/+* mutants are similar to wild type and do not show striking heart defects. Pericardin networks are detectable in these larvae. *prc/TM6* mutants are also similar to +/+ and produce Pericardin. In some *prc/+* and *prc/TM6* mutants large clumps of hemocytes (arrow) were observed at the DV. *prc/prc* mutants do not express any immunodetectable Pericardin. Pericardial cells are detached from the heart lumen, which has a smaller diameter than the wild type heart lumen. HP hemocytes (arrowhead) from the dorsally located epidermis extend towards the heart and flank the posterior end of DV, however no hemocytes are associated with the DV. (B) Virtually constructed transverse cross section of a *prc/prc* mutant. Dorsal side (top) of the DV are in close contact with epidermal HPs (arrowhead). Two small hemocyte clusters (h) are in close contact with the heart lumen and the pericardial cell. Asterisk: pericardial cell, hl: heart lumen. . Magenta: Hemocytes expressing HmldsRed. Blue: α-BPS integrin, helps see the outline of cardiomyocytes and pericardial cells. Scale bar: (A) 75 μm, (B) 25 μm. Genotypes: *+/+: HmldsRedvkg/+; +/+.prc/+: HmldsRed/+; prc/+. prc/TM6: Dot Gal4/HmldsRed+; prc/TM6. prc/prc: HmldsRed/+; prc/prc.*

A

α-βPS-integrin α-Pericardin

+/+

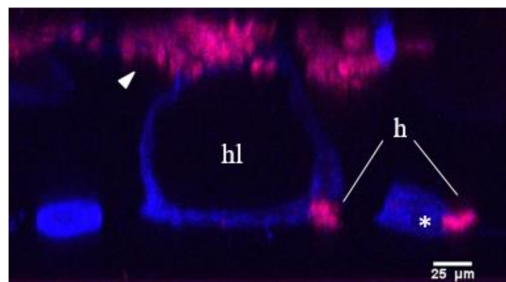
prc/+

prc/TM6

prc/prc

hl

B



3.7 Collagen IV of the DV is produced by fat body and hemocytes.

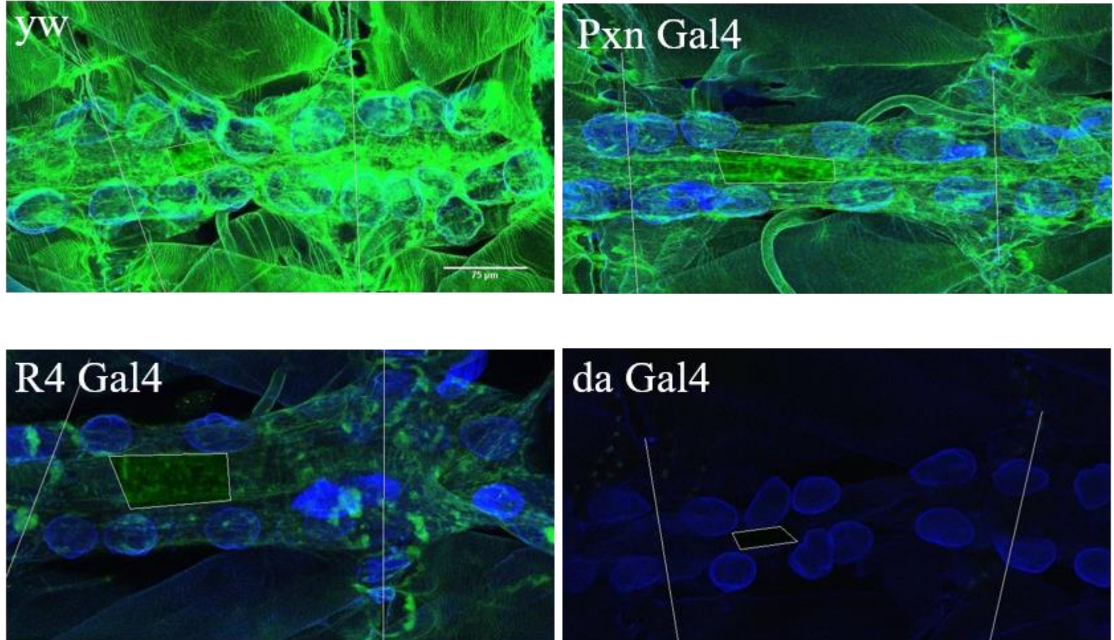
One of the main aims of this project was to identify the cell type that produces the DV ECM. To do this, we tested if hemocytes or fat cells produce one of the major ECM proteins: Collagen IV. GFP tagged Vkg can be efficiently knocked down with ubiquitous expression of GFP RNAi with the driver *daughterless (da)* Gal4 (Fig 3.8). Ubiquitous depletion of Vkg-GFP almost completely abolished the GFP at the DV. Using the same *vkg-GFP* GFP RNAi tool, I inhibited the GFP tagged Collagen IV production in hemocytes and fat body using drivers *Pxn Gal4* and *R4 Gal4* respectively. GFP intensity at the DV decreased dramatically when Vkg-GFP production in the fat body was inhibited. This shows that most of the Vkg that is found at the DV is produced by the fat body. Depleting the Vkg-GFP produced at the hemocytes also significantly decreased Vkg that is found at the DV according to a t-test. However, this decrease was less in magnitude than the effect of depleting Vkg that is secreted from the fat body. It is possible that both cell types contribute to Vkg synthesis.

Figure 3.8: Hemocytes and the fat bodies contribute to the production of Collagen IV at the DV.

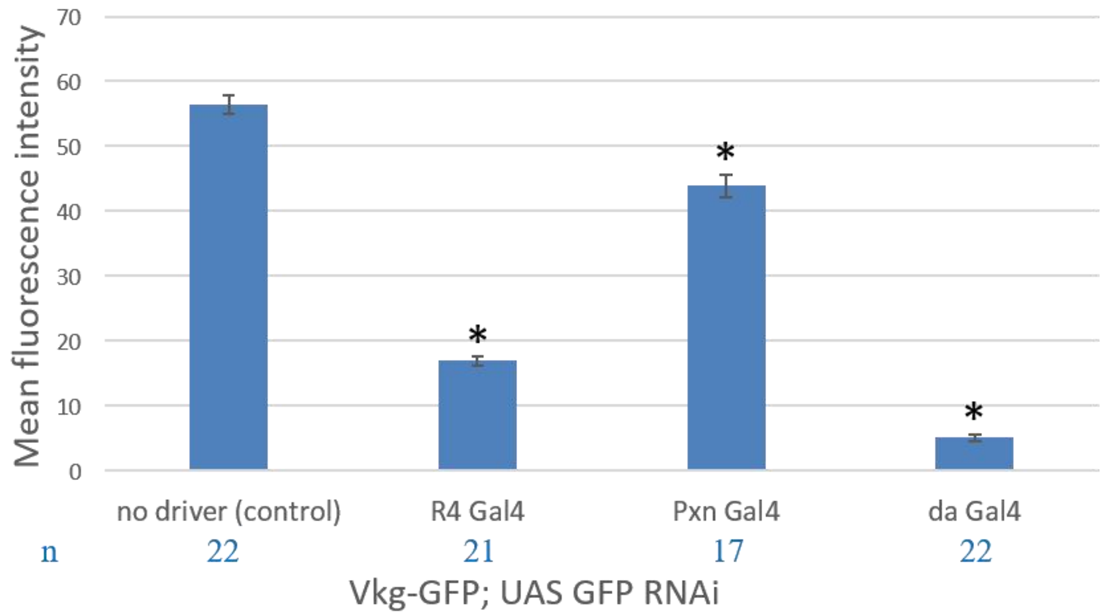
Fluorescence detection of vkg-GFP in 3rd instar larvae that express *UAS GFP RNAi* using drivers that are active in hemocytes (*Pxn Gal4*), fat body (*R4 Gal4*) or ubiquitously (*da-Gal4*). Control larvae are *UAS GFP RNAi/+*. (A) Representative images showing the boxed areas that were used to measure GFP intensity in each heart. The boxed areas were always chosen between 2 pairs of contralateral pericardial cells that are located on part of the DV on segment A5. White lines: Segmental boundaries. Scale bar: 75 µm.

(B) Quantification of vkg-GFP intensity of the boxed areas represented in (A). Knocking down GFP ubiquitously and in fat bodies and hemocytes significantly (*) reduces vkg-GFP intensity of the DV compared to the control. The reduction is greater when fat cells express GFP RNAi relative to when hemocytes do. Blue: βPs integrin antibody. Error bars: SEM. Sample sizes are on X axis. Two-tailed t-test, $P<0.001$.

A



B



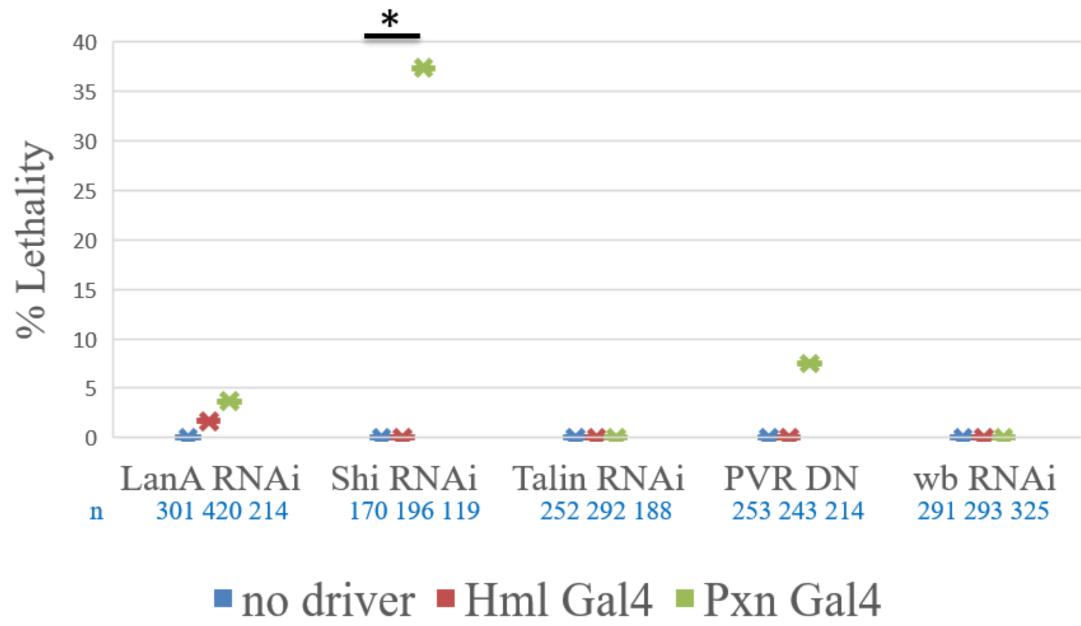
3.8 Expression of *shi RNAi* in hemocytes increases pupal lethality.

I have used various UAS lines to ablate hemocytes or impair their ability to secrete ECM proteins using 2 different hemocyte drivers, Hml Gal4 and Pxn Gal4 (Fig 3.9). In spite of important functions of hemocytes in the developing *Drosophila*, all flies of the ablation made it into adulthood. None of the larvae had gross defects at the DV (results not shown). However, there can be mild DV defects in these larvae, which can be investigated in the future. No tests were done to detect lethality rates during embryonic or larval stages, but only the pupal stages. Therefore I may have only assessed the escapers and missed the *Drosophila* that died during embryogenesis or larval development. When Dynamin (*shi*), the protein required for vesicle production and microtubule bundling was depleted in hemocytes using Pxn Gal4, the lethality rates of pupa increased significantly compared to the control. This encouraged me to use the Pxn Gal4 driver in other experiments instead of the Hml Gal4 driver, because Pxn Gal4 was more effective. Another unrelated experiment that encouraged me to do so was using UAS fluorescent reporters with Hml Gal4 or Pxn Gal4, which showed that fluorescence in the hemocytes is much stronger in Pxn Gal4 larvae. However, it should be noted that the expression of Hml Gal4 might be more specific to hemocytes than Pxn Gal4.

The effect of Dynamin depletion in hemocytes was not investigated further in this project, however it is interesting to note that Dynamin production in hemocytes is critical to the organism during metamorphosis. High lethality rates may be reflective of the important function of Dynamin and endocytosis during pupation when hemocytes engulf the tissues that are being broken down for remodeling.

Figure 3.9: Pupae with hemocytes that are Dynamin deficient have high lethality rates.

Using hemocyte drivers to activate Laminin A, Wing blister (wb) and Talin RNAi or dominant negative PVR did not result in significantly increased rates of lethality during pupation. *Shi* (Dynamin) RNAi causes significantly (*) more lethality during pupation relative to the control (no driver) when activated with Pxn Gal4 driver. Sample sizes are on X-axis. Chi square test, $P<0.001$.


*
40
35
30
25
20
15
10
5
0
% Lethality
LanA RNAi
Shi RNAi
Talin RNAi
PVR DN
wb RNAi
n
301 420 214
170 196 119
252 292 188
253 243 214
291 293 325
no driver
Hml Gal4
Pxn Gal4

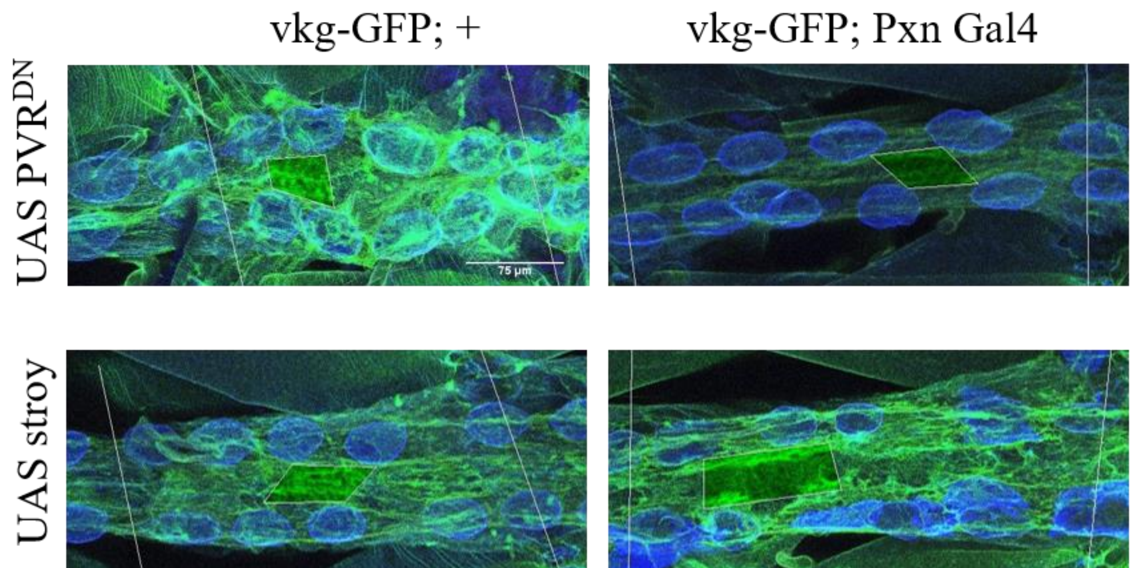
3.9 Impaired expression of PVR^{DN} in hemocytes decreases Vkg content of the L3 DV.

I sought to determine the contribution of hemocytes to ECM of the DV by performing a second GFP intensity measurement experiment. This time, instead of using the *UAS GFP RNAi* tool, I impaired hemocytes with constructs that cause hemocyte survival defects. This experiment not only tests if hemocytes produce Vkg for the DV, but also tests if hemocytes are responsible for carrying Vkg with a different cell origin to the DV. Using Vkg-GFP to detect the amount of Collagen IV at the DV, we observed the changes in GFP when the expression of *UAS* PVR^{DN} (dominant negative PVR) was regulated with *Pxn Gal4* (Fig 3.10). A t test showed that these larvae had significantly less GFP accumulation at the DV compared to the control. I also repeated the same experiment with *UAS stroy*, which is an apoptosis triggering gene. However, in this case the larvae with apoptotic hemocytes did not have less GFP than the control at the DV, in fact the control had less GFP (no statistical test was performed). The *stroy* experiment had a much smaller sample size than the other fluorescence intensity experiments, so this observation requires further validation. I observed that both *UAS stroy* and *UAS* PVR^{DN} have fewer hemocytes than wild type when *Pxn Gal4* driver is used (results not shown and not quantified), but not all of the hemocytes are killed.

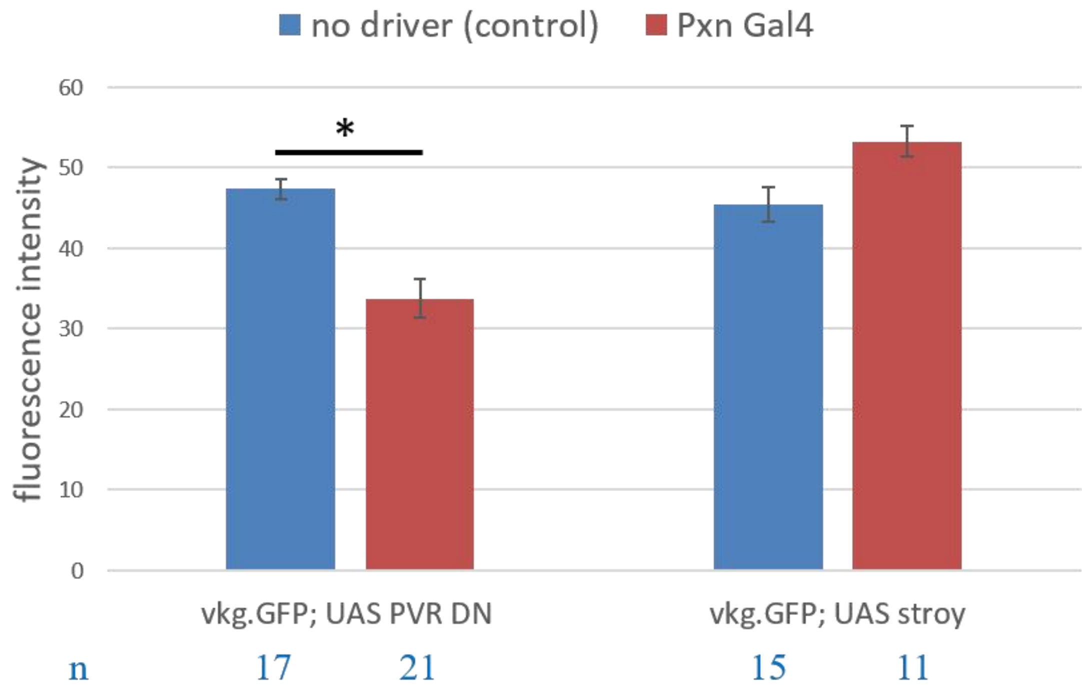
Figure 3.10: Hemocytes contribute to Collagen IV deposition at the DV.

Fluorescence detection of vkg-GFP in 3rd instars that express *UAS stroy* or UAS PVR^{DN} using the hemocyte driver Pxn Gal4. Control larvae have no Gal4 driver. (A) Representative images showing the boxed areas that were used to measure GFP intensity in each heart. The boxed areas were always chosen between 2 pairs of contralateral pericardial cells that are located on part of the DV on segment A5. White lines: Segmental boundaries. Scale bar: 75 µm. (B) Quantification of Vkg-GFP intensity of the boxed areas represented in (A). Expressing dominant negative PVR in hemocytes significantly (*) reduces Vkg-GFP intensity of the DV compared to the control. Expressing apoptosis activator (*stroy*) in hemocytes did not decrease the Vkg-GFP intensity of the DV compared to the control. Blue: βPs integrin antibody. Error bars: SEM. Sample sizes are written in X axis. Two-tailed t-test, $P<0.001$. Genotypes: UAS stroy/+ (or Y); *HmldsRed, vkg-GFP/+; Pxn Gal4/+*. *HmldsRed, vkg-GFP/+; Pxn Gal4/UAS* PVR^{DN}. Controls: *UAS stroy/+* (or Y); *HmldsRed, vkg-GFP/+*. *HmldsRed, vkg-GFP/+; UAS* PVR^{DN}*/+*.

A



B



Chapter 4.0: Discussion & Future Directions

In this thesis I sought to elucidate two main questions: 1) Do hemocytes cluster at the DV and respond to immune stimulation? 2) Do either fat body cells or hemocytes secrete Collagen IV for the DV up until 3rd instar stage? First part of the thesis focused on characterizing the behavior of DV associated hemocytes. Now that we are familiar with where most DV hemocytes are found, we can do more experiments to test if the hemocyte clusters of the DV are hematopoietic sites. This research can open new avenues to understanding hemocyte migration, cell signaling, cell-cell adhesion, cell-ECM adhesion and new functions for pericardial cells or hemocytes. The second part addressed the quantity of Collagen IV, a major ECM protein at the DV, in order to identify the cell type that produces a component of ECM found at the DV. ECM networks are intricate and dynamic, and they change and develop as the tissue that they surround grows in size. Identifying the source of such an elaborate structural component can elucidate how macromolecules are transported, assembled and regulated in a synchronized manner according to developmental stage and size of the organism.

Vertebrate hematopoietic sites are systems where blood cells can self-renew or proliferate in tissue specific microenvironments or in the bone marrow (Gold & Bruckner 2014). ECM proteins such as Laminin A (Gu et al. 2003; Siler et al. 2000), Collagens including Collagen IV and Fibronectin (Nilsson et al. 1998) are found in the vertebrate bone marrow (Ghosh et al. 2015). Drosophila hematopoietic sites such as adult hematopoietic hubs, larval HPs and the lymph gland provides a powerful genetic model to characterize hematopoiesis (Ghosh et al. 2015; Márkus et al. 2009; Makhijani et al. 2011; Leitã O & Sucena 2015; Bretscher et al. 2015). DV hemocyte

clusters may well be a newly discovered hematopoietic site. Even though each hematopoietic site has its unique quality there are some common themes in hematopoiesis: Hemocytes can increase in number in 2 ways at hematopoietic sites: By hemocyte progenitors differentiating into mature hemocytes (Ghosh et al. 2015) or proliferation of mature hemocytes themselves (Makhijani et al. 2011). Additionaly, transdifferentiation of mature hemocytes such as plasmatocytes transdifferentiating into crystal cells (Leitã O & Sucena 2015) is a cell activity that can be observed in hematopoietic sites. Hematopoietic sites receive signals from their environment (Makhijani et al. 2011) and hemocytes interact with the ECM proteins (Ghosh et al. 2015; Comber et al. 2013; Urbano et al. 2009; Sampson & Williams 2012). Lastly, these sites have the ability to respond to immune challenges (Márkus et al. 2009; Ghosh et al. 2015). We can further explore DV hemocyte clusters and their possible function in hematopoiesis by designing experiments to address these points.

4.1 Dorsal vessel associated hemocytes are specific to 3rd instar larvae

So far we have only detected, categorized and quantified DV associated hemocytes of the 3rd instar larvae. Even if there are a few individual hemocytes in close proximity of the DV in 1st and 2nd instars, no clusters are observed. However, 1st and 2nd instar DVs are not readily dissected, so I can not examine the DVs as closely as I did in 3rd instars. In adults, no hemocyte clusters similar to the ones I observed in 3rd instar DVs were detected. In summary, hemocytes specifically associate with 3rd instar larvae, suggesting that accumulation of hemocytes at this stage might have a crucial role for the pre-metamorphosis larvae.

4.2 DV associated hemocytes of 3rd instars may be capable of proliferation and transdifferentiation like a hematopoietic hub.

Even though no cell tracking experiments were done to find which cell group DV hemocytes originate from, transverse cross section images taken a few micrometers apart suggests that HPs can extend ventrally and colonize the DV. Perhaps the hemocyte clusters of the DV share similar hematopoietic functions with HPs such as capacity for proliferation or transdifferentiation. Anti-phospho histone H3 mitosis marker or BRDU can be used to detect proliferation. It is possible that DV plasmatocytes are capable of proliferation just like the HP plasmatocytes of 3rd instars (Makhijani et al. 2011) and hematopoietic hub plasmatocytes of immune challenged adults (Ghosh et al. 2015). The function of DV associated hemocytes as a proliferative niche would explain why they would appear during 3rd instar development and not during other stages. Because 3rd instar is the stage before metamorphosis, it would make sense to observe more hematopoietic sites where hemocyte populations can expand. Following the expansion, hemocytes can be released into circulation in pupa and play their important role in engulfing apoptotic cells (Lanot et al. 2001).

Proliferation experiments can not detect the crystal cell production that happens through differentiation, because the progenitor cell is not dividing. For example, In 3rd instar HPs, plasmatocytes differentiate into crystal cells (Leitã O & Sucena 2015), whereas in adults, hemocyte progenitors differentiate into crystal cells or plasmatocytes (Ghosh et al. 2015). The latter is similar to how all 3 kinds of hemocytes are produced from prohemocytes in the lymph gland (Jung et al. 2005), which is sensible because the hemocyte progenitors of adults descend from the lymph gland (Ghosh et al. 2015). However under normal conditions, in 3rd instars, the lymph

gland hemocytes are still separated from the rest of the sessile hemocytes (Makhijani & Brückner 2012), so the progenitor hemocytes that are available to adults is not available in 3rd instar larvae. Therefore, it is likely that the crystal cells at the DV hemocyte clusters originate from transdifferentiated plasmatocytes and not from progenitors. Sustained activation of Notch (Duvic et al. 2002), followed by the expression of *lozenge (lz)* gives rise to crystal cells (Lebestky et al. 2000; Leitã O & Sucena 2015). By tracing the activity of Notch transcription factors such as *suppressor of hairless*, we can detect the cells that give rise to crystal cells (Ghosh et al. 2015; Duvic et al. 2002). Observing Lz together with Hml is also useful, because plasmatocytes that are in transition to becoming mature crystal cells are both Hml^+ and Lz^+ (Leitã O & Sucena 2015). Switching to a nuclear HmldsRed marker, which should appear more punctate than the one we are using currently or finding ways to overcome laser power limitations can help us detect the transitioning cells in the future.

4.3 DV associated hemocytes of 3rd instars may be capable of an immune response like a hematopoietic hub.

Detecting lamellocyte production can be a way to assess if DV hemocytes are capable of an immune response. Fig. 3.6 suggests that DV hemocyte clusters possibly contain lamellocytes. There is not much data on the relationship between the adult hematopoietic hubs and lamellocytes, but HPs of the larvae are known to contain and produce lamellocytes, especially following an immune challenge caused by a parasitic wasp or mechanical injury (Márkus et al. 2009). Very few lamellocytes can be detected under normal conditions in late 3rd instars (Márkus et al. 2009). We have observed unidentified cells in the DV clusters in a larva grown on plates that are

treated with antibiotic. Confirming that these cells are lamellocytes would be unexpected, because the antibiotic plates should be free of microbial infection. However, it is possible that this larva had an injury while being transferred from plate to plate, which has triggered an injury response and caused lamellocytes to be produced. There is evidence to suggest that HP hemocytes are capable of transdifferentiating into lamellocytes upon an immune challenge (Márkus et al. 2009), so it would be interesting to see if DV associated hemocytes respond similarly. Lamellocytes can be labeled with the L1 antibody (Kurucz, Váczi, et al. 2007).

Adult hematopoietic hub hemocytes proliferate and are released into circulation upon an immune challenge (Ghosh et al. 2015). In this project, we saw that immune challenged larvae had more hemocytes at the DV. This may be an indicator for the presence of proliferating hemocytes. The microbial agent used in this project should be identified using sequencing. We can confirm the results we obtained with the microbial agent by using other immune challenges such as parasitic wasps or injury induction. I have three postulations of what could happen to DV hemocyte clusters upon encountering any of these immune challenges. The first is that DV hemocytes would not be affected by an immune reaction, based on what Babcock et al. 2008 had observed when introducing a wound and tracing the nearby tissue-bound hemocytes. These hemocytes were unresponsive to injury and remained sessile and tissue bound. It is unlikely that this observation would apply to larval HPs, because Márkus et al. 2009 observed that the HP banding pattern is weakened following the introduction of a parasitoid wasp. Therefore the 2nd postulate is that DV hemocytes woud decrease in number due to being released into circulation. The 3rd postulate is that DV hemocytes woud increase in number because they proliferate faster than they

get realeased into the circulation. So far, the last option is consistent with our data. In previous studies, it was observed that following a disturbance to the HP bands, the number of hemocytes at the DV increased, while the HP bands re-formed in time (Makhijani et al. 2011). It was suggested that hemocytes accumulate at the DV as a result of a circulation build up (Babcock et al. 2008). Our data can be partially explained by this prediction, meaning the larvae exposed to microbial infection have more DV associated hemocytes because there are more hemocytes in circulation compared to the control. However, another aspect of our data contradicts this prediction. It was previously observed that there are more hemocytes found in circulation as 3rd instar development progresses (Lanot et al. 2001). According to the hemocyte build-up postulate, our data should show that the most amount of hemocytes found at the DV should be immediately before pupation. In contrast, our data shows a decrease in DV associated hemocytes right before pupation both in immune challanged and unchallanged larvae. This suggests that the circulation build-up is not the sole reason for DV associated hemocytes to form and these hemocytes may detach from the DV immediately before pupation in order to prepare for metamorphosis.

4.4 DV associated hemocytes are found close to the heart tube, pericardial cells and ECM networks.

Characterizing the location where hemocytes associate with the DV was one of the aims of this project, because studying the environment that surrounds a potential hematopoietic site can reveal how blood cells interact with dynamic structures such as ECM proteins or other cells such as pericardial cells. The DV is not an organ that is known to be involved in hematopoiesis, but just like vertebrates that have

hematopoietic microenvironments in various tissues, *Drosophila* might have an easily accessible and visible hematopoietic microenvironment at its DV.

Various hematopoietic sites of *Drosophila* share similarities and differences in their location. DV hemocytes are either found inside the ECM pockets that surround pericardial cells or at the posterior-most tip of the DV, covered by the extensions of DV Collagen IV network. Adult hematopoietic hubs share more similarities with HPs of the 3rd instars than the DV clusters in terms of their location, because they repeat segmentally and are clustered closer to the dorsal side of the DV (Ghosh et al. 2015). Adult hematopoietic niches also differ from both the HP and DV hemocytes of 3rd instars because the hemocyte clusters sit in a network of ECM, where hemocytes are interspersed among the ECM proteins (Fig. 1.5C; Ghosh et al. 2015). However in 3rd instars the hemocytes are found in clusters contacting each other and not distributed across an ECM network (this thesis). When I immunolabelled for ECM proteins and also observed the Vkg-GFP distribution both in HPs and DV associated hemocytes, I did not see an abundance of ECM proteins distributed between the hemocytes. However, the hemocyte clumps are closely associated with the basement membrane that ensheath them, which suggests that these cells could transmit signals to and from the ECM. Moreover, it is possible that DV associated hemocytes are in such close proximity of each other that they form cellular interdigitations, structures that were observed in previous studies when 2 hemocytes are in close contact (Lanot et al. 2001).

Because hemocytes are found in the hemolymph that flows into the DV through ostia, it would be sensible to observe few hemocytes that are located in these areas or in the heart lumen. Our data shows that hemocytes are also found in locations

that are not immediately on the path of ostia flow. The DV hemocytes somehow migrate under the basement membrane and Pericardin networks, and form continuous clusters adjacent to pericardial cells and the heart tube. Hemocytes are surrounded by a basement membrane (labeled with Vkg-GFP and Laminin antibody) and a network of Pericardin.

The nephrocyte function of pericardial cells suggests they filter the hemolymph that passes through the basement membrane. However, the hemocyte clumps are adjacent to pericardial cells, possibly reducing the contact between nephrocyte diaphragms (Weavers et al. 2009) and the hemolymph. If there is an advantage to pericardial cells being surrounded by hemocytes, it may involve cross talk between the two cell types. Because pericardial cells have secretory functions (Chartier et al. 2002; Crossley 1972), it is possible that it could be advantageous for hemocytes to be in close proximity of pericardial cells if hemocytes are capable of picking up pericardial cell secretions and utilizing these for unknown purposes.

In some larvae, hemocytes were observed inside the heart lumen. It is unlikely that hemocytes accumulate inside the heart lumen to become sessile in the heart tube, because such clumps in the DV might obstruct the hemolymph flow. Another reason for hemocytes to be found in the heart lumen could be that they migrate into the ECM pockets where pericardial cells reside by passing through the heart tube. This is unlikely even if single but not clustered hemocytes pass through the heart tube. Such a migration route would require the hemocytes to not only pass through the basement membrane of the heart, but also to pass in between cardiomyocytes that are connected to each other. Furthermore, because these luminal hemocyte clumps are found at the

valves, it is likely that they are formed by individual hemocytes accumulating at the valves as a result of the heart tube collapsing and losing its original shape.

4.5 DV associated hemocytes may adhere to the heart tube, pericardial cells and ECM networks.

I have observed that there are not a lot of ECM proteins between the hemocytes and pericardial cells, which suggests that hemocytes are in close contact with the pericardial cells. Perhaps pericardial cells attach to their environments including the hemocytes through integrin or SJ proteins. The importance of these two kinds of proteins in proper cell localization of pericardial cells was characterized in previous studies (Stark et al. 1997; Yi et al. 2008). The barely detectable Pericardin immunolabel between hemocytes and the pericardial cells supports the model that Yi et al. 2008 suggested (Fig. 1.4B). In their model, pericardial cells were connected to cardioblasts through SJs and ECM molecules such as Pericardin. The same model could apply to hemocytes and pericardial cells.

It is also probable that integrin is important for DV associated hemocytes to attach to their surroundings during 3rd instar stage, since it has this function at embryonic stages (Comber et al. 2013) and the integrin expression can be detected in 3rd instar larval hemocytes with βPS integrin antibody. It would be interesting to study some of the gain of function mutants discussed in Stofanko et al. 2008 that have unusual amounts of hemocytes accumulating at the DV, especially the ones regarding integrin. One of the mutants is associated with high levels of βPS integrin, whereas another is the overexpression of αPS3, yet they both have unusual amounts of DV associated hemocytes. This suggests that integrin has an effect on the number of

hemocytes found at the DV in 3rd instar larvae. Another transmembrane receptor that may play a role in adhesion of DV associated hemocytes to their surroundings is Eater. Because this receptor is crucial for HPs to form (Bretscher et al. 2015), it may have the same function in DV associated clusters.

4.6 DV associated hemocytes may receive signals from pericardial cells that attract them to the DV.

Because DV hemocytes are found so close to pericardial cells, they might be receiving signals from the pericardial cells that guide them towards the heart. PVR/PVF pathway is one of the candidate signals. We can test this idea by driving *UAS PVF RNAi* with a pericardial cell specific promoter (Dot Gal4), then quantifying the amount of hemocytes found at the DV. This experiment can be conducted in the future using a transgenic line that I have already created. Additionally, we can test the same idea by using the mutant of a nephrocyte gene *dKlf15,* which is required for pericardial cell fate (Ivy et al. 2015). We can observe if hemocytes still accumulate at the DV even then pericardial cells are not there.

4.7 Disruption of the Pericardin network affects the hemocyte accumulation at the DV

Pericardin mutants can be utilized to learn more about the homing behavior of DV hemocytes. For example, *prc/TM6* larvae might have more hemocytes at the DV because the ECM pockets are still there, but there is more space for hemocytes to fill up between the pericardial cells and the heart tube. Moreover a specific locus on the

TM6 balancer might have secondary effects on the phenotype, raising more opportunities to study new ECM genes. For example mutations of *twdlA* (also called *tubby*), are used as a dominant marker on TM6 (Guan et al. 2006). *twdlA* is from a gene family called Tweedle that affects the cuticle and changes the body shape (Guan et al. 2006). It is expressed on the dorsal epidermis of late stage embryos (Guan et al. 2006). Because the expression pattern is so close to the DV, it may interact with the DV ECM, be a part of ECM itself or simply affect the shape of the DV by changing the shape of the organism altogether. If homozygous *prc* or *twdlA* mutants have more space between the pericardial cells and the heart tube or have more hemocytes than wild type for other reasons, we can quantify this difference.

Prc homozygous mutants do not have DV associated hemocytes, perhaps a result of the diminished Pericardin network. HPs still exist in homozygous *prc* mutants, which shows that the mutation did not prevent hemocytes from forming clusters and taking part in hematopoietic sites. We do not know if the basement membrane is disturbed in any way in homozygous *prc* mutants, but previous research shows that basement thickness is not affected in late-embryonic *prc* mutants (Drechsler et al. 2013). In one homozygous *prc* mutant hemocytes were found on the abluminal side of the pericardial cells whereas in wild type, the hemocytes are usually found on the luminal side. Pericardial cells usually neighbor the basement membrane on their abluminal side. Therefore, it may be that the basement membrane is disrupted in *prc/prc* larvae, allowing the hemocytes to locate to ectopic positions. Moreover, the pericardial cells are so detached from the heat tube in homozygous mutants that it would be very surprising to find that the structural integrity of the basement membrane is not affected by this change.

4.8 Fat body and hemocytes are required for proper Collagen IV deposition at the DV.

The fluorescence intensity measurements subsequent to RNAi knockdown show that hemocytes and fat body produce DV Vkg. Ubiquitous depletion of Vkg-GFP almost completely abolished the GFP found at the DV, however we did not get the full effect when we depleted Vkg-GFP secreted from the fat body or hemocytes. This shows that dsRNAi levels did not deplete all vkg-GFP mRNA, or their combined effect is required to completely abolish Vkg-GFP at the DV. It could also be that that there is another cell expressing Vkg. The data suggests that the fat body produces most of the Vkg. We can study the possible stage specific expression of Vkg from these tissues by using the Gal80^{ts}-UAS system. Gal80^{ts} is a temperature sensitive inhibitor of Gal4, which we can use to control the activity of Gal4 in a temperature sensitive manner. We can also test the R4 Gal4 and Pxn Gal4 drivers together to see if there is an additive effect. Another experiment we can do to confirm our results is to use Hml Gal4 or embryonic drivers of hemocytes to see if we get the same fluorescence reduction as we did when we used Pxn Gal4 with UAS GFP RNAi, because these drivers might be more specific to hemocytes than Pxn Gal4.

The decrease in the fluorescence intensity of Vkg-GFP at the DV in larvae with PVR^{DN} expressing hemocytes suggest that when hemocytes have migration and survival defects, there is less Vkg at the DV. This could be because hemocytes secrete Vkg for the DV themselves or because they pick up the Vkg secreted by the fat body to deliver it to the DV. However, considering the fluorescence intensity experiment discussed above, it is possible that hemocytes are responsible for secreting some of the Vkg found at the DV. I did not observe the same decrease in DV Vkg-GFP

fluorescence when apoptosis was triggered in hemocytes using *UAS stroy*. This experiment should be repeated with a bigger sample size. Also we can grow the larvae at higher temperatures than the room temperature in order to better activate Gal4 and possibly detect small differences between the control and treatments.

For all the fluorescence intensity experiments, I only used controls for UAS lines, and detecting their fluorescence without a driver. In the future, we can also control for all the drivers that were used. Another future experiment would be to test if fat body and hemocytes produce laminin for the DV by designing similar experiments to the ones discussed above.

4.9 Future directions

This thesis characterized a new location where hemocytes are found, demonstrated that fat body cells contribute to DV Collagen IV composition the most and identified new analysis techniques that can be used in future hemocyte work. However, it is still not known which tissues produce other ECM proteins such as Laminin. Moreover, it is still to be explored whether DV associated hemocytes are a part of a hematopoietic site that is capable of proliferation and immune reaction. It is yet to be confirmed whether HP hemocytes migrate ventrally and colonize the heart. If there is a hematopoietic site at the DV, then does it produce lamellocytes and crystal cells? Answers to these questions can elucidate how proliferating niches form and how basement membrane, a structure that is universally important to all animals relate to this structure.

References

Alayari, N.N. et al., 2009. Fluorescent labeling of Drosophila heart structures. *Journal of visualized experiments : JoVE*, (32), pp.1–5.

Ashburner, M., 1989. Drosophila. In *SpringerReference*. Berlin/Heidelberg: Springer-Verlag, p. 174. Available at: http://www.springerreference.com/index/doi/10.1007/SpringerReference_95313.

Babcock, D.T. et al., 2008. Circulating blood cells function as a surveillance system for damaged tissue in Drosophila larvae. *Proceedings of the National Academy of Sciences of the United States of America*, 105(29), pp.10017–22. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2474562&tool=pmcentrez&rendertype=abstract.

Badylak, S.F., Freytes, D.O. & Gilbert, T.W., 2009. Extracellular matrix as a biological scaffold material: Structure and function. *Acta Biomaterialia*, 5(1), pp.1–13.

Bogatan, S. et al., 2015. Talin Is required continuously for cardiomyocyte remodeling during heart growth in Drosophila. *PLoS ONE*, 10(6).

Brandt, R. & Paululat, A., 2013. Microcompartments in the Drosophila heart and the mammalian brain: general features and common principles. *Biological chemistry*, 394(2), pp.217–30. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23314534 [Accessed July 27, 2014].

Bretscher, A.J. et al., 2015. The Nimrod transmembrane receptor Eater is required for hemocyte attachment to the sessile compartment in Drosophila melanogaster. *Biology open*, 4(3), pp.355–63. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4359741&tool=pmcentrez&rendertype=abstract.

Brückner, K. et al., 2004. The PDGF/VEGF receptor controls blood cell survival in Drosophila. *Developmental cell*, 7(1), pp.73–84. Available at: http://www.cell.com/article/S1534580704002072/fulltext [Accessed March 29, 2016].

Bunt, S. et al., 2010. Hemocyte-secreted type IV collagen enhances BMP signaling to guide renal tubule morphogenesis in Drosophila. *Developmental Cell*, 19(2), pp.296–306. Available at: http://dx.doi.org/10.1016/j.devcel.2010.07.019.

Chartier, A. et al., 2002. Pericardin, a Drosophila type IV collagen-like protein is involved in the morphogenesis and maintenance of the heart epithelium during dorsal ectoderm closure. *Development (Cambridge, England)*, 129(13), pp.3241–53. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12070098.

Cho, N.K. et al., 2002. Developmental control of blood cell migration by the Drosophila VEGF pathway. *Cell*, 108(6), pp.865–76. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11955438.

Clark, R.I. et al., 2011. Multiple TGF-B superfamily signals modulate the adult drosophila immune response. *Current Biology*, 21(19), pp.1672–1677.

Clauss, M. et al., 1990. Vascular permeability factor: a tumor-derived polypeptide that

induces endothelial cell and monocyte procoagulant activity, and promotes monocyte migration. *The Journal of experimental medicine*, 172(6), pp.1535–45. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2188755&tool=pmcentrez&rendertype=abstract.

Colognato, H. & Yurchenco, P.D., 2000. Form and Function : The Laminin Family of Heterotrimers. *Developmental dynamics*, 234(January), pp.213–234.

Comber, K. et al., 2013. A dual role for the βPS integrin myospheroid in mediating Drosophila embryonic macrophage migration. *Journal of cell science*, 126(Pt 15), pp.3475–84. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3730248&tool=pmcentrez&rendertype=abstract.

Cripps, R.M. & Olson, E.N., 2002. Control of cardiac development by an evolutionarily conserved transcriptional network. *Developmental biology*, 246(1), pp.14–28.

Crossley, A., 1985. *Comprehensive insect physiology, biochemistry and pharmacology* G. Kerkut & L. Gilbert, eds., Oxford: Pergamon Press.

Crossley, A.C., 1972. The ultrastructure and function of pericardial cells and other nephrocytes in an insect: Calliphora erythrocephala. *Tissue and Cell*, 4(3), pp.529–560. Available at: http://dx.doi.org/10.1016/S0040-8166(72)80029-6.

Damke, H., 1996. Dynamin and receptor-mediated endocytosis. In *FEBS Letters*. pp. 48–51.

Das, D. et al., 2008. Gene expression analysis in post-embryonic pericardial cells of Drosophila. *Gene Expression Patterns*, 8(3), pp.199–205.

Davies, L.C. et al., 2013. Tissue-resident macrophages. *Nature immunology*, 14(10), pp.986–95. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4045180&tool=pmcentrez&rendertype=abstract.

Denholm, B. & Skaer, H., 2009. Bringing together components of the fly renal system. *Current opinion in genetics & development*, 19(5), pp.526–32. Available at: http://www.sciencedirect.com/science/article/pii/S0959437X09001403 [Accessed March 8, 2016].

Drechsler, M. et al., 2013. The conserved ADAMTS-like protein lonely heart mediates matrix formation and cardiac tissue integrity. *PLoS genetics*, 9(7), p.e1003616. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3708815&tool=pmcentrez&rendertype=abstract [Accessed August 8, 2014].

Duvic, B. et al., 2002. Notch Signaling Controls Lineage Specification during Drosophila Larval Hematopoiesis. *Current Biology*, 12(22), pp.1923–1927. Available at: http://www.cell.com/article/S0960982202012976/fulltext [Accessed April 16, 2016].

Evans, C.J., Hartenstein, V. & Banerjee, U., 2003. Thicker than blood: Conserved mechanisms in Drosophila and vertebrate hematopoiesis. *Developmental Cell*,

5(5), pp.673–690. Available at: http://linkinghub.elsevier.com/retrieve/pii/S1534580703003356.

Ferrara, N., Gerber, H.-P. & LeCouter, J., 2003. The biology of VEGF and its receptors. *Nature medicine*, 9(6), pp.669–76. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12778165.

Fossett, N. et al., 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. *Proceedings of the National Academy of Sciences of the United States of America*, 98(13), pp.7342–7.

Frasch, M., 1999. Intersecting signalling and transcriptional pathways in Drosophila heart specification. *Seminars in cell & developmental biology*, 10(1), pp.61–71. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10355030.

Furuse, M. & Tsukita, S., 2006. Claudins in occluding junctions of humans and flies. *Trends in Cell Biology*, 16(4), pp.181–188.

Ghosh, S. et al., 2015. Active hematopoietic hubs in Drosophila adults generate hemocytes and contribute to immune response. *Developmental cell*, 33(4), pp.478–88. Available at: http://www.sciencedirect.com/science/article/pii/S1534580715001823 [Accessed June 4, 2015].

Gold, K.S. & Bruckner, K., 2014. Drosophila as a model for the two myeloid blood cell systems in vertebrates. *Experimental Hematology*, 42(8), pp.717–727.

Gotwals, P.J. et al., 1994. Drosophila integrins and their ligands. *Current opinion in cell biology*, 6(5), pp.734–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7833052.

Gu, Y.C. et al., 2003. Laminin isoform-specific promotion of adhesion and migration of human bone marrow progenitor cells. *Blood*, 101(3), pp.877–885. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12393739.

Guan, X. et al., 2006. Mutation of TweedleD, a member of an unconventional cuticle protein family, alters body shape in Drosophila. *Proceedings of the National Academy of Sciences of the United States of America*, 103(45), pp.16794–9. Available at: http://www.pnas.org/cgi/content/long/103/45/16794 [Accessed April 16, 2016].

Gutierrez, E. et al., 2007. Specialized hepatocyte-like cells regulate Drosophila lipid metabolism. *Nature*, 445(7125), pp.275–280.

Haag, T. a et al., 1999. The role of cell adhesion molecules in Drosophila heart morphogenesis: faint sausage, shotgun/DE-cadherin, and laminin A are required for discrete stages in heart development. *Developmental biology*, 208(1), pp.56–69. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10075841.

Hammonds, A.A.S. et al., 2013. Spatial expression of transcription factors in Drosophila embryonic organ development. *Genome biology*, 14(12), p.R140.

Hoffmann, J.A. et al., 1999. Phylogenetic perspectives in innate immunity. *Science*

(New York, N.Y.), 284(5418), pp.1313–1318.

Hollfelder, D., Frasch, M. & Reim, I., 2014. Distinct functions of the laminin β LN domain and collagen IV during cardiac extracellular matrix formation and stabilization of alary muscle attachments revealed by EMS mutagenesis in Drosophila. *BMC developmental biology*, 14(1), p.26. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4068974&tool=pmcentrez&rendertype=abstract [Accessed July 27, 2014].

Holz, A. et al., 2003. The two origins of hemocytes in Drosophila. *Development (Cambridge, England)*, 130(20), pp.4955–62. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12930778 [Accessed November 11, 2013].

Honti, V. et al., 2010. Cell lineage tracing reveals the plasticity of the hemocyte lineages and of the hematopoietic compartments in Drosophila melanogaster. *Molecular Immunology*, 47(11-12), pp.1997–2004.

Honti, V. et al., 2014. The cell-mediated immunity of Drosophila melanogaster: hemocyte lineages, immune compartments, microanatomy and regulation. *Developmental and comparative immunology*, 42(1), pp.47–56. Available at: http://www.sciencedirect.com/science/article/pii/S0145305X13001687 [Accessed March 28, 2016].

Hudson, B.G., Reeders, S. & Tryggvason, K., 1993. Type IV Collagen: Structure, Gene Organization, and Role in Human Diseases. *Journal of Biological Chemistry*, 238(35), pp.26003–036.

Hynes, R.O. & Zhao, Q., 2000. The evolution of cell adhesion. *Journal of Cell Biology*, 150(2).

Ivy, J.R. et al., 2015. Klf15 is critical for the development and differentiation of drosophila nephrocytes. *PLoS ONE*, 10(8), pp.1–17.

Jeltsch, M. et al., 1997. Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. *Science (New York, N.Y.)*, 276(5317), pp.1423–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9162011 [Accessed August 24, 2015].

Jung, S.-H. et al., 2005. The Drosophila lymph gland as a developmental model of hematopoiesis. *Development (Cambridge, England)*, 132(11), pp.2521–33. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15857916 [Accessed August 7, 2014].

Kalluri, R., 2003. Basement membranes: structure, assembly and role in tumour angiogenesis. *Nature reviews. Cancer*, 3(June), pp.422–433.

Knibiehler, B. et al., 1987. Haemocytes accumulate collagen transcripts during Drosophila melanogaster metamorphosis. *Roux's Archives of Developmental Biology*, 196(4), pp.243–247. Available at: http://link.springer.com/10.1007/BF00376348 [Accessed August 14, 2015].

Kocks, C. et al., 2005. Eater, a transmembrane protein mediating phagocytosis of bacterial pathogens in Drosophila. *Cell*, 123(2), pp.335–346.

Kowalevsky, A., 1889. Ein Beitrag zur Kenntnis der Excretions-organe. *Biol. Centralbl.*, ix, pp.74–79.

Krzemien, J. et al., 2010. Hematopoietic progenitors and hemocyte lineages in the Drosophila lymph gland. *Developmental Biology*, 346(2), pp.310–319.

Krzemień, J. et al., 2007. Control of blood cell homeostasis in Drosophila larvae by the posterior signalling centre. *Nature*, 446(7133), pp.325–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17361184 [Accessed August 7, 2014].

Kukk, E. et al., 1996. VEGF-C receptor binding and pattern of expression with VEGFR-3 suggests a role in lymphatic vascular development. *Development (Cambridge, England)*, 122(12), pp.3829–37. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9012504 [Accessed August 24, 2015].

Kurucz, E., Váczi, B., et al., 2007. Definition of Drosophila hemocyte subsets by cell-type specific antigens. *Acta biologica Hungarica*, 58 Suppl, pp.95–111.

Kurucz, E., Markus, R., et al., 2007. Nimrod, a Putative Phagocytosis Receptor with EGF Repeats in Drosophila Plasmatocytes. *Current Biology*, 17(7), pp.649–654.

Kusche-Gullberg, M. et al., 1992. Laminin A chain: expression during Drosophila development and genomic sequence. *The EMBO journal*, 11(12), pp.4519–27. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=557027&tool=pmcentrez&rendertype=abstract.

Lanot, R. et al., 2001. Postembryonic Hematopoiesis in Drosophila. *Developmental Biology*, 230(2), pp.243–257. Available at: http://www.sciencedirect.com/science/article/pii/S0012160600901234.

Lebestky, T., 2000. Specification of Drosophila Hematopoietic Lineage by Conserved Transcription Factors. *Science*, 288(5463), pp.146–149. Available at: http://www.sciencemag.org/cgi/doi/10.1126/science.288.5463.146 [Accessed August 6, 2014].

Lebestky, T. et al., 2000. Specification of Drosophila hematopoietic lineage by conserved transcription factors. *Science (New York, N.Y.)*, 288(5463), pp.146–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10753120 [Accessed August 24, 2015].

Lebestky, T., Jung, S.H. & Banerjee, U., 2003. A Serrate-expressing signaling center controls Drosophila hematopoiesis. *Genes and Development*, 17(3), pp.348–353.

Lehmacher, C., Abeln, B. & Paululat, A., 2012. The ultrastructure of Drosophila heart cells. *Arthropod structure & development*, 41(5), pp.459–74. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22426062 [Accessed July 10, 2014].

Leitã O, A.B. & Sucena, E., 2015. Drosophila sessile hemocyte clusters are true hematopoietic tissues that regulate larval blood cell differentiation. *Elife*, 4, p.e06166.

Li, S. et al., 2005. Laminin-sulfatide binding initiates basement membrane assembly and enables receptor signaling in Schwann cells and fibroblasts. *Journal of Cell Biology*, 169(1), pp.179–189.

Locke, M. & Russell, V., 1998. *Microscopic anatomy of invertebrates.* F. Harrison & M. Locke, eds., Wiley.

Makhijani, K. et al., 2011. The peripheral nervous system supports blood cell homing and survival in the Drosophila larva. *Development (Cambridge, England)*, 138(24), pp.5379–91. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3222213&tool=pmcentrez&rendertype=abstract [Accessed November 14, 2013].

Makhijani, K. & Brückner, K., 2012. Of blood cells and the nervous system: Hematopoiesis in the Drosophila larva. *Fly*, 6(4), pp.254–260. Available at: http://www.landesbioscience.com/journals/fly/2011FLY0091R.pdf [Accessed August 7, 2014].

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

Márkus, R. et al., 2009. Sessile hemocytes as a hematopoietic compartment in Drosophila melanogaster. *Proceedings of the National Academy of Sciences of the United States of America*, 106(12), pp.4805–4809.

Masur, S.K., Kim, Y.T. & Wu, C.F., 1990. Reversible inhibition of endocytosis in cultured neurons from the Drosophila temperature-sensitive mutant shibirets1. *Journal of neurogenetics*, 6(3), pp.191–206. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2113575.

McKee, K.K. et al., 2007. Role of laminin terminal globular domains in basement membrane assembly. *The Journal of biological chemistry*, 282(29), pp.21437–47. Available at: http://www.jbc.org/cgi/content/long/282/29/21437 [Accessed March 17, 2016].

Meister, M. & Lagueux, M., 2003. Drosophila blood cells. *Cellular Microbiology*, 5(9), pp.573–580.

Mills, R.P. & King, R.C., 1965. The pericardial cells of Drosophila melanogaster. *The Quarterly journal of microscopical science*, 106(3), pp.261–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/5865262.

Monier, B. et al., 2005. Steroid-dependent modification of Hox function drives myocyte reprogramming in the Drosophila heart. *Development (Cambridge, England)*, 132(23), pp.5283–5293.

Mukherjee, T. et al., 2011. Interaction between Notch and Hif-alpha in development and survival of Drosophila blood cells. *Science (New York, N.Y.)*, 332(6034), pp.1210–3. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21636775.

Munier, A. et al., 2002. hemocyte proliferation in Drosophila larvae. , 3(12), pp.1195–1200.

Natzle, J., Monson, J. & McCarthy, B., 1982. Cytogenic location and expression of collagen-like genes in Drosophila. *Nature*, 296, pp.368–371.

Nelson, K.S. & Beitel, G.J., 2009. Cell Junctions: Lessons from a Broken Heart. *Current Biology*, 19(3).

Nelson, R.E. et al., 1994. Peroxidasin: a novel enzyme-matrix protein of Drosophila development. *The EMBO journal*, 13(15), pp.3438–3447.

Nilsson, S.K. et al., 1998. Immunofluorescence characterization of key extracellular matrix proteins in murine bone marrow in situ. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society*, 46(3), pp.371–377.

Ocorr, K., Vogler, G. & Bodmer, R., 2014. Methods to assess Drosophila heart development, function and aging. *Methods (San Diego, Calif.)*, 68(1), pp.265–72. Available at: http://www.sciencedirect.com/science/article/pii/S1046202314001339 [Accessed July 10, 2014].

Olofsson, B. & Page, D.T., 2005. Condensation of the central nervous system in embryonic Drosophila is inhibited by blocking hemocyte migration or neural activity. *Developmental Biology*, 279(1), pp.233–243.

Pastor-Pareja, J.C. & Xu, T., 2011. Shaping cells and organs in Drosophila by opposing roles of fat body-secreted Collagen IV and perlecan. *Developmental cell*, 21(2), pp.245–56. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21839919 [Accessed July 9, 2014].

Reed, B.H., McMillan, S.C. & Chaudhary, R., 2009. The preparation of Drosophila embryos for live-imaging using the hanging drop protocol. *Journal of visualized experiments : JoVE*, (25), p.e1206.

Rehorn, K.P. et al., 1996. A molecular aspect of hematopoiesis and endoderm development common to vertebrates and Drosophila. *Development (Cambridge, England)*, 122(12), pp.4023–31. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9012522.

Rizki, T.M., 1978. The circulatory system and associated cells and tissues. In *The Genetics and Biology of Drosophila*. pp. 397–452. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7834751.

Rugendorff, A., Younossi-Hartenstein, A. & Hartenstein, V., 1994. Embryonic origin and differentiation of the drosophila heart. *Roux's Archives of Developmental Biology*, 203, pp.266–280.

Sampson, C.J. & Williams, M.J., 2012. Real-time analysis of Drosophila post-embryonic haemocyte behaviour. *PloS one*, 7(1), p.e28783. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3252279&tool=pmcentrez&rendertype=abstract [Accessed November 14, 2013].

Sellin, J. et al., 2006. Dynamics of heart differentiation, visualized utilizing heart enhancer elements of the Drosophila melanogaster bHLH transcription factor Hand. *Gene Expression Patterns*, 6(4), pp.360–375.

Shah, A.P. et al., 2011. Cardiac remodeling in Drosophila arises from changes in actin gene expression and from a contribution of lymph gland-like cells to the heart musculature. *Mechanisms of Development*, 128(3-4), pp.222–233.

Shibuya, M. et al., 1990. Nucleotide sequence and expression of a novel human receptor-type tyrosine kinase gene (flt) closely related to the fms family. *Oncogene*, 5(4), pp.519–524.

Shpetner, H.S. & Vallee, R.B., 1989. Identification of dynamin, a novel mechanochemical enzyme that mediates interactions between microtubules. *Cell*,

59(3), pp.421–432.

Siekhaus, D. et al., 2010. RhoL controls invasion and Rap1 localization during immune cell transmigration in Drosophila. *Nature cell biology*, 12(6), pp.605–610.

Sieweke, M.H. & Allen, J.E., 2013. Beyond stem cells: self-renewal of differentiated macrophages. *Science*, 342(6161), p.1242974. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24264994.

Siler, U. et al., 2000. Characterization and functional analysis of laminin isoforms in human bone marrow. *Blood*, 96(13), pp.4194–4203.

Sinenko, S.A. & Mathey-Prevot, B., 2004. Increased expression of Drosophila tetraspanin, Tsp68C, suppresses the abnormal proliferation of ytr-deficient and Ras/Raf-activated hemocytes. *Oncogene*, 23(56), pp.9120–9128.

Smyth, N. et al., 1998. The targeted deletion of the LAMC1 gene. *Annals of the New York Academy of Sciences*, 857, pp.283–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9917858.

Sorrentino, R.P., Gajewski, K.M. & Schulz, R.A., 2005. GATA factors in Drosophila heart and blood cell development. *Seminars in Cell and Developmental Biology*, 16(1), pp.107–116.

Stark, K. a et al., 1997. A novel alpha integrin subunit associates with betaPS and functions in tissue morphogenesis and movement during Drosophila development. *Development (Cambridge, England)*, 124(22), pp.4583–94. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9409675.

Stofanko, M., Kwon, S.Y. & Badenhorst, P., 2008. A misexpression screen to identify regulators of Drosophila larval hemocyte development. *Genetics*, 180(1), pp.253–67. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2535679&tool=pmcentrez&rendertype=abstract [Accessed August 9, 2014].

Stramer, B. et al., 2005. Live imaging of wound inflammation in Drosophila embryos reveals key roles for small GTPases during in vivo cell migration. *The Journal of cell biology*, 168(4), pp.567–73. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2171743&tool=pmcentrez&rendertype=abstract [Accessed August 24, 2015].

Sultan, R. et al., 2001. Drug resistance of bacteria commensal with Drosophila melanogaster in laboratory cultures. *Dros. Inf. Serv.*, 84, pp.175–180.

Tao, Y. & Schulz, R. a, 2007. Heart development in Drosophila. *Seminars in cell & developmental biology*, 18(1), pp.3–15. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17208472 [Accessed September 21, 2013].

Tepass, U. et al., 1994. Embryonic origin of hemocytes and their relationship to cell death in Drosophila. *Development (Cambridge, England)*, 120(7), pp.1829–37. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7924990.

Tepass, U. et al., 2001. Epithelial cell polarity and cell junctions in Drosophila. *Annual review of genetics*, 35(1), pp.747–784.

Tepass, U. & Hartenstein, V., 1994. The development of cellular junctions in the Drosophila embryo. *Developmental biology*, 161(2), pp.563–596.

Terman, B.I. et al., 1991. Identification of a new endothelial cell growth factor receptor tyrosine kinase. *Oncogene*, 6(9), pp.1677–1683.

Tögel, M., Pass, G. & Paululat, A., 2008. The Drosophila wing hearts originate from pericardial cells and are essential for wing maturation. *Developmental Biology*, 318(1), pp.29–37.

Tomancak, P. et al., 2007. Global analysis of patterns of gene expression during Drosophila embryogenesis. *Genome biology*, 8(7), p.R145.

Tomancak, P. et al., 2002. Systematic determination of patterns of gene expression during Drosophila embryogenesis. *Genome biology*, 3(12), p.RESEARCH0088.

Urbano, J.M. et al., 2009. Drosophila laminins act as key regulators of basement membrane assembly and morphogenesis. *Development*, 136(24), pp.4165–4176. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19906841\nhttp://dev.biologists.org/content/136/24/4165.full.pdf.

Vanderploeg, J. et al., 2012. Integrins are required for cardioblast polarisation in Drosophila. *BMC Developmental Biology*, 8(February).

Vogler, G. & Ocorr, K., 2009. Visualizing the beating heart in Drosophila. *Journal of visualized experiments : JoVE*, (31), pp.6–8. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3150055&tool=pmcentrez&rendertype=abstract.

Ward, E.J. & Skeath, J.B., 2000. Characterization of a novel subset of cardiac cells and their progenitors in the Drosophila embryo. *Development (Cambridge, England)*, 127(22), pp.4959–69. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11044409.

Wasbrough, E.R. et al., 2010. The Drosophila melanogaster sperm proteome-II (DmSP-II). *Journal of Proteomics*, 73(11), pp.2171–2185.

Weavers, H. et al., 2009. The insect nephrocyte is a podocyte-like cell with a filtration slit diaphragm. , 457(7227), pp.322–326.

Wodarz, A. et al., 1995. Expression of crumbs confers apical character on plasma membrane domains of ectodermal epithelia of drosophila. *Cell*, 82(1), pp.67–76.

Wood, W., Faria, C. & Jacinto, A., 2006. Distinct mechanisms regulate hemocyte chemotaxis during development and wound healing in Drosophila melanogaster. *Journal of Cell Biology*, 173(3), pp.405–416.

Wood, W. & Jacinto, A., 2007. Drosophila melanogaster embryonic haemocytes: masters of multitasking. *Nature reviews. Molecular cell biology*, 8(7), pp.542–51. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17565363 [Accessed July 11, 2014].

Yarnitzky, T. & Volk, T., 1996. Laminin is Required for Heart, Somatic Muscles, and Gut Development in the Drosophila Embryo. *Developmental Biology*, 169, pp.609–618.

Yasothornsrikul, S. et al., 1997. viking: identification and characterization of a second type IV collagen in Drosophila. *Gene*, 198(1-2), pp.17–25. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9370260 [Accessed August 24, 2015].

Yi, P. et al., 2008. Heterotrimeric G Proteins Regulate a Noncanonical Function of Septate Junction Proteins to Maintain Cardiac Integrity in Drosophila. *Developmental Cell*, 15(5), pp.704–713. Available at: http://dx.doi.org/10.1016/j.devcel.2008.10.001.

Yurchenco, P.D., 2011. Basement membranes: Cell scaffoldings and signaling platforms. *Cold Spring Harbor Perspectives in Biology*, 3(2), pp.1–27.

Zaffran, S., 2002. Early Signals in Cardiac Development. *Circulation Research*, 91(6), pp.457–469. Available at: http://circres.ahajournals.org/cgi/doi/10.1161/01.RES.0000034152.74523.A8 [Accessed September 23, 2013].

Zeitouni, B. et al., 2007. Signalling pathways involved in adult heart formation revealed by gene expression profiling in Drosophila. *PLoS genetics*, 3(10), pp.1907–21. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2014791&tool=pmcentrez&rendertype=abstract [Accessed June 5, 2014].

Zhang, F., Zhao, Y. & Han, Z., 2013. An in vivo functional analysis system for renal gene discovery in Drosophila pericardial nephrocytes. *Journal of the American Society of Nephrology : JASN*, 24(2), pp.191–7. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3559487&tool=pmcentrez&rendertype=abstract [Accessed August 8, 2014].