CCM OF CARDIOBLASTS DURING HEART MORPHOGENESIS

COLLECTIVE CELL MIRATION DURING HEART MORPHOGENESIS IN *DROSOPHILA* REQUIRES GUIDANCE SIGNALING AND EXTRACELLULAR MATRIX REMODELLING

BY

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ABSTRACT

Collective cell migration is a defining feature of many morphogenetic processes. Congenital heart diseases and cancer arise due to mis-regulation of collective migratory behaviour and animal models have played a pivotal role in dissecting the molecular mechanisms which underlie this process. During embryonic heart development, cardiac precursors undergo a period of collective migration in both vertebrates and invertebrates. We developed a paradigm to quantitatively assess collective cell migration of cardiac precursors in live embryos of *Drosophila,* which is the simplest genetic model organism with a heart. We studied processes which are commonly observed in most collective cell migration models such as guidance signalling and extracellular matrix remodelling. Our results demonstrate that the leading edge of migrating cardioblasts is highly active and that this behaviour is regulated by guidance cues, Slit and Netrin and their respective receptors Robo/Robo2 and Frazzled/Un-coordinated5. These molecules cooperatively promote leading edge motility and epithelial characteristics of the cardioblasts. Next, we determined that matrix restructuring around the cardioblasts requires proteases Mmp1 and Mmp2, which are members of the highly conserved Matrix Metalloproteinase family. We demonstrate that Mmp1 and Mmp2 have distinct roles during lumen formation, however, both Mmp1 and Mmp2 are required for collective motility of the cardioblast leading edge. Hence, we propose that embryonic heart development in *Drosophila* is an effective and amenable model of collective cell migration which can be applied to discover unique mechanisms which coordinate cell movement in groups.

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LIST OF ABBREVIATIONS

α	alpha- or anti-
Abl	Abelson tyrosine kinase
ADAM	A disintigrin and metalloproteinase
AS	Amnioserosa
BC	Border cells
BM	Basement membrane
BMP	Bone morphogenetic protein
CB	Cardioblast
CCM	Collective cell migration
CHD	Congenital heart disease
C-Slit	C terminal Slit
DC	Dorsal closure
DCC	Deleted in colorectal cancer
Dg	Dystroglycan
Dlg	Discs-large
Dscam	Down syndrome cell adhesion protein
DV	Dorsal vessel
E-Cad	E-Cadherin
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor

EMT	Epithelial-to-mesenchymal transition
Ena	Enabled
FGF	Fibroblast growth factor
FNIII	Fibronectin type 3
Fra	Frazzled
F-Slit	Full length Slit
GAG	Glycosaminoglycan
GFP	Green fluorescent protein
GPI	Glycosylphosphotidylinositol
HSPG	Heparin sulphate proteoglycan
Ig	Immunoglobulin
Kuz	Kuzbanian
Lan	Laminin
LE	Leading edge
Mef2	Myocyte enhancer factor 2
MET	Mesenchymal-to-Epithelial transition
Mmp	Matrix Metalloproteinase
Mp	Multiplexin
MT-Mmp	Membrane tagged-Matrix metalloproteinase
Net	Netrin
NGS	Normal goat serum
N-Slit	N terminal Slit

PBS	Phosphate buffer solution
PBT	Phosphate buffer solution with 0.1% Triton X
PI3K	Phosphotidylinositol 3 kinase
PIP_2	Phosphotidylinositol-(4,5)-biphosphate
PIP_3	Phosphotidylinositol-(3,4,5)-triphosphate
pLL	Posterior lateral line
Prc	Pericardin
Sdc	Syndecan
srGAP	Slit-Robo GTP-ase activating protein
Svp	Seven-up
Timp	Tissue inhibitor of metalloproteinases
TGF-β	Transforming growth factor – β
TNF	Tumour necrosis factor
Tup	Tail-up
Unc5	Uncoordinated-5
VASP	Vasodilator-stimulated phosphoprotein
VEGF	Vascular endothelial growth factor
Vkg	Viking
WASP	Wiskott-Aldrich Syndrome Protein
WAVE	WASP-family verprolin-homologous protein

Introduction

CHAPTER ONE

What comes to mind when you hear the word “migration”? Some would describe a flock of migrating birds. Others might describe the behaviour of salmon swimming upstream. I for instance, think about humans who migrate in search of a more suitable environment. It is an undeniable fact of life that all of the living things that exist rely profoundly on migration, not only on a macroscopic but on a microscopic scale as well. The initial life forms on our planet such as bacteria evolved the ability to use migration as a survival tool. By using flagella as motors and chemotaxis as their guide, a bacterium can steer itself towards a source of nutrition or away from a toxic environment. As metazoans evolved, the eukaryotic migratory mechanisms became more sophisticated and meticulous. Membrane protrusions became the primary way cells developed traction for movement. Cells learned to cooperate with one another and migrate in a collective manner towards a mutual destination by providing support to their neighbours. Remarkably, this primitive migratory behaviour is conserved amongst most migrating eukaryotic cells. Immune cells within an organism circulate throughout the body in search of pathogens. Migration of cells within the embryo gives rise to complex tissue and organ systems. Migration is even the primary reason for some of the most deadly diseases that affect humans, such as cancer.

Anybody who has travelled in his life can agree that even if a migration route is perfectly laid down, obstacles can appear unexpectedly. Maybe your travel agent will misspell your name on the ticket or perhaps the airline will misdirect your luggage. In either case, the travelling plans will be affected and this nuisance will cause delays.

Similarly, during migration cells must overcome barriers which are present on their path or otherwise the migration will be hindered.

But the questions still remains, how does a cell know where its final destination is? What mechanism does the cell employ to reach point "B" and how does the cell overcome barriers that are set in the way? Substantial research is being conducted on various *in vitro* and i*n vivo* models of cell migration to answer these questions. Intriguingly, most of the migratory machineries present in a single celled organism such as *Dictostyleum* exist in multicellular eukaryotic organisms as well (Friedl and Gilmour, 2009). And despite the difference in the types of cells, mechanisms of migration are evolutionarily conserved. To truly understand migratory behaviour, we have to evaluate the differences and similarities between various cell migration models and dissect the molecular mechanisms that govern these processes.

By employing an embryonic cell migration model, this thesis will offer some insights into how cells are able to regulate migration. Morphogenetic processes such as organ formation often depend upon cell migration. During embryogenesis, precursor cells must migrate to specific embryonic coordinates which will serve as their final destination. Not only do these cells will have to reach that point and form an organ, sometimes they will be required to complete the journey while adhered to other partner cells. This type of migration is termed Collective Cell Migration (CCM). Let's explore this mode of migration in more detail.

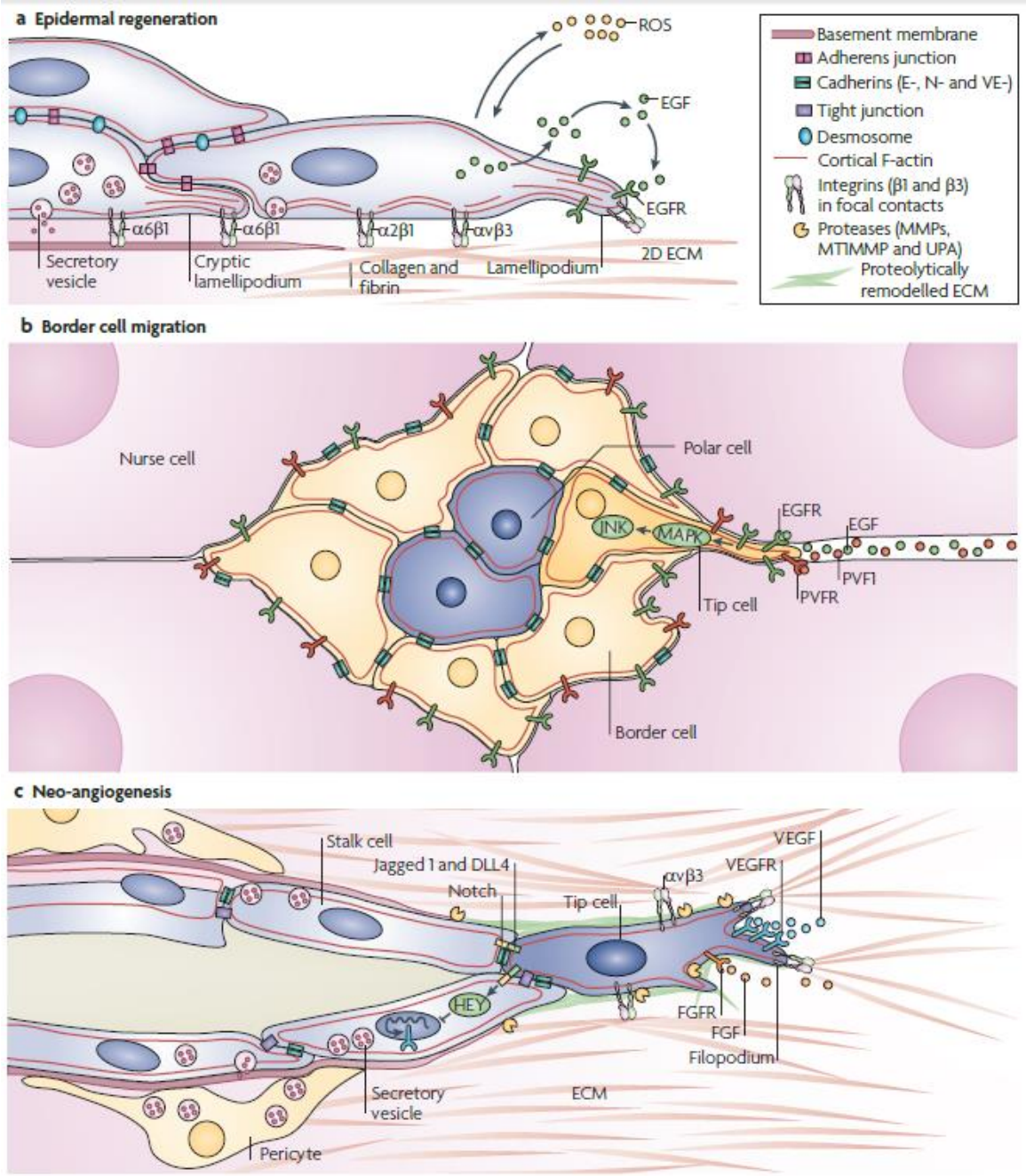
1.1 Collective Cell Migration

CCM is the coordinated movement of cohesive cell groups which share the direction of migration. CCM underlies many morphogenetic, regenerative and diseases related processes. Three main criteria define CCM (Friedl and Gilmour, 2009). First, cell groups must maintain adhesions with neighbouring cells such that the physical contact is maintained throughout the duration of migration. Second, polarization of the actin cytoskeleton dictates the formation of protrusions which generate traction and accelerative forces. Also, polarized targeting of adhesion molecules maintains cell-cell junctions. Lastly, in most cases, cells must create a migration path by structurally modifying tissue, by degrading or depositing Extracellular Matrix (ECM). Even though CCM has been extensively studied in *in vitro* models, such as the 2d scratch assay (Fig 1.1 A) (Vitorino and Meyer, 2008) and invasion assays involving 3D ECM scaffolds (Gaggioli et al., 2007; Wolf et al., 2007), few *in vivo* models exist which can help us uncover the morphogenetic regulation of this complex process (Fig 1.1). Below, I will describe well established *in vitro* and *in vivo* models of CCM which have only just began to augment our understanding of this elaborate process.

1.1.1 Border Cell Migration in *Drosophila*

During oogenesis, border cells (BC) from the anterior follicular epithelium recruit their neighbours and form a cluster of 8-10 cells which detaches from the remaining epithelia. Subsequently, this cluster collectively migrates towards the posteriorly located oocyte (Fig 1.1 B). As the cluster reaches the anterior side of the oocyte, it curves dorsally and arrives at it final destination. BCs cluster consist of 2 polar cells

Figure 1.1. Mechanisms of Collective Cell Migration. (A) Mechanism of cell migration during a 2d scratch assay. Leading edge (LE) cells extend protrusion towards the direction of migration and localize the guidance receptor, epidermal growth factor receptor (EGFR), which responds to local stimuli such as epidermal growth factor (EGF). Integrin forms adhesions with the underlying ECM. Adherens junctions present between the cell-cell interface maintain adhesion as the cells migrate collectively. (B) Molecular mechanism of BC Migration during *Drosophila* oogenesis. A cluster of cells composed of 2 polar cells, 5-7 BCs and 1 tip cell migrate collectively within the developing egg chamber. All BCs and tip cells localize guidance receptors, EGFR and PVFR which respond to gradient of chemokines. However, only tip cells respond to the stimuli by extending cytoplasmic protrusions. E-Cadherin (E-Cad) mediated junctions maintain cluster integrity. (C) Molecular mechanism of neo-angiogenesis. Angiogenesis initiates when vascular endothelial cells respond to fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) which specifies polarity and promotes differentiation of the tip cell. The tip cell has the ability to extend cytoplasmic protrusions in the form of filopodia and lamellopodia and guides CCM. Interaction with the ECM is mediated by Integrin which is expressed in the tip cell along with proteases which clear ECM obstructions. Adhesion between trailing cells is maintained by vascular endothelial Cadherins. Figure adopted from (Friedl and Gilmour, 2009) and used with permission from Nature Publishing Group.


a Epidermal regeneration
ROS
EGF
EGFR
α6β1
α6β1
α2β1
αvβ3
Secretory vesicle
Cryptic lamellipodium
Collagen and fibrin
Lamellipodium
2D ECM
Basement membrane
Adherens junction
Cadherins (E-, N- and VE-)
Tight junction
Desmosome
Cortical F-actin
Integrins (β1 and β3) in focal contacts
Proteases (MMPs, MTIMMP and UPA)
Proteolytically remodelled ECM
b Border cell migration
Nurse cell
Polar cell
JNK
MAPK
EGFR
EGF
PVF1
PVFR
Tip cell
Border cell
c Neo-angiogenesis
Stalk cell
Jagged 1 and DLL4
Notch
Tip cell
αvβ3
VEGF
VEGFR
HEY
FGFR
FGF
Filopodium
Secretory vesicle
ECM
Pericyte

which localize to the inner part and are surrounded by migratory BCs. The cluster often rearranges as it migrates however the inner polar cells always maintain central localization. Additionally, the outward oriented cells take turn as leaders guide the cluster towards the anterior dorsal part of the egg chamber from the posterior side. Once at their final destination, BCs secrete signals which sculpt the micropyle which is essential for sperm entry. Hence, defective migration of BCs leads to infertility. BC migration is reviewed by (Montell et al., 2012).

Before initiation of migration, apical-basal polarity is specified in the cell cluster by polarity markers such as Par-3 and Par-6 (Tanentzapf et al., 2000). These markers localize to the apical region of the cell and positively regulate the protrusive behaviour of the cell membrane and facilitate cohesion within the cluster. However, only one cell out of the cluster has the ability to extend protrusions which ensures that unidirectional migration towards the oocyte is maintained (Theveneau and Mayor, 2010). Furthermore, BCs express the EGFR and VEGFR like protein Pvr which are required for guidance of the cluster to its final destination (McDonald et al., 2006). This *in vivo Drosophila* model has been useful in determining the role of important actin cytoskeletal remodelling and cell polarization molecules such as Rac (Wang et al., 2010), Cdc42 (Llense and Martin-Blanco, 2008), E-Cad and β-catenin (Niewiadomska et al., 1999) which function similarly during embryogenesis and disease causing processes such as metastasis (Li et al., 2014; Schmalhofer et al., 2009; van Roy, 2014).

1.1.2 **Lateral line primordial migration in *Danio***

The Lateral Line is a sensory organ which is found in most fish and amphibians. Formation of this organ begins during embryogenesis and requires CCM. Specifically, the posterior lateral line (pLL) consists of over 100 primordial cells which are specified at the pLL placode adjacent to the ear. These cells detach from the underlying basement membrane (BM) and form a cohesive cluster which migrates to the posterior side of the embryonic trunk. As the cell cluster travels, the trailing edge periodically deposits cells which form into eight garlic bulb-shaped rosettes and mature into mechanosensory organs called neuromasts. Cells which are located at the LE display more mesenchymal characteristics whereas trailing edge cells appear to have epithelial morphology. Lateral line migration is reviewed by (Chitnis et al., 2012).

pLL primordial cell migration relies heavily on chemokine signalling. LE cells secrete FGF, which activates FGF signalling in the trailing edge and induces epithelial characteristics. Additionally, LE cells express the guidance receptor CXCR4, which is activated by the gradient of CXCL12 ligand presented by the underlying muscle progenitors (Haas and Gilmour, 2006). Adhesion between the cells of the pLL is mediated by both N-Cadherin and E-Cad in a spatial manner. The LE cells express N-Cadherin whereas cells which are deposited for the formation of rosettes form E-Cad mediated adhesions (Aman and Piotrowski, 2009). The role of ECM in promoting pLL primordial migration has not been fully understood, however, an ECM protein Anosmin-1a (homologue of human Kal-1) has been shown to play a key role in activation of CXCR4/CXCL12 signalling (Yanicostas et al., 2008). Hence, studies conducted on this

vertebrate model have enhanced our understanding of mechanisms which promote CCM and growth of organ systems during development and disease.

1.1.3 **Other collective cell migration models**

Dictyostelium discoideum

One of the best studied examples of CCM is the social amoeba, *Dictyostelium sp*. During starvation conditions the amoeba aggregate into a multicellular slug containing several thousands of cells. Migration and adhesion of cells in these cohorts requires front-back symmetry which is internally regulated via chemoattractants (Weijer, 2004).

2D sheet migration

When epidermal cells are transplanted on a custom made 2D ECM, the cells collectively spread while maintaining lateral adhesions (Fig 1.1 A). The role of molecules such as Integrins, Cadherins and cytoskeletal regulators Rho GTPases have been uncovered using this model (Farooqui and Fenteany, 2005; Nobes and Hall, 1999; Vitorino and Meyer, 2008).

Vascular sprouting

The sprouting of a vessel gives rise to the branched network which is shaped during angiogenesis. In this model, a single tip cell initiates the invasion process by cleaving the ECM and protruding multiple actin-rich filopodia (Fig 1.1 C). The endothelial stalk cells maintain lateral adhesions and follow the tip cell which expresses growth factors such as VEGF. Angiogenesis models, such as the murine retina have demonstrated how VEGF and other growth factors regulate extracellular gradients by binding to the ECM and cell surface (Gerhardt, 2008).

Cancer invasion

Since slow and long-term processes which give rise to cancer are not readily amenable to *in vivo* microscopic observation, migration and collective cell dynamics of metastatic cells are not fully understood. However, direct visualization of cancer cells undergoing metastasis can be achieved in 3D ECM explant models (Alexander et al., 2008). Additionally, development of 3D injections allows for generation of mouse or human cancers cell xenografts in the dermis of a living animal to study the invasion characteristics (Ding et al., 2010). Due to this technique, it has become possible to track the route and depth of invasion in various cancer cell lines. Neverthless, *in vivo* regulation of this highly invasive and adaptable process is still unclear.

Dorsal closure in Drosophila

Dorsal closure (DC) is a morphogenetic event during *Drosophila* embryogenesis during which ectodermal sheets migrate over the internalizing Amnioserosa (AS) and results in formation of a continuous ectoderm around the embryo. DC initiates after germ band retraction as the ectodermal LE migrates towards the dorsal midline while maintaining adhesions with the AS perimeter cells. The DC LE cells protrude multiple filopodia towards the direction of migration and also maintain lateral and basal adhesions with adjacent ectodermal cells. Actin dynamics with respect to cytoskeletal adaptors which are involved in filopodia and lamellopodia formation have been studied in this model. Additionally, live imaging experiments have identified intracellular signalling pathways which function through small GTPases and zippering mechanism of wound healing in this model (Reviwed by Jacinto et al. 2002).

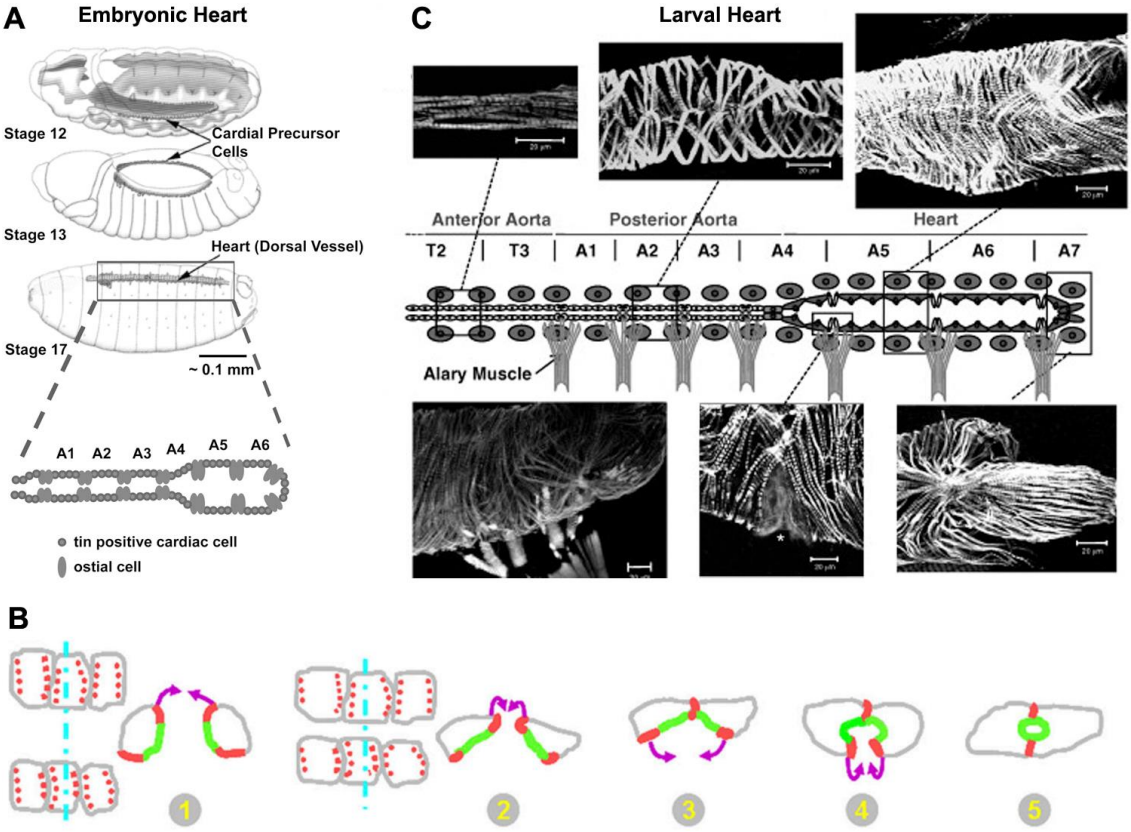
Tracheal cell migration in Drosophila

Tracheal progenitor cells migrate collectively to form a tubular organ during Drosophila embryogenesis. This process is reviewed by (Affolter et al, 2003).

1.2 Heart development in *Drosophila*

The heart in *Drosophila*, also known as the dorsal vessel (DV), is a tubular organ which pumps hemolymph around the larval and adult body (Fig 1.2). The development of the DV undergoes stages of CCM and hence can be utilized as a powerful model to elucidate genetic requirements. *Drosophila* heart development has been well established as a valuable embryonic, larval and adult model of cardiogenesis and has been used to study clinically relevant genetic cardiac diseases (Bier and Bodmer, 2004; Piazza and Wessells, 2011; Qian and Bodmer, 2012). Accessibility of transgenic and mutant flies along with recent advances in live imaging and immunolabelling techniques have made dissecting of molecular mechanisms regulating heart development relatively simple. The DV is composed of two rows of longitudinally arranged contractile cardiac cells flanked by non-contractile pericardial cells (Fig 1.2). DV is also polarized on the anterior-posterior axis. The posterior region of the heart encompasses a larger lumen which provides most of the contractile power to pump the hemolymph whereas the anterior region serves as the outflow tract. Cardial and pericardial cells are segmentally patterned. Each segment of the heart contains six cardial cells among which 4 posterior muscular cells express the transcription factor *tinman* whereas the remaining two express *seven-up*. Seven-up expressing cardioblasts (CBs) are destined to become the inflow cells of the larval and adult heart. Heart structure is reviewed by (Tao and Schulz, 2007)

Figure 1.2. Embryonic and larval heart development in *Drosophila*. (A) After germ band retraction, CBs form rows on the lateral sides of the embryo and collectively migrate to the dorsal midline along with the ectoderm. (B) Cross sectional images show the stages of lumen formation. Red outline marks the adhesive domains and green outline marks the luminal domain. 1) As CBs approach the midline, they extend dorsal protrusions towards the midline. 2) Contact between contralateral CBs is established and results in the formation of dorsal adhesion. 3) The ventral domain of the CBs extends and curves medially to form a ventral adhesion. 4,5) Formation of the ventral adhesion encloses a medial lumen. (C) Larval heart. Post embryogenesis, the heart lumen significantly increases in diameter as the larvae grows. Transverse fibers of the cardiomyocytes wrap around the heart and their simultaneous contraction leads to the pumping action of the heart. Pericardial cells flank the heart at the basal side. Images adopted from (Helenius and Beitel, 2008; Medioni et al., 2009; Wolf and Rockman, 2011) and used with permission from "Rockefeller University Press", "Elsevier" and "American Heart Association".


A
Embryonic Heart
Stage 12
Cardial Precursor Cells
Stage 13
Heart (Dorsal Vessel)
Stage 17
~ 0.1 mm
A1 A2 A3 A4 A5 A6
tin positive cardiac cell
ostial cell
C
Larval Heart
Anterior Aorta
Posterior Aorta
Heart
T2 T3 A1 A2 A3 A4 A5 A6 A7
Alary Muscle
B
1
2
3
4
5

1.1.4 **Stages of development**

Despite the basic morphology of the mature DV, the sequence of events which give rise to the heart are intricate and require precise regulation of developmental cues. Cardiac precursors arise from the dorsal mesoderm which requires input from the surrounding tissue. Signals such as FGF, Decapentaplegic and Wingless released by the overlying ectoderm specify the cardiogenic mesoderm (Zaffran and Frasch, 2002). Before germ band retraction, CBs undergo a mesenchymal to epithelial transition (MET) and subsequently go through stages of CCM and lumen formation (Borkowski et al., 1995; Fremion et al., 1999).

Collective cell migration

After germ band retraction, CBs align into bilateral rows on each side of the embryo and migrate towards the dorsal midline along with the overlying ectoderm (Fig 1.2 A) (Haack et al., 2014) . DC, which is the process where ectodermal LE migrates over an extra-embryonic tissue called the As, occurs simultaneously during CB migration. At stage 14 of embryogenesis, CBs migrate a few cell diameters behind the ectodermal LE and display epithelial characteristics (MacMullin and Jacobs, 2006). Lateral adhesion of CBs is maintained through the localization of proteins such as E-Cad and β-catenin which promote CB-CB adhesion (Haag et al., 1999; Medioni et al., 2008). As the ectoderm fuses at stage 16, CBs migrate over the internalizing AS, however cannot make contact with the contralateral partner until AS detaches from the ectoderm. At this stage CBs display partial mesenchymal characteristics and extend protrusion between ectoderm and the AS while maintaining lateral adhesions (Appendix D) (Fig 1.2 B) (Macabenta et

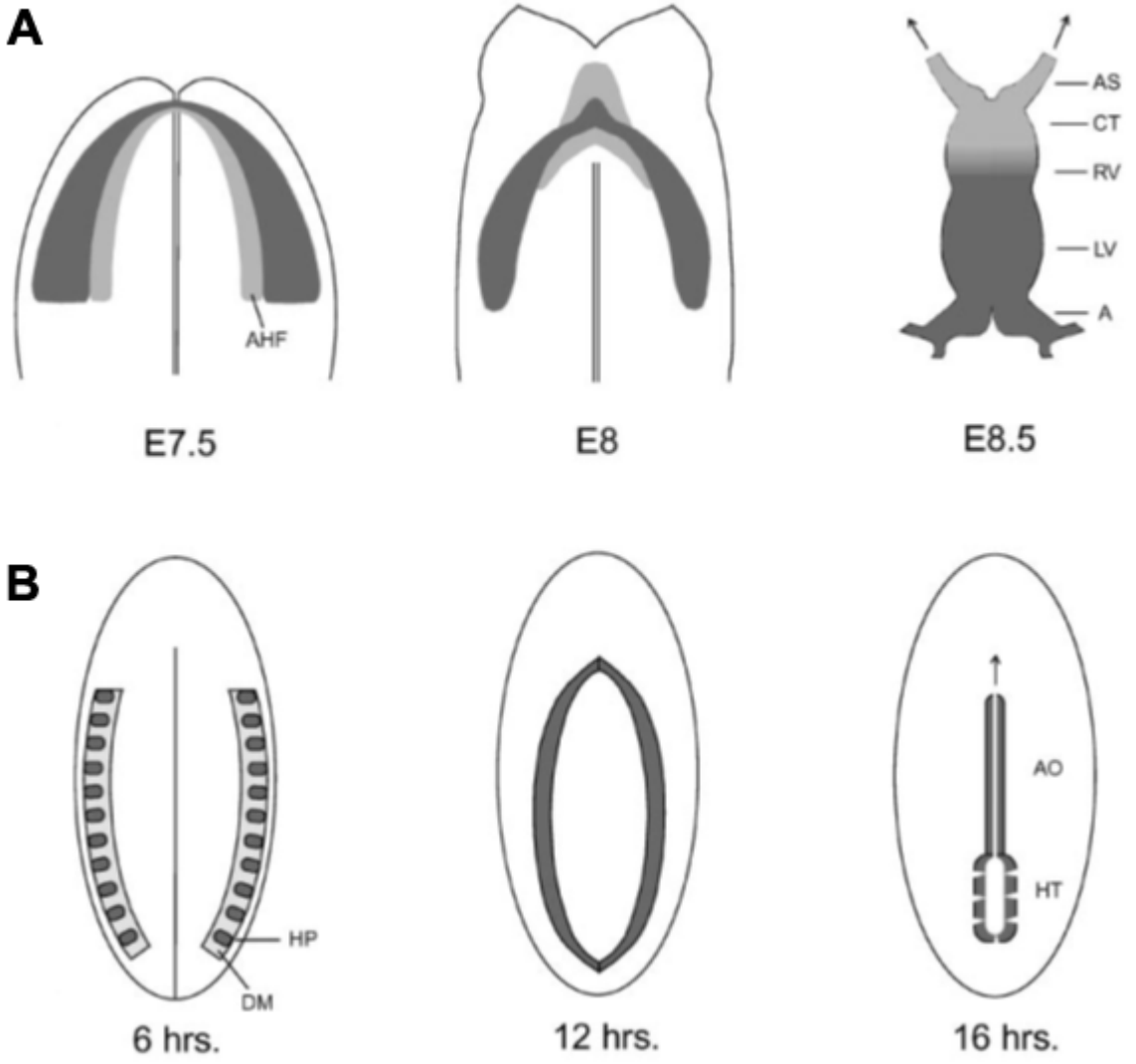
al., 2013; Swope et al., 2014; Vogler et al., 2014). Once the AS is internalized completely, contact between contralateral CBs is established.

Lumen formation

The primary contact and adhesion between contralateral CBs is made through apical outgrowths which localize E-Cad (Fig 1.2 B) (Haag et al., 1999). Subsequently, CBs adopt a crescent shape and form ventral adhesion which encloses a medial lumen and complete tubulogenesis (Fig 1.1 C). Integrin, an ECM receptor, localizes to the luminal domain and provides instructional cues for the localization of guidance signalling molecules Slit and Robo. A model presented by Santiago-Martinez et al suggests that Slit-Robo signalling at the midline restricts adhesion between contralateral CBs to the dorsal and ventral contact points leaving a non-adhesive medial luminal domain (Santiago-Martinez et al., 2008).

An additional guidance signalling pathway mediated by Netrin (Net), Frazzled (Fra) and Uncoordinated-5 (Unc5) is also required for the formation of the lumen. Fra and Unc5, which are Net receptors, are expressed by the CBs and occupy non-overlapping domains, where the former localizes to the adhesion domain and latter localizes to the luminal domain. Fra facilitates cell outgrowth and attachment formation during lumen formation whereas Unc5, similarly to Robo, is required to restrict CB attachments to the dorsal and ventral sides (Albrecht et al., 2011; Macabenta et al., 2013).

Figure 1.3. Early cardiac morphogenetic events in vertebrates and *Drosophila*. (A) Early stages of heart tube formation in mouse embryos. After cardiomyocyte specification, bilateral heart primordia are generated. These cells migrate towards the midline along with the ectoderm and undergo tubulogenesis. The intermediate tubular heart consists of external myocardial cells and an internal layer of endocardial cells. Subsequently, the tubular heart undergoes a process called rightward looping and form distinct chambers. (B) Embryonic stages of *Drosophila* heart formation. CBs form bilateral rows and migrate towards the midline along with the ectoderm. Once at the midline, CBs undergo tubulogenesis. Figure adapted from (Zaffran and Frasch, 2002) and used with permission from "American Heart Association".


A
AHF
E7.5
E8
AS
CT
RV
LV
A
E8.5
B
HP
DM
6 hrs.
12 hrs.
AO
HT
16 hrs.

1.1.5 Conservation of morphogenetic mechanism with vertebrates

The common purpose of the invertebrate or vertebrate heart is to circulate the hemolymph or blood around the body. Nevertheless, the tubular structure of the *Drosophila* does not resemble the chambered heart of vertebrates. Despite the morphological differences the early events that govern heart development are conserved between vertebrates and *Drosophila*. In both organisms, cardiomyocytes are derived from the first mesoderm to migrate away from the midline (Bodmer, 1995). After gastrulation, signals from the endoderm and ectoderm have been shown to play a key role in vertebrates and *Drosophila*, respectively (Zaffran and Frasch, 2002). In vertebrates, the endoderm is not only important for inducing specific cardiac cell fate but is also required for cardiac mesoderm migration (Aleksandrova et al., 2015). A strikingly similar role for the ectoderm in driving CB migration has been described in *Drosophila* (Haack et al., 2014). Furthermore, the signals from the epithelial tissue give rise to two bilateral regions of heart precursors which collectively migrate medially in both organisms. Once at the midline, in vertebrates, the heart tube forms and resembles the structure of the DV in *Drosophila*. However, further cardiac looping events in mammals give rise to a more complex four chambered heart which contains two atria and two ventricles. The DV maintains its tube morphology but the lumen expands significantly as the organism grows.

Transcription factor networks regulating cardiomyocyte identity and differentiation are evolutionarily conserved between *Drosophila* and vertebrates. In both models, secretion of Wnts, FGFs and Bone morphogenetic protein (BMP -homologue of

Decapentaplegic in *Drosophila*), by the overlying epithelium induces expression of transcription factor *Nkx2.5* in vertebrates and *tinman* in *Drosophila* (Tao and Schulz, 2007). The expression of Tinman in the *Drosophila* CB is essential for its specification and is expressed throughout cardiac development (Bodmer, 1993). Similarly, in mouse, chick, frog and zebrafish, *Nkx2.5* is expressed in the cardiogenic mesoderm and its transcripts are found in cardiomyocytes throughout heart development (Zaffran and Frasch, 2002). Additionally, Slit and Robo are expressed in vertebrate and *Drosophila* hearts and function to promote tubulogenesis (Fish et al., 2011; MacMullin and Jacobs, 2006; Medioni et al., 2010). In conclusion, there is a remarkable conservation of developmental mechanisms during heart formation between *Drosophila* and vertebrates.

1.1.6 *Drosophila* heart development as a congenital heart disease model

Congenital heart diseases (CHDs) are the top cause of infant mortality. About 1% of newborns are born with cardiovascular defects which account for 1.35 million newborns worldwide (van der Linde et al., 2011). Even though medical advances have better prepared physicians and surgeons to confront these diseases, late-onset cardiac complications frequently occur during adulthood and pose a significant threat. *Drosophila* model system has been previously used to elucidate molecular mechanisms which underlie human diseases. These diseases include but are not limited to Alzheimer's and Parkinson's disease, metabolic disorders, CHDs and metastasis. Additionally, 75% of human genes have orthologues in *Drosophila* while a third of these genes are functionally equivalent (Chintapalli et al., 2007; Pandey and Nichols, 2011). Mutant and Gal4/UAS system mediated misexpression analysis are readily conducted in *Drosophila* due to

availability of loss-of-function alleles and transgenic lines (Duffy, 2002). Since the discovery of homologous mechanism which control both vertebrate and invertebrate cardiac development, this model organism has been used to study early developmental stages of heart formation and molecular mechanism which give rise to CHDs in

vertebrates. For example, mutations in the gene which codes for the ATP-dependent potassium channel results in similar cardiac specific defects in both mice and *Drosophila* (Bier and Bodmer, 2004). Additionally, the transcription factor Evx2 is expressed in the epicardium of the mouse. The orthologs of this protein in *Drosophila*, even-skipped, is expressed in a subset of pericardial cells as well. Mutations in this gene result in deletion of the pericardial cell lineage and result in cardiac specific defects in adults including elevated arrhytmicity, alterations in diastolic width and demonstrate an overall restrictive cardiomyopathy phenotype in *Drosophila* (Piazza and Wessells, 2011). Therefore, the function which Eve and its vertebrate ortholog Evx2 share during heart development is most likely conserved and therefore the molecular mechanism can be extrapolated.

1.2.4 CCM migration of CBs as a model for cancer metastasis

Cancerous cells mimic morphogenetic movements by re-activating similar invasion patterns and mechanisms which are active during embryonic CCM (Micalizzi et al., 2010). Therefore, universal mechanistic themes of CCM, on molecular and cellular level, must be revealed by comparing cellular behaviours in various *in vivo* models. Acquisition of such information will facilitate development of strategies that either suppress or enhance collective movements of cells. Clarifying common rules and differences between

invasion programmes and embryonic development will lead to improvement of strategies which are currently being used to suppress tumour metastasis. *Drosophila* embryonic heart development is an emerging *in vivo* system to study CCM; however, to this point, it has been underutilized in this capacity. All three behaviours which define CCM are displayed by CBs during migration. First, CBs are receptive to positional cues (Macabenta et al., 2013; MacMullin and Jacobs, 2006; Medioni et al., 2008; Santiago-Martinez et al., 2006; Santiago-Martinez et al., 2008). Second, CBs maintain lateral adhesion with adjacent cells while migrating (Haag et al., 1999; Santiago-Martinez et al., 2008). Lastly, CBs form adhesions with the surrounding ECM while migrating (Vanderploeg et al., 2012; Volk et al., 2014). Even though models such as BC and posterior lateral line migration have significantly increased our knowledge about CCM, it is still unclear how exactly cells coordinate multicellular movements. Therefore, CB migration in *Drosophila* is the newest addition to the list of established genetic models of CCM and must be employed for future studies. Although CCM of CBs shares many common features with other models, some unique features are also noted. Unlike other models, where 1 or 2 cells guide the cluster, multiple Tinman expressing CBs are able to function as leaders. Disruption in the function of the small GTPase, Cdc42, in these cells leads to failure in the medial migration of the CBs (Swope et al., 2014). Similarly, in cancers such as colorectal adenocarcinomas, Cdc42 is overexpressed and favours tumour growth and migration (Gomez Del Pulgar et al., 2008; Qadir et al., 2015). Inhibition of Cdc42 in these cancers suppresses progression of intestinal tumours (Sakamori et al., 2014). Hence, similar molecular mechanisms discovered in migratory CBs might apply to

CCM in cancers such as epithelial-to-mesenchymal (EMT) transition and ECM degradation.

1.2 **Guidance signalling**

So far we have considered overall CCM. Now, let us zoom in and have a closer look at the specific mechanisms with which cells regulate their behaviour. In *Drosophila*, a lot of what we know about guidance signalling and its mode of activity contributes to axonal guidance. These studies have identified vital guidance cue such as Wnt, Semaphorins, Ephrins, Slits and Nets. Specifically, Slit and Net have been shown to be key players which establish heart development in *Drosophila*. Therefore, the role of these morphogens and their respective receptors Robo/Robo2 and Fra/Unc5 is explored below in more detail.

1.2.1 **Slit-Robo-Robo2 signalling**

Robo receptor was initially identified in a mutant screen for defects in the midline crossing of the commissural axons in *Drosophila* (Kidd et al., 1998) whereas Slit ligand was identified as a glycoprotein which is secreted by the midline glia (Rothberg et al., 1990). Since then, the role of this guidance signalling ligand-receptor pair has been demonstrated in numerous both vertebrate and invertebrate tissues and organs (Dickinson and Duncan, 2010; Fujiwara et al., 2006; Liao et al., 2010; Mommersteeg et al., 2015; Yang et al., 2013). Their mammalian orthologs perform important roles in the development of heart, kidney, lung, mammary gland and have been implicated in human diseases such as cancers and chronic inflammation (Mehlen et al., 2011). Human genome

encodes 3 Slit and 4 Robo receptor genes. In *Drosophila*, only one Slit and 3 Robo receptors exist (Dickson and Gilestro, 2006).

Biochemistry and protein structure

Drosophila Slit contains conserved domains including EGF like domains, Laminin G-like domain and a C-terminal cysteine knot. At the N terminal, Slit contains 4 leucine-rich repeats which are required for binding to the Robo receptors (Brose et al., 1999). *in vivo* cleavage of Slit is required for its activation. Once cleaved, the N-terminal Slit binds to all Robo receptors in *Drosophila* whereas the function of C-terminal Slit is unknown. Robo receptors belong to the immunoglobulin (Ig) superfamily of cell adhesion molecules (CAMs). The extracellular region of the Robo receptors consists of five Ig like domains followed by three fibronectin type 3 (FNIII) repeats. At the cytosolic region, Robo1 contain 4 conserved linear motifs labelled CC0-CC3, whereas Robo2 and Robo3 lack CC2 and CC3 domains (Hohenester, 2008). Activation of Robo/Slit signalling in *Drosophila* requires the binding of the heparin sulphate proteoglycans (HSPG) Syndecan (Sdc) (Ypsilanti et al., 2010). Sdc binds Slit at the N terminal region and its role as a co-factor is crucial in promoting Slit/Robo signalling (Chanana et al., 2009; Steigemann et al., 2004).

Signalling mechanism during axonal guidance

Slit/Robo signalling guides migrating axons. Slit has been characterized as a repellent signal when bound to Robo1 (Kidd et al., 1999). When migrating neurons encounter Slit, cytoskeletal rearrangement at the contact domain is inhibited or activated dependent on the intracellular recruitment of adaptor proteins such as Rho GTPases

(Wong et al., 2001). In mice, downstream effectors such as the member of the Slit-Robo GTPase Activating Proteins (srGAPs), srGAP3, bind Robo1 at the CC3 intracellular domain. This binding results in the recruitment of the Wiskott Aldrich syndrome protein (WASP)/ WASP-family verprolin-homologous protein (WAVE) scaffolding complex which plays an important role in promoting actin cytoskeletal reorganisation and promotes neuronal morphogenesis and cell migration (Ypsilanti et al., 2010). SrGAP1, on the other hand, mediates repulsive signalling when Slit is present by inactivating Cdc42 and RhoA but nor Rac1 (Wong et al., 2001). Robo2 which contains only the CC0 and CC1 domain at cytoplasmic tail mediates attractive signalling when bound to Slit via an unknown mechanism (Lopez-Bendito et al., 2007; Ma and Tessier-Lavigne, 2007). Therefore, the attractive or repulsive function of Slit-Robo signalling is mediated by the intracellular adaptor proteins which are recruited to the plasma membrane by the cytoplasmic domain of the activated Robo receptor.

Signalling mechanism during heart development

Slit and Robo are expressed in the cardiac tissue during early heart developmental stages and are autonomously required for the formation of the heart in zebrafish, mice and *Drosophila* (Fish et al., 2011; MacMullin and Jacobs, 2006; Medioni et al., 2010; Mommersteeg et al., 2015). Specifically in *Drosophila*, Slit is uniformly distributed on the plasma membrane during early CBs migration and apicalizes as the CBs initiate lumen formation (MacMullin and Jacobs, 2006). Loss of Robo or Slit results in the extension of the cell adhesion domains, which localize junctional proteins E-Cad and β-catenin, to the entire apical surface leading to a lumen-less phenotype (Santiago-Martinez

et al., 2008). Additionally, in *slit* and *robo,robo2* mutants, CBs remain rounded as they approach the midline, indicating Slit and Robo regulate cell shape changes during heart development (Medioni et al., 2008). Additionally, overexpression of Slit in the CBs induces formation of ectopic lumens suggesting that Slit/Robo signalling not only antagonizes adhesion formation at the apical surface but also specifies luminal characteristics (Helenius and Beitel, 2008). Even though Slit and Robo are required for lumen formation, how this guidance signalling system modulates CCM during heart development is not fully understood.

1.2.2 **Netrin-Frazzled-Uncoordinated-5 signalling**

A significant advancement in the field of neuroscience was the discovery of unc-5, unc-6 and unc-40 genes in *C. elegans* during the early 90s (Hedgecock et al., 1990). Since then, homologues of these cell-guidance molecules have been identified in vertebrates and invertebrates. Unc-6, also known as Netrin (Net) is a secreted morphogen which binds its receptor Unc-40/Fra/DCC and Unc-5 and function to regulate guidance of migratory cells. The *Drosophila* genome encodes two Nets, Net-A and Net-B (from here on collectively referred to as Net for simplicity), one Fra and one Unc5 receptor, whereas the vertebrate genome has 4 Nets, one Fra/DCC and four Unc5 (A-D) homologs (Lai Wing Sun et al., 2011).

Biochemistry and Protein Structure

Drosophila Nets are secreted molecules which have the ability to bind ECM protein Laminin (Lan) and transmembrane receptors Fra/Deleted in Colorectal Cancer (DCC) and Unc5 (Lai Wing Sun et al., 2011). All known vertebrate and invertebrate Nets contain

Lam like G domains, IV and V, at the N terminal, followed by three EGF repeats and C-terminal which interacts with HSPGs and Integrins (Barallobre et al., 2005). Like Robo, Net receptor Fra/DCC contains multiple Ig and FNIII like domains with evidence to suggest that Net binds to the fourth and fifth FNIII repeats. Intracellular domain of Fra contains three conserved P domains, P1, P2 and P3. Unc5 contains two Ig domains. However instead of FNIII like domain, Unc5 has two thrombospondin type-1 domains and the cytoplasmic tail includes three well conserved motifs: ZU-5, a DB motif and carboxy-terminal death domain (DD) (Lai Wing Sun et al., 2011) .

Signalling mechanism during axonal guidance

During axonal guidance in *Drosophila*, Net acts as a chemoattractant when bound to Fra through its Ig domain (Garbe and Bashaw, 2007) whereas Net/Unc5 complex induces a repulsive signal similar to Robo (Keleman and Dickson, 2001). Unc5 and Fra, are both required for guiding the axonal projections during commissure formation during embryonic CNS development in *Drosophila* (Harris et al., 1996; Hiramoto et al., 2000; Keleman and Dickson, 2001; Kolodziej et al., 1996; Mitchell et al., 1996). In vertebrates, activation of Fra homolog, DCC, promotes assembly of intracellular cytoskeletal remodelling complex including Cdc42, Rac1 and N-WASP. This recruitment event leads to directed actin reorganisation which is required for growth cone expansion and migration (Shekarabi et al., 2005).

Signalling mechanism during heart development

Both Fra and Unc5 are expressed by the CBs during early and late cardiac development in the embryo. Both these receptors occupy non overlapping domains,

where the former localizes to the adhesion domain and latter is present at the luminal domain. Both these receptors are distinctly required for lumen formation during heart development. In *fra* mutants, CBs fail to extend cytoplasmic outgrowth and form initial contact with contralateral partners (Macabenta et al., 2013). In *unc5* mutants, adhesion domains encompass the entire apical region of the CBs and lumen fails to form (Albrecht et al., 2011). Accordingly, in *Net* deficient embryos, lumen formation is suggested to be disrupted. Even though the role of Net, Fra and Unc5 is well established during lumen formation, however their relationship with Slit and Robo and how these molecules collectively promote CCM is unknown.

1.2.3 **Inter-regulation of Slit and Netrin dependent signalling**

Attractive or repulsive guidance is dependent on the type of receptor and the downstream proteins that are activated spatially at the plasma membrane of a migrating cell or cell cluster. Once again most of our understanding of how Slit and Net signalling pathways converge together to regulate organ formation comes from studies conducted in morphogenetic models. For example *Drosophila* embryonic CNS and salivary gland morphogenesis requires opposing independent functions of Slit/Robo and Net/Fra signalling which regulate cell migration (Garbe and Bashaw, 2007; Kolesnikov and Beckendorf, 2005). Nevertheless, during tracheal development, Robo 2 conveys attractive signalling when bound to Slit (Englund et al., 2002). Also, cooperative Slit and Net functions have been demonstrated during contralateralization of facial somatosensory neurons in mice (Mirza et al., 2013). Furthermore, in vertebrates, Slit/Robo signalling is implicated in both branch-promoting and branch-repelling actions of peripheral

developing sensory axons (Ma and Tessier-Lavigne, 2007). Interestingly, the physical interaction between Robo and Fra/DCC is mediated through the CC1 domain of Robo in Xenopus (Stein and Tessier-Lavigne, 2001). However, in this example, Slit dependent Robo binding of Fra results in silencing of the attractive Net signalling. However, in *Drosophila*, a genetic study conducted on Robo-Fra chimeric receptors demonstrated that the repulsive or attractive function of the receptors is strictly mediated by cytoplasmic tail of Robo and Fra, and recruits specific intracellular effectors (Bashaw and Goodman, 1999).

Neurons and other developing tissues often express both attractive and repulsive receptors at the same time. Additionally, Net and Slit receptors both contain similar Ig and FNIII domains. One way to modulate the guidance response is to differentially attract cytoplasmic adaptor proteins to the intracellular tail of receptors which could either inhibit or promote migration. Although during heart development opposing functions for Fra and Unc5 have been proposed, it is not known how Slit and Net receptors interact with each other to promote CCM in the CBs.

1.4 Extracellular matrix

ECM is the dense meshwork of fibrous proteins which is present on the outside perimeter of cells which functions like glue to holds cells together. Due to the various essential functions ECM provides, it has been termed a “non-cellular component” of tissues (Frantz et al., 2010). Not only does ECM serves as a scaffold which gives cells a rigid or flexible structure for support, it can also influence cell positioning, vesicular trafficking and migration (Kular et al., 2014). ECM is composed of multiple building

blocks, including structural proteins such as Collagen and non-structural proteins such as Lans, proteoglycans and growth factors. ECM can be classified in to two distinct classes, the interstitial ECM (not discussed further) and basement membrane (BM). The structure, function and composition of specialized BM vary across tissues and organ system and play a crucial role in maintaining cellular identity and function. In most cases, cells interact physically with the ECM through receptors such as Integrins and Dystroglycan (Dg), which convey bidirectional positional input (Berrier and Yamada, 2007). Therefore, ECM can be thought of as an "extension of the plasma membrane" which offers additional protection from disorderly physical stresses and provides an interface with the outside environment and the cell (Yurchenco, 2011).

1.4.1 Constituents of the extracellular matrix

Collagen-IV

One of the most abundant proteins found in the body of an organism are collagens. Specifically type-IV collagen makes up about 50% of the BM composition and provides not only tensile strength but also functions in cell adhesion and migration (Yurchenco, 2011). In vertebrates, there are 6 Collagen-IV chains (Ricard-Blum, 2011) whereas in *Drosophila* there are two Collagen IV chains, Viking (Vkg) and Cgc25 which produce a heterotrimer consisting of one Vkg chain and two Cgc25 chains (Myllyharju and Kivirikko, 2004). During heart development, Vkg is required to stabilise the cardiac ECM however its exact role is unclear (Hollfelder et al., 2014).

Laminins

Laminin (Lan) are the second most abundant proteins found in the BM. Their typical structure includes three chains α, β and γ which are inter-twined and resemble a three-pronged fork. More importantly, Lan are the first ECM molecules expressed in the embryo and recruited to the BM in *Drosophila* and vertebrates. Lan are crucial for embryonic formation and play a vital part in several cell processes including differentiation, migration and adhesion (Yurchenco, 2011). Defects in the gene coding for Laminin α and β chains result in formation of serious BM defects affecting multiple organ development (Chen et al., 2010; Hochgreb-Hagele et al., 2013; Ichikawa-Tomikawa et al., 2012; Nguyen and Senior, 2006). During heart development in *Drosophila* Lan is required for cardiac ECM assembly. Specifically, defects in alary muscle attachment and lumen formation are continuously noted in hypomorphic mutants of Lan β chain. Additionally, apicalisation of Slit does not occur in these mutants (Hollfelder et al., 2014).

Heparin Sulphate Proteoglycans

HSPGs are crucial constituents of the ECM which have distinct structures. These proteoglycans contain a core protein structure from which are many covalently linked chains of complex carbohydrates, termed glycosaminoglycans (GAGs) (Princivalle and de Agostini, 2002). In vertebrates, HSPGs sequester growth factors such as TGF-β via GAGs chains into the ECM (Rider, 2006). Similar roles are proposed for *Drosophila* HSPGs, Sdc and Perlecan (Baeg and Perrimon, 2000; Lin and Perrimon, 2000). Sdc has

been specifically implicated in driving cardiac morphogenesis in *Drosophila* by promoting CB specification and lumen formation (Knox et al., 2011).

1.4.2 Extracellular matrix receptors

Communication of signals from ECM to the cells is mediated by transmembrane receptors. These receptors bind various ECM constituents and are able to transduce mechanosensory and biochemical stimuli via recruitment of specific cytoplasmic adapters to the intracellular domain which regulates ECM adhesion, cytoskeletal dynamics, cell migration, differentiation, polarization and cell death during embryogenesis (Bonnans et al., 2014; Gullberg and Ekblom, 1995).

Integrins

Integrins are heterodimeric receptors which link the ECM to the actin cytoskeleton of the cell. Activation of this receptor by binding of ligands induces conformational change which exposes binding sites at the cytoplasmic tail. Integrin ligands include but are not limited to Collagens, Lans, HSPGs, cell adhesion molecules and other components of the ECM (Larsen et al., 2006). During *Drosophila* heart development, Integrins are required for inducing LE motility and lumen formation. Furthermore, Integrins accumulation stabilizes the morphogen Slit and its receptor Robo at the pre-luminal and luminal domain and modulates cell polarity (Vanderploeg et al., 2012).

Dystroglycan

Dg, a core member of the dystrophin-associated complex, is another transmembrane receptor which links the actin-cytoskeleton to the ECM by binding molecules such as Lans and HSPGs (Gullberg and Ekblom, 1995). During heart development, Dg localizes

to the luminal and basal ECM and its activity is required to maintain the normal size of the medial luminal space (Medioni et al., 2008).

1.4.3 Function of the extracellular matrix during development

ECM serves diverse functions which shape tissues and promote organogenesis reviewed by (Kular et al., 2014). First, it can serve as an anchorage point which immobilizes the cell through functioning as a migration barrier. Second, ECM can sequester guidance cues and present them to the cell receptors and concurrently function as a low affinity co-factor. Third, mechanosensory information can be communicated to the cell via complexes. Lastly, degradation of the ECM by proteases releases ECM fragments, bound cytokines and GF which induce cell polarity, migration and fate specification. Collectively, these roles influence development of the embryo and contribute to progress of diseases such as cancer (Bonnans et al., 2014).

1.4.4 Extracellular matrix around the *Drosophila* heart

Usually, apical-basal polarity is dictated by the BM, which specifies the basal domain of the cell. The apical domain of polarised cells usually does not localize ECM markers. ECM surrounding the CBs is unique in the sense that both apical (luminal) and basal domains localize components of the ECM such as Collagen-IV, Lans and Sdc (Fig 1.4) (Haag et al., 1999; Hollfelder et al., 2014; Knox et al., 2011). However, the composition of the basal and luminal ECM differs with respect to a few proteins. The basal ECM, localizes a Collagen-IV like protein Pericardin (Prc), which is secreted by the pericardial cells and is absent from the apical ECM (Chartier et al., 2002). The apical

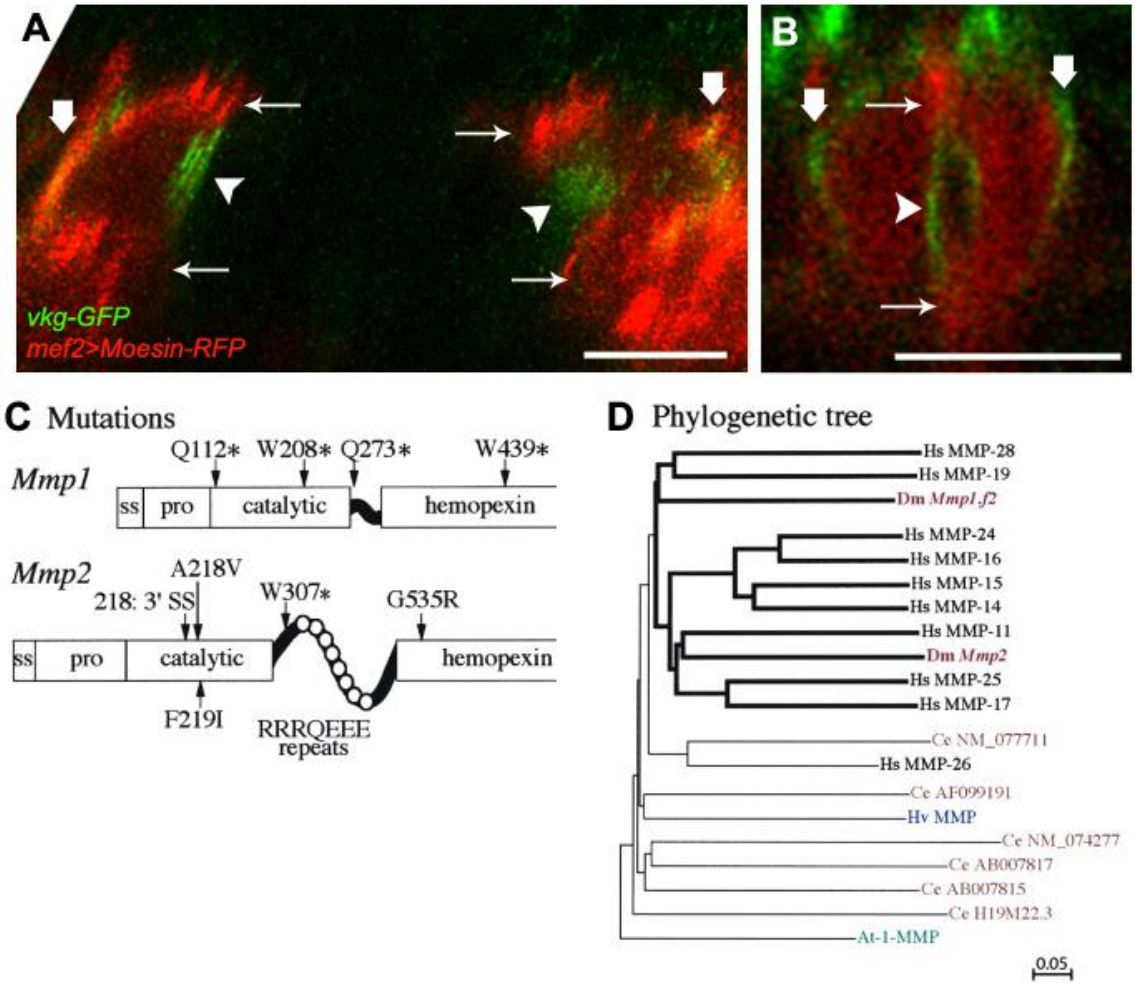
ECM localizes a Collagen-XV/XVIII like protein Multiplexin (Mp). However, Mp localizes strictly to the apical ECM of the posterior heart and is absent from the aorta. Such anterior-posterior segregation of Mp results in formation of a larger lumen between CBs at the heart proper compared to the aorta (Harpaz et al., 2013).

CBs interact with the surrounding ECM by localizing receptors such as Integrins and Dg at the basal and luminal domains which appear to stabilize the ECM at those domain. By binding ECM components through these receptors, CBs maintain adhesions with the ECM which is required for cell migration and lumen formation (Volk et al., 2014). In addition, cell polarity is modulated through the interaction of these receptors with the ECM components (Vanderploeg et al., 2012). Additionally, it appears that CBs produce and deposit their own ECM. So it seems that ECM plays many important roles during heart formation, however several questions regarding its role in regulating CCM still remain unanswered. How do CBs regulate the localisation of the ECM surrounding the heart during migration? How is polarized localisation of ECM constituents maintained during CCM? Are ECM remodelling proteases required for heart formation?

1.5 Matrix metalloproteinases

Matrix Metalloproteinases (MMPs) are zinc dependent proteases from the metzincin superfamily which are found all across the kingdom animalia. These proteases have the ability to collectively degrade all of the components of the ECM and by doing so promote various ECM related processes such as cell migration, cell adhesion and tumour invasion. MMPs are often regulated by endogenous Tissue Inhibitors of

Figure 1.4. Matrix Metalloproteinases and ECM of the heart. (A-B) Cross sectional images of the heart demonstrating localization of Collagen-IV during migratory and late stages of heart development. The localisation of Vkg is reported by the *vkg*-GFP trap construct. The localisation of Actin is reported by the expression of UAS-moesin-mCherry under the control of *myocyte enhancer factor 2*-GAL4 (*mef2*) driver. ECM is detected at the basal and apical side of the CBs. Presence of CollagenIV at the basal side of CBs in observed throughout heart development (A, B thick arrows). At the apical domain, during migratory stages, CollagenIV is present at the pre-luminal domain of the CBs (A arrowhead) and is absent from the dorsal and ventral junctional domains (A arrow). During late stages, CollagenIV localizes to the luminal perimeter (B arrowhead) and is absent from the jucntional domain (B arrows). (C) MMP1 and MMP2 domain structure and mutant alleles. MMP1 and MMP2 share conserved pro-peptide, catalytic and hemopexin domains. In MMP2 linker region between catalytic and hemopexin domain is much larger compared to MMP1. Null mutant alleles $mmp1^{q112*}$ and $mmp2^{w307*}$ were used for MMP analysis. (D) Phylogenetic analysis of MMP in vertebrates and *Drosophila*. Phylogenetic tree suggests that *Drosophila* MMPs are more closely related to human MMPs than to each other, suggest an ancient divergence event in the MMP gene family. Micron scale is 10μm. Panel A and B were generated during the studies reported in this thesis. Panel C and D adopted from (Page-McCaw et al., 2003) and used with permission from "Cell Press".


A
B
vkg-GFP
mef2>Moesin-RFP
C Mutations
Mmp1
Q112*
W208*
Q273*
W439*
ss
pro
catalytic
hemopexin
Mmp2
A218V
218: 3' SS
W307*
G535R
ss
pro
catalytic
hemopexin
F219I
RRRQEEE
repeats
D Phylogenetic tree
Hs MMP-28
Hs MMP-19
Dm Mmp1.f2
Hs MMP-24
Hs MMP-16
Hs MMP-15
Hs MMP-14
Hs MMP-11
Dm Mmp2
Hs MMP-25
Hs MMP-17
Ce NM_077711
Hs MMP-26
Ce AF099191
Hv MMP
Ce NM_074277
Ce AB007817
Ce AB007815
Ce H19M22.3
At-1-MMP
0.05

Metalloproteinases (Timps). Timps are able to inhibit MMP activity by blocking substrate recognition (Nagase et al., 2006).Apart from degrading the ECM and removing physical barriers, MMPs are known for cleaving other non-ECM related proteins such as GF and cell surface receptors. MMPs in mammal have overlapping ECM substrate. For example, Collagen IV can be degraded by seven different MMPs, whereas Lan is a substrate for eight MMPs. Other MMP substrates include HSPGs such as Sdc, growth factors, such as TGF-β, FGF, VEGF, adhesion molecules such as Cadherins and Integrins and other cell signalling related molecules (Sternlicht and Werb, 2001). Therefore, MMPs modulate diverse physiological and pathological processes including tubulogenesis, CCM, cell polarity and cancer metastasis (Lemaitre and D'Armiento, 2006).

1.5.1 Structure and homology

All MMPs contain common conserved domain structure including a catalytic domain which has a conserved Methionine residue along with a zinc-binding active site (met-zincin family) and a hemopexin domain which contributes to substrate recognition, enzyme activation and inhibition. To date, 23 MMPs and 4 Timps have been identified in humans. Initial genetic characterization for 14 MMPs has been conducted in mice. These studies have demonstrated that MMPs functions are genetically redundant and many MMPs have overlapping substrates both *in vitro* and *in vivo*. Surprisingly, most single *mmp* mutants mice survive birth, which suggests that MMP activity is dispensable for embryonic development. However, the more likely explanation is that in single *mmp* mutants functional compensation by other MMPs might be sufficient for embryogenesis (Page-McCaw et al., 2007). This phenomenon has raised difficulties in determining

specific roles of MMPs. On the other hand, in *Drosophila*, only two MMPs, MMP1 and MMP2, and one Timp, are encoded. By generating double mutants or inhibiting both MMPs at the same time, the concern about genetic redundancy and compensation can be eliminated in *Drosophila*. Additionally, the domain structure, activity and substrates of *Drosophila* MMPs are evolutionarily conserved in mammalian MMPs; however, fly MMPs do not have any orthologues in mammals (Page-McCaw et al., 2007). Nevertheless, fly Timp has the ability to inhibit mammalian MMPs and reversely mammalian Timps can inhibit fly MMPs (Wei et al., 2003). Finally, MMPs can be separated into two categories, secreted MMPs and transmembrane MMPs.

Secreted Mmp

Most of the human MMPs are predicted to be secreted. These MMPs contain the SS domain which targets them to the extracellular space. Subsequent extracellular cleavage of the pro-domain activates protease activity. In *Drosophila* MMP1 is a secreted protease which has all the conserved features typical of mammalian MMPs (Llano et al., 2000). *in vitro* analysis confirmed that MMP1 is able to degrade common vertebrate MMP substrates including Collagen and gelatin. However, MMP1 do not possess the ability to cleave Lan and Fibronectin (Llano et al., 2000). In *Drosophila, mmp1* gene transcription is up-regulated during embryonic development, diminishes during larval stages and is virtually absent in adults. Nevertheless, a significant increase in MMP1 levels during pupation is demonstrated. *mmp1* mutant embryos survive embryogenesis but die during late larval stages suggesting that MMP1 activity is crucial for larval growth but not embryogenesis (Page-McCaw et al., 2003). Additionally, MMP1 is required for

tracheal air sac development and wound healing during larval morphogenesis (Glasheen et al., 2010; Stevens and Page-McCaw, 2012). The role of MMP1 in regulating cell adhesion has also been demonstrated in two independent developmental models in *Drosophila*. MMP1 dependent cleavage of NinjurinA promotes liberation of cell adhesion the tracheal cells in a cell-nonautonomous manner (Zhang et al., 2006). Also, during fat body disintegration in pupae, MMP1 is specifically required to cleave E-Cad (Jia et al., 2014). A specific role of MMP1 during embryogenesis, specifically heart development, is unknown.

Transmembrane Mmp

6 of the 23 MMPs in humans are membrane tagged MMPs (MT-MMPs). The transmembrane nature of these proteases is dictated by the presence of a glycosylphospotidylinositol anchor site (GPI) or of a type1 transmembrane pass sequence. MT-MMPs are tethered to the plasma membrane and perform cleavage of pericellular ECM. MT1-MMP, also known as MMP14, is the most studied MMP in humans and its role in many morphogenetic and disease related processes have been the focus of several reviews (Bai et al., 2005; Coyle et al., 2008; Itoh, 2006, 2015; Kajita et al., 2001; Sato and Seiki, 1996). In *Drosophila*, a single transmembrane MMP2 containing a GPI anchor was identified by sequence alignment with mammalian MT-MMP (Llano et al., 2002). MMP2 contains similar catalytic and hemopexin domains, and the substrate specificity between mammalian MT-MMPs and *Drosophila* MMP2 is also conserved. RT-PCR analysis of *mmp2* gene expression suggests that this protease is expressed at low levels throughout embryonic, larval and adult stages. Similar to MMP1,

MMP2 expressing during early pupation is significantly increased compared to other developmental stages (Page-McCaw et al., 2003). Even though *mmp2* mutant *Drosophila* survives embryogenesis and larval growth, several morphogenetic processes require MMP2 activity. MMP2 promotes motor axon fasciculation via matrix molecule Faulty Attraction (Miller et al., 2011; Miller et al., 2008). Air sac tubulogenesis requires the activity of MMP2 (Guha et al., 2009). In addition, fat body disintegration requires MMP2 activity to cleave component of the BM (Jia et al., 2014). The role of MMP2 during heart development in *Drosophila* is currently unknown.

1.5.2 The role of MMPs in cell migration

MMPs have the ability to influence cell migration by cleaving cell-cell adhesion proteins, releasing bioactive ECM molecules and cleavage of signalling receptors. Many tissues and cells, such as endothelial cell, keratinocytes, leukocytes, and metastatic tumour cells rely on the ability of MMPs to promote cell migration. There are multiple ways MMPs can induce cell migration. First, MMPs provoke migration by loosening the ECM barrier which requires its collagenase activity. Consistently, MT-MMPs are observed to localize at the LE membranes of migrating cells which clear a path for migration (Poincloux et al., 2009). Second, by degrading the ECM, MMPs trigger release of embedded growth factors and signalling molecules which promote migration. Examples of such ECM embedded molecules include TGF-β, FGF and VEGF. Specifically, MMP dependent shedding of HSPGs such as Sdc releases bound growth factors which increase migratory capability of the cell (Endo et al., 2003). Additionally, cleavage of Lans and collagen IV results in generation of ECM fragments with cryptic

sites which can promote migration (Favreau et al., 2014; Giannelli et al., 1997; Pirila et al., 2003; Xu et al., 2001). Lastly, MMPs disrupt cell-cell adhesion by degrading proteins which are responsible for holding cells together (Jia et al., 2014). Absence of cell-cell adhesion induces migratory capacity by triggering EMT (Micalizzi et al., 2010).

1.6 Role of guidance signalling and MMPs in formation of congenital heart diseases and cancer

Both guidance signalling and MMP promote heart formation in mammals. These key molecules also play a significant role in promoting development of cancer and metastasis. What follows below is a review of the current literature about the role of guidance signalling and MMP in shaping the embryonic heart and how these molecules regulate cancer formation.

1.6.1 Congenital heart diseases

Infants born with CHDs display several heart related defects including malformation in ventricles, atria, valves or blood vessels. Ventricular or atrial septum defects are the most common type of CHDs, which arise when the muscular walls of the septum separating the two ventricles develop holes (Gruber and Epstein, 2004). Detection of these defects before birth by ultrasound imaging has significantly decreased infant morbidity, however subtle heart malformations are difficult to diagnose by using imaging techniques and can cause late-onset complications. Therefore, discoveries of genetic pathways which regulate heart formation can allow the use of genetic testing to diagnose CHDs before birth. Vertebrate heart development models, such as zebrafish, chick and

mouse, have led to a detailed understanding of ventro-atrial septation defects (Eisenberg and Markwald, 1995; Nakajima et al., 2000; Stainier, 2001). Since early stages of heart development are conserved between vertebrates and invertebrates, *Drosophila* can be used as a model to gain insight in to the genetic mechanisms of CHD (Bodmer and Venkatesh, 1998).

Guidance signalling molecules play an essential role during invertebrate and vertebrate heart development. In zebrafish, a regulatory loop, which includes Slit, Robo and microRNA miR-218, is required for heart tube formation (Fish et al., 2011). Using morpholino knockdowns, Fish et al. demonstrated that endocardial and myocardial migration is disrupted in *slit2* and *robo1* morphants. Additionally, Slit and Robo are expressed in the mouse heart during looping morphogenesis and disruption of Slit/Robo signalling results in ventricular septum and valvular defects at birth (Medioni et al., 2010; Mommersteeg et al., 2015). Interestingly, defects such as thickening of the aortic valves or septum are also underlying causes for CHDs in humans. Whether Net, Fra/DCC and Unc5 are expressed in the vertebrate heart during embryogenesis or whether these molecules play a role during early cardiac assembly is currently unknown. However, studies conducted on *Drosophila* heart developmental models have suggested potential roles for Net and its receptors (Albrecht et al., 2011; Macabenta et al., 2013). Hence, in the future, their roles during vertebrate heart development might be uncovered .

Cardiac ECM undergoes extensive remodelling in vertebrates via the activity of MMPs2, MMP3, MMP7 and MMP9 which are upregulated in cardiomyocytes during early stages of development (Alexander et al., 1997; Brauer and Cai, 2002; Cai et al.,

2000; Cantemir et al., 2004). Temporally inhibiting or performing multiple MMP gene knockouts lead to formation of severe cardiac defects during development. For instance, inhibition of MMP2 activity by neutralizing monoclonal antibodies and broad spectrum inhibitors results in *cardia bifida* and other developmental defects in heart tube closure (Linask et al., 2005). Additionally, absence of MMP activity leads to narrowing of the aortic valve which results in CHDs such as aortic stenosis and regurgitation (Fondard et al., 2005). These defects are also commonly observed in humans when fibrinolytic within the heart valves is impaired (Natorska et al., 2013). Post heart tube formation, temporal inhibition of MMP2 resulted in a decrease in ECM remodelling in the mesocardium which disrupts looping morphogenesis in the heart (Linask et al., 2005). Therefore, it appears that several MMPs converge together and their autonomous activity is required to remodelling of the cardiac ECM in vertebrates. The role of MMPs during heart development in *Drosophila* however is currently unknown. However, due to the conservation of early developmental mechanism of formation, we hypothesize that MMP have a significant role in shaping cardiac morphogenesis in *Drosophila* as well.

1.6.2 Cancer metastasis

The axon guidance cues, Net and Slit, are implicated in the formation of human cancers (Mehlen et al., 2011). Misregulation of these signalling molecules has been reported in large number of human tumours and multiple studies conducted in mouse cancer models have demonstrated that the misexpression of Slits and Nets is associated with tumour invasion. Similar to their roles during axon guidance, Slit and Net and their respective receptors regulate tumour cell migration. The exact role of Net, Fra/DCC or

Unc5 in promoting tumour formation is not clear however Net-1 has been shown to stimulate cell migration in human melanoma, glioblastoma and pancreatic adenocarcinoma cells. Substantial evidence exists that Slit and its receptor Robo regulate tumour cell migration. Again however, the exact mechanisms are unknown. In some tumours, such as breast cancer and medulloblastoma cells, ectopic expression of Slit inhibits cell migration (Dickinson et al., 2004; Marlow et al., 2008). Conversely, in lung cancer cells and gliomas, Slit expression promotes cell migration in a Robo dependent manner (Mertsch et al., 2008). Therefore, genetic models in animals can greatly assist our understanding of how these molecules control CCM.

One of the trademarks of metastatic cancer cells is their ability to degrade their surrounding ECM and invade adjacent tissues. MMPs have been associated with cancer-cell invasion since early 60s. Strikingly, the mechanisms which promote MMP dependent cell migration during morphogenesis are conserved in tumour cell migration as well (Egeblad and Werb, 2002). For example, MMP dependent degradation produces Lan and collagen fragments which reveal cryptic sites within these molecules and induce tumour cell migration (Favreau et al., 2014; Giannelli et al., 1997; Xu et al., 2001). Additionally, degradation of the cell-cell junctional protein E-Cad by MMPs leads to EMT, which is the first step during metastasis (Noe et al., 2001; Zheng et al., 2009). Nevertheless, clinical trials which employ MMP inhibitors to combat tumour progression have been unsuccessful (Coussens et al., 2002). This is in primarily due to the limitation of our understanding of how multiple MMPs function collectively during this extremely complex process which occurs *in vivo*.

Nevertheless, the mechanisms behind how these molecules mediate migration have not been well established in morphogenetic models. It is likely that more specific therapeutic agents which target these molecules will be developed in the future and might contribute to halting the progression of cancer. However, to design effective drugs, more information is required about their cooperative activity under physiological conditions.

1.7 Objectives

The primary objective of my thesis was to characterise heart development as a model to dissect the mechanisms of CCM. As outlined in chapter 3, we set out to elucidate the mechanism of guidance signalling and its effect on LE behaviour during cardiac migration. By conducting fluorescent time-lapse imaging analysis, I developed an assay to quantitatively assess CCM during heart development. Since Slit and Robo are required for cell migration and these molecules promote heart morphogenesis in vertebrates, we analysed mutants of guidance signalling molecules, Slit, Net and their respective receptors to determine their roles during CCM of CBs. Literature suggests that Slit and Net activity is tightly inter-regulated. We additionally determined whether Slit and Net receptors interact genetically by carrying out time-lapse analysis in embryos where gene dosage of each receptor in *trans* was reduced by half. Following this, we carried out rescue experiment with receptor transgenes to establish the hierarchy between guidance signalling receptors. Since ECM remodelling plays a significant role in establishing the vertebrate heart, we wanted to determine its role during CCM and lumen formation and our results are presented in chapter 4. We applied our previously mentioned quantitative strategy to assess cell behaviour of CBs during CCM in wildtype

and *mmp* mutant embryos. By performing immunohistochemistry labelling of various polarity, luminal and ECM markers, we analysed MMP mutant embryos to determine whether cell polarity and lumen formation were disrupted. Further, we tested whether MMP activity is autonomously required in CBs by conducting RNAi based knockdown and rescue experiments. We also analysed larval heart morphogenesis in MMP mutants to determine whether defects accumulated during embryonic develop persist in later stages.

Methods and Materials

CHAPTER TWO

2.1 Fly Maintenance

All fly stocks were kept at room temperature (23-27°C) in specialized fly vials (Fisher, AS519) plugged with rayon (Fisher, 12 640 41). Flies were fed yeast agar medium containing: 1.8% of agar, 0.8% sodium potassium tartarate, 0.1% of dipotassium hydrogen orthophosphate and 0.05% each sodium chloride, calcium chloride, magnesium chloride and ferric sulphate was added to a 10% solution of sucrose. In a separate flask, dried yeast was added with deionized and distilled water to a final concentration of 5%. Both solutions were autoclaved and cooled down to 55°C and treated with methyl 4-hydrozybenzoate and acid mix solution [42% proprionic and 4.5% phosphoric acid]. Amplified stocks were flipped twice a week whereas a copy of each stock was flipped every 7-10 days.

2.2 *Drosophila* stocks

The following table lists all the fly stocks used in the study.

Table 2.1: List of all stocks used

Stock	Source	Stock Number
y^{1}, w^{1118}		
sli^{2}/CyO^{lacZ}	Bloomington Stock Center	3266
$robo^{1}/CyO^{lacZ}$	Bloomington Stock Center	8755
lea^{2}/CyO^{lacZ}	Bloomington Stock Center	3102
$robo^{1}$,$leak^{54-14}/CyO^{lacZ}$	Greg Bashaw	
fra^{3}/CyO^{GFP}	Bloomington Stock Center	8813
fra^{4}/CyO^{GFP}	Bloomington Stock Center	8743
UAS-fra	Bloomington Stock Center	8814
UAS-fra RNAi	Vienna RNAi Center	14401
Df(1)NP5 [*netA*,*netB*]	Bloomington Stock Center	2179
UAS-liveActin-GFP	Bloomington Stock Center	35544
mef2-Gal4/TM3	Bloomington Stock Center	50742
UAS-*moesin*-mCherry	Thomas Millard	

Stock	Source	Stock Number
tail-up-F4-GFP	Robert Schulz	
basigin-GFP trap	Bruce Reed	
yet-1-GFP trap	Bruce Reed	
unc5^{8}/CyO^{GFP}	Greg Bashaw	
UAS-*unc5* RNAi	Greg Bashaw	
UAS-*robo1, robo2, robo3*-RNAi (referred to as RoboRNAi)	Greg Bashaw	
UAS-slit dsRNA	Vienna RNAi Center	20210
MMP1^{2}/CyO^{lacZ}	Andrea Page-McCaw	
MMP1^{Q112}/Cyo^{lacZ}	Andrea Page-McCaw	
MMP2^{DFuba1}/Cyo^{GFP}	Andrea Page-McCaw	
MMP2^{W307}/CyO^{GFP}	Andrea Page-McCaw	
UAS-*timp*	Andrea Page-McCaw	
UAS-*mmp2*	Andrea Page-McCaw	
UAS-*mmp2* RNAi	Andrea Page-McCaw	
viking-GFP-trap	Andrea Page-McCaw	
sevenup-GAL4	Bloomington Stock Center	49682
UAS-*gfp* RNAi	Bloomington Stock Center	9331
pi3k92e/TM3^{YFP}	Bloomington Stock Center	25900
UAS-*phospholipaseC*-PH	Smith Harlan	
UAS-*grp1*-GFP	Herve Guillou	

2.3 Live imaging protocol

Wildtype and mutant embryos were imaged in *tup-f4-GFP, mef2GAL4, UAS-Moesin-mCherry* background to visualize the actin cytoskeleton at the LE (mCherry) and CB nuclei (GFP). The Hanging Drop Protocol (Reed et al., 2009) was used to mount embryos at stages 14, 15 and 16. Time-lapse movies and still images of live embryos were acquired using Leica SP5 confocal microscope. Every 1 minute, z-stack of 20-30 sections at 1 μm intervals was acquired. Images were de-speckled and projected using Leica LSM software. There images were later extracted from live movies and further processed and quantified using ImageJ and Adobe Photoshop.

2.4 Quantification of filopodial activity, lamellopodial activity and migration velocity protocol

To quantify the filopodial activity, images from movies were extracted from at least 5 minutes apart. Segment boundaries were identified and numbers of filopodia extended by segments A2-A7 were quantified. Distance from the center of the segment to the dorsal midline was measured as well to quantify the relationship between filopodial activity and distance between bilateral LE.

To measure the migration velocity of each bilateral row, distance between the most central CB on each LE was measured at stage 15 (d_O) and stage 16 (d). To calculate the velocity of migration (assuming both LEs migrated with equal velocities), d_O was subtracted by d and divided by twice the length of the movie (30 mins).

Active membrane of the LE was identified by the presence of cellular extensions extended towards the midline and length of this apical domain was measured. Length of active LE was obtained by summing the cross-sectional diameter of apical membranes in active cells separately for each bilateral row. LE activity is presented as a percentage of active LE over the sum of total LE.

2.5 Embryo staining protocol

2.5.1 Embryo collection

30-50 virgin females and 20-30 male flies were placed in houses and allowed to mate and lay eggs on small agar plates with small quantities of yeast to promote egg laying. Agar plates were switched twice a day for the duration of 4 days and aged at 25°C

for a total of 20-22 hours from the start of egg laying and then stored at 4^0C for a maximum of 72 hours. Prior to fixation embryos were removed from the 4^0C fridge and were incubated at 25^0C for 90 minutes. For RNAi experiments, embryos were collected for 8 hours at 25°C and then aged for 8 hours at 29°C.

2.5.2 Fixation

Embryos were soaked in 50% bleach solution for 5 minutes. Using sieves, embryos were collected and rinsed with distilled water. Embryos were set in a rotator for 20 minutes in the fixation solution (0.5ml 37% formaldehyde, 4.5ml phosphate buffer solution (PBS), 5 ml Heptane). Subsequently, embryos were cracked and washed in methanol to remove fixative and rehydrated in PBS containint 0.1% Triton-X (PBT). Occasionally embryos were stored in methanol at 4°C for 2-3 weeks prior to rehydration.

2.5.3 Immunolabelling

The protocol used for immunostaining embryos and larvae was adopted from Patel et al. (Patel, 1994) . Rehydrated and fixed embryos were incubated in 1/15 Normal Goat Serum (NGS) in PBT for 30 minutes. Primary antibody was added and the sample was incubated in a shaker overnight or over the weekend in 4°C. The embryos were then, washed in PBT for 4 to 6 hours with periodic washes every 30-45 minutes. Embryos were again blocked with 1/15 NGS in PBT for 30 minutes. Secondary antibody was added and the mixture was incubated at 25°C on an orbital shaker for 2 hours. To protect the fluorescent antibody from light, labeling tunes were wrapped in tinfoil. Embryos were further washed with PBT and placed on a rotator overnight. If multiple primary antibodies

which were raised in the same animal species were used, sequential reactions were performed and antibody concentrations were adjusted. Finally, PBT was replaced with 70% glycerol in which embryos were stored until mounted on microscope slides.

2.5.4 Antibodies used

Polyclonal primary antibodies

Polyclonal primary antibodies were used at the following dilutions: rabbit anti-Mef2 (1:5000) (Vanderploeg, 2014), rabbit anti-Dg (1:600) (Vanderploeg, 2014), chicken anti-GFP (1:1000) (Cedarlane, ab13970).

Monoclonal primary antibodies

Following mouse monoclonal antibodies were used at 1:30 dilution: anti-βPS (Brower D. DF.6G11-s), anti-Discs-large (Dlg) (Goodman C. 4F3 anti-discs large), anti-Pericardin (Gratecos D. EC11 anti-Pericardin), anti-Slit (Artavanis-Tsakonas S. C555.6D-s) and anti-Robo (Goodman C. 13C0 antiRobo-s).

Secondary antibodies

The following fluorescent secondary antibodies were used: Alexa 488 anti-Chicken, Alexa 488 anti-Rabbit (Invitrogen A11034), Alexa 546 anti-Mouse (Invitrogen A11030), Alexa 546 anti-Rb (Invitrogen A11010), Alexa 594 anti-Mouse (Invitrogen A11032), Alexa 594 anti-Rabbit (Invitrogen A11037), Alexa 594 anti-Rat (Invitrogen A11007), Alexa 647 anti-Chicken (Invitrogen A21449), Alexa 647 anti-Rabbit (Invitrogen A21245).

2.5.5 Mounting

Embryos were mounted on a specialized microscope glass slide. To modify the glass slide, two coverslips placed in parallel 5mm apart from each other. The coverslips were fixed with a small amount of nail polish on the outer edges. 20µl of glycerol solution containing immunolabelled embryos was placed in the gap between coverslips. A 22x22 No. 1.5 coverslip was placed on top of the gap containing glycerol-embryo solution.

2.5.6 Imaging and processing

Fluorescent labelled embryos were imaged using Leica SP5 upright confocal microscope. Z stacks which were 1 µm apart were acquired. Fixed specimen frontal images displayed here are a merge of 3-10 sections. Images of z-sections are single slice images taken at the heart tin-man expressing CBs. All images were blurred using the Leica LSM software and further processed using ImageJ.

2.6 Larval staining protocol

2.6.1 Larvae collection

Larvae were collected on apple juice agar plates and aged to first or second instar larvae. Larvae were genotyped using YFP-marked balancer chromosomes and fifty larvae per genotype were transferred to food plates. Larvae were aged until third instar and then collected for dissections.

2.6.2 Fixation and immunolabelling

Third instar larvae were dissected by hand and fixed in a solution containing 4% paraformaldehyde, 4% sucrose in PBS buffer. The larvae were transferred to a well and incubated with fixative solution for 2 hours at room temperature. The protocol used for immunostaining larvae was adopted from Patel et al. and Bogatan et al. (Bogatan et al., 2015; Patel, 1994). The larvae were then transferred to a well containing PBT and placed on a shaker for 3 hours with periodic washes every hour. Following the washes, larvae were placed in fresh 100µl PBT with 10µl NGS and larvae were incubated for 30-60 mins. 5 µl of αβPS antibody was added to the solution and larvae were incubated at 4° overnight. Next day, larvae were washed again for 3 hours with periodic washes every hour after which they were blocked for 30-60 mins in 100µl PBT with 10µl NGS. 1µl of secondary anti Mouse Alexa-647 and Phalliodin Alexa-546 were added and larvae were incubated overnight. Next day, larvae were washed with PBT for a minimum of 2 hours. The larvae were then transferred into a solution containing 50% glycerol and 50% PBS and stored for a maximum of one week.

2.6.3 Mounting, imaging and processing

Imaging chambers made for embryos were used for larvae as well. For confocal imaging larvae were placed in this space and the chamber was sealed by another coverslip on the top and nail polish on the sides. Similar to embryos, fluorescent labelled larvae were imaged using Leica SP5 upright confocal microscope. Z stacks were acquired which were 2 µm apart. Frontal images are a merge of 15-20 z sections. All images were blurred using the Leica LSM software and further processed using ImageJ.

2.7 Western blot analysis

For the Western blots, ten stage 17 embryos were genotyped based on the presence balancer expressing GFP and collected. Membranes were probed with primary antibodies mouse anti-Slit (1:100, DHSB) or anti-βTubulin (1:500, DHSB E7) and horseradish peroxidase-conjugated anti-mouse secondary antibody (1:5,000, Jackson ImmunoResearch 115-035-166). Western blots were developed using a Pierce ECL Western Blotting Substrate (Thermo Scientific 32106) and Amersham Hyperfilm ECM (GE Healthcare 28906838) and scanned. Images were analyzed and quantified using ImageJ, with βTubulin used as a loading control reference.

Slit and Netrin signalling is required for leading edge motility of the cardioblasts during embryonic heart development

CHAPTER THREE

3.1 Abstract

CCM is the coordinated movement of cells which organizes tissue morphogenesis. The embryonic development of the vertebrate and *Drosophila* hearts are both characterised by the coordinated medial migration of a bilateral cluster of mesodermal cells. In *Drosophila*, the CBs form cohesive bilateral rows and migrate as a unit towards the dorsal midline to form the DV. Here, we characterized the behaviour of the LE of migrating CBs. Filopodial activity increase as the CBs approach the midline. In the absence of Slit and Net signalling, filopodial and lamellopodial activity is significantly reduced. Robo and Fra functionally complement each other to induce filopodial and lamellopodial dynamics. Embryos with reduced function in both the Slit and Net pathways have a more than additive phenotype, suggestive of a common function in promoting LE motility of the CBs. Additionally, Slit/Robo signalling is required for promoting both migratory dynamics and lumen formation, whereas Net/Fra signalling is required to induce only protrusive activity. Removing Slit, Robo and Fra in CBs increases the frequency to EMT revealing their role in stabilization of lateral junctions during CB migration. Overall, we propose that by functioning through a common pathway, Slit and Net signals coordinate the collective behaviour of CBs prior to lumen formation at the midline.

3.2 Introduction

CCM is a process by which cells or tissues migrate as a group towards a destination. Well studied examples of CCM include migration of BC during oogenesis (Bianco et al., 2007) and migration of tracheal progenitors in *Drosophila* (Schottenfeld et al., 2010), pLL migration in Zebrafish (Dona et al., 2013), formation of mammary ducts in mice (Affolter et al., 2003), and tumour invasion strands (Friedl and Gilmour, 2009). Although CCM classification are still in discussion (Aman and Piotrowski, 2009), three basic prerequisites define CCM: a common direction of migration dictated by positional cues, adhesion between cells in a cluster or LE, and structural modification of the ECM (Friedl and Gilmour, 2009). Heart development in vertebrates and *Drosophila* also involves stages of CCM of cardiomyocytes and therefore can be utilized as a genetic model (Bier and Bodmer, 2004; Bodmer and Venkatesh, 1998).

Drosophila heart development initiates after progenitor cells from the lateral mesoderm are specified to become CBs, and then form bilateral rows on each side of the embryo. The CBs collectively migrate towards the dorsal midline in tandem with the dorsal ectoderm to meet their contralateral partners and form the DV (Tao and Schulz, 2007). During migratory stages, Slit and Robo mediated signalling is required to maintain lateral adhesions in CBs (MacMullin and Jacobs, 2006; Santiago-Martinez et al., 2008). The role of Net and its repulsive receptor, Unc5, during migratory stages is not known. However, Fra, an attractive receptor for Net, and a homolog of vertebrate DCC (Shekarabi et al., 2005), localizes to the apical junctions of CBs and is required for the formation of dorsal and ventral outgrowths (Macabenta et al., 2013). During late stages of

DV formation both Slit and Net mediated signalling play an essential roles during lumen formation through their respective repulsive receptors Robo and Unc5. These receptors localise at the luminal surface and promote formation of non-adhesive domain which encompasses the lumen (Albrecht et al., 2011; Macabenta et al., 2013; MacMullin and Jacobs, 2006; Qian et al., 2005; Santiago-Martinez et al., 2008). During axonal guidance Robo through direct associate with Fra silences Net based attraction to establish the ladder like structure of the commissures (Hiramoto and Hiromi, 2006; Stein and Tessier-Lavigne, 2001). This type of hierarchical relationship between guidance receptors Robo and Fra/DCC has been demonstrated in zebrafish as well (Zhang et al., 2012). Nevertheless, the role of Slit and Net signalling in promoting CCM of CBs is unclear, and whether these pathways intersect to direct CB migration is not known.

In this study, we employed an *in vivo* approach to study the role of guidance signalling molecules in promoting CCM of CBs during embryogenesis. Recently developed live imaging and non-invasive embryo handling techniques (Kanca et al., 2014; Reed et al., 2009), enabled us to track the migration of CBs in guidance signalling mutants and quantitatively evaluate CB protrusive activity. We demonstrate that in wildtype embryos the filopodial and lamellopodial activity of the CB LE increases as the distance between the contralateral rows decreases. Our data suggests a role for guidance cues in promoting LE motility. We demonstrate that the increase in filopodial and lamellopodial activity in CBs is dependent on Slit, Robo, Robo2 and Net, Fra, Unc5 mediated signalling. Additionally, we provide evidence that both Robo and Fra function in a common pathway to promote CB LE motility. We demonstrate that in the absence of

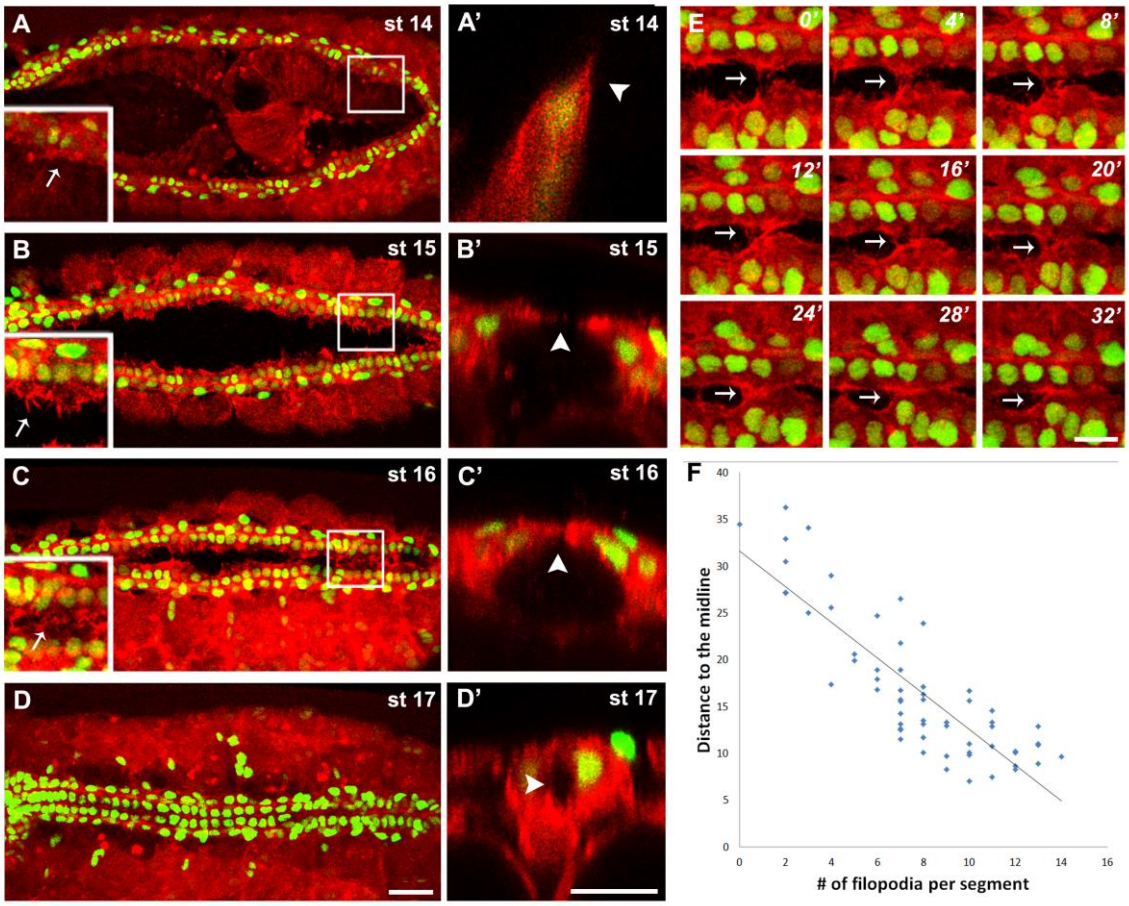
guidance signalling, migratory capacity of the CB is affected and can lead to EMT of cardiac cells. Altogether, our results demonstrate a requirement for both Slit and Net based signalling during CCM of CBs in *Drosophila*.

3.3 Results

3.3.1 Extension of cytoplasmic processes increases as the cardioblasts approach the midline

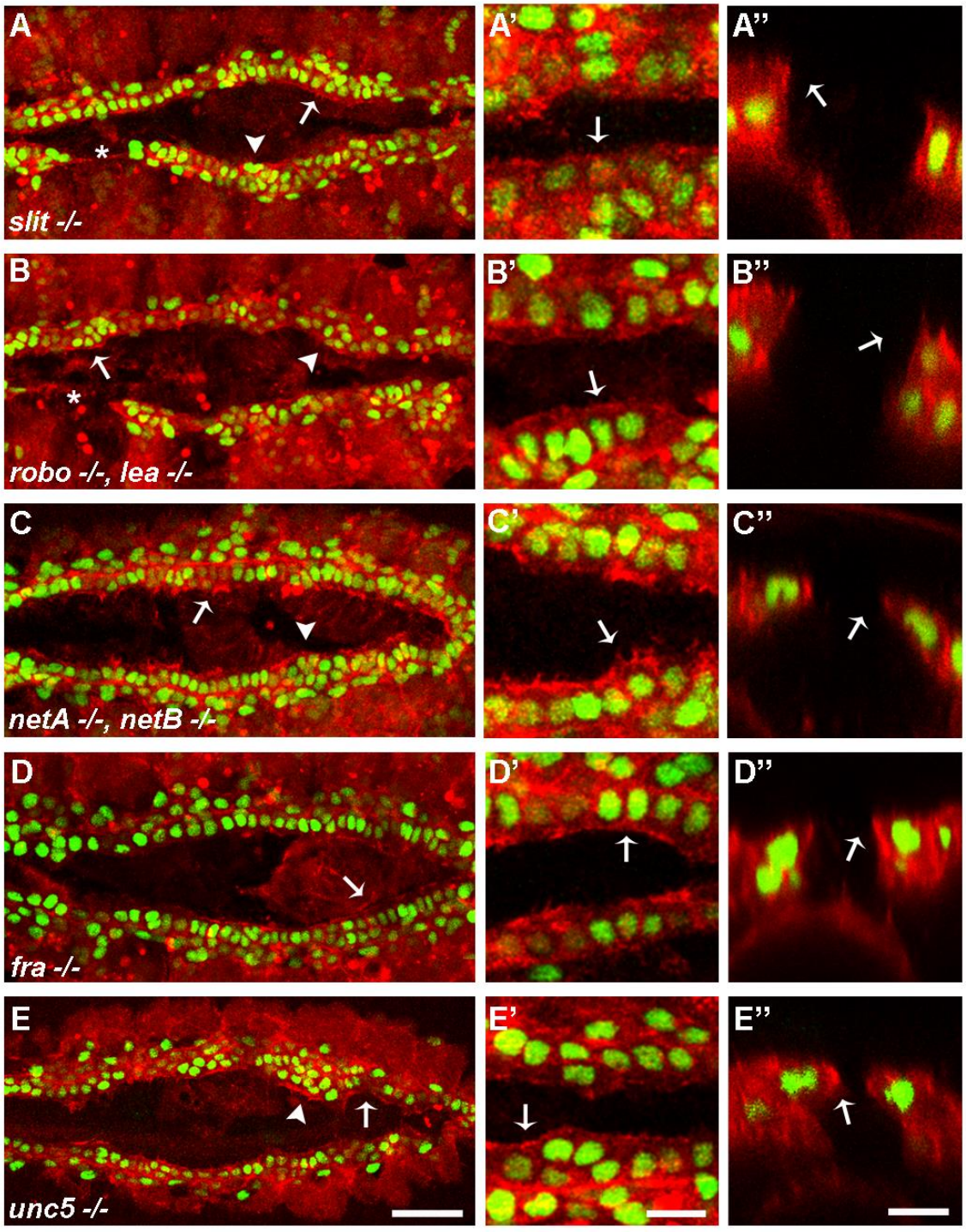
An important hallmark of migratory cells is the presence of protrusive activity at the LE of the cells. Protrusions are of 2 major types: filopodia - thin finger-like structures comprised of parallel bundles of actin, and lamellopodia - thin sheet-like structures filled with branched networks of actin (Mattila and Lappalainen, 2008). The presence or absence of cytoplasmic protrusion at the LE is interpreted as a response to attractive or repulsive guidance cues (Albuschies and Vogel, 2013; Gallo and Letourneau, 1998, 2004). To visualize the protrusive activity of the CBs we expressed a mCherry tagged C-terminal actin binding domain of Moesin (Millard and Martin, 2008). We opted to not employ tagged actin due to reports of dominant effects (personal observations; (Geisbrecht and Montell, 2004) (Fulga and Rorth, 2002)) and established that the sensitivity and distribution of each marker was the same (Fig 3.7, A). We observed no dominant effects from overexpressing Moe-mCherry constructs in wild-type embryos as also reported by others (Millard and Martin, 2008). We generated time-lapse movies of wildtype embryos expressing *tup*-GFP and UAS-*moesin*-mCherry under the control of *mef2*GAL4 (Fig 3.1). At stage 14 when CBs are on the lateral side of the embryo,

Figure 3.1. The cardioblasts leading edge extends multiple filopodia and lamellopodia. Timelapse images of live embryos expressing tailup-GFP and UAS-*moesin*-mCherry under the control of *mef2*GAL4 driver in wildtype embryos. (A,A') At stage 14, the LE of CBs is minimally active with few filopodia and lamellopodia extended towards the midline (arrow). Dorsal apical domain protrudes towards the ectodermal LE (arrowhead). (B,B') As the CBs migrate closer to the midline at stage 15, LE activity increases at the most posterior and anterior parts of the bilateral rows (arrow). CBs adopt a pear-shaped morphology and extend cytoplasmic processes from the dorsal-apical side of the cell towards the midline (arrowhead). (C, C') At stage 16, when the contralateral CBs are adjacent, activity of the LE is highest (arrow). Initial contact occurs between contralateral filopodia at this stage (arrowhead). Enriched localization of actin reporter, Moe-mCherry, is observed at the basal and apical sides of (arrows B', C', D'). (E) Time-lapse images of the A3 embryonic heart segment are shown before and after the initial contact between bilateral LEs. CBs extend lamellopodia and filopodia towards the contralateral partner in anticipation of contact (arrows). (F) Scatter plot demonstrating the inverse linear relationship between 'distance to the midline' and 'number of filopodia' extended by cells within heart segments (R^2=0.713). In this and subsequent dorsal view images posterior of the embryo is to the right and micron scale is 25 μm. In all cross sectional images dorsal is upwards and micron scale is 10 μm. In all high magnification images micron scale is 10 μm.


A
st 14
A'
st 14
B
st 15
B'
st 15
C
st 16
C'
st 16
D
st 17
D'
st 17
E
0'
4'
8'
12'
16'
20'
24'
28'
32'
F
Distance to the midline
# of filopodia per segment
0
5
10
15
20
25
30
35
40
0
2
4
6
8
10
12
14
16

filopodial activity of the LE was low and few filopodia were observed (Fig 3.1, A, F). Cross sectional images further demonstrated that apical extensions were short and CBs had a rounded morphology (Fig 3.1, A'). At stage 15, we observed increased filopodial activity at the most posterior and anterior segments of the LE whereas the central segments, which were further apart, remained relatively inactive (Fig 3.1, B,). At stage 16, most of the CBs were active with multiple filopodial and lamellopodial extensions along the entire length of the LE (Fig 3.1, C, Movie 3.1). Also, at stage 16, all CBs had adopted a pear-shaped morphology with dorsal-apical extensions oriented towards the midline, whereas at stage 15 only the most anterior and posterior CB had an extended morphology. At the pre-luminal (Vanderploeg et al., 2012) and luminal domain however Moe-mCherry intensity was low, compared to the junctional domain (Fig 3.1, B', C', D'). To quantify the filopodial activity, we counted the number of filopodia extended by segments of the CB at a given time and plotted against the distance of that segment from the midline (Fig 3.1, F). We noted a negative correlation between the distance to the midline and number of filopodia at a given segment ($R^2 = 0.713$). We did notice that when the cardiac segments were further than 25μm from the midline, the average number of filopodia per segment was 2.8. However, when the cells crossed the 25 μm threshold point, the average number of filopodia per segment increased to 6.7. We did not notice any difference in filopodial extension frequency when we compared segments from the heart proper and aorta (data not shown).

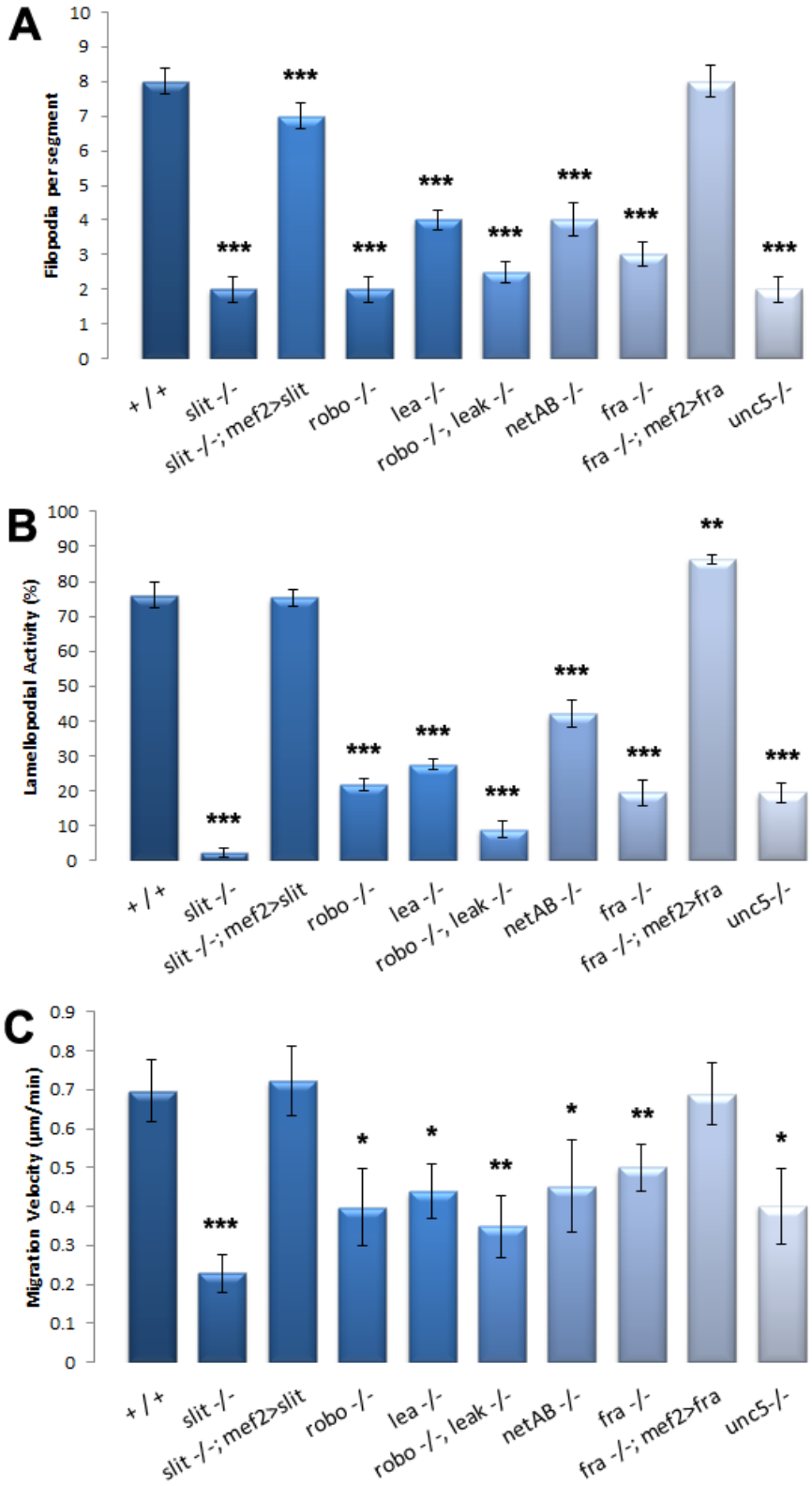
Figure 3.2. Slit/Robo and Netrin/Frazzled/UNC5 signalling is required for LE activity of the cardioblasts.. Timelapse images of living embryos expressing *tailup-GFP* and *UAS-Moesin*-mCherry under the control of *mef2GAL4* driver. (A-E) Dorsal images of the developing heart at stage 15. *slit, robo,leak, fra*, *netA,netB* and *unc5* mutants display reduced filopodial and lamellopodial activity (A-E arrows). The sequential localization of cardiac nuclei relative to their lateral partner CBs is disrupted in mutants and cell clusters are observed at several positions (A-E arrowheads). Gaps within the LE are observed in both *slit* and *robo,leak* mutants embyos (A,B astericks). (A'-E') High magnification images of the most posterior segment (A2-A3) in mutant embyos at stage 16. (A"-E") Cross sectional images of CBs during stage 16. CBs are rounded and fail to extend outgrowth in anticipation of contact with the contralateral partner cell (A''-B'', D''-E'' arrows). In *netA,netB* mutant embryos not all CBs were rounded and some were capable of extending small apical protrusions (C" arrow).


A
A'
A''
slit -/-
B
B'
B''
robo -/-, lea -/-
C
C'
C''
netA -/-, netB -/-
D
D'
D''
fra -/-
E
E'
E''
unc5 -/-

3.3.2 Slit/Robo/Robo2 and Netrin/Frazzled/Uncoordinated-5 are required for extension of cytoplasmic protrusions by the CBs

Signalling via Slit and Net is required for CB polarization and lumen formation (Albrecht et al., 2011; Macabenta et al., 2013; MacMullin and Jacobs, 2006; Qian et al., 2005). Expression of the secreted morphogen, Slit, increases as the CB approach the dorsal midline (MacMullin and Jacobs, 2006; Qian et al., 2005; Santiago-Martinez et al., 2008). As this parallels the increase in LE activity, we inquired whether the increase in LE activity was dependent upon guidance signals. We analysed time-lapse movies of *slit*, *robo*, *leak* (encoding Robo2), and *robo,lea* double mutants. Filopodial and lamellopodial activity of the CBs was significantly reduced in all *slit*, *robo* and *lea* mutant combinations tested (Fig 3.2, Fig 3.3, Fig 3.7, B, Table 3.1, Movie 3.2 and 3.3). Cross sectional images of *slit,* and *robo,lea* mutants revealed the rounded morphology of the CBs with some visible extension towards the overlaying ectoderm (Fig 3.2 A"-B"). Additionally, gaps along the LE were commonly observed in *sli* and *robo,lea* mutants (Fig 3.2 A,B). The inverse relationship between distance to the midline and number of filopodial extensions is also absent in *slit* and *robo,lea* mutants (Fig 3.8 A,F). Next we reasoned that if cytoplasmic protrusions assist the migrating cell by generating traction for movement (Mattila and Lappalainen. 2008), the velocity of the CB LE should be reduced in guidance signalling mutants. Accordingly, we observed a significant drop in migration velocity in *slit*, *robo*, *lea* and *robo,lea* mutants (Fig 3.3, B, Table 3.1). Furthermore, overexpressing full length Slit transgene in a *slit* mutant background rescued filopodial activity, lamellopodial activity, migration velocity and lumen formation (Fig 3.4 A-A').

Figure 3.3. Quantification of the migratory behaviour of cardioblasts. (A) Filopodial density is significantly reduced in *slit, robo, Net, Fra* and *unc5* mutants. Overexpression of Slit in *slit* mutant background rescues filopodial numbers relative to *slit* mutants. Overexpressing Fra in *fra* mutant background rescues filopodial numbers to wildtype levels. (B) LE activity is significantly reduced in *slit, robo, Net, Fra* and *unc5* mutants. Overexpressing Slit in *slit* mutant background rescues lamellopodial activity to wildtype levels. Overexpressing Fra in *fra* mutant background significantly increases lamellopodial activity relative to wildtype levels. (C) Migration velocity is significantly reduced in *slit, robo, Net, Fra* and *unc5* mutants. Overexpressiong Slit and Fra in *slit* and *fra* mutant background, respectively, rescues migration velocity to wildtype levels. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.Here and in subsequent graphs error bars represent standard error.


A
Filopodia per segment
0
1
2
3
4
5
6
7
8
9
10
***
B
Lamellopodial Activity (%)
0
10
20
30
40
50
60
70
80
90
100
**
***
C
Migration Velocity (µm/min)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
*
**
+ / +
slit -/-
slit -/-; mef2>slit
robo -/-
lea -/-
robo -/-, leak -/-
netAB -/-
fra -/-
fra -/-; mef2>fra
unc5-/-

These results demonstrate the absolute requirement of Slit mediated signalling in promoting LE motility during CCM of CBs.

Next we tested the requirement of another guidance signalling pathway mediated by Net and its receptors Fra and Unc5 in promoting LE motility. In *fra* and *unc5* mutants, CBs appear rounded with minimal extensions towards the midline (Fig 3.2 D-D", E-E"). In *netA,netB* mutants, we noted the presence of small apical extensions (Fig 3.2 C"). Furthermore, we noted a significant reduction in filopodial activity, lamellopodial activity and migration velocity in *netA,netB, fra* and *unc5* mutants which suggested to us that these molecules are required for inducing LE motility similar to Slit, Robo and Robo2 (Fig 3.2, Table 3.1, Movie 3.4, 3.5, 3.6). Consistently, the inverse relationship between distance to the midline and number of filopodial extensions was also absent in *netA,netB, fra* and *unc5* mutants (Fig 3.8, C,E). However, in *netA,netB* mutans, filopodial and lamellopodial activity was higher compared to *fra, unc5, slit* and *robo,lea* mutants (Fig 3.2 C, Table 3.1). These results suggested that filopodia and lamellopodia can be induced in the absence of Net. However, Net is required for maximal protrusive activity of the LE. Overexpressing a Fra transgene in the CBs in *fra* mutant background restored filopodial activity, lamellopodial activity, migratory velocity and lumen formation (Fig 3.3 A-C, Fig 3.4 B-B"). However, lamellopodial activity was significantly increased compared to wildtype in rescued embryos (Fig 3.3 B). Other defects such as gaps (data not shown), cell clumping, and improper linear alignment of the LE were observed in *netA,netB, fra* and *unc5* mutants which further indicates that, similar to Slit and Robo signalling, Net signalling is required to promote CCM of the CBs (Fig 3.2 C-E).

To verify these results we conducted RNAi mediated protein depletion. Severe reduction in migration velocity and filopodial/lamellopodial extension was observed subsequent to Robo and Robo2 depletion (Fig 3.9 D-D'). In embryos where Slit dsRNA was expressed in the CBs, filopodial and lamellopodial activities were significantly reduced however migration velocity was unaffected. Nevertheless, gaps were formed interrupting the continuity of the LE (Fig 3.9 C-C'). Expression of Fra dsRNA in CBs resulted in decreased filopodial and LE activity, but did not affect migration velocity (Fig 3.9 F,F'). When Unc5 dsRNA was expressed in the CBs, a decrease in filopodial activity, lamellopodial activity and migration velocity was not observed (Fig 3.9 G, G').

3.3.3 Robo and Frazzled synergistically promote extension of cytoplasmic protrusions

Cross talk between attractive and repulsive guidance signals has been demonstrated in many morphogenetic processes (Derijck et al., 2010; Krishnan and Alam-Nazki, 2011; Ruediger et al., 2013; Ye et al., 2010). For example, during salivary gland morphogenesis Slit and Net antagonistically regulate guidance of migratory cells (Kolesnikov and Beckendorf, 2005). The interplay of these signalling pathways has not been studied in the context of heart development. Since both Slit and Net are required for apical membrane motility during heart development, we hypothesized that Slit and Net signalling pathways converge to promote filopodial/lamellopodial activity and lumen formation. To address this hypothesis, we examined whether a synergistic interaction between Slit receptor Robo and Net receptors, Fra and Unc5, exists

Figure 3.4. Rescue of outgrowth and lumen formation in guidance signalling mutants. Transgenes were expressed using *mef2GAL4* in mutant backgrounds. (A-A'') Expressing full length Slit in *slit* mutant embryos rescues filopodial behaviour (A arrow), outgrowth (A' arrowhead) and lumen formation (A'' arrow). (B-B'') Expressing Fra in *fra* mutant rescues filopodial extensions (B arrow), outgrowth (B' arrowhead) and lumen formation (B'' arrow). (C-C'') Overexpressing Robo in *fra* mutant rescues filopodial extensions (C arrow) outgrowth (C' arrowhead) and lumen formation (C'' arrow). (D-D'') Overexpressing Fra in *robo* mutant rescues filopodial extensions (D arrow), outgrowth (D' arrowhead) but not lumen formation (D'' arrow).

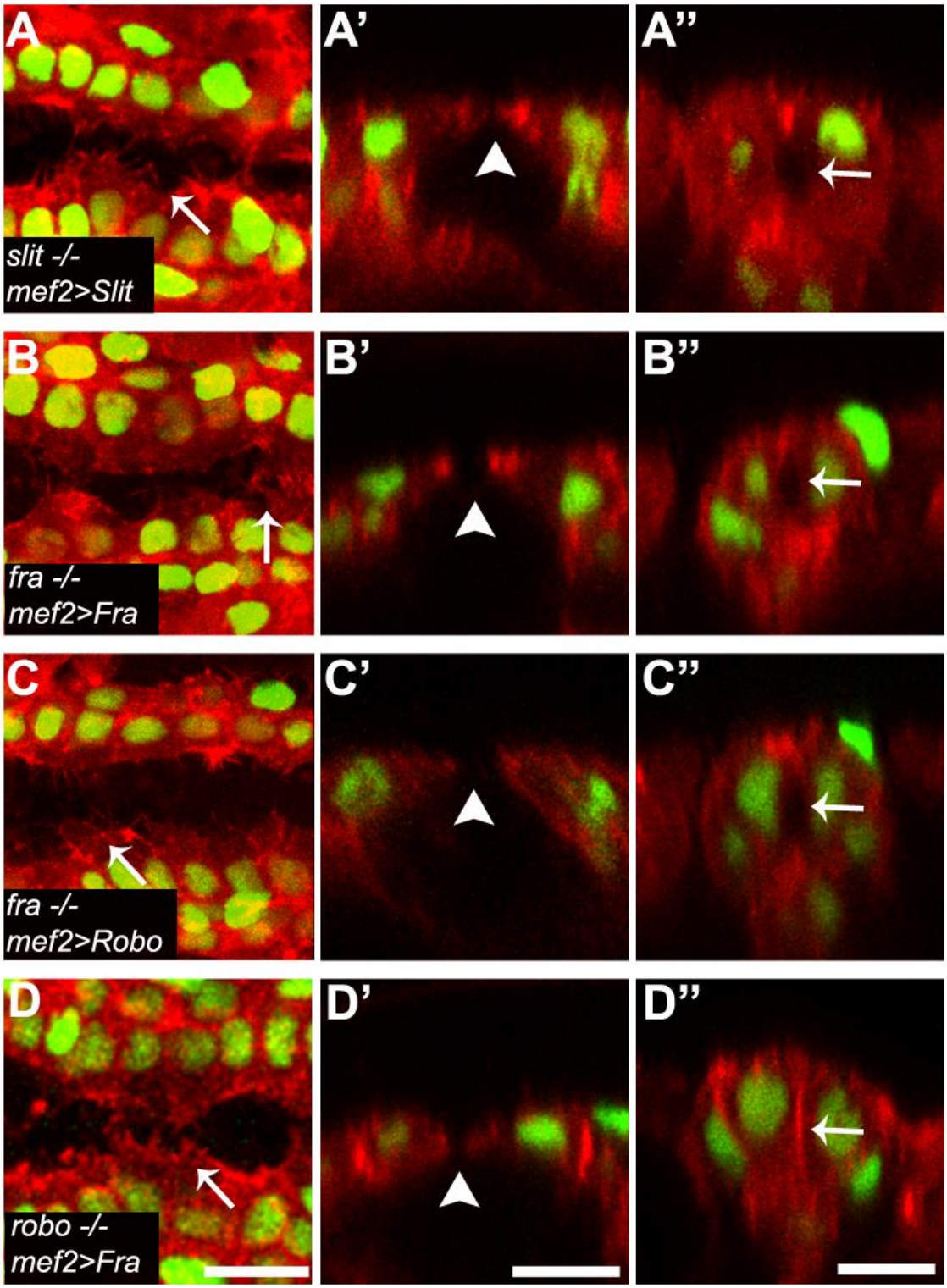
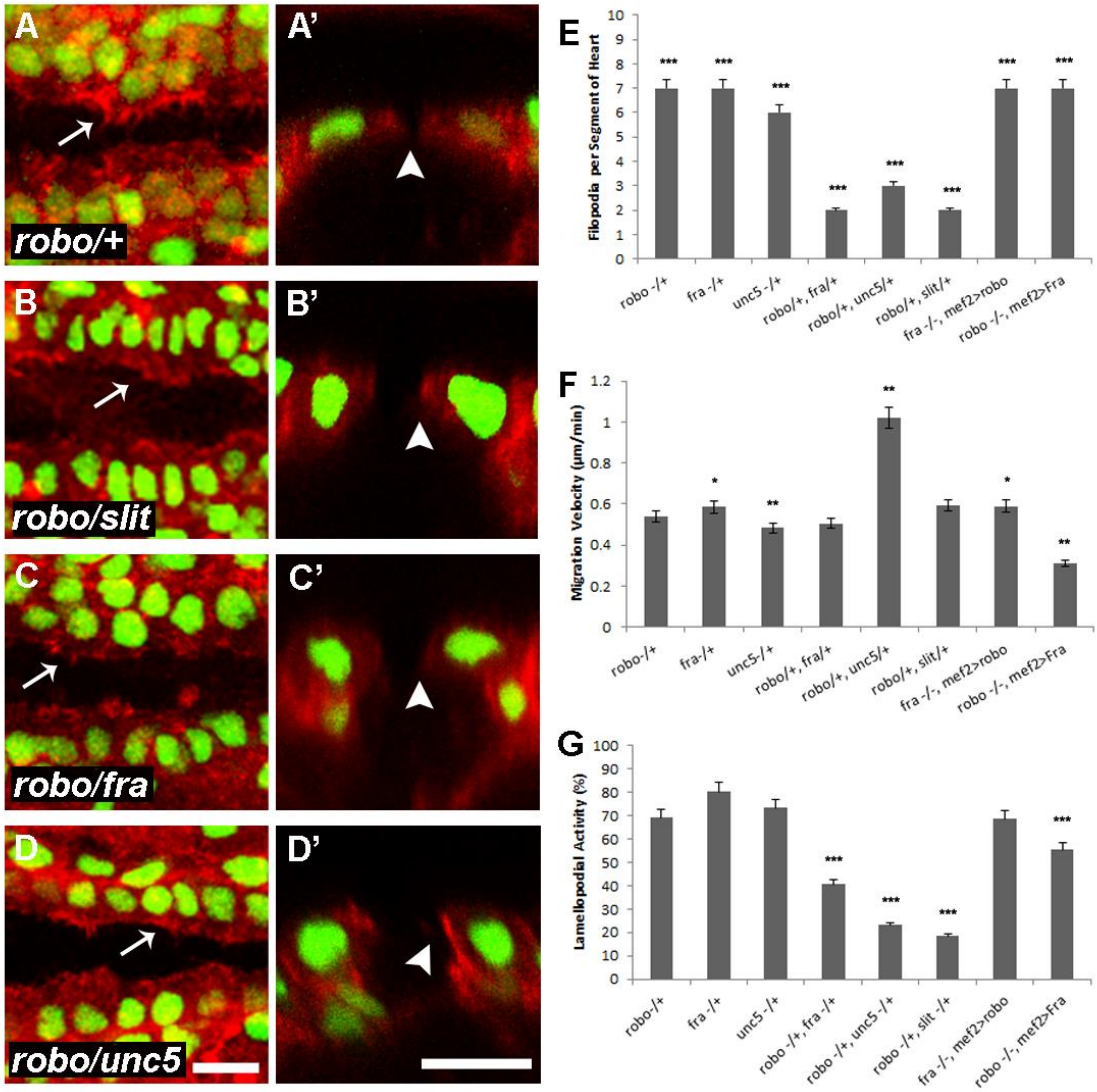

A
A'
A''
slit -/-
mef2>Slit
B
B'
B''
fra -/-
mef2>Fra
C
C'
C''
fra -/-
mef2>Robo
D
D'
D''
robo -/-
mef2>Fra

Figure 3.5. Slit receptor Robo synergistically promotes CB migratory behaviour by interacting with Netrin receptors Frazzled and Unc5. (A,B,C,D) High magnification images of *robo -/+, robo -/+,slit -/+, robo -/+,fra -/+* and *robo -/+,unc5 -/+* transheterozygotic mutants. Filopodial and lamellopodial activity is reduced (arrows). (A',B',C',D') Cross sectional images of *robo/slit* (B'), *robo/fra* (C') and *robo/unc5* (D') show shorter dorsal apical protrusion towards partner cells compared to *robo/+* control (A'). (E,F,G) Quantification of filopodial density, migration velocity and LE activity in transheterozygotic mutants. Filopodial and lamellopodial activity is significantly reduced in *robo/slit, robo/fra* and *robo/unc5* double heterozygotes but not in *robo -/+, fra-/+* and *unc5 -/+*single heterozygotes. Migration velocity was not affected in *robo -/+, robo -/+,slit -/+, robo -/+,fra -/+* and *robo -/+,unc5 -/+* however a significant reduction was observed in *fra -/+*and *unc5 -/+* embryos compared to wildtype. Overexpressing Robo in *fra* mutant background rescued filopodial and lamellopodial activity, and migration velocity relative to *fra* mutants. Overexpressing Fra in *robo* mutant background rescued filopodial and lamellopodial activity relative to *robo* mutants.


A
A'
robo/+
B
B'
robo/slit
C
C'
robo/fra
D
D'
robo/unc5
E
Filopodia per Segment of Heart
robo -/+
fra -/+
unc5 -/+
robo/+, fra/+
robo/+, unc5/+
robo/+, slit/+
fra -/-, mef2>robo
robo -/-, mef2>Fra
F
Migration Velocity (µm/min)
G
Lamellopodial Activity (%)
robo-/+, fra -/+
robo -/+, unc5 -/+
robo -/+, slit -/+

and whether the interaction between these receptors is required for migration and lumen formation in the CBs. We opted to use a gene dosage approach to probe for non-additive phenotypes suggestive of functional interaction between gene products. Embryo heterozygous for *robo* served as a sensitized background since embryos missing one copy of the *robo* gene do not exhibit noticeable migratory defects, however the filopodial activity is significantly reduced compared to wild-type levels in these embryos (Table 3.1). Double heterozygotes for *robo* and *slit* served as a positive control for epistatic relationship since they interact genetically, and are mapped functionally to the same pathway (MacMullin and Jacobs, 2006). In *robo-/+,slit-/+* embryos a significant reduction in filopodial and lamellopodial activity, but not migration velocity is detected (Fig 3.5, A-A',B- B'). We introduced one copy of null mutant alleles of *fra* and *unc5* into the *robo -/+* background and assessed whether the phenotype of reduced LE motility was enhanced. Filopodial and lamellopodial activities were reduced in *robo-/+,fra-/+* and *robo-/+,unc5-/+* double heterozygotic mutants relative to *robo-/+, fra-/+, unc5-/+* embryos and were comparable to what was observed in *robo-/+,slit-/+* (Fig 3.5, Table 3.1). Again, significant reduction in migration velocity was not observed in *robo-/+,slit-/+, robo-/+,fra-/+* and *robo-/+,unc5-/+* mutants. A significant increase in migration velocity was observed in *robo/unc5* mutants (Fig 3.5 F, Table 3.1). Additionally, cross sectional images of *robo-/+,slit-/+, robo-/+,fra-/+* and *robo-/+,unc5-/+* mutants illustrate the absence of the wildtype pear shaped morphology (Fig 3.5 B', C', D'). This data supports a model where Robo along with Fra and Unc5 synergistically promote LE motility of the CBs. Since these results suggest that Robo and Fra mediated signalling

pathways converge together to promote LE dynamics, we tested whether overexpression of Robo and Fra transgenes in *fra* and *robo* mutants, respectively, would rescue filopodial and lamellopodial activity. Overexpression of Robo in *fra* mutant background significantly increased filopodial activity, lamellopodial activity and migration velocity. Similarly, overexpression of Fra in a *robo* mutant background significantly increased filopodial activity, lamellopodial activity (Fig 3.5 E,F). However, migratory velocity was not rescued in these mutants (Fig 3.5 G). Furthermore, overexpression of Robo in *fra* null mutants rescues lumen formation (Fig 3.4 C'-C"). Conversely, when Fra is overexpressed in *robo* null mutants, lumen formation does not occur (Fig 3.4 D'-D"). These data suggest a non-additive but not an epistatic interaction between robo and fra in LE activity, that does not extend to the lumen formation phenotype.

3.3.4 Cardiac cells undergo EMT in the absence of Slit or Netrin mediated signalling

Like other collectively migrating cells, CBs have properties of both epithelial and mesenchymal cells. After specification during embryogenesis, CBs undergo a MET to form bilateral rows which migrate collectively (Fremion et al., 1999). Stable lateral adhesions between ipsilateral CBs and apical/basal ECM are characteristics of epithelial behaviour. However, CBs display protrusive capabilities at the LE suggesting that mesenchymal characteristics are also present in these otherwise epithelial cells (Fig 3.1). The presence of gaps along the CB bilateral rows in *slit, robo, netA,netB, fra* and *unc5* mutants suggests that epithelial characteristics of CBs might be disrupted in the absence of guidance signalling. Our time-lapse movies demonstrate that in embryos mutant for

Figure 3.6. Cardioblasts undergo EMT and independently migrate away from the leading edge in *slit, robo,lea* and *fra* mutants. (A-C) Dorsal view of heart development during migratory stages in *slit, robo,lea* and *fra* mutants. (A'-A'''', B'-B'''') In *slit* and *robo,lea* mutants, CB flanking a gap in the LE undergoes EMT, by detaching from the ipsilateral cell, and migrates laterally (asterick). (C-C'''') In *fra* mutant, CB emerges from the LE and migrate into the embryo (asterick). CBs undergoing EMT extend protrusions towards the direction of migration (arrows). The CBs which are part of the LE do not extend protrusions (arrowheads).

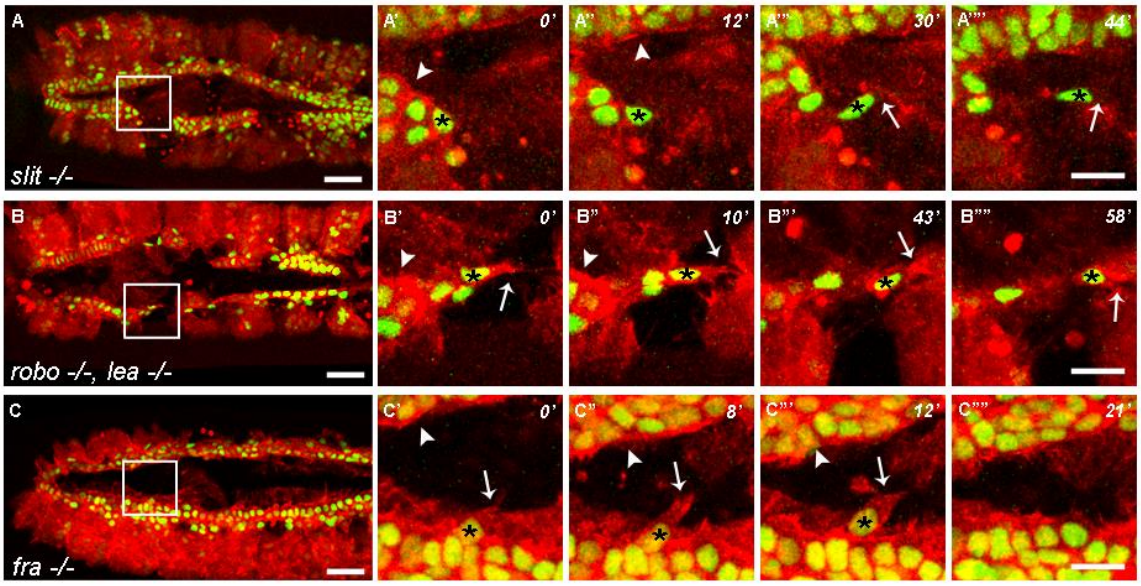

A
A'
0'
A''
12'
A'''
30'
A''''
44'
slit -/-
B
B'
0'
B''
10'
B'''
43'
B''''
58'
robo -/-, lea -/-
C
C'
0'
C''
8'
C'''
12'
C''''
21'
fra -/-

Table 3.1. One-tailed T test *p* values for filopodial density, LE activity and migration velocity conducted between wildtype and mutant genotypes.

Mutants	Filopodial Activity (wt)	Filopodial Activity (robo/+)	Migration Velocity (wt)	Migration Velocity (robo/+)	Lamellopodial Activity (wt)	Lamellopodial Activity (robo/+)
slit -/-	$<10^{-5}$	-	$2.2x10^{-5}$	-	$<10^{-5}$	
robo -/-	$<10^{-5}$	$<10^{-5}$	0.01	0.14	$<10^{-5}$	$<10^{-5}$
lea -/-	$<10^{-5}$	$<10^{-5}$	0.001	0.06	$<10^{-5}$	$<10^{-5}$
robo -/- lea -/-	$<10^{-5}$	$<10^{-5}$	0.001	0.04	$<10^{-5}$	$<10^{-5}$
netA -/- netB -/-	$<10^{-5}$	-	0.038	-	$<10^{-5}$	-
fra -/-	$<10^{-5}$	-	0.008	-	$<10^{-5}$	-
unc5 -/-	$<10^{-5}$	-	0.01	-	$<10^{-5}$	-
robo -/+	$3.0x10^{-5}$	-	0.08	-	0.36	-
fra -/+	10^{-4}	-	0.01	0.30	0.06	0.007
unc5 -/+	$<10^{-5}$	0.04	0.003	0.17	0.44	0.28
robo -/+ slit -/+	$<10^{-5}$	$<10^{-5}$	0.21	0.32	$<10^{-5}$	$<10^{-5}$
robo -/+ fra -/+	$<10^{-5}$	$<10^{-5}$	0.12	0.48	$<10^{-5}$	$<10^{-5}$
robo -/+ unc5 -/+	$<10^{-5}$	$<10^{-5}$	0.07	0.009*	$<10^{-5}$	$<10^{-5}$
slit -/- mef2>slit	$4.0x10^{-4}$	-	0.38	-	0.16	-
fra -/- mef2>fra	0.43	-	0.35	-	0.001*	-
fra -/- mef2>robo	$1.77x10^{-5}$	-	0.02	-	0.43	-
robo -/- mef2>fra	$<10^{-5}$	-	0.002	-	$2.1x10^{-5}$	-

Genotype included in brackets served as reference for T-test conducted for filopodial activity, migration velocity and lamellopodial activity. Table cells with a significant p value ($P<0.05$) are shaded in grey. * - Value is significantly higher compared to wildtype.

Figure 3.7. Moesin-mCherry co-localizes with Actin cytoskeleton. Filopodia extended by CBs at stage 16. (A-A') *UAS-Moesin-mCherry* and *UAS-liveAct-GFP* were expressed using dmefGAL4 driver concurrently. Both the actin binding domain of Moesin and Live-Act co localized at the filopodia (arrow). (B) Graph representing the percentage of all measured segments divided into subsections according to the number of filopodia extended. "F" refers to the number of filopodia extended by a single heart segment. The number of segments extending less than or equal to 2 filopodia is increased in guidance signalling and transheterozygotic mutants. Measured segments were used for the bar graph ranging from st 14 to late st 16.

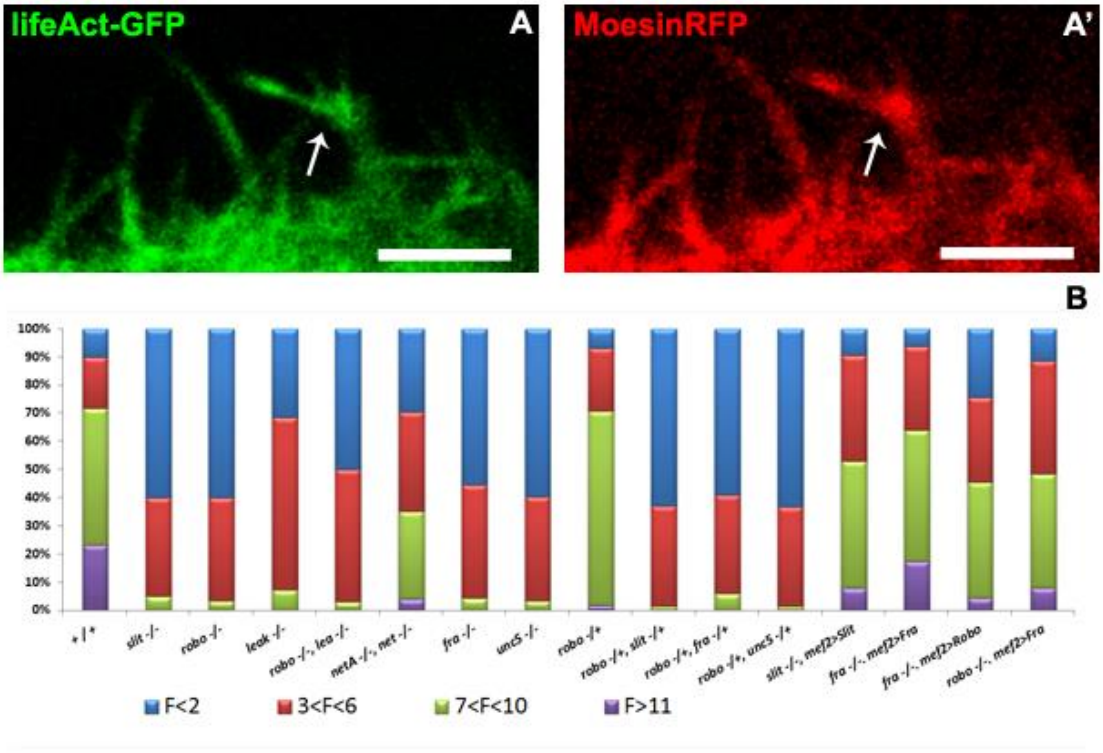

lifeAct-GFP
A
MoesinRFP
A'
B
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
+/+
slit -/-
robo -/-
leak -/-
robo -/-, lea -/-
netA -/-, net -/-
fra -/-
unc5 -/-
robo -/+
robo -/+, slit -/+
robo -/+, fra -/+
robo -/+, unc5 -/+
slit -/-, mef2>Slit
fra -/-, mef2>Fra
fra -/-, mef2>Robo
robo -/-, mef2>Fra
F<2
3<F<6
7<F<10
F>11

Figure 3.8. Scatter plot of distance to the midline and the number of filopodia extended by CB segments. Coefficient of correlation of all genotypes demonstrates the absence of linear relationship between 'distance to the midline' and the 'number of filopodia', unlike observed in wildtype where a linear relationship exists (R^2=0.713) (Fig 3.1).

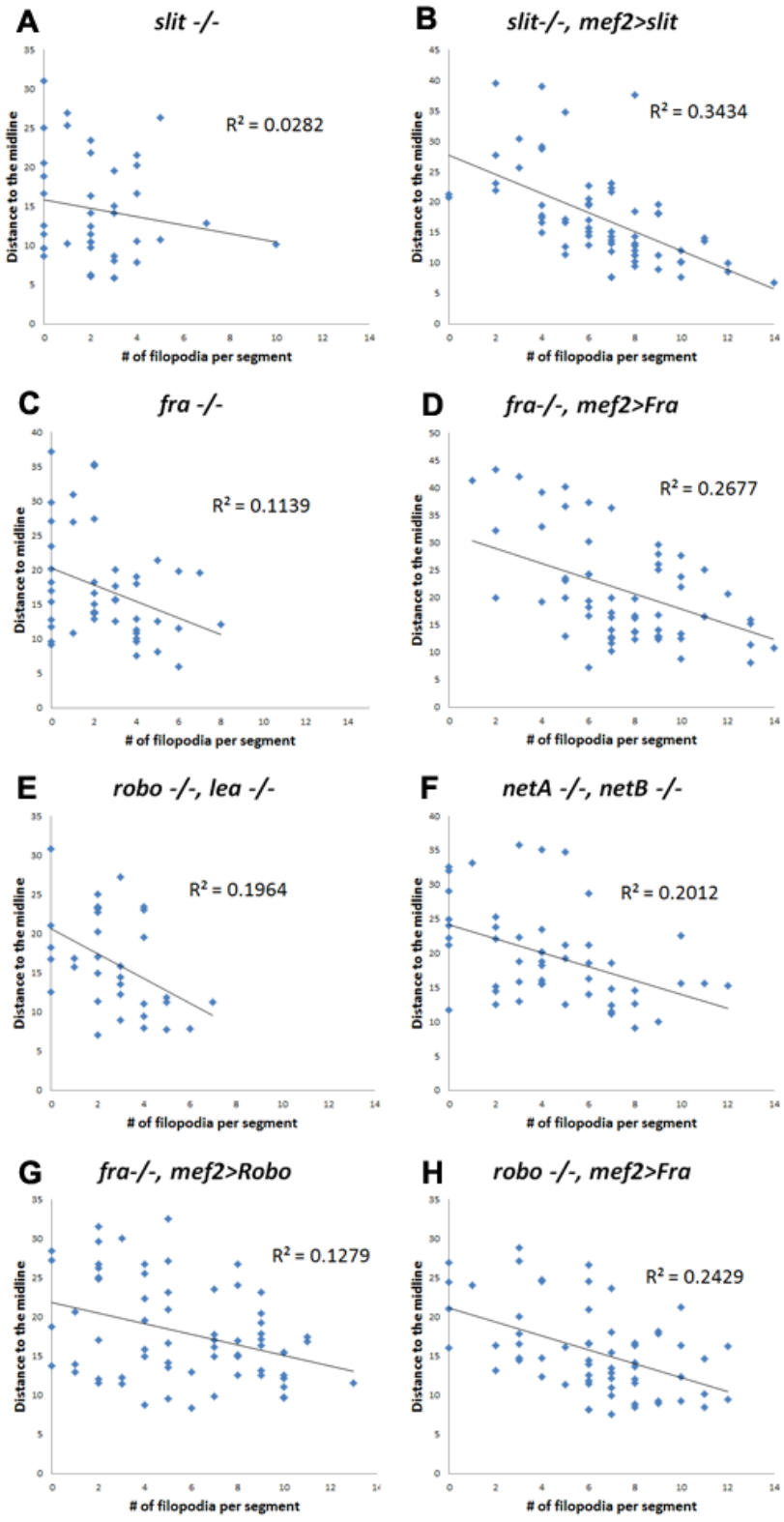
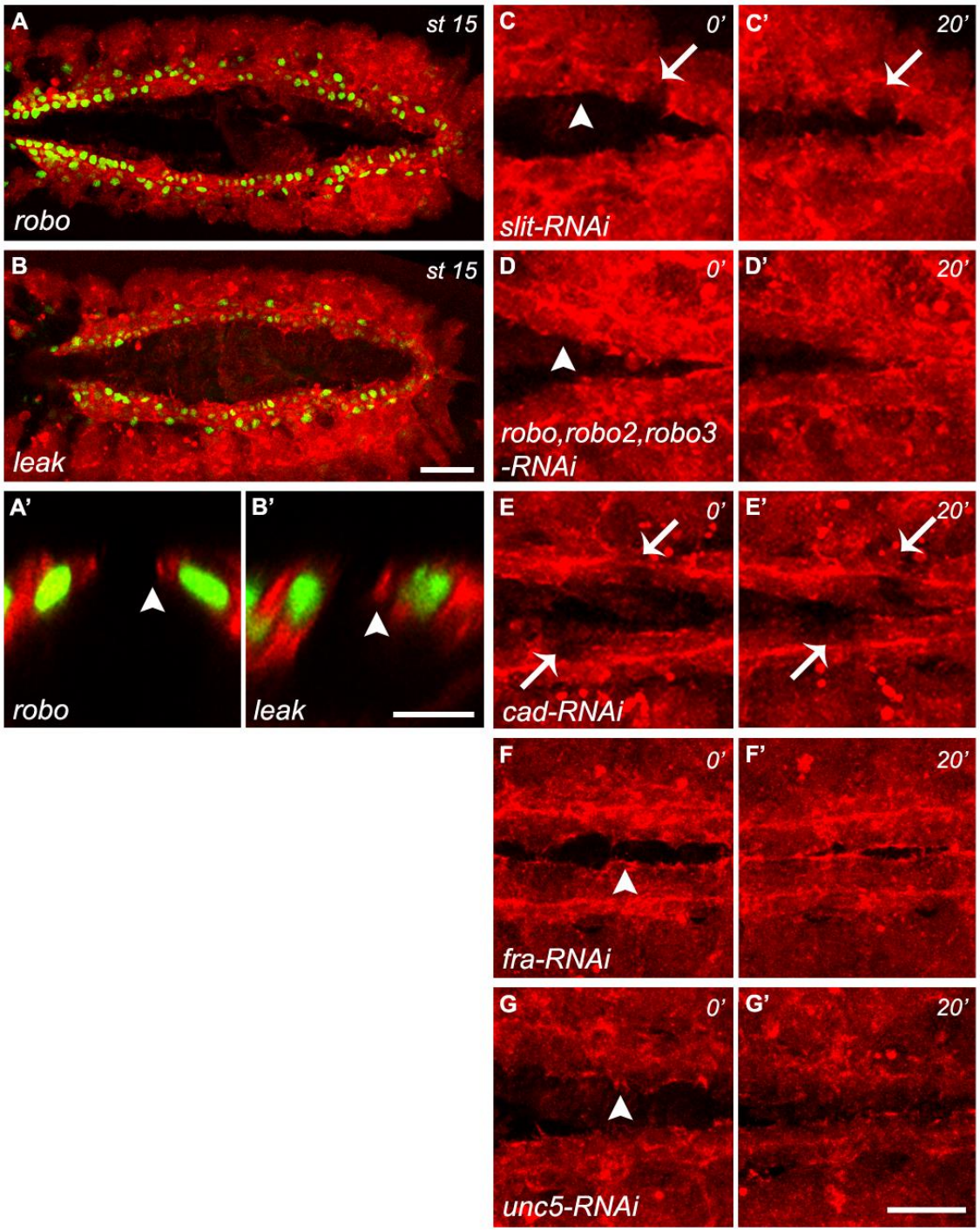

A
slit -/-
R² = 0.0282
B
slit-/-, mef2>slit
R² = 0.3434
C
fra -/-
R² = 0.1139
D
fra-/-, mef2>Fra
R² = 0.2677
E
robo -/-, lea -/-
R² = 0.1964
F
netA -/-, netB -/-
R² = 0.2012
G
fra-/-, mef2>Robo
R² = 0.1279
H
robo -/-, mef2>Fra
R² = 0.2429
Distance to the midline
# of filopodia per segment

Figure 3.9. Filopodial activity and cardioblast extensions are reduced in *robo* and *leak* mutants. (A,A') Dorsal and cross sectional view of CB migration in a *robo* mutant background. (B,B') Dorsal and cross sectional view of CB migration in a *leak* mutant background. Filopodial and lamellopodial activity of the LE is reduced. Wildtype pear-shaped morphology is partially affected in both *robo* and *leak* mutants. (C-E') Downregulation of Slit (C-C'), Robo/Leak (D-D') and Cadherin (E-E') using dmefGAL4 resulted in reduced filopodial and lamellopodial activity (arrowheads). Gaps within the LE were also observed in multiple cases (arrows). Gaps were identified by the presence of lateral filopodia being extended into the adjacent empty space. (A,B) Micron scale is 25 μm. (A',B') Micron scale is 5 μm. (C-G') Micron scale is 10 μm.


A
st 15
robo
B
st 15
leak
A'
robo
B'
leak
C
0'
slit-RNAi
C'
20'
D
0'
robo,robo2,robo3
-RNAi
D'
20'
E
0'
cad-RNAi
E'
20'
F
0'
fra-RNAi
F'
20'
G
0'
unc5-RNAi
G'
20'

slit, *robo,lea* and *fra*, CBs lose adhesion with ipsilateral partners and migrate independently of the LE (Fig 3.6). In *slit* and *robo,lea* mutants, we observed that CBs flanking gaps along the LE were able to separate from their partners and migrate away (Fig 3.6, A-B’’’, Movie 3.7, 3.8). These CB extended protrusions towards the CB at the opposite ipsilateral edge of the gap (Fig 3.6, B). In *netA,netB*, *fra*, and *unc5* mutants, free migrating CBs expressing Moe-mCherry and tupGFP were observed in the AS, ectoderm and near the LE (Fig 3.6, C-C’’’, Movie 3.9, data not shown). Persistent expression of tupGFP and Moe-mCherry markers suggests that a loss of CB cell fate is not the cause of gap formation. Rather, we hypothesize that reduced lateral adhesion with ipsilateral CBs disinhibits the mesenchymal aspects of the CB phenotype – enhancing active apical membrane activity and allowing for independent migratory behaviour. Furthermore, the migration of independent CBs is predominantly towards the midline, suggesting that they retain autonomous sensitivity to other cues that orient motility, even in the absence of the Slit or Net signal. Interestingly, in guidance signalling mutants, detached CBs are capable of extending filopodia and lamellopodia (Fig 3.6, arrows), whereas CBs which maintained lateral adhesions failed to extend protrusions (Fig 3.6, arrowheads).

3.4 Discussion

Many in vivo models, such as the BC and pLL, have been used to study the mechanical and chemical factors that coordinate CCM. Here we have characterised embryonic cardiac development in *Drosophila* as a unique model to quantitatively study CCM. During heart development, all CBs behave as leader cells, yet they move in coordination, indicating they communicate with each other. Our study demonstrates that

during cardiac CCM, all CBs extend filopodia and lamellopodia which can reach up to 10μm, oriented towards the dorsal midline. We show that once CBs pass a threshold point, which is predicted to be 25 μm from the midline, LE activity increases. Based on our mutant and RNAi analysis, Net and Slit promote formation of filopodial and lamellopodial protrusions during migratory stages. Filopodial and lamellopodial activity is reduced, but not absent, in all mutant genotypes tested. *slit* mutants display the most severe and penetrant LE motility phenotype. Net deficient hearts display a less dramatic reduction in filopodial and lamellopodial activity. We propose that Slit is the primary guidance cue for LE protrusive capability, however both Slit and Net, in conjunction, are required for inducing maximal levels of LE activity. This is supported by the following observations. Our experiments suggest that cross-talk between Slit and Net receptors, Robo, Fra and Unc5 likely exists. The collective signalling input from these receptors likely converges and activates intracellular cytoskeletal rearrangement machinery and leads to the stimulation of LE dynamics. We noted that overexpressing Robo in *fra* mutants, rescues all migratory defects, suggesting that increasing guidance input through Robo is sufficient to induce LE activity and lumen formation. However, when Fra levels are elevated in *robo* mutants, only filopodial and lamellopodial activities are rescued. Fra, however, is not essential for lumen formation and for maintaining normal migratory velocity during CCM of CBs. The essential requirement of Fra in establishing apical CB attachments but not lumen formation has been demonstrated previously (Macabenta et al., 2013). Therefore, we conclude that both Slit and Net signalling through Robo and Fra, likely converge on the same final CB pathway that induces LE membrane protrusive

activity. Nevertheless, lumen formation requires both Slit/Robo and Net/Unc5 signalling independently (Albrecht et al., 2011). The repulsive function of Slit ligand is well established during axonal guidance (Brose et al., 1999; Kidd et al., 1999). Growth cones arrest their forward motility when Slit signal is encountered. In the CBs, Slit and Netrin are not required for their forward migration to the midline, but are required for LE motility. Slit predominantly localises to the luminal domain in CBs and this localisation is required to define CB polarity (MacMullin and Jacobs, 2006; Qian et al., 2005). Perhaps, by promoting CB polarisation Slit modulates the formation of a dorsal protrusive domain and indirectly promotes Net/Fra attractive signalling.

Our data suggests that during CCM CBs are very sensitive to the levels of guidance signalling which control filopodial and lamellopodial activity. However, are cytoplasmic protrusions required for heart development? In other models such as endothelial cell migration and growth cone guidance, integration of signal from guidance cues does not require filopodia (Dwivedy et al., 2007; Wacker et al., 2014). However, during anastomosis of blood vessel, induction of filopodial activity requires guidance signalling which facilitates contralateral adhesion formation (Phng et al., 2013). Interestingly, when cytoplasmic protrusions are reduced, CBs are still able to reach the dorsal midline likely by maintaining adhesions with the overlaying BM of the ectoderm (Haack et al., 2014). We propose that CBs do not require filopodia to reach the midline, however primary contact formation between contralateral CBs depends on the ability of the cells to extend filopodia and lamellopodia.

Disruption of E-Cad based lateral junctions result in the formation of gaps within rows of CBs (Macabenta et al., 2013; MacMullin and Jacobs, 2006; Qian et al., 2005; Santiago-Martinez et al., 2008). We suggest that absence of guidance signalling mutants results in weakening of lateral adhesions between CBs during migration. Therefore, it might be possible that the mechanical tension can overwhelm lateral adhesions at the weakest point thereby breaking contacts between ipsilateral CBs. According to our analysis, the CBs which flank these formed gaps are more likely to undergo EMT. Therefore, we propose that guidance signalling through Slit and Netrin is required to stabilize the E-Cad mediated adheren junctions around the CBs. We suggest that guidance signalling regulates the balance between mesenchymal and epithelial characteristics of CBs and functions to suppress total EMT. Consistent with our conclusion, in vertebrates Slit and Nets promote epithelial characteristics of cells prone to EMT and the tumour suppressive roles for their receptors Robo, Fra/DCC and Unc5 have been proposed. (Hagedorn et al., 2013; Mehlen et al., 2011).

3.5 Conclusion

We have characterized embryonic cardiac development in *Drosophila* as a unique quantitative genetic model to study CCM. We show that CBs LE is highly dynamic and guidance signalling plays an important role in regulating CB LE motility. Slit and Net signalling receptors, Robo and Fra, genetically and functionally compensate for each other to induce LE activity. Furthermore, this study reinforces a strong link between guidance signalling and EMT, and provides evidence that guidance cues and their receptors stabilize lateral adhesions during CB collective migration.

Matrix Metalloproteinases regulate cardiac cell migration, polarisation and lumen formation in *Drosophila*

CHAPTER FOUR

4.1 Acknowledgement of contributions

Dr. J Vanderploeg performed immunolabelling and imaging for the following figures.

Figure 4.1 – Cross sectional images of heart stained with αDg, αDlg and αMef

Figure 4.3 – Frontal images of embryos stained with αβPS and αMef.

Frontal and cross sectional images of αPrc and αMef

Figure 4.4 – Frontal and cross sectional images of the heart stained with αSlit and αMef

Figure 4.8 – All panels

Dr. J Vanderploeg also performed the western blot analysis as presented in Figure 4.10

4.2 Abstract

MMPs are enzymes which are able to degrade components of the ECM and perform essential functions during morphogenesis. These proteases and their endogenous inhibitors are expressed during early cardiac development in vertebrates. *Drosophila* genome encodes two copies of MMPs, MMP1 and MMP2 whereas in humans up to 25 MMPs have been identified with overlapping functions. We investigated the role of MMPs during embryonic heart development in *Drosophila*, a morphogenetic process which includes stages of CCM, cell polarization and lumen formation. We have demonstrated that MMP1 and MMP2 are each required for embryonic heart development. MMP2 is expressed in the CBs and is required to breakdown the ECM at the apical domain to allow filopodia and lamellopodia formation. Additionally, MMP2 is required for regulating the apical identity of ECM including targeting of guidance signalling molecules to the apical cell membrane. MMP1 is required for luminal expansion by limiting the junctional domain to the dorsal and ventral adheren junctions. Inhibition of MMP activity through ectopic expression of Timp in the ectoderm blocks lumen formation. Larval hearts in *mmp* mutants display myofibrillar disorganization and adhesion defects. Defects accumulated during embryogenesis in *mmp* mutant hearts are not repaired during larval development. Lastly, we report that larval heart function is not required for larval development in *Drosophila*. Altogether we demonstrate that modulation of ECM remodelling and guidance signalling via MMPs promotes CCM, cell polarization and lumen formation during heart morphogenesis in *Drosophila*.

4.3 Introduction

Tissue remodelling is a hallmark of development and disease related processes such as cancer. These processes are regulated by a variety of secreted and transmembrane protease such as MMPs. MMPs are zinc dependent proteases which break down components of the ECM and embedded signalling molecules and thereby regulate morphogenesis and contribute to disease progression of cardiovascular malformation and tumour invasion (Lemaitre and D'Armiento, 2006). Due to high level of genetic redundancy in the mammalian genome, which encodes 25 mammalian MMPs and 4 TIMPs (Sternlicht and Werb, 2001), it is challenging to examine the role of MMPs since genetic compensation can affect mutant analysis. However, MMP functions are conserved across the animal kingdom, including in amenable genetic models (Egeblad and Werb, 2002; Nagase et al., 2006; Page-McCaw et al., 2007). *Drosophila* genome encodes 2 MMPs, a secreted protease MMP1 (Llano et al., 2000), a transmembrane protease MMP2 (Llano et al., 2002), and 1 copy of TIMP (Wei et al., 2003).

MMPs, through proteolysis of ECM and cell surface proteins, regulate many morphogenetic processes such as cell migration, proliferation, differentiation and ECM degradation (Sternlicht and Werb, 2001). For example, in an *in vitro* model of human vasculogenesis, lumen formation between endothelial cells requires the activity of a MT1-MMP for luminal expansion and formation of vascular guidance tunnels (Davis et al., 2011). Branching morphogenesis in organs such as lungs, mammary and submandibular glands requires activity of MMPs for cell motility which is regulated through ECM degradation (Bonnans et al., 2014). During tumour invasion, MT1-MMP, targeted to the

invadopodia, promote metastasis by degrading ECM barriers (Jacob and Prekeris, 2015; Poincloux et al., 2009). Although vertebrate MMPs have received much attention, their contributions to embryonic morphogenesis are largely unknown (Egeblad and Werb, 2002; Khokha et al., 2013; Overall and Lopez-Otin, 2002; Page-McCaw et al., 2007). By using *Drosophila* embryogenesis as a genetic model we can study the effect of complete elimination or inhibition of MMP activity *in vivo,* a feat difficult to achieve in other model organism systems. Conservation of activity in *Drosophila* homologues of MMPs has been well established (Llano et al., 2002; Llano et al., 2000). However, a study conducted by Page-McCaw et al. suggested that MMP activity is not required for embryogenesis since single or double *mmp* mutant embryos hatch and survive until mid or late larval stages (Page-McCaw et al., 2003). Nevertheless, developmental processes such as motor axon fasciculation during embryogenesis (Miller et al., 2011; Miller et al., 2008), airway remodelling during larval tracheal development (Glasheen et al., 2010) and re-epithelialization (Stevens and Page-McCaw, 2012) require MMP activity in *Drosophila*. Additionally, MMPs and Timps are expressed in the cardiomyocytes during early heart tube assembly in vertebrate (Cai et al., 2000; Cantemir et al., 2004) and modulate cardiac morphogenetic events such as heart tube formation, directional looping (Linask et al., 2005) and differentiation of ostial cells (Alexander et al., 1997). Therefore, we tested the genetic requirement of MMPs during embryonic heart development in *Drosophila*, a process which involves CCM, cell polarization and lumen formation. To form the embryonic heart, CBs form bilateral rows and collectively migrate towards the dorsal midline. Upon reaching the midline, CBs form specific adhesions with the

contralateral cells and form a medial lumen which expands as the embryo transitions into larval stages (Tao and Schulz, 2007).

We demonstrate that both secreted (MMP1) and transmembrane (MMP2) MMPs contribute to CCM of CBs by promoting LE activity. However, both MMPs play distinct roles during cell polarisation and lumen formation stages. MMP2 is essential for forming apical adhesion with contralateral CBs, whereas MMP1 activity is required to limit the adhesive domain to the dorsal and ventral apical regions and for luminal expansion. Additionally, our results indicate that MMP2 specifically regulates apicalization of guidance signalling molecules Slit and Robo. Furthermore, positioning and composition of the CB apical ECM is modulated by MMP activity. However, MMPs are not required to locate ECM receptors, Integrin and Dg. Maturation of the embryonic heart into a contractile larval heart requires MMPs. Overall, we propose that both MMP1 and MMP2 play significant roles in promoting cell migration, polarisation and lumen formation during *Drosophila* cardiogenesis.

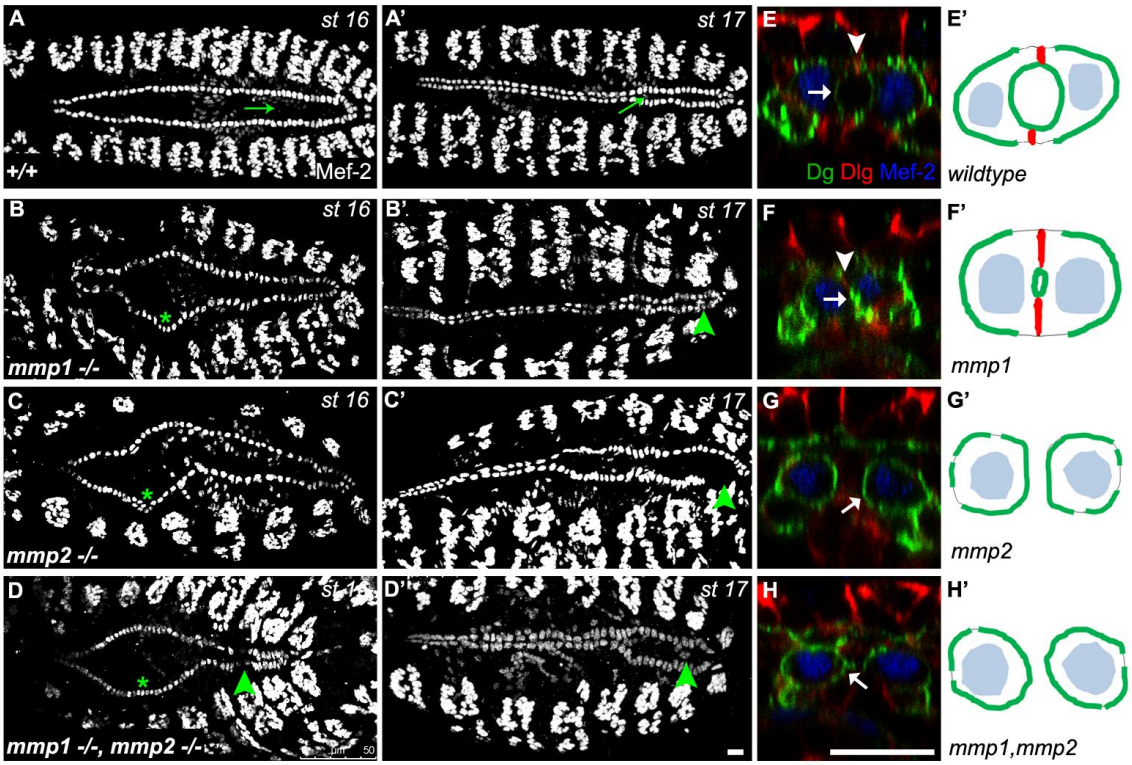
4.4 Results

4.4.1 MMP1 and MMP2 are required for lumen formation during embryonic morphogenesis

MMPs are required for lumen formation in two independent tubulogenesis models. First, during endothelial cell migration, MT1-MMP is required for fusion of the intercellular vacuoles which merge medially to form a lumen (Davis et al., 2011). Second, heart tube formation during early embryonic development in chick embryo requires MMP

activity (Linask et al., 2005). Therefore, we asked the following question: Does lumen formation in the *Drosophila* heart require MMP activity? To address this question we labelled wildtype and mutant embryos with luminal, junctional and nuclear markers and evaluated the structure of the heart (Fig 4.1). Wildtype embryos at stage 16 maintain aligned lateral contacts along the bilateral rows and migrate to the midline collectively (Fig 4.1 A). At stage 17, contralateral CBs change shape to enclose a medial lumen (Fig 4.1 A'). Dg, an ECM receptor, localizes to the apical domain which becomes the lumen, whereas Dlg, an apical polarity scaffold protein, localizes to the junctional domains at the apical attachment sites (Fig 4.1 E, E'). Loss of either or both *mmp1* and *mmp2* result in disorganized CB arrangement along the bilateral rows, reflecting variations in migration rate, however CBs eventually reach the midline (Fig 4.1 B-B',C-C',D-D'). Following migration, *mmp1* and *mmp2* mutant phenotype diverge. In *mmp1* mutants, a Dg rich lumen forms, but it is reduced in size and is enclosed by extended Dlg-marked junctions (Fig 4.1 F,F'). In *mmp2* and *mmp1,mmp2* double mutants lumenogenesis fails to occur (Fig 4.1 G-G',H-H'). Instead of a wildtype crescent shape, CBs appear rounded and Dg localization is observed around the entire cell membrane, whereas Dlg-marked junctions are absent (Fig 4.1 G-G',H-H'). To quantify lumen formation defects, we scored cross sectional images of the posterior hearts labelled with a luminal marker for 20 or more embryos (Table 4.1). Results confirmed that approximately 75% of the embryos mutant for either *mmp2* or both *mmp1,mmp2* displayed rounded morphology and did not contact the contralateral cell, whereas most prevalent phenotype in *mmp1* mutants was the presence of a small apical lumen.

Figure 4.1. MMP1 and MMP2 are required for embryonic heart development and lumen formation. . (A-D') Embryos were labelled with anti Mef-2 antibodies to visualize the nuclei of the CBs. (A) In wildtype embryos, CBs are organized into bilateral rows and migrate collectively towards the dorsal midline (arrow). (A') At stage 17, contralateral CBs segmentally align at the midline and form a lumen (arrow). (B-B') In *mmp1* mutants, organization of CB rows is uneven and some delayed migration of CBs is observed (asterisk) although CBs reach the midline (arrow). (C-D') In *mmp2* and *mmp1,mmp2* mutants, CBs do not form straight bilateral rows and delayed migration of CBs is observed (asterisk). Misplaced CBs are observed, however most of them reach the midline (arrow). Although most CBs migrate in tandem, gaps from CB displacement are observed (arrowheads). (E-H) Cross sectional view stage 17 hearts labelled with anti Mef-2 (blue), anti Dg (green) and anti Dlg (red) are shown. (E) In wildtype embryos, Dg localizes to the CB domain which encompasses the lumen (arrow) whereas Dlg localizes to the junctional domains (arrowhead). (F) In *mmp1* mutants, a reduced lumen is formed (arrow). Dg localizes to the reduced lumen (arrowhead) and Dlg localizes to the entire apical domain (arrow). (G) In *mmp2* and *mmp1,mmp2* mutants, lumen formation does not occur, and CBs fail to contact contralateral partners (arrow). CBs exhibit rounded morphology and Dg localization is extended over the entire apical domain (arrow). (E'-H') Cartoon representation of CBs at stage 17 in wildtype and MMP mutants where green represent Dg, red represent Dlg, blue represent Mef2 localization and black represents plasma membrane. Scale bars are 10 μm.


A
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F
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mmp1
C
st 16
mmp2 -/-
C'
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G
G'
mmp2
D
st 16
mmp1 -/-, mmp2 -/-
D'
st 17
H
H'
mmp1,mmp2

Table 4.1. Scoring of luminal defects of embryonic heart development in *mmp* mutants.

Genotypes	Normal Lumen (%)	Reduced Lumen* (%)	No Lumen† (%)	Gap between CBs‡ (%)	Embryos Scored (n)
wildtype	95	5	0	0	21
mmp1	16	68	8	8	25
mmp2	0	9	14	77	22
mmp1,mmp2	5	15	5	75	20

* - A decreased size lumen between CBs which localizes luminal markers

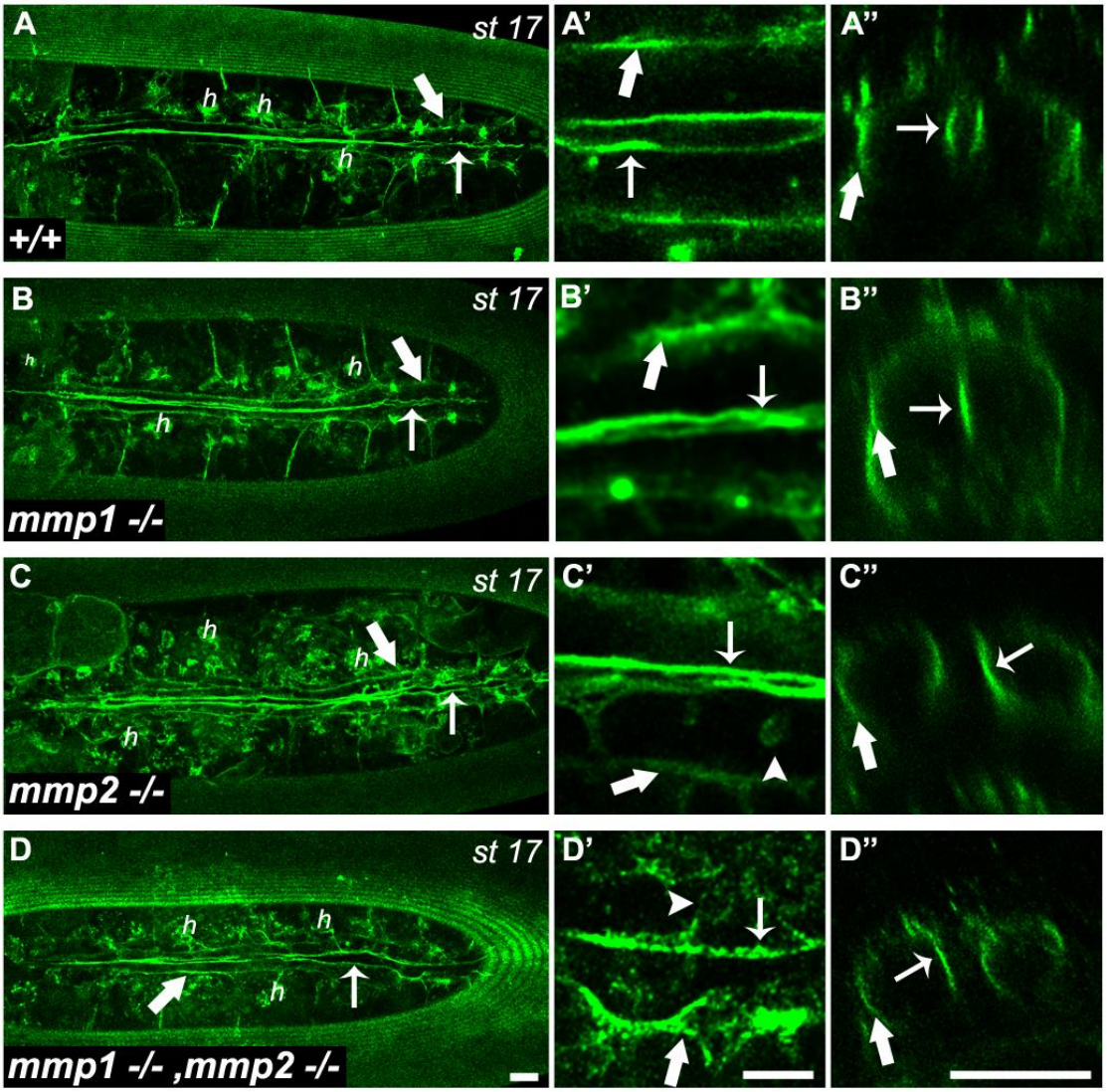
† - The apical medial region doesn't localize luminal markers

‡ - Contralateral CBs do not make contact

4.4.2 Restructuring of apical and basal ECM requires MMP1 and MMP2 activity in the CBs

In *Drosophila,* MMP2 is required to cleave components of the BM and therefore regulates its polarised deposition (Deady et al., 2015; Glasheen et al., 2010; Jia et al., 2014; Miller et al., 2011; Stevens and Page-McCaw, 2012). We speculated that MMP2 might regulate placement of the ECM around the CBs. To determine whether MMPs are required for Collagen-IV degradation, we analysed time-lapse movies of embryos expressing Collagen-IV reporter, Vkg-GFP in wildtype and *mmp* mutant embryos. In wildtype embryos, Collagen-IV is present both at the basal and apical side of the CBs during CCM and lumen formation (Fig 4.2 A, Supp Fig 1 G,H, Movie 4.1). At the apical side of CBs, Collagen-IV is absent from the dorsal and ventral apical extensions and restricted to the pre-luminal domain during CCM and subsequently to the lumen (Fig 4.2 A'', Supp Fig 1 G,H). In *mmp1* mutants, Collagen-IV localizes to luminal domain however the lumen fails to expand (Fig 4.2 B-B'', Movie 4.2). In *mmp2* and *mmp1,mmp2* double mutants, Collagen-IV localization is extended to the entire apical region of the CBs (Fig 4.2 C-C'',D-D'',Movie 4.3, 4.4). Lateral accumulation of Collagen-IV is also observed in *mmp2* and *mmp1,mmp2* mutants (Fig 4.2 C',D'). The broad localization of ECM at the apical domain of CBs suggests that MMP2 is required to limit the localization of ECM to the pre-luminal and luminal domains. If MMP2 limits the extent of the luminal ECM, does it also play a role in defining ECM identity? To answer this question we examined the distribution of Prc, an exclusive component of the basal ECM, which is expressed and deposited by the pericardial cells at the basal side of CBs

Figure 4.2. ECM restructuring requires MMP activity. Live embryos expressing collagenIV-GFP (vikingGFP) trap in wildtype, *mmp1*, *mmp2*, and *mmp1,mmp2* mutant background. (A-D) Dorsal view of the stage 17 hearts. (A-D') Magnified frontal image of the CBs at stage 17. (A''-D'') Cross sectional images heart at stage 17. (A-A'') In wildtype embryos, Collagen-IV localizes to the luminal (arrow) and basal domains (A' thick arrow). (B-B'') In *mmp1* mutants, Collagen-IV localizes to the luminal (arrow) and basal domains (thick arrow). In *mmp2* and *mmp1,mmp2* mutants, Collagen-IV localizes to the entire apical domain (C''',D''' arrow) and basal domain (thick arrow). Ectopic lumens between lateral domains of CBs are also observed in *mmp2* and *mmp1,mmp2* mutants (C'-D' thick arrow). Few embryonic hemocytes are marked with "h". Scale bars are 10 μm.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h
h
mmp2 -/-
C'
C"
D
st 17
h
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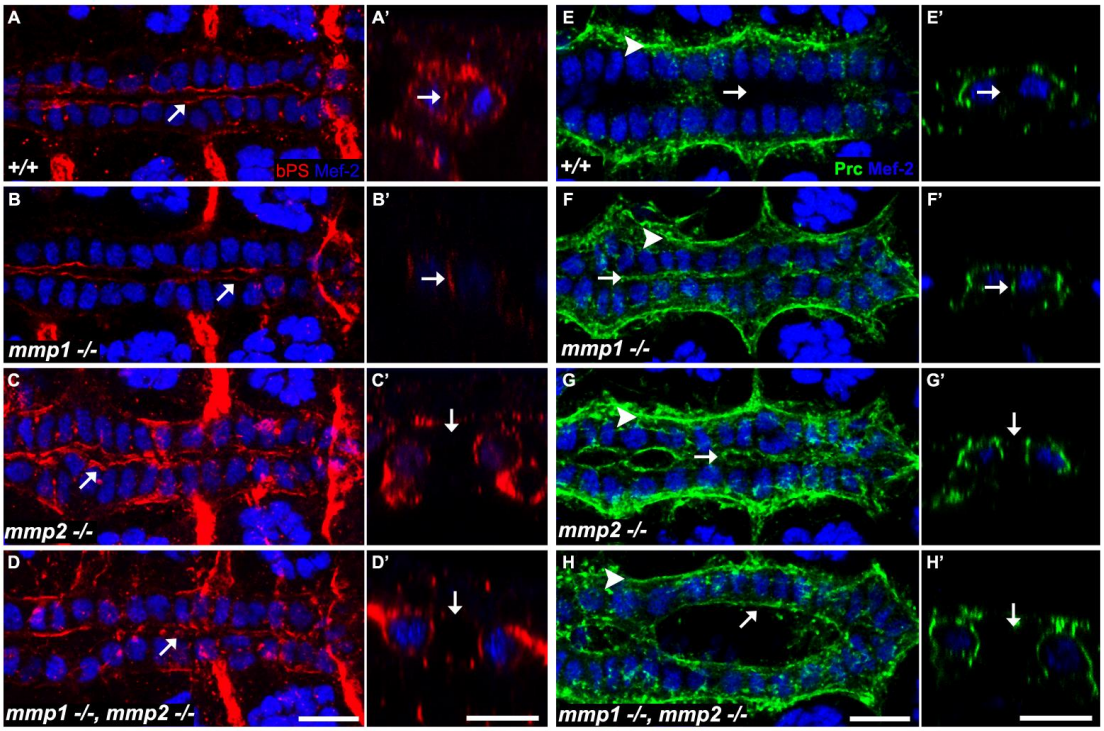
h
mmp1 -/- ,mmp2 -/-
D'
D"

Figure 4.3. Polarity markers are mislocalized in MMP1 and MMP2 mutants. (A-H) Dorsal view of the posterior heart is shown. (A'-H') Cross sectional view of the posterior heart through the tinman cells is shown.(A-A') In wildtype, βPS localizes to the luminal domain (arrow). (B-B') In *mmp1* mutant embryos, βPS is localized to the reduced lumen (arrow). (C-D') In *mmp2* and *mmp1,mmp2* mutant embryos, βPS localizes at the entire medial apical surface of the CBs (arrow). (E-E') In wildtype embryos, Pericardin localizes exclusively to the basal domain (arrowhead). In *mmp1* (F-F'), *mmp2* (G-G'') and *mmp1,mmp2* (H-H'') mutants, Pericardin localizes to the entire medial surface (arrow) as well as the basal side (arrowhead) of the CBs. CB nuclei are labelled with anti-Mef-2 antibody (blue) in all images. (A-D') Wildtype and *mmp* mutant embryos labelled with anti-Integrin (βPS) (red) are shown. (E-H') Wildtype and MMP mutants embryos labelled with anti-Pericardin (green) are shown. Scale bars are 10 μm.


A
A'
+/+
bPS Mef-2
E
E'
+/+
Prc Mef-2
B
B'
mmp1 -/-
F
F'
mmp1 -/-
C
C'
mmp2 -/-
G
G'
mmp2 -/-
D
D'
mmp1 -/-, mmp2 -/-
H
H'
mmp1 -/-, mmp2 -/-

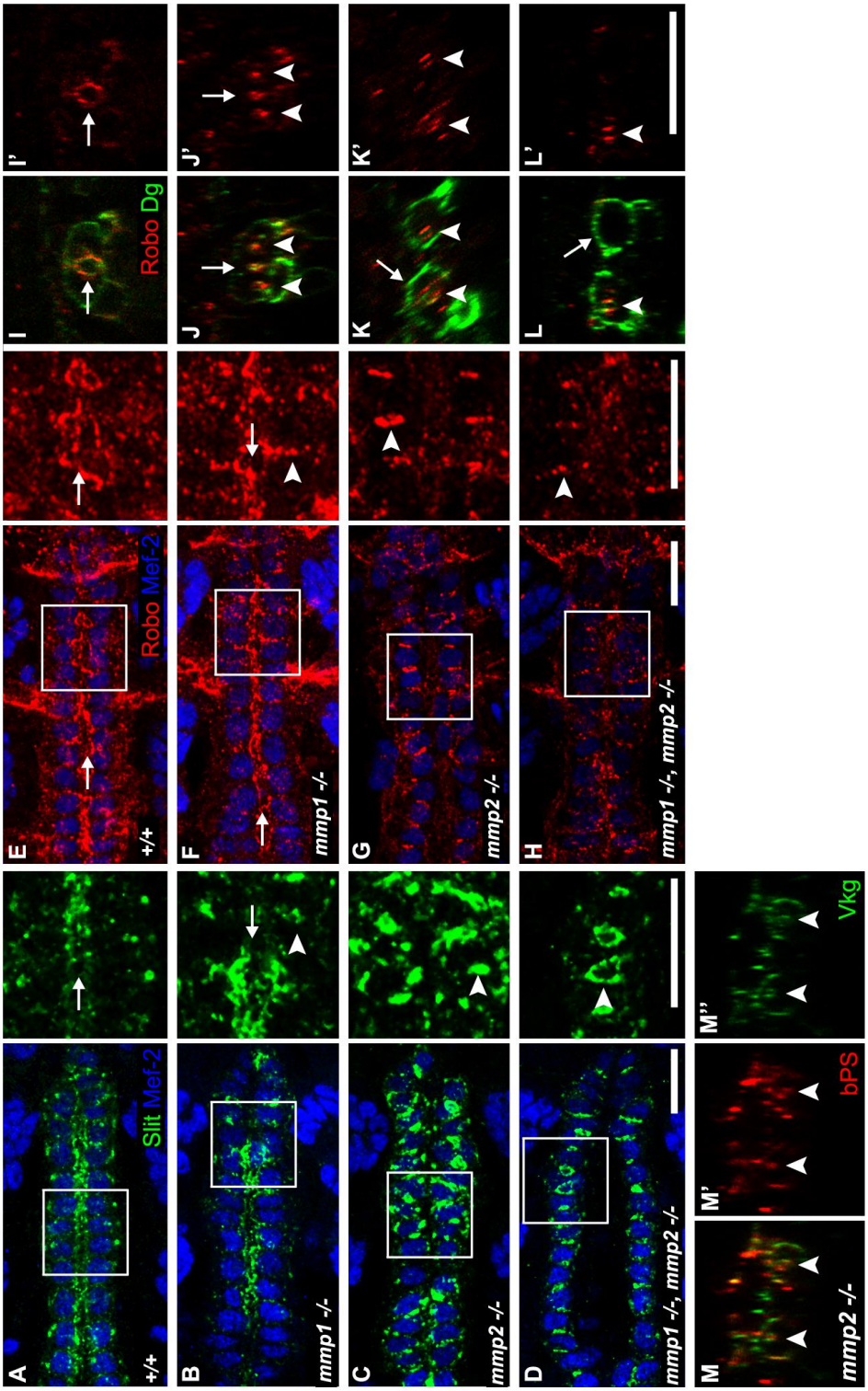
(Chartier et al., 2002). In contrast to wildtype, where Prc is excluded from the lumen (Fig 4.3 E,E'), we found Prc localisation at the apical ECM as well as the basal ECM in *mmp1*, *mmp2* and *mmp1,mmp2* mutants (Fig 4.3 F-F', G-G', H-H'). These results suggest that MMP1 and MMP2 collectively regulate the identity of the apical ECM, whereas MMP2 is required to limit ECM to the pre-luminal and luminal domain.

4.4.3 CB polarisation is disrupted in MMP1 and MMP2 mutants

Apicalisation of Integrin is an early step in CB polarisation. Integrin apicalisation instructs the CBs to stabilise polarising morphogens such as Slit and its receptor, Robo, which are required for lumen formation (MacMullin and Jacobs, 2006; Santiago-Martinez et al., 2008; Vanderploeg et al., 2012). Observing that Integrin ligand, Collagen-IV, accumulates ectopically in *mmp2* mutants (Fig 4.2 C-C'', Fig 4.3 G-G'), we sought to determine whether Integrin localisation is similarly affected. We labelled wildtype and *mmp* mutant embryos with an antibody specific to beta subunits of Integrin, βPS. In wildtype hearts, βPS localizes to the luminal domain (Fig 4.3 A,A'). In *mmp1* mutants, βPS is restricted to the reduced lumen (Fig 4.3 B,B'), whereas in *mmp2* and *mmp1,mmp2* mutants, βPS accumulates at the apical and basal sides of the CBs and some lateral locations (Fig 4.3 C-D'). This suggests that, similar to Collagen-IV distribution, Integrin is extended to the entire apical region of the CBs.

Next, since Integrin dependent stabilization of Slit and Robo complex at the luminal domain is a defining factor for lumenogenesis, we sought to determine whether the apical domain retains Slit and Robo in *mmp* mutants. In wildtype stage 17 CBs, Slit and Robo localize to the luminal domain and are absent from lateral domain

Figure 4.4. Slit and Robo are mislocalized to ectopic lumens in MMP1 and MMP2 mutants. (A-H) Wildtype and *mmp* mutant embryos labelled with anti-Slit (green) and anti-Robo (red) antibodies**.** (I-L) Cross-sectional view of wildtype and *mmp* mutant embryos labelled with anti-Dg (green) and anti-Robo (red) antibodies**.** (A) In wildtype embryos, Slit localizes to the luminal domain of the stage 17 CBs (1 arrow). (B) In *mmp1* mutants, Slit localization is observed at the luminal domains (arrow), however lateral mislocalization also takes place at some positions (arrowhead). (C,D) In *mmp2* and *mmp1*,*mmp2* mutants, Slit primarily localizes to the ectopic domains at the lateral sides of CBs (insets arrowheads). (E, I-I') In wildtype embryos, Robo localizes to the luminal domain of the CBs (arrow). (F,J-J') In *mmp1* mutants, apical (arrow) and lateral (arrowhead) distribution of Robo is observed. In *mmp2* (G,K-K') and *mmp1*,*mmp2* (H,L-L') mutants, Robo primarily localizes to concentrated ectopic domain which form between laterally adjacent CBs (arrowheads), whereas Dg localizes throughout the plasma membrane (arrows). (M-M'') Distribution of both Collagen-IV and βPS is observed at the concentrated ectopic domain in *mmp2* mutants (arrowheads) (For control localization of Vkg and βPS, refer to Fig 4.2 A'' and Fig 4.3 A').Scale bar is 10 μm.


A
Slit Mef-2
+/+
B
mmp1 -/-
C
mmp2 -/-
D
mmp1 -/-, mmp2 -/-
E
Robo Mef-2
+/+
F
mmp1 -/-
G
mmp2 -/-
H
mmp1 -/-, mmp2 -/-
I
Robo Dg
J
K
L
I'
J'
K'
L'
M
mmp2 -/-
M'
bPS
M''
Vkg

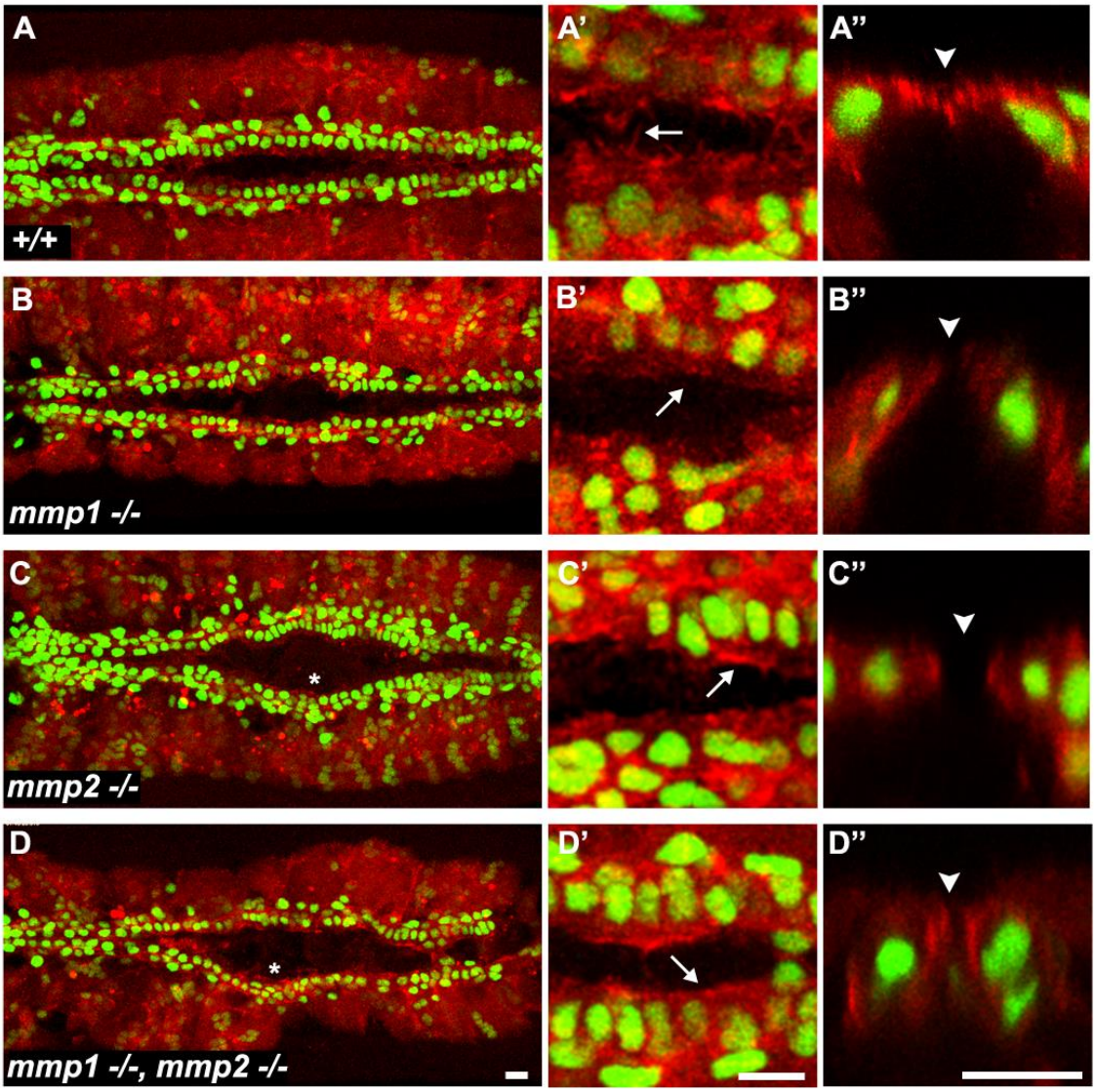
(Fig 4.4 A, E, I-I'). In *mmp1* mutants, Slit and Robo localize predominantly to the luminal domain, however lateral accumulation is observed (Fig 4.4 B, F, J-J'). In *mmp2* mutants, similar to *mmp1,mmp2* mutants, Slit and Robo do not apicalise, but accumulate at the lateral domains (Fig 4.4 C, D, H, G, K-K', L-L'). These lateral domains resemble small pockets of luminal space (Vanderploeg et al., 2012). To confirm that these pockets are ectopic lateral lumens, we determined whether Integrin and Collagen-IV are present at these domains. As predicted, both Integrin and Collagen-IV are present in the ectopic lumens in *mmp2* mutants, confirming the luminal characteristics of these lateral structures (Fig 4.4 M-M''). Even though Integrin is targeted to the apical domain in *mmp* mutants, it appears that the ability to target Slit and Robo to the apical domain is completely lost in *mmp2* mutants and is somewhat retained in *mmp1* mutants. Nevertheless, MMP1 activity is required for luminal expansion independent of Slit and Robo. Collectively these results suggest that targeting of guidance molecules Slit/Robo to the luminal domain, downstream of Integrin, requires MMP2 activity specifically.

Since, Slit accumulates on the CB cell surface in *mmp2* mutants, we hypothesized that Slit is a substrate for MMP2. We overexpressed MMP2 using a *tubulin*-GAL4 driver and performed western blot on whole embyo lysates to measure the levels of C-terminal Slit which is a product of Slit cleavage. We observed no differences in C-Slit levels in our control and experimental lanes (Fig 4.10), therefore these results failed to disprove our hypothesis.

4.4.4 MMP1 and MMP2 are required for collective cell migration of the CBs

Heart formation requires CBs to collectively migrate towards their final destination - the dorsal midline. Since MMPs in vertebrates regulate cell migration (Endo et al., 2003; Itoh, 2006; Kajita et al., 2001) and the apical ECM domain is extended in *mmp2* mutants, we hypothesized that the CCM of CBs may be affected in these mutants. To observe CCM, we developed time-lapse movies of heart development in embryos expressing a nuclear marker *tup*-GFP, and a fluorescent actin binding protein, *moesin*-mCherry, specifically in the heart cells (Fig 4.5) which allowed us to quantify aspects of CCM such as migration velocity, filopodial and lamellopodial activity. We noticed a significant reduction in migration velocity in both *mmp2* and *mmp1,mmp2* mutant CBs, however, a non-significant reduction was noted in *mmp1* mutants (Fig 4.6 A). These results indicated to us that perhaps MMP2 dependent ECM modification is required for normal migratory phenotype of CBs. Furthermore, as the CBs approach the midline, extension of multiple Moesin-labelled filopodia and lamellopodia occurs at the LE (Fig 4.5 A-A', Movie 3-1). These actin rich protrusions form apical adhesions with the contralateral CBs upon medial contact (Fig 4.5 A'') (Santiago-Martinez et al., 2008). In *mmp1 mmp2* and *mmp1,mmp2* mutants, the number of cytoplasmic extensions, in the form of lamellopodia and filopodia, present at the apical side of the CBs were significantly reduced (Fig 4.5 B - B', C-C',D-D', Fig 4.6 B, C, Movies 4.5, 4.6, 4.7.). Consistent with our previously mentioned model, this suggests that activity of MMP1 and MMP2 is required for clearing ECM barrier at the junctional domain to permit protrusive activity.

Figure 4.5. MMP1 and MMP2 are required for the formation of filopodia and lamellopodia at the leading edge. Timelapse images of live embryos expressing *tailup-GFP* and *UAS-Moesin-mCherry* under the control of *dmef-GAL4* driver. Thin cytoplasmic protrusions were identified as filopodia, whereas sheet-like extensions were categorized as lamellopodia. (A-A'') In wildtype embryos, CBs extend filopodia and lamellopodia (A' arrow) at the dorsal apical domain towards their contralateral partner cells to form contacts (A'' arrowhead). In *mmp1* mutants, numbers of filopodia and lamellopodia present at the LE are reduced (B' arrow) and CBs form small outgrowths towards their contralateral partners (B'' arrowhead). (C-D'') In *mmp2* and *mmp1,mmp2* mutants delayed migration is commonly observed (asterisk). CBs extend reduced numbers of filopodia and lamellopodia (C',D' arrow). CBs appear rounded without any apical extensions towards the midline (C''',D''' arrow). Scale bar is 10 μm


A
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+/+
B
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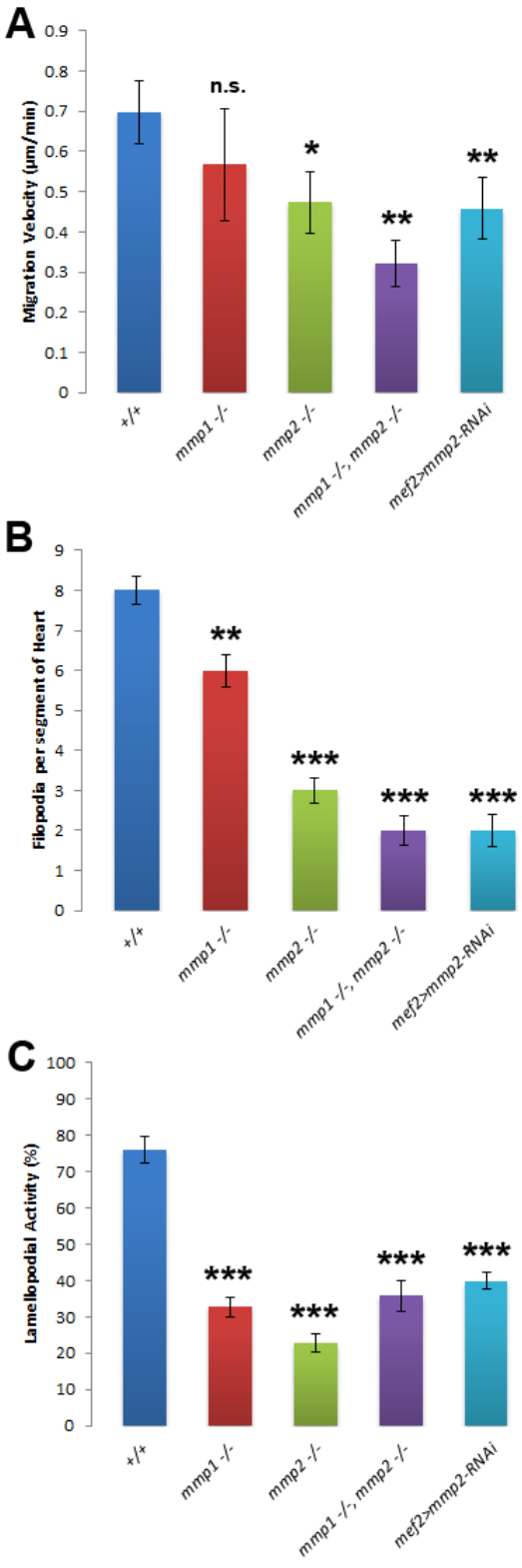
B''
mmp1 -/-
C
C'
C''
*
mmp2 -/-
D
D'
D''
*
mmp1 -/-, mmp2 -/-

Figure 4.6. Migration velocity, filopodial and lamellopodial activity of CBs is reduced in *mmp* mutant embryos. Data was collected by analysing time-lapse movies of embryos expressing cardiac markers tup-GFP (nuclear) and reporter for filamentous actin, Moesin-mCherry (cytoskeleton). **(A)** Migration velocity was calculated by measuring the distance travelled by cardiac nuclei labelled with tup-GFP over 30 minutes. Migration velocity is significantly reduced in *mmp2, mmp1,mmp2* and *mmp2-RNAi* knockdown embryos. MMP2-RNAi was driven under the control of mef-2GAL4 driver. (B) Filopodial activity was measured by counting the number of thin finger like projections at the LE for individual segments (6 CBs) of the heart (n > 50). (C) Lamellopodial activity was measured by summing the length of the bilateral rows (n > 10) which were protruding sheet like extensions and dividing it by the length of the full bilateral row. Filopodial and lamellopodial activities are significantly reduced in *mmp1, mmp2, mmp1,mmp2 mutant* and *mmp2-RNAi* knockdown embryos.. n.s. – not significant, * - P<0.05, ** - P<0.01, *** - P<0.001. Error bars represent standard error.

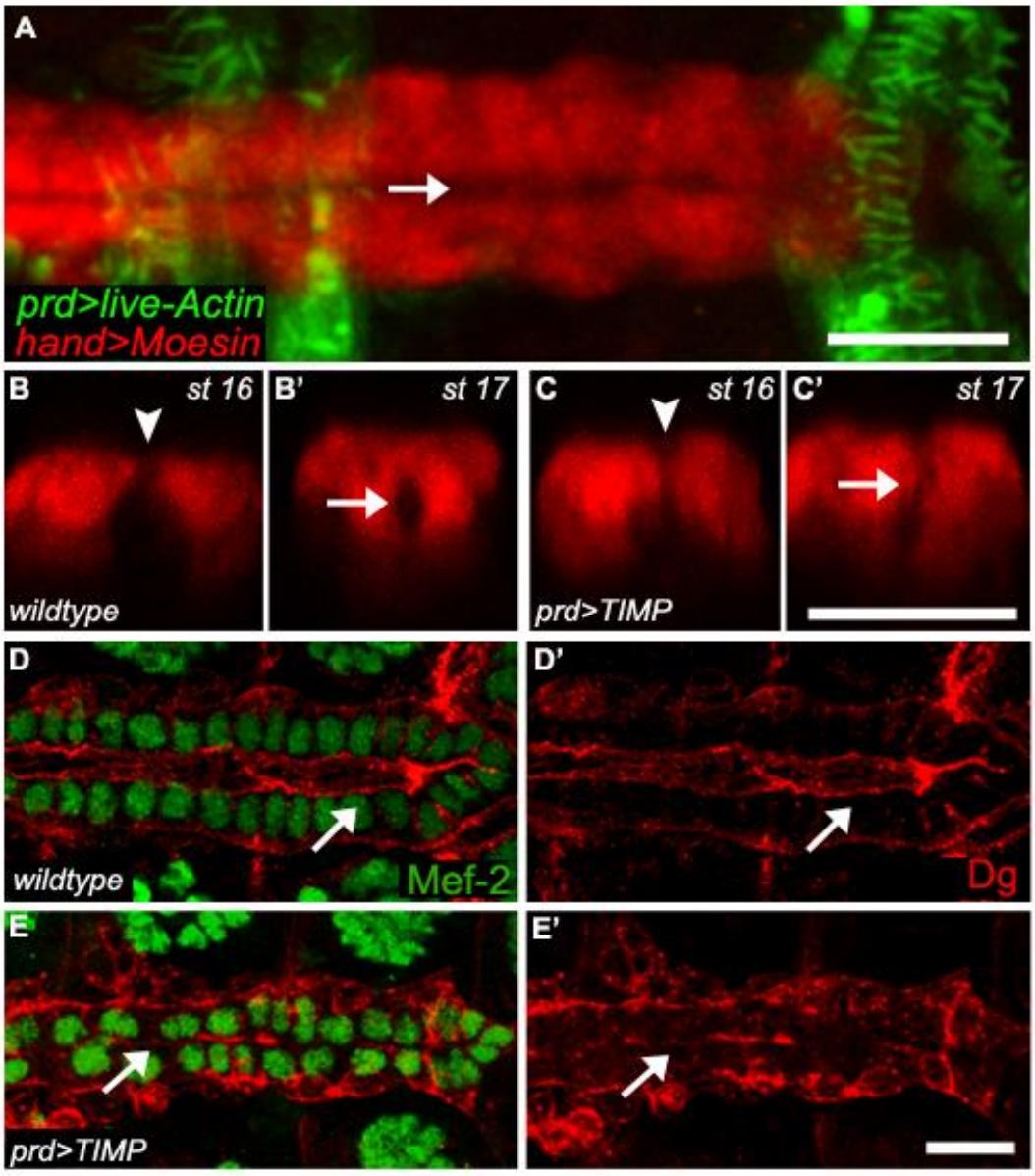

A
Migration Velocity (µm/min)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
n.s.
*
**
**
+/+
mmp1 -/-
mmp2 -/-
mmp1 -/-, mmp2 -/-
mef2>mmp2-RNAi
B
Filopodia per segment of Heart
9
8
7
6
5
4
3
2
1
0
**
***
***
***
+/+
mmp1 -/-
mmp2 -/-
mmp1 -/-, mmp2 -/-
mef2>mmp2-RNAi
C
Lamellopodial Activity (%)
100
90
80
70
60
50
40
30
20
10
0
***
***
***
***
+/+
mmp1 -/-
mmp2 -/-
mmp1 -/-, mmp2 -/-
mef2>mmp2-RNAi

Next, we wanted to test the following question: Do CBs regulate ECM degradation autonomously? To answer this question, we reduced MMP2 levels in the CBs by driving an UAS-*mmp2*-RNAi construct using a *mef2*GAL4 driver. In these embryos, CCM was affected which was apparent by the reduced LE activity (Fig 4.6 B,C, Fig 9 B,D) and reduced migration velocity to levels comparable to those in *mmp2* mutants (Fig 4.6 A). In addition, lumen formation does not occur upon expression of *mmp2*-RNAi in CBs (Fig 4.9 F), suggesting that CBs regulate their own ECM by expressing MMP2 during heart development. Altogether, these results indicate that both MMP1 and MMP2 activity is required to promote LE activity and outgrowth formation during CCM stages of heart development.

4.4.5 Ectopic expression of Timp in the ectoderm inhibits lumen formation in CBs

MMP activity is locally regulated by endogenous inhibitors (Wei et al., 2003). Timps are expressed in both the mammalian and *Drosophila* hearts during cardiac development (Brauer and Cai, 2002; Godenschwege et al., 2000). Expression of Timps in the heart tissue suggests that CBs might regulate MMP activity. Therefore, we decided to overexpress Timp in the CBs to test whether over-inhibition of MMP activity might lead to cardiac formation defects similarly observed in MMP1 or MMP2 mutants. Overexpression of Timp in the CBs did not affect lumen formation (data not shown). Therefore, we chose to express Timp in the tissue surrounding the developing heart and inhibit protease activity ectopically. We used the *paired*-GAL4 driver which is expressed in ectodermal stripes since it allowed us to inhibit MMP activity in specific segments of the heart (Fig 4.7 A). When Timp was expressed in paired stripes, CBs failed to extend

Figure 4.7. Ectopic expression of Timp in the ectodermal stripes affects lumen formation between CBs. (A) PairedGal4 expression is reported by UAS-live-Actin-GFP (green) and CBs are expressing Moesin-mCherry under the control of *hand* promoter. (B-C') Cross sectional view of embryos across the posterior heart tinman cells. (B-C') Cross sectional images taken at the tinman expressing cells under the non-paired expressing ectoderm (A dotted line). (B-B') In wildtype embryos, at stage 16, CBs extend protrusions (arrowhead) towards the contralateral partner cells and by stage 17 form a enclosed lumen (arrow). (C-C') When TIMP is expressed in the ectodermal stripes, apical protrusions (arrowhead) do not form and subsequently lumen formation does not occur (arrow). (D-D') In wildtype embryos, Dg localizes to the apical luminal domain (arrow). (E-E') In embryos expressing ectopic TIMP, apical distribution of Dg is disrupted and a luminal space does not exist between contralateral CBs (arrow). Micron scale is 10 μm.

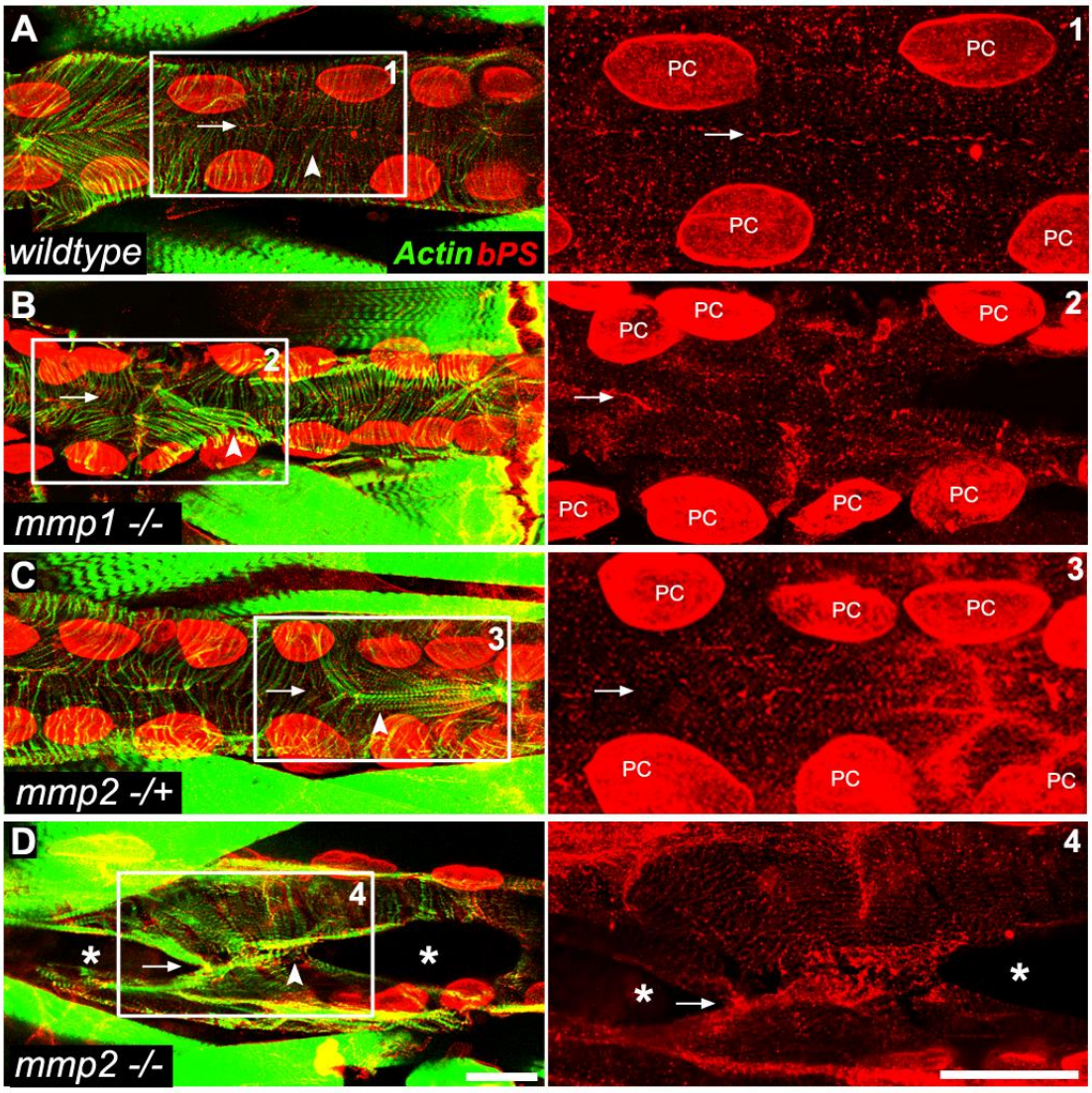

A
prd>live-Actin
hand>Moesin
B
st 16
wildtype
B'
st 17
C
st 16
prd>TIMP
C'
st 17
D
wildtype
Mef-2
D'
Dg
E
prd>TIMP
E'

apical protrusions (Fig 4.7 C). In addition, lumen formation was blocked across the heart (Fig 4.7 C'). We labelled ectopic Timp expressing embryos with a luminal (Dg) and nuclear marker (Mef2) to observe whether luminal markers localize to the apical domain. In the control embryo we see the presence of a lumen between contralateral CBs as revealed by Dg label (Fig 4.7 D-D'). In ectopic Timp expressing embryos, the proximity of the contralateral CBs indicates that lumen formation did not occur (Fig 4.7 E-E'). However, Dg label was present in few places along the heart suggesting that a reduced lumen formed (Fig 4.7 E-E'). These results suggest that segmentally patterned ectopic expression of Timp inhibits MMP activity in the entire heart.

4.4.6 Development of the larval heart is compromised in *MMP1* and *MMP2* mutants

Although we have demonstrated that both MMP1 and MMP2 are required for normal embryonic heart development, mutants are able to complete embryogenesis and hatch into larvae. Interestingly, *mmp2* mutant larvae reach third instar and die during pupation whereas *mmp1* mutant larvae rarely reach third instar (Page-McCaw et al., 2003). Therefore we were interested in assessing how a deficiency in either protease affected ECM restructuring in the growing heart. To monitor the morphology of the heart we labelled larvae with Phalloidin, which reports actin localization, and Integrin (βPS), which localizes to the cell attachment sites in the larval heart. In wildtype larvae, transverse myofibers project perpendicularly to the midline and encompass the heart circumferentially (Fig 4.8 A). Myofibers within ostial cells curve towards the inflow

F**igure 4.8. Larval heart development is affected in *mmp* mutants.** (A-D) Third instar larvae labelled with anti-Phalliodin (green) and anti-βPS antibodies. (A-A') In wildtype larvae, βPS accumulates at the midline of the heart, where contractile myofibrils connect (arrows). Transverse myofibers are organized perpendicularly towards the midline (arrowhead). (B-B') In *mmp1/mmp1* mutants, midline apposition of βPS cell adhesions is disrupted and the diameter of the heart is reduced (arrow). Transverse myofibrils re-oriented longitudinally (arrowhead). (C-C') In *mmp2*/+ positioning of the midline, as reported by βPS localization, at the center of the heart is affected (arrows) and transverse myofibrils extend longitudinally (arrowhead). (D-D') In *mmp2/mmp2* mutants, openings at the midline within the heart are frequently observed (asterisk). Disorganization of transverse myofibrils is also frequently noted (arrowhead). Pericardial cells are marked with "PC". Posterior of the heart is to the right. Micron scale is 50 μm.


A
1
wildtype
Actin bPS
PC
PC
PC
1
B
2
mmp1 -/-
PC
PC
PC
PC
PC
PC
2
C
3
mmp2 -/+
PC
PC
PC
PC
PC
PC
3
D
4
*
*
mmp2 -/-
*
*
4

tracts (Fig 4.8 A). Integrin localizes to the costameres, as well as the terminations of the myofibrils at the midline (Fig 4.8 A). In *mmp1* mutants, larval hearts have a smaller luminal diameter (Fig 4.8 B), consistent with the small overall size of *mmp1* mutant larvae (data not shown). In addition, abnormal lateral extension of transverse myofibers was observed and integrin localization at the midline was diffuse or interrupted (Fig 4.8 B). Interestingly, in *mmp2* homozygous mutants, large gaps in myofibril coverage were observed and overall heart morphology was severely defected (Fig 4.8 D). Since we observed a severe heart phenotype in MMP2 mutants, we decided to see whether larval heart development was disrupted in *mmp2* heterozygotes which develop into viable and fertile adults (Page-McCaw et al., 2003). Heterozygous *mmp2* larvae exhibited defects in transverse myofiber extension and medial integrin localization, however the diameter of the heart was normal (Fig 4.8 C). This strongly suggests that, cardiovascular defects which arise during embryogenesis are not repaired during larval development.

Figure 4.9. Expression of MMP2-RNAi in CBs results in reduced filopodial and lamellopodial activity and disrupts lumen formation. (A-F) Images of live embryos expressing *tup*-GFP and UAS-*moesin*-mCherry under the control of *mef2*GAL4 driver are shown here. (A) In wildtype embryos, filopodia and lamellopodia are abundant over the entire LE of CBs (arrow). (B) In CBs where MMP2 levels are reduced, many fewer of filopodia and lamellopodia are extended at the LE (arrow). (C) CBs form protrusions which contact the contralateral partner cells once they are near the midline (arrowhead). (D) When MMP2 levels are depleted, outgrowths do not form (arrowhead). (E) Actin, as reported by Moesin-mCherry, accumulates at the junctional domains in wildtype hearts (arrowhead). (F) Upon MMP2 depletion, lumen formation does not occur (arrow) and Actin accumulation is not observed at the junctional domain (arrowhead). (G) Apical ECM, as reported by *vkgGFP* is limited to the pre-luminal domain (arrow). Vkg does not localize to the lamellopodial extensions (arrowhead). (H) Vkg resides in the luminal ECM (arrow) and is absent from the junctional domain (arrowhead).

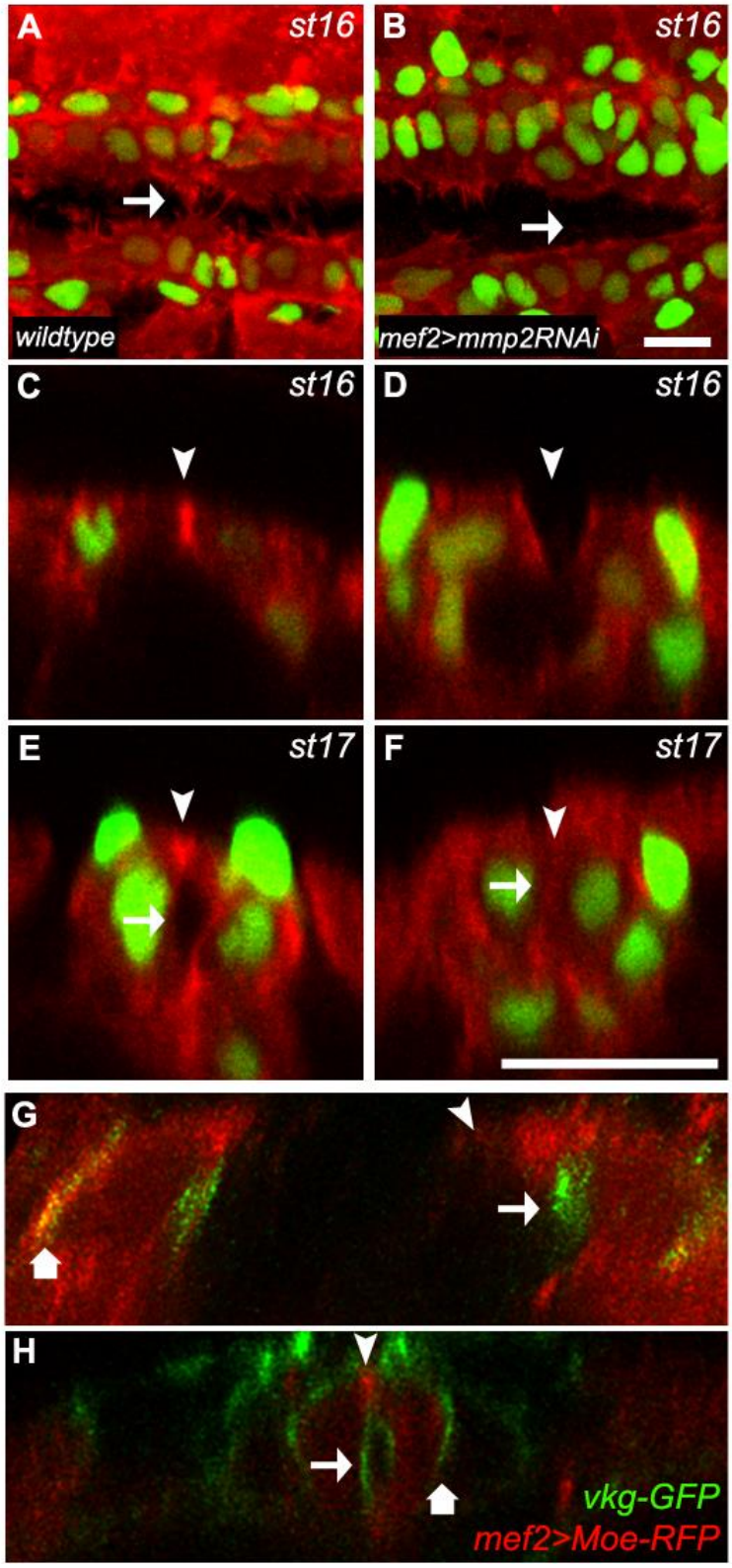
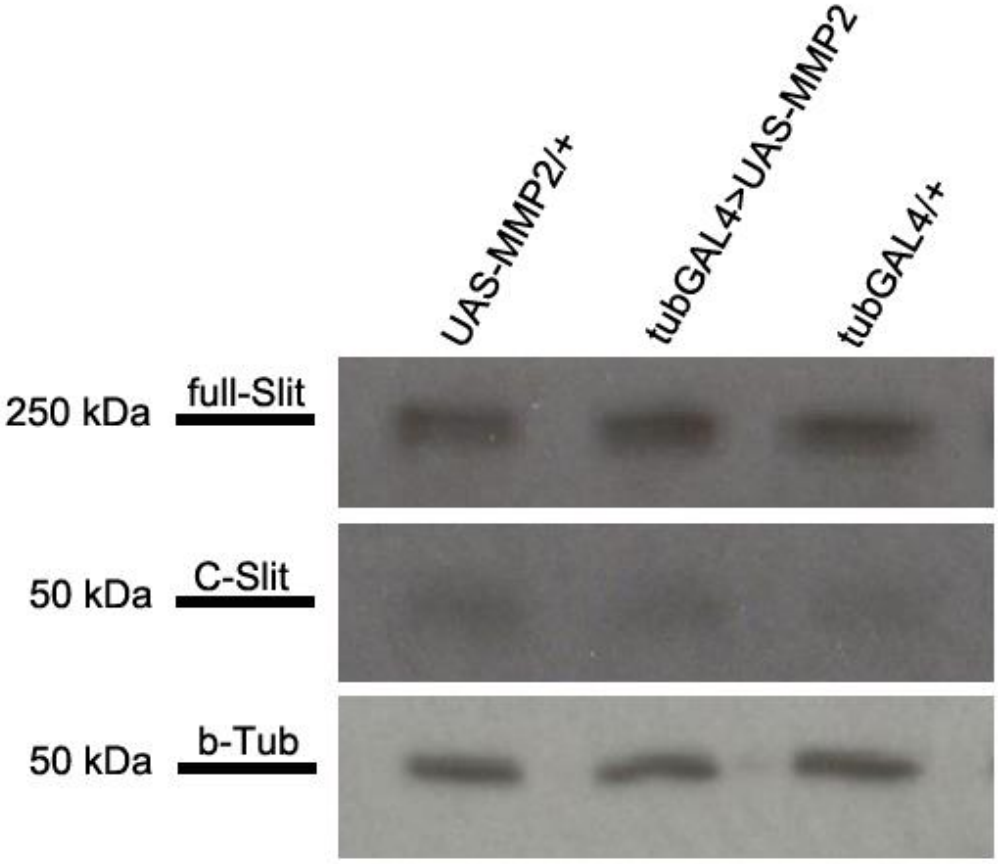

A
st16
wildtype
B
st16
mef2>mmp2RNAi
C
st16
D
st16
E
st17
F
st17
G
H
vkg-GFP
mef2>Moe-RFP

Fig 4.10. Overexpressing MMP2 throughout the embryo does not reduce Slit levels.

MMP2 was overexpressed under the control of *tubulin*-GAL4 to access whether Slit is a potential protease substrate. To evaluate Slit levels, a Western blot was performed by using C-Slit specific antibody. No difference in quantity was observed in full length Slit and C-Slit levels in control and experimental crosses. β-Tubulin blot was used as a control.


UAS-MMP2/+
tubGAL4>UAS-MMP2
tubGAL4/+
250 kDa
full-Slit
50 kDa
C-Slit
50 kDa
b-Tub

4.5 Discussion

During early stages of heart development in both vertebrates and *Drosophila* extracellular proteases play important roles during heart morphogenesis. A Disintegrin And Metalloprotease (ADAM) protein, Kuzbanian (Kuz) through cleavage of Notch promotes lateral inhibition within the cardiac primordium in *Drosophila* (Albrecht et al., 2006). *Drosophila* MMPs and ADAM proteins contain conserved regulatory and catalytic domains suggesting analogous substrate specificity and preserved ability to cleave components of the ECM (Page-McCaw et al., 2007). In mice, overexpression of tumour necrosis factor (TNF), which is a major substrate of TACE/ADAM17, results in hypertrophic hearts (Dibbs et al., 2003). Other defects such as valvular and cardiovascular malformations and septal defects are also commonly observed in the absence of both MMPs and ADAMs in mice (Zhou et al., 2004). In addition to ADAMs, several MMPs and Timps are expressed in mammalian heart precursor cells and are required for the development of the heart (Cai et al., 2000; Cantemir et al., 2004). However, the role MMPs play during *Drosophila* heart development is still largely unknown (Linask et al., 2005). Therefore, we investigated whether MMPs are required for heart morphogenesis in *Drosophila*. We have demonstrated that heart development in *Drosophila* requires the activity of both MMP1 and MMP2 during embryonic development. We have shown that MMP2 activity in CBs is autonomously required, whereas Timp expression can affect the morphogenesis of adjacent tissue. We propose that MMPs regulate both vertebrate and invertebrate early cardiac development via homologous mechanisms, and that *Drosophila* provides a tractable genetic model of cardiac ECM remodelling.

4.5.1 MMP1 and MMP2 have distinct roles during embryonic heart development

Our data indicates that MMP1 and MMP2 perform distinct functions during heart development. At first, both MMP1 and MMP2 are required for LE activity during CCM. Subsequently, their roles during apical outgrowth and lumen formation diverge. In the absence of MMP1 activity, apical junctions form, however extend into the luminal zone which highlights the requirement of this secreted protease during luminal expansion. Consistently, we noted a considerable reduction in cardiac luminal diameter in *mmp1* mutant larvae. Due to the extreme nature of the *mmp2* mutant hearts, it is difficult to interpret whether MMP2 might contribute to luminal expansion. Nevertheless, MMP2 activity is crucial for dorsal and apical adhesion formation since in *mmp2* mutants CBs are completely rounded and do not make medial contacts. During fat body disintegration in *Drosophila* pupae, MMPs serve distinct functions, where MMP2 is primarily required to cleave components of the BM to disable cell-ECM interaction and MMP1 disrupts cell-cell adhesions by cleaving cell adhesion molecule, E-Cad (Jia et al., 2014). The differential substrate specificities of MMP1 and MMP2 might explain their distinctive roles during heart development. For instance, *Drosophila* MMP1 cleaves mammalian Fibronectin and non-fibrillar Collagen-IV, whereas fibrillar collagens and Lan are resistant to its proteolytic activity (Llano et al., 2000). Whether Lan is a substrate of MMP2 is not known but its collagenase activity is conserved in *Drosophila* (Llano et al., 2002). Since Lan is a substrate for mammalian MT1-MMP (Koshikawa et al., 2004) and Lan is a crucial ECM components required for CB lumenogenesis (Haag et al., 1999) and vertebrate cell migration (Koshikawa et al., 2000), absence of MMP2 dependent cleavage

of Lan-A might account for extreme cardiac phenotype observed in MMP2 loss-of-function mutant.

4.5.2 MMPs promote cell polarization by modulating guidance signalling

The first polarizing event during CCM of CBs is the targeting of Integrin and ECM components to the apical pre-luminal domain. Subsequently, Integrin presence at the luminal domain stabilizes the localisation of guidance molecules, Slit and Robo, which are required to expand the lumen (Vanderploeg et al., 2012). CB polarization is severely compromised in the absence of MMP2, whereas in the absence of MMP1, polarization defects are relatively less pronounced. Specifically, Integrin, Dg and Col-IV accumulate over the entire apical domain in *mmp2* mutants, whereas in *mmp1* mutants these proteins localize normally to a reduced lumen. MMP2 is not required to target Integrin apically, rather, is required to limit the extent of the ECM within the apical domain. During fat body disintegration in *Drosophila*, MMP2 is also not required for proper targeting of Integrin (Jia et al., 2014). In addition, in the absence of MMP2 activity, ectopic lumens (Vanderploeg and Jacobs, 2015; Vogler et al., 2014) form between lateral CBs which localize luminal markers, Integrin, Dg, Slit, Robo and Col-IV. Therefore, the complex of initial luminal determinants can assemble in the absence of MMP2. However, stabilisation of late luminal markers, Slit and Robo, is MMP2 dependent. Furthermore, presence of Prc at the apical domain in *mmp1* and *mmp2* mutants suggests that even though the apical cell membrane localizes Integrin and Dg, apical ECM identity is lost in absence of MMP2 and partially lost in absence of MMP1. Therefore, we propose that MMP2 regulates apical assembly of ECM which modulates

apicalisation of guidance signalling molecules Robo and Slit and limiting the localization of ECM constituents and receptors to the luminal apical domain. Since it is still not clear whether Slit is a substrate for MMP2, one possible way of how ECM remodelling might modulate Slit/Robo signalling is through regulation of the heparin sulphate proteoglycan, Sdc (Sdc). Sdc is required for stabilizing the Slit/Robo signalling complex and might function as a factor which localizes Slit and Robo to the luminal domain (Knox et al., 2011). Consistently, Sdc contains conserved cleavage sites recognized by membrane tagged MMPs (MT-MMPs) (Manon-Jensen et al., 2013) and shedding of Sdc by MT-MMPs promotes cell migration *in vitro* (Endo et al., 2003). Therefore, LE motility of CBs might be locally stimulated through release of Sdc bound GF which in turn induce actin cytoskeletal rearrangement.

4.5.3 MMP1 and MMP2 are required for formation of cytoplasmic protrusions at the LE

MT1-MMP, which is the most studied membrane type MMP in vertebrates, has been shown to accumulate at invadopodia of metastasizing cancer cells and is responsible for tumour invasion (Jacob and Prekeris, 2015; Poincloux et al., 2009; Watanabe et al., 2013). Significant reduction of filopodia and lamellopodia at the LE of CBs in *mmp* mutants suggests that both MMP1 and MMP2 play an essential role in regulating both the formation of filopodia and lamellopodia. Nevertheless, significant reduction in migration velocity of CBs in *mmp2*, but not *mmp1*, mutants indicates that MMP2 dependent ECM remodelling might be required to promote CCM. How do MMPs regulate filopodial and lamellopodial extension in CBs? Low level of filopodial and lamellopodial activity is

detected in *mmp1*, *mmp2* double mutants indicating that MMPs might not have a direct role in promoting LE activity. Rather, MMPs may be required to remove the apical ECM barrier which creates space for the formation of normal protrusive activity. In addition, cleavage of the ECM component, Sdc, which is abundant in the cardiac ECM (Knox et al., 2011), might release embedded signalling molecules such as Slit and Net. Both Slit and Net contain domains which interact with ECM components and are required for inducing LE activity in the CBs (MacMullin and Jacobs, 2006; Syed, 2011) (Chapter Three). We propose the MMP2 dependent cleavage of the ECM at the dorsal and ventral apical domain creates the right environment for the formation of protrusions and releases guidance signalling molecules embedded in the ECM which induce filopodial and lamellopodial dynamics. ECM at the pre-luminal and luminal apical domains however might be shielded from MMP degradation by Timp which is endogenously expressed in the CBs during CCM and lumen formation (Godenschwege et al., 2000). This means that in the absence of Timp, lumen formation presumably will not occur. We can test this by labelling *timp* mutant embryos with an Integrin and ECM specific antibodies.

4.6 Conclusion

In summary, we propose that MMP1 and MMP2 cooperatively regulate LE dynamics of the CBs during CCM and identity of the apical ECM. During lumen formation, MMP2 activity regulates outgrowth formation, cell polarisation and lumen formation whereas MMP1 limits the medial extension of the adhesion domain and promotes luminal expansion. We conclude that both cooperative and distinct roles of MMPs regulate embryonic heart development in *Drosophila*.

Discussion

CHAPTER FIVE

In this thesis, I developed a new genetic model to study CCM. The data presented here demonstrates that all the characteristics which are observed in other cell migration models such as common direction of migration, stabilization of lateral adhesions and ECM remodelling are also observed during CB migration in *Drosophila*. First, we developed a paradigm to study the LE kinetics of the migrating CBs. By employing time-lapse imaging coupled with GAL4/UAS mediated expression of fluorescent reporters, I have qualitatively and quantitatively assessed the migratory behaviour of the CBs. Analysis presented here reveals characteristics of the LE which would go undetected in experiments which use fixed embryo imaging techniques. The CB LE is highly dynamic and is capable of extending multiple apical protrusions in the form of filopodia and lamellopodia which are in constant change. Protrusive behaviour progressively increases as the CBs reach their midline destination. During the initial stages of lumen formation, which occurs after the CBs have migrated to the midline, filopodia and lamellopodia mediate contact formation between the contralateral partner cells which leads to stabilization of the apical attachment. Furthermore, the CB LE is highly sensitive to the levels of guidance signalling which is mediated through Robo/Robo2 and Fra/Unc5 and their respective ligands Slit and Net. In *Drosophila* and other higher organisms, these guidance receptors display a hierarchical relationship where Robo in physical association with Fra attenuates Net based attractive guidance (Stein and Tessier-Lavigne, 2001). Nevertheless, a concrete model for their collective activity has not been established and a context dependent repulsive and/or attractive role for both Slit and Netrin guidance cues have been advocated. We propose that during heart development, both Robo and Fra

function cooperatively to induce actin cytoskeletal rearrangement at the LE presumably through recruitment of cytoplasmic adaptor proteins. Second, we demonstrate that CBs autonomously regulate ECM remodelling by modulating members of the highly conserved family of proteases, MMPs. In vertebrates, membrane tethered and secreted MMPs perform various function through cleavage of both ECM and non-ECM components (Itoh, 2015; Lemaitre and D'Armiento, 2006). In the absence of either MMP1 or MMP2, several processes which are required for heart development are compromised. Membrane tethered MMP2 and secreted MMP1 are required to establish the apical motility of the CB LE. Cell polarization and lumen formation require MMP2 whereas the luminal expansion is dependent on the activity of MMP1. Finally, our data suggests that the interplay of guidance signalling and ECM remodelling is required to mould the embryonic heart in *Drosophila*. In vertebrates, similar relationship between ECM remodelling and growth factors signalling has been established in promoting processes such as cell migration, cell polarization and overall organogenesis (Bonnans et al., 2014; Brown, 2011). In mammals, ECM components such as cell surface-tethered proteoglycans sequester GFs, FGF, TGF-β and BMP by forming non-covalent associations (Baeg and Perrimon, 2000; Matsuo and Kimura-Yoshida, 2014). Such accumulation of latent GFs results in the formation of signal reservoirs within the ECM (Kular et al., 2014). The release of these trapped GFs is achieved through proteolysis of the ECM by MMPs, which functions upstream of GF mediated signalling in multiple models (Sternlicht and Werb, 2001). Our data suggests that a similar mechanism takes place during heart morphogenesis in *Drosophila* where MMP2 functions upstream of

Slit/Robo signalling to induce LE dynamics, cell polarity and lumenogenesis. Slit localizes to the CB surface and accumulates within the cardiac ECM. MMP2 dependent degradation of the apical ECM releases Slit and Net signal which (1) induces formation of the filopodia and lamellopodia during CB migration; (2) promotes polarised retention of ECM components; (3) formation of apical adhesions between contralateral CBs and (4) preservation of lateral adhesion between ipsilateral CBs.

5.1 Heart development in *Drosophila* as a Collective Cell Migration model

To be classified as a model of CCM, three criteria must be preserved (Friedl and Gilmour, 2009). These criteria include (1) the maintenance of adhesions between cells during migration; (2) organization of multicellular polarity which ensure unidirectional migration by directing the LE; and (3) remodelling of the surrounding ECM by extracellular proteases. CBs rely on two sets of cellular adhesions to reach the midline. First, CBs form adhesions with ipsilateral partners which localize adheren junction related markers E-Cad, β-catenin, Dlg and PIP_2 (Haag et al., 1999; Qian et al., 2005; Santiago-Martinez et al., 2008) (Appendix A). Second, during early stages of migration, CBs depend on adhesions with the overlaying ectoderm (Haack et al., 2014). This satisfies criterion (1). Time-lapse analysis presented in this thesis effectively demonstrates that CBs form a polarised LE which is directed towards the direction of migration. Furthermore, guidance signalling through Slit and Net is required for protrusive behaviour of CBs. This satisfies criterion(2). Lastly, CBs form integrin mediated adhesion with the basal and apical ECM (Vanderploeg and Jacobs, 2015; Vanderploeg et al., 2012) and our results further reveal that remodelling of the ECM surrounding the CBs via

MMPs is essential for normal migratory behaviour of the CBs. This satisfies criterion (3). Therefore, the experiments reported here provide sufficient evidence that *Drosophila* embryonic heart development, like other well established CCM models (Dona et al., 2013; Montell et al., 2012), can be employed to study dynamics of collective movements of cells. Nevertheless, two unique features are displayed during CB CCM. First, all CBs function as leader cells during migration. This behaviour requires proper coordination between ipsilateral cells and is achieved through autonomous regulation by secreted morphogens such as Slit (Appendix B). Nevertheless, reduction of Slit and Robo levels in ostial CB progenitors results in disruption of heart development between muscular CBs as well. Even though this mechanism is not fully understood, it is possible that by signalling to their neighbours, CBs are able to coordinate cell-cell adhesion, cell-ECM adhesion and cell polarisation. Second, CBs are encompassed by both apical and basal ECM. These ECM domains differ in composition of proteins which are deposited by the CBs.

5.2 Interplay between Slit and Netrin mediated guidance signalling

CBs physically interact with the AS perimeter cells, which are the last cells to lose contact with the ectoderm, and are able to extend protrusions between the AS-ectoderm interface (Appendix C) (Haack et al., 2014). Contacts with the AS perimeter cells are maintained up until interaction between contralateral CBs is established. This mechanism of migration is also noted during avian heart tube assembly, where anterior displacement of the myocardium requires both contraction of the endoderm and autonomous myocardial deformation (Aleksandrova et al., 2015). In *Drosophila* protrusive activity of the CB LE significantly increases as the cells approach the midline. Possibly, the

activation of LE dynamics in the CBs is a turning point when autonomous signalling via Slit and Net assists in promoting migration. A similar role for Slit and Net has not been demonstrated in vertebrate hearts however expression of Slit, but not Net, has been reported in heart tissue during early stages of tube formation in zebrafish and mice (Fish et al., 2011; Medioni et al., 2010). Therefore, Slit and Net might function via analogous mechanisms to promote cardiomyocyte migration and early tube assembly during vertebrate heart formation as discussed below.

In the developing *Drosophila* heart, we demonstrate that the LE is highly sensitive to the levels of guidance signalling. Activity of the LE and migration velocity of the CBs analysed in any of the single guidance signalling mutants were noticeably reduced. Additional heart defects such as weakened lateral adhesions, delayed migration and absence of migratory morphology of CBs are commonly noted in all mutant genotypes. Therefore, our data disputes the conclusion stated by Vogler et al. which claimed that Slit/Robo signalling is not required for filopodial extension in the CBs (Vogler et al., 2014). To quantify filopodial number, Vogler et al. overexpressed *actin5C*-GFP construct in the CBs to visualise the filopodia. Overexpression of Actin5C can lead to dominant phenotypes in multiple models of cell migration ((Fulga and Rorth, 2002; Geisbrecht and Montell, 2004), personal observation in the AS) and therefore we suspect that the high number of filopodia in *robo,robo2* mutants might be due to overexpression of Actin5C in the CBs. To avoid this problem, we used an alternative actin reporter *moesin*-mCherry, which co-localizes with Actin at the LE and has been previously validated to not cause dominant effects (Millard and Martin, 2008).

Cooperation between attractive Net and repulsive Slit signalling establishes the positioning of the axonal outgrowths during neuronal development (Ypsilanti et al., 2010). In *Drosophila*, Robo functions upstream of Fra/DCC and suppresses responsiveness to Net which leads to axons crossing the segment boundary rather than turning medially (Hiramoto and Hiromi, 2006). A similar hierarchical organisation of Robo and Fra receptor is noted in vertebrates as well (Zhang et al., 2012). Nevertheless, in some cases Robo conveys an attractive signal during cell migration. For example, in mice, both branch-promoting and branch-repelling role of Slit/Robo signalling have been suggested in the developing sensory axon (Ma and Tessier-Lavigne, 2007). Additionally, during *Drosophila* tracheal development Robo functions as a repulsive receptor and Robo2 promotes attractive signalling when Slit is present (Englund et al., 2002). During heart development, both Slit, Net and their receptors are required to promote LE activity. Since evidence for their cooperative function has been suggested in other models, we hypothesized that Slit and Net based signalling converges together to establish the LE activity of the CBs. Our analysis suggests that an epistatic relationship between Slit receptor Robo and Net receptors Fra and Unc5 exists. Reducing gene dosage of both Robo and one of the Net receptors, Fra or Unc5, in trans leads to reduction of LE dynamics. This suggests that Robo along with Fra and Unc5 function to promote the same cellular output which is to increase LE motility. Interestingly, studies have demonstrated that Robo via its CC1 intracellular domain physically interacts with Fra P3 domain, and this interaction is required for Robo mediated silencing of Fra attraction (Stein and Tessier-Lavigne, 2001). Therefore, we hypothesized that if Robo and Fra function

collectively to induce LE dynamics, then overexpression of either transgenes in *robo* or *fra* mutant should at least partially rescue LE motility. Indeed, overexpression of Fra in *robo* mutant background and overexpression of Robo in *fra* mutants rescued both filopodial and lamellopodial activities. This clearly suggests that Robo and Fra function cooperatively to induce LE motility of the CBs during migratory stages. Furthermore, when *robo* mutants were rescued by overexpressing Fra, migration velocity and lumen formation were not restored. This is consistent with results from a previous study which demonstrated that Fra contributes to lumen formation by establishing the apical adhesive junctions only. In *fra* mutants, CBs do not form apical adhesion however a fraction of embryos displays a phenotype where the luminal domain is expanded at the expense of the apical adhesive domains (Macabenta et al., 2013). To summarize, these results suggest that function of Robo and Fra converge together to promote filopodial and lamellopodial dynamics of the CB LE, however lumen formation strictly requires Robo whereas Fra activity is dispensable for this process.

The extent to which the filopodial or lamellopodial activity is reduced in the absence of either Slit or Net differs considerably. For instance, most dramatic reduction in LE activity is observed in *slit* mutants and both filopodial and lamellopodial activities are significantly higher in *netA,netB* mutants compared to *slit* mutants. Therefore, we concluded that Net is not essential for promoting LE activity. This means that Fra might be functioning independent of Net since our quantitative data further demonstrates the number of heart segments extending 7 or more filopodia is higher in *netA,netB* mutants compared to *fra* mutants. Even though Slit and Net distinctly regulate levels of LE

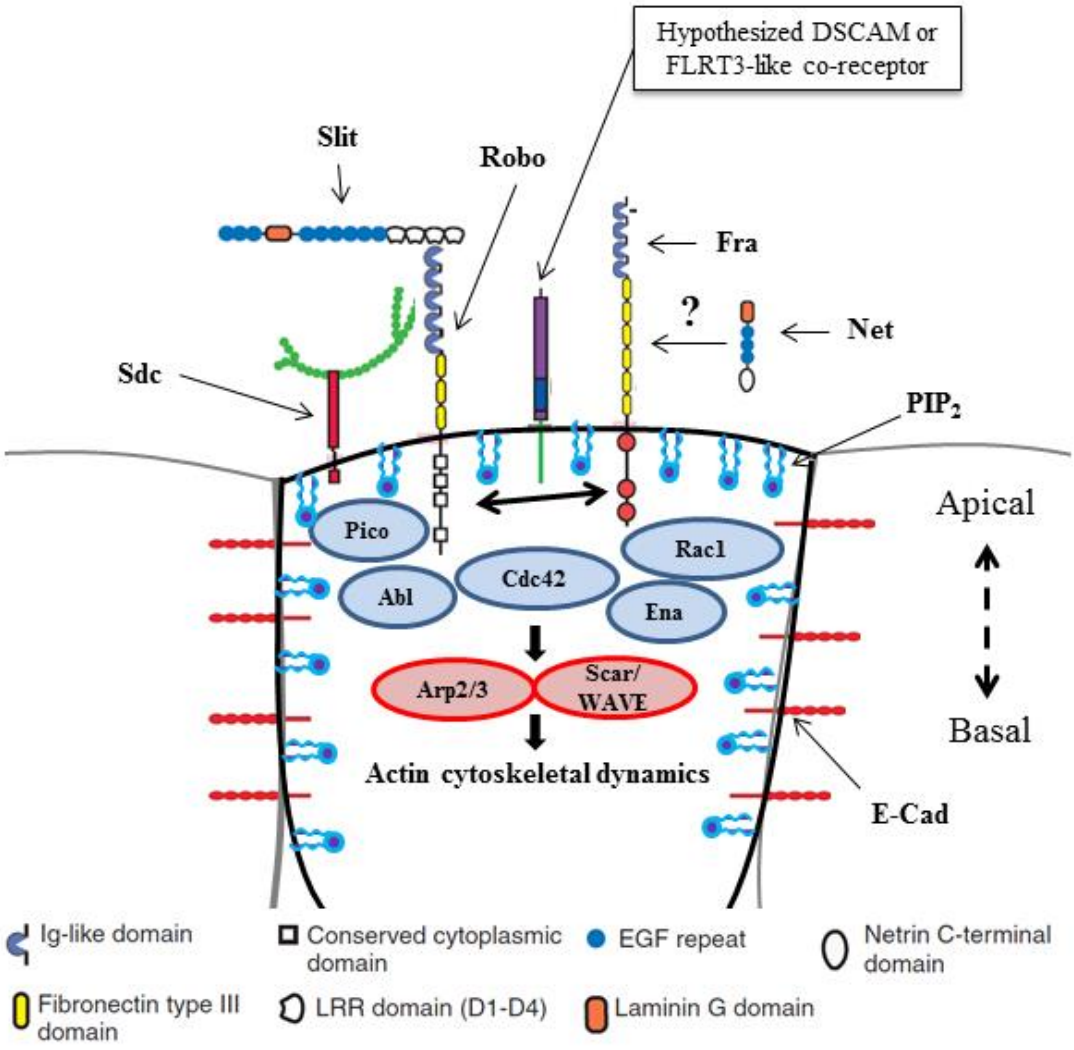
activity, both of these guidance cues are simultaneously required for promoting maximal protrusive behaviour of the CB LE. Similar requirements for Slit and Net are noted during lumen formation. Absence of Slit results in the complete loss of luminal characteristics at the apical domain along the entire heart. In *netA,netB* mutants, although a continuous lumen fails to form across the heart, presence of apical luminal pockets localizing luminal markers are reported here (Appendix D) and in other studies(Albrecht et al., 2011; Macabenta et al., 2013). Lumen formation however is reported to require the activity of repulsive Net receptor, Unc5 which function independent of Slit/Robo signalling. However, in the absence of Unc5, markers such as Slit, Robo and Dg are present at the luminal domain however the lumen fails to expand (Albrecht et al., 2011). Despite what is proposed by Albrecht et al., it appears that similar to Net, Unc5 might be required for luminal expansion since localisation of key luminal determinant is normal in *unc5* mutants. Therefore, we finally conclude that Slit is the primary guidance cue which establishes LE motility and lumen formation during heart development, whereas Net is partially required. In addition, Unc5 might function independent of Slit/Robo for luminal expansion whereas Fra might work alongside Robo to induce outgrowth formation.

The traditional model of guidance signalling during axon guidance states that Slit is a repulsive ligand for Robo whereas Net is an attractive cue for Fra and a repulsive cue for Unc5. When Robo binds Slit, negative regulation of actin cytoskeletal rearrangement molecules such as Cdc42, Rac1 and RhoA leads to deactivation of protrusive capacity at the domain which comes in contact with the Slit ligand and hence the membrane activity in the axon is reduced and actin cable growth is inhibited (Ypsilanti et al., 2010). On the

other hand, when Fra receptors encounters Net ligands, Cdc42, Rac1, Pak1 and N-WASP are recruited to the cytoplasmic domain and activate cytoskeletal rearrangement which leads to formation of filopodial and lamellopodial protrusions in the direction of Net signal (Shekarabi et al., 2005). Our results do not directly demonstrate a hierarchical organisation of Robo and Fra. Rather both Robo and Fra functions converge together to activate formation of cytoplasmic protrusions and therefore both of these receptors are functioning attractively. This means that, unlike its role during axonal guidance, Robo function as an attractive receptor during CB migration and might be required to promote cytoskeletal rearrangement. Similarly, during *C. elegans* embryogenesis, both Sax-3/Robo and Unc-40/DCC/Fra function to polarise filamentous Actin by regulating the Scar/WAVE actin nucleation complex (Bernadskaya et al., 2012). In mice, Robo1 interacts with the member of the fibronectin leucine-rich repeat transmembrane receptor FLRT3 and this interaction promotes Net based attraction by upregulation of DCC levels at the plasma membrane of migrating axons (Leyva-Diaz et al., 2014). Therefore, it might be possible that when Robo physically or indirectly associates with attractive co-receptors, such as FLRT3 and Fra, adapter proteins which induce cytoskeletal rearrangement are recruited. Consistently, the intracellular domains of both Fra and Robo are able to bind Enabled (Ena)/Vasodilator-associated phosphoprotein (VASP) and Abelson tyrosine kinase (Abl) which are adaptor proteins with conserved functions in inducing cytoskeletal dynamics (Forsthoefel et al., 2005; O'Donnell and Bashaw, 2013). Additionally, a study conducted on Robo-Fra chimeric receptors demonstrated that attractive or repulsive signalling solely depends on the intracellular domain of the

receptor and not the ligand (Bashaw and Goodman, 1999). One possible candidate, is the *C.elegans* ortholog of the mammalian Lamellopodin (Lpd), MIG-10 (Chang et al., 2006). In migrating fibroblasts, Lpd localizes to the lamellopodia and by physically interacting with Ras GTPases via Ras association domain and PIP_2 via pleckstrin homology (PH) domain is recruited to the intracellular localisation at the plasma membrane (Michael et al., 2010). Both mammalian Lpd and its *Drosophila* homolog Pico physically interact with Ena/VASP proteins and thereby positively regulate lamellipodial protrusion dynamics at the LE of migrating cells (Law et al., 2013). Additionally, reducing levels of Pico leads to an increased Globular:Filamentous Actin ratio whereas increasing Pico levels lead to a decrease in Globular:Filamentous Actin ratio in an EGFR dependent manner (Lyulcheva et al., 2008). In *Xenopus*, Lpd cell autonomously regulates neural cell migration via Scar/WAVE complex. In *Drosophila*, Pico regulates CCM of the BC by physically binding Scar (Law et al., 2013). These studies suggest that Pico and it homologs are evolutionary conserved regulators of LE dynamics during cell migration. Therefore, we hypothesize that Pico functions downstream of Robo and Fra in the CBs to induce formation of filopodia and lamellopodia by recruiting Ena/VASP, Abl and Scar/WAVE complexes (Krause et al., 2004; Michael et al., 2010). The recruitment of proteins to the LE such as Cdc42, ARP2/3 complex and ENA/VASP is accomplished by Phophotidylinositol-(4,5)-biphosphate (PIP_2) (Yin and Janmey, 2003). Furthermore, PIP_2 directly, and also as a secondary messenger, modulates filopodia and lamellopodia formation at the LE (Mattila and Lappalainen, 2008; Suetsugu et al., 2014). PIP_2 localizes at the filopodial and lamellopodia in the CBs (Appendix A). Since Pico contains a PH

Figure 5.1. Model for guidance signalling function during migratory stages of CBs. The following model developed based on evidence presented in this thesis and information provided by studies conducted in other cell migration models. At the apical edge, activation of Slit and Net receptors, Robo and Fra, results in localisation of PIP_2 to the LE domain. PIP_2 and the cytoplasmic tails of Robo and Fra receptors recruit adaptor proteins Cdc42, Rac1, Abl, Ena and Pico which activate cytoskeletal rearrangement. These adaptor proteins further recruit scaffolding complexes Arp2/3 and Scar/WAVE, which nucleate Actin filaments. Bundling of F-Actin in to long thin and branched fibers results in formation of cytoplasmic protrusions such as filopodia and lamellopodia. Slit and Net physically interact with Dscam or an unknown FLRT3 like co-receptor which further amplifies cytoplasmic localisation of PIP_2 and adaptor proteins at the LE. Dscam might also mediate the physical interaction between Robo and Fra and this association is required for LE activity. Net might work independent of Fra to induce LE activity, whereas both Slit and Sdc are required to promote signalling via Robo. Lateral domains also localize PIP_2 in response to guidance signalling, which stabilizes E-Cad mediated adhesions.


Hypothesized DSCAM or FLRT3-like co-receptor
Slit
Robo
Fra
?
Net
Sdc
PIP2
Apical
Basal
Pico
Rac1
Cdc42
Abl
Ena
Arp2/3
Scar/ WAVE
Actin cytoskeletal dynamics
E-Cad
Ig-like domain
Conserved cytoplasmic domain
EGF repeat
Netrin C-terminal domain
Fibronectin type III domain
LRR domain (D1-D4)
Laminin G domain

domain, it might be possible that PIP_2 mediated recruitment of cytoskeletal rearrangement adaptor proteins, such as Pico, Cdc42, Rac1, ARP2/3, Ena/VASP and Scar/Wave downstream of Robo and Fra is required for formation of filopodia and lamellopodia in the CBs (Fig 5.1). In support of this model, expression of a Cdc42 dominant negative construct in the CBs results in reduction of filopodial processes at the LE (Swope et al., 2014).

One way to further test this model is to reduce Pico levels in the CBs by RNAi mediated knockdown and determine whether LE dynamics in the CBs are disrupted. Additionally, we can determine whether Pico co-localizes with PIP_2 at the LE. Whether PIP_2 functions downstream of Dscam, Robo and Fra mediated signalling in the CBs to recruit cytoskeletal rearrangement adaptor proteins and maintain E-Cad based lateral adhesions is currently unknown. We hypothesize that PIP_2 mediated signalling input from Integrin recruits guidance signalling molecules and their downstream cytoplasmic adaptors which activate actin cytoskeletal rearrangement machinery in the CBs. In addition, we think that PIP_2 dependent stabilization of lateral adhesion junctions is crucial to maintain the balance between epithelial and mesenchymal characteristics in CBs. Therefore, to test this idea, we can determine whether PIP_2 localization at the apical protrusions and lateral domains of CBs is disrupted in Integrin mutants.

Another molecule which functions as an attractive receptor when bound to Net is Down Syndrome Cell Adhesion Protein (Dscam). Dscam, like Fra/DCC and Robo, contains FNIII and Ig domains and has the ability to bind Net with the same affinity as Fra/DCC (Liu et al., 2009). Dscam function in parallel with Fra/DCC to promote

attractive signalling via Net (Ly et al., 2008). Evidence that Dscam also binds the N-terminal product of Slit cleavage was presented at the recent *Drosophila* Research Conference. According to Alavi et al. presence of N-Slit promotes formation of a Robo-Dscam complex which conveys attractive signalling (Alavi et al. 2015). This could explain the attractive role for Slit and Robo presented in this thesis. It is possible that Dscam, Robo and Fra collectively promote actin cytoskeletal rearrangement at the CB LE. It is not currently known whether cleavage of Slit is required for LE motility of the CBs or whether Dscam is expressed in the CBs. Future studies can focus on elucidating the localisation of Dscam during early and late stages of heart development. Additionally, by studying the migratory and lumen formation phenotypes exhibited by CBs in *dscam* mutant embryos, we can determine whether Dscam and its homologues are required for filopodial and lamellopodial activity. To demonstrate that combinatorial attractive input from Dscam, Robo and Fra receptors is required to increase CB LE activity, we can test whether introduction of a mutant allele of these receptors into a sensitized background such as *robo/+* might result in an more than additive LE motility phenotype.

In the guidance signalling mutants, even though LE activity was affected, CBs eventually reach the midline. In addition, in transheterozygotic mutants of Slit and Net receptors, migration velocity was unaffected even though filopodial and lamellopodial activity was significantly reduced. This suggests that absence of filopodia and lamellopodia alone does not lead to a reduction in migration velocity of CBs. However, absence of either Slit or Net dependent signalling does lead to a significant reduction in migration velocity. During sprouting angiogenesis in zebrafish, filopodia are not essential

for mediating guidance signals but are required to facilitate rapid migration of endothelial tip cells and anastomosis of blood vessels (Phng et al., 2013; Wacker et al., 2014). Similarly, in *C. elegans* filopodia are dispensible for accurate axonal guidance downstream of Slit and Net signalling (Chang et al., 2006). Finally, we conclude that, similar to endothelial cell migration and axonal guidance, cytoplasmic outgrowths are dispensable for CB medial migration, however, are required for establishing apical attachments which is crucial for heart formation.

5.3 MMP activity is required for ECM remodelling during heart development

Drosophila MMPs were first described 15 years ago (Llano et al., 2002; Llano et al., 2000). Since then several processes have been discovered which require MMP activity during morphogenesis (Deady et al., 2015; Glasheen et al., 2010; Jia et al., 2014; Miller et al., 2011; Page-McCaw, 2008; Stevens and Page-McCaw, 2012). Research on vertebrate MMPs has been ongoing since the early 60s. Hence, a lot of what we know about the molecular mechanisms of these proteases comes from *in vitro* and *in vivo* vertebrate studies. For example, studies conducted on mice could not establish that MMPs play critical roles during development. These vertebrate studies underestimated the importance of coordinated activity of multiple MMPs in shaping embryogenesis and thus applied a single gene knockout strategy which was ineffective. In *Drosophila*, however, only two MMPs exist which have divergent functions and are shown to have both overlapping and non-overlapping substrates (Jia et al., 2014; Llano et al., 2002; Llano et al., 2000). Therefore, the traditional approach of studying single and double MMP gene knockout in *Drosophila* can allow for complete elimination of MMP activity.

Nonetheless, in *Drosophila* double mutants of MMPs survive embryogenesis and die during mid to late larval stages. This observation led to the conclusion that MMPs are not essentially required for embryogenesis (Page-McCaw et al., 2003). In this thesis, I demonstrate that MMPs play crucial roles during the formation of the *Drosophila* embryonic heart.

Cell polarisation is regulated by physical interactions with the basal ECM (Yurchenco, 2011). Polarised cells regulate their apico-basal polarity by depositing ECM components such as Collagens and Lans into the basal ECM. In turn, the cell localizes ECM receptors such as Integrin and Dg which sense biomechanical and biochemical cues and relay the information via intracellular adaptor proteins (Gullberg and Ekblom, 1995). ECM surrounding the CBs during migration has distinctive structure and composition. Both apical and basal domains localize components of the BM such as Collagen-IV and Lan (Haag et al., 1999; Hollfelder et al., 2014). However, certain constituents are either targeted to the basal or the apical ECM. The apical ECM encompasses a luminal domain and localises Mp whereas pericardial cells secrete Prc specifically into the BM (Chartier et al., 2002; Volk et al., 2014). In vertebrate, MMPs dependent remodelling of the ECM surrounding the embryonic and adult heart is tightly regulated and its misregulation causes formation of CHDs which arise due to atrio-ventricular and/or valvular formation defects (Linask et al., 2005; Liu et al., 2006; Spinale, 2007). In *Drosophila*, both secreted MMP1, and transmembrane MMP2, perform overlapping and non-overlapping functions during heart development. Both of these proteases promote the LE motility of the CBs and hence are required for CCM. During lumen formation, MMP2 is required for

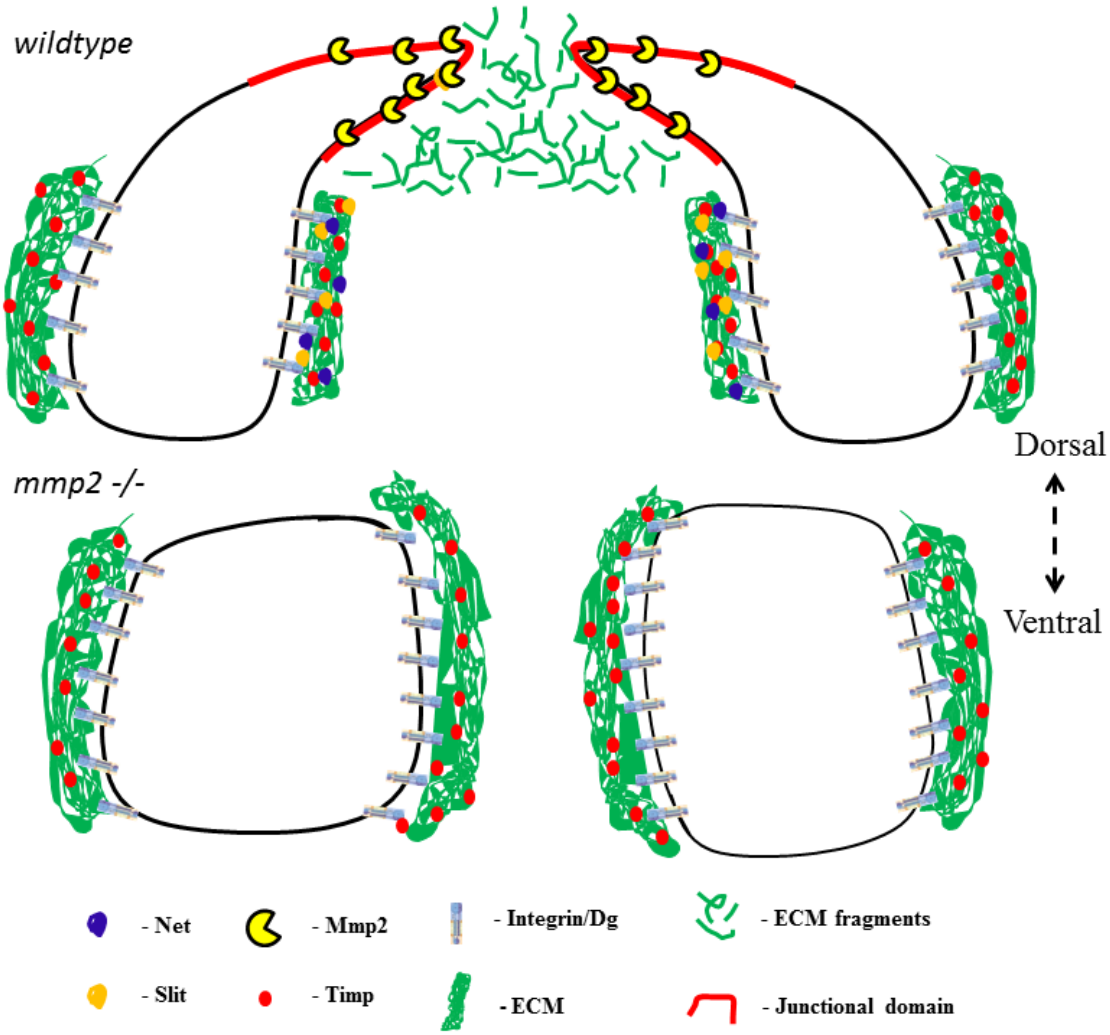
formation of the apical outgrowths and contralateral attachments, whereas MMP1 is required for mediating luminal expansion.

Secreted MMPs work over long distances whereas transmembrane MMPs act on substrates which are in the immediate vicinity of the cell membrane. MMP1 is required for inducing maximal LE activity, expansion of the lumen and regulation of the apical ECM identity. However, we observe that in the absence of secreted MMP1, migration velocity of the CBs and the apical targeting of polarity markers is relatively unaffected compared to control. MMP1 might regulate luminal expansion by increasing the levels of Mp at the apical ECM. This hypothesis can be tested by immunolabelling *mmp1* and *mmp2* mutant embryos with αMp antibody to determine whether Mp is present in the luminal ECM in posterior heart. Whether CBs secrete MMP1 is not known, hence, the primary source of MMP1 at the dorsal midline should also be identified in future immunolabelling studies. On the other hand, embryonic heart development is severely perturbed in the absence of MMP2. In the CBs MMP2 is autonomously required to increase LE activity, the pace of CB migration and for the formation of apical outgrowths. Similarly, in vertebrates MT1-MMP, which shares common structural features with MMP2, is required for cell migration (Coyle et al., 2008; Itoh, 2006; Kajita et al., 2001; Koshikawa et al., 2000). Specifically, MT1-MMP is targeted to the lamellopodia in multiple cell lines *in vitro* and studies suggest that these proteases have important conserved roles which promote cell migration by removing ECM barriers (Gilles et al., 2001; Mori et al., 2002). Additionally, studies conducted on human cancer cell lines clearly demonstrate that MT1-MMP is targeted to the Cdc42/N-WASP/Arp2/3 mediated

invadopodia where its activity leads to degradation of the ECM and causes EMT of tumour cells (Watanabe et al., 2013; Yamaguchi et al., 2005). Hence, targeting of MT-MMPs to the domain of the cell where actin cytoskeletal rearrangement is activated might constitute a universal mechanism. By utilizing a MMP2::GFP construct (Deady et al., 2015), we were able to determine the localisation of MMP2 in the CBs. Since in the absence of MMP2 luminal ECM extends over the entire apical side of the CBs, we hypothesize that MMP2 localises to the filopodia and lamellopodia during migration and limits the positioning of the luminal ECM by cleaving it at the dorsal part of the apical domain (Fig 5.2). To test this, we can develop an antibody against MMP2 and determine whether it localises to the lamellopodia of CBs. Additionally, to test whether MMP2 cleaves ECM around the CBs, we can overexpress MMP2 transgene under the control of *mef2*-GAL4 driver and determine whether apical identity or positioning of the apical and basal ECM is affected.

In vertebrates MT1-MMP activates secreted MMP2 by cleaving its pro-peptide domain (Sato et al., 1994; Strongin et al., 1995). Additionally, MT1-MMP dependent activation of MMP2 is required for cardiac development in vertebrates (Alexander et al., 1997; Koenig et al., 2012; Stawowy et al., 2004). Activation of MMP2 by MT1-MMP is applied as a strategy to amplify the collective proteolytic activity of MMPs. Therefore it is possible that in *Drosophila* MMP2 functions upstream of MMP1 and regulates its activity by cleaving its proactive domain. Consistently, in double mutants of *mmp1,mmp2*, migratory pattern of CBs and overall heart morphology highly resembles

Figure 5.2. Model for MMP2 function during heart development. Based on the evidence presented in this thesis and functions suggested in other vertebrate and *Drosophila* model, we propose the following mechanism of MMP2 function during late stages of heart development. In wildtype hearts, MMP2 is targeted to the LE of the CBs where its activity degrades ECM barriers. Timp is expressed by the CBs and is deposited at the apical and basal ECM. Presence of Timp in the apical and basal ECM protects it from MMP2 dependent degradation. Slit and Net specifically localise at the luminal ECM. In the absence of MMP2, localisation of ECM and its receptors is extended across the entire apical domain. Outgrowths do not form and ECM barrier are not degraded at the LE.


wildtype
mmp2 -/-
Dorsal
Ventral
- Net
- Mmp2
- Integrin/Dg
- ECM fragments
- Slit
- Timp
- ECM
- Junctional domain

mmp2 mutant hearts. However, in *mmp1* mutants, CBs are able to form small outgrowths and a luminal domain which suggests that MMP2 activity is sufficient for inducing outgrowth formation and cell polarisation in the absence of MMP1. Based on this, we propose that MMP2 functions primarily to regulate all aspects of heart development upstream of MMP1. By activating MMP1, MMP2 regulates LE activity of the CBs as well as expansion of the luminal domain.

Another possibility is that MMP1 and MMP2 cleave different ECM constituents during heart development. For instance, both MMPs recognize Collagen-IV as a substrate but only MMP2 has the ability to cleave Lan (Llano et al., 2002; Llano et al., 2000). Lan is a key player in establishing both apical and basal ECM assembly and heart development in *Drosophila* (Haag et al., 1999), whereas Collagen-IV is required for ECM stabilization and is not required for establishment of a luminal domain (Hollfelder et al., 2014). Additionally, *mmp2* mutant hearts display phenotypes which are also observed in *lanA* mutant hearts (Haag et al., 1999; MacMullin and Jacobs, 2006). In *lanb1, lanA* and *mmp2* mutants, cell polarity defects are commonly noted in the CBs and the apical ECM fails to localise apical polarity marker Slit (Hollfelder et al., 2014). This suggests that MMP2 might additionally regulate heart development, independent of MMP1, by modulating Lan cleavage around the CBs. Similarly, MT-MMPs in vertebrates are demonstrated to cleave Lans which regulate epithelial cell morphology and migration (Koshikawa et al., 2000; Koshikawa et al., 2004). In *Drosophila*, biochemical analysis has suggested that MMP2 preferentially cleave components of the BM such as Lans, whereas MMP1 favourably cleaves cell-cell adhesion proteins such as E-Cad (Jia et al.,

2014). During heart development apical attachment between CBs are formed via E-Cad based adheren junctions and in *mmp1* mutants these apical junctions are medially extended (Santiago-Martinez et al., 2008). This suggests that MMP1 might function to limit the medial extension of apical junctional domains and hence regulate the size of the heart lumen. In addition, in the absence of MMP1, Collagen-IV is limited to the luminal domain whereas in *mmp2* and *mmp1,mmp2* mutants, it accumulates over the entire apical domain. This suggests that MMP1 is not required for limiting the apical ECM to the pre-luminal and luminal domains. Therefore, we propose a model where MMP2 pleiotropically modulates heart development by regulating ECM remodelling whereas MMP1, downstream of MMP2 at the molecular level, supplements LE motility and is required for luminal expansion.

5.4 Interaction between guidance signalling and proteolytic activity of MMPs modulates heart formation in *Drosophila*

It is evident from our analysis that both guidance signalling and ECM remodelling collectively orchestrate heart development. Cell polarity, LE activity and migration velocity of CBs are significantly affected in the absence of both MMP2 and guidance signalling molecules. In addition, the rounded morphology of the CBs, which is observed in the guidance signalling and *mmp2* mutants, suggests that both types of molecules are required for outgrowth formation separately. Yet, careful analysis of the late stage mutant heart phenotype reveals an interesting hierarchy. In *slit* and *robo* mutants, CBs form apical contacts however the entire medial domain localizes E-Cad mediated junctions (Santiago-Martinez et al., 2008). In *mmp2* mutants, CBs are incapable of making the

medial contact. Additionally, adhesion marker Dlg, which co-localizes with E-Cad adaptor protein β-catenin in wildtype hearts (Haag et al., 1999; Medioni et al., 2008; Santiago-Martinez et al., 2008; Vanderploeg and Jacobs, 2015), is not targeted to the cell periphery in the absence of MMP2. These results suggest that MMP2 functions upstream of Slit/Robo to establish apical outgrowths. In support of this statement, we established that targeting of Slit and Robo to the apical domain is dependent on MMP2 activity.

Interestingly, the ECM receptor Integrin also functions upstream of Slit/Robo signalling (Vanderploeg et al., 2012). In the absence of MMP2, Integrin and Dg localisation is extended across the entire apical domain and consistently co-localises with the apical ECM. Both Integrin and Dg are also mistargeted to the lateral ectopic domains which form between ipsilateral CBs in *mmp2* mutants. These ectopic pockets localize Slit, Robo, Integrin, Dg and Collagen-IV thus confirming their luminal identity. Presence of ectopic lumens between ipsilateral CBs also indicates that the overall CB polarisation is disrupted in the absence of MMP2, but not MMP1. A major step in polarisation of the CBs is the apicalisation of Slit and Robo complex to the luminal domain of the CBs (MacMullin and Jacobs, 2006). It is evident in *mmp2* mutants that Slit, Robo and Dlg fail to be targeted to the peripheral cell membrane hence confirming that MMP2 is required to promote cell polarisation during late stages of heart development. Targeting of ECM components and receptors to the pre-luminal domain during migratory stages however does not require MMP1 or MMP2. Therefore, we propose that MMP2 functions downstream of Integrin signalling but upstream of Slit and Robo signalling to establish luminal identity of the apical domain in CBs during late stages of development.

Significant reduction in LE motility when both guidance signalling molecules and MMPs are absent is noted. But does the interplay of these molecules regulate filopodial and lamellopodial dynamics and migration? In vertebrates, both secreted and transmembrane MMPs are able to activate latent growth factor embedded in the ECM (Hinkle et al., 2003; Yu and Stamenkovic, 2000). Additionally, cleavage of ECM components such as Collagen-IV and Laminin reveals intramolecular cryptic sites which induce migratory capabilities in multiple models (Favreau et al., 2014; Giannelli et al., 1997; Pirila et al., 2003; Xu et al., 2001). MMPs also induce the release of growth factors which are trapped in the ECM. This is achieved by MMP dependent cleavage of ECM component, specifically HSPGs, which are known to sequester guidance cues (Rider, 2006; Suzuki et al., 1997). MMP dependent degradation of HSPGs, such as Sdc, releases growth factors which are able to induce cell migration, polarisation and tumour invasion (Manon-Jensen et al., 2013; Yu and Stamenkovic, 2000). Therefore, one possible mechanism of MMP action might be that degradation of the ECM at the LE results release of trapped guidance cues which promotes filopodial and lamellopodial activity of the CBs. Consistent with the model, in *Drosophila*, guidance cues, Slit and Net, contain Lan-like and EGF-like domains which bind ECM components (Brose et al., 1999; Lai Wing Sun et al., 2011). Slit also genetically interacts with ECM components Collagen-IV and Lan during nervous system and heart development in a dose dependent way (MacMullin and Jacobs, 2006; Stevens and Jacobs, 2002). More importantly, Sdc is a co-receptor of Slit and is essentially required to promote signalling through Robo during heart formation (Knox et al., 2011). Therefore, we propose a model where MMP2

dependent cleavage of ECM components such as Collagen-IV, Sdc and Lan releases Slit and Net which function to promote filopodial and lamellopodial dynamic at the LE. In addition, release of these molecules from the ECM might promote lateral cell adhesion and induce cell polarisation. To test this model, we can determine whether an epistatic relationship between Sdc and MMPs exist. We can introduce a single mutant copy of *mmp1, mmp2, slit, robo, net* and *fra* into a sensitized *sdc*/+ background and observe whether an additive LE motility phenotype is exhibited. Additionally we can test whether Sdc is required for inducing LE motility separately by quantifying LE behaviour in *sdc* null mutants. MMP2 might regulate Slit/Robo signalling is by proteolytically processing Slit. Interestingly, divergent biological role for both full length Slit (F-Slit) and N-Slit have been previously proposed. F-Slit or the un-cleavable forms of Slit when bound to Robo elicit a repulsive response which deters axonal outgrowths (Coleman et al., 2010). N-Slit however has been proposed to function as an attractant in *in vitro* cultured rat neurons and during muscle migration by associating with Dscam ((Nguyen Ba-Charvet et al., 2001), unpublished data). Our attempt to determine whether Slit is cleaved by MMP2 did not provide concrete evidence of this mechanism. Whether MMP2 recognizes Slit as a substrate remains an open question. Although it is possible that F-Slit by localizing to the luminal domain during late stages promotes repulsive signalling required for lumen formation, whereas N-Slit serves as an attractant to induce protrusive activity at the LE.

5.5 Heart development as a model of congenital heart disease

Congenital defects of the heart are the leading cause of infant mortality in humans (Gruber and Epstein, 2004). Vertebrate animal models, such as mice and zebrafish, have

provided molecular functions of many genes which are implicated in the formation of cardiovascular defects. However, due to high genetic redundancies and long inter-generation time, testing genetic requirement of molecules can be time consuming and single gene knockout analysis can be unfruitful. *Drosophila* is the simplest genetic model organism that contains a fluid pumping heart. We have uncovered novel roles for guidance signalling molecules and ECM remodelling enzymes during heart development. Since the functions of these proteins are conserved between *Drosophila* and mice, it is likely homologs of these genes in human share the same function and act via similar molecular mechanisms. The observation that CBs undergo EMT in the absence of guidance signalling demonstrates a possible mechanism by which atrioventricular septum defects such as formation of holes with in the walls of the heart might occur. Additionally, absence of membrane tagged MMP2 activity in *Drosophila* hearts leads to misdirected placement of the ECM and bears strong resemblance to cardiac fibrosis phenotype in vertebrates (Cheitlin et al., 1980; Ho et al., 1996). Therefore, we recommend that *Drosophila* must be added to the list of genetic models such as mice, zebrafish and xenopus which are utilized to study molecular mechanisms of cardiac specific congenital defects.

5.6 Collective cell migration of Cardioblasts as a model for metastasis

Cancer is one of the leading causes of adult human deaths. In *Drosophila*, embryonic cells exhibit features which are common in metastatic cells. Additionally, *Drosophila* models have deeply enhanced our understanding of signalling pathways such as Ras/MAPK, Notch, Wnt/wingless and BMP which are activated in tumours (Botas,

2007). Tumour suppressive roles of guidance cues Slit and Net and their receptors have been recently demonstrated in multiple cancer (Mehlen et al., 2011). Fra, homologue of vertebrate DCC, is a known oncogene which is downregulated in most patients who present with colorectal cancer (Kikuchi-Yanoshita et al., 1992). Additionally, Slit and Robo have been discovered to be misexpressed in multiple human tumours and directly cause metastasis (Gara et al., 2015). Furthermore, overwhelming amount of evidence suggests possible functions for MMPs in both cancer progression and inhibition (Egeblad and Werb, 2002). We have revealed potential roles for guidance signalling molecules and MMPs in regulating CCM, a homologous process by which cancer cell collectively invade adjacent tissues. In the past, several therapeutic agents which target both MMPs and Timp have been developed and failed in clinical trials due to limited knowledge of their activity in intact physiological environment (Coussens et al., 2002). Therefore, *Drosophila* embryogenesis can be used as a tool to test the effects of these therapeutic agents. Due to the advances in the field of microfluidics, new devices are available which allow for rapid microinjection of potential drugs in to embryos. This technique coupled with time-lapse confocal microscopy and readily available GFP-trap lines, will allow us to determine the physiological effects of novel therapeutic agents *in vivo*. Additionally, these affects can be interpreted by quantitatively assessing processes which involve a combination features which are common to cancer such as CCM, proliferation, differentiation, survival and apoptosis.

5.7 Conclusions

Collective migration of cells is a complex phenomenon. Therefore to understand this process multiple animal models should be utilized and common developmentally mechanistic themes must be identified. Here we classified heart development during *Drosophila* embryogenesis as a quantitative *in vivo* model to study CCM. We validated the functions of guidance signalling molecules and ECM remodelling proteases during migratory and non-migratory stages of heart development. CBs display many characteristics which are commonly observed in other CCM models, however some unique features are noted and described here. In vertebrates, spatiotemporal expression of guidance signalling molecules and matrix metalloproteinases is required for early stages of heart development and since heart development in vertebrate and *Drosophila* share striking resemblance during early stages, it is likely that these proteins have conserved functions in shaping organogenesis of the heart. Therefore, due to less genetic redundancy and quick generation time, *Drosophila* model of heart development can be used to test homologues of human genes which potentially cause CHDs. Furthermore, even though heart development does not morphologically resemble tumour metastasis, the underlying features such cell adhesion, guidance signalling and ECM remodelling are conserved in both processes. Therefore, CCM of *Drosophila* CBs can be used as metastasis model to uncover novel mechanism of migration and test the therapeutic potential of drugs.

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Phosphoinositol Signalling during Heart development

Appendix A

A.1 Introduction

Phosphoinositols (PI) are secondary messengers which function to establish actin cytoskeletal rearrangement, intracellular vesicular trafficking and adhesion formation between the cell-cell or cell-ECM interface (Thapa and Anderson, 2012; Tsujita and Itoh, 2015). There are 7 possible combinations of phosphorylated PI and each molecule differs in the phosphorylation pattern on the 3^{rd}, 4^{th} or 5^{th} –OH group of the inositol ring. These molecules are embedded into the plasma membrane via a lipid chain and their inositol heads are intracellularly exposed for interactions with adaptor proteins (Ling et al., 2006). Two PI species, phosphoinositol (4,5) biphosphate (PIP_2) and phosphoinositol (3,4,5) triphosphate (PIP_3) have been implicated in modulating cell migration and adhesion in vertebrates (Ling et al., 2006). In *Drosophila*, PIP_2 has been specifically implicated in inducing cell polarity, migration and protrusion formation in the mesoderm during gastrulation (Fabian et al., 2010; Murray et al., 2012). Therefore, we investigated the localisation of these molecules within the migrating mesodermal CBs and conducted preliminary analysis on the function of enzyme, PI3K which converts PIP_2 to PIP_3 by phosphorylating the 3^{rd} hydroxyl group on the inositol head.

A.2 Results

To visualize PIP_2, we employed the GAL4/UAS system and expressed the plekstrin homology (PH) domain of Phospholipase-C containing a CFP tag, which is known to specifically bind PIP_2 (Harlan et al., 1994). To visualize PIP_3, we expressed an adaptor protein Grp1containing a GFP tag which specifically binds PIP_3 (Klarlund et al., 1997). In the migrating CBs PIP_2 localised at the lateral and basal domains (A

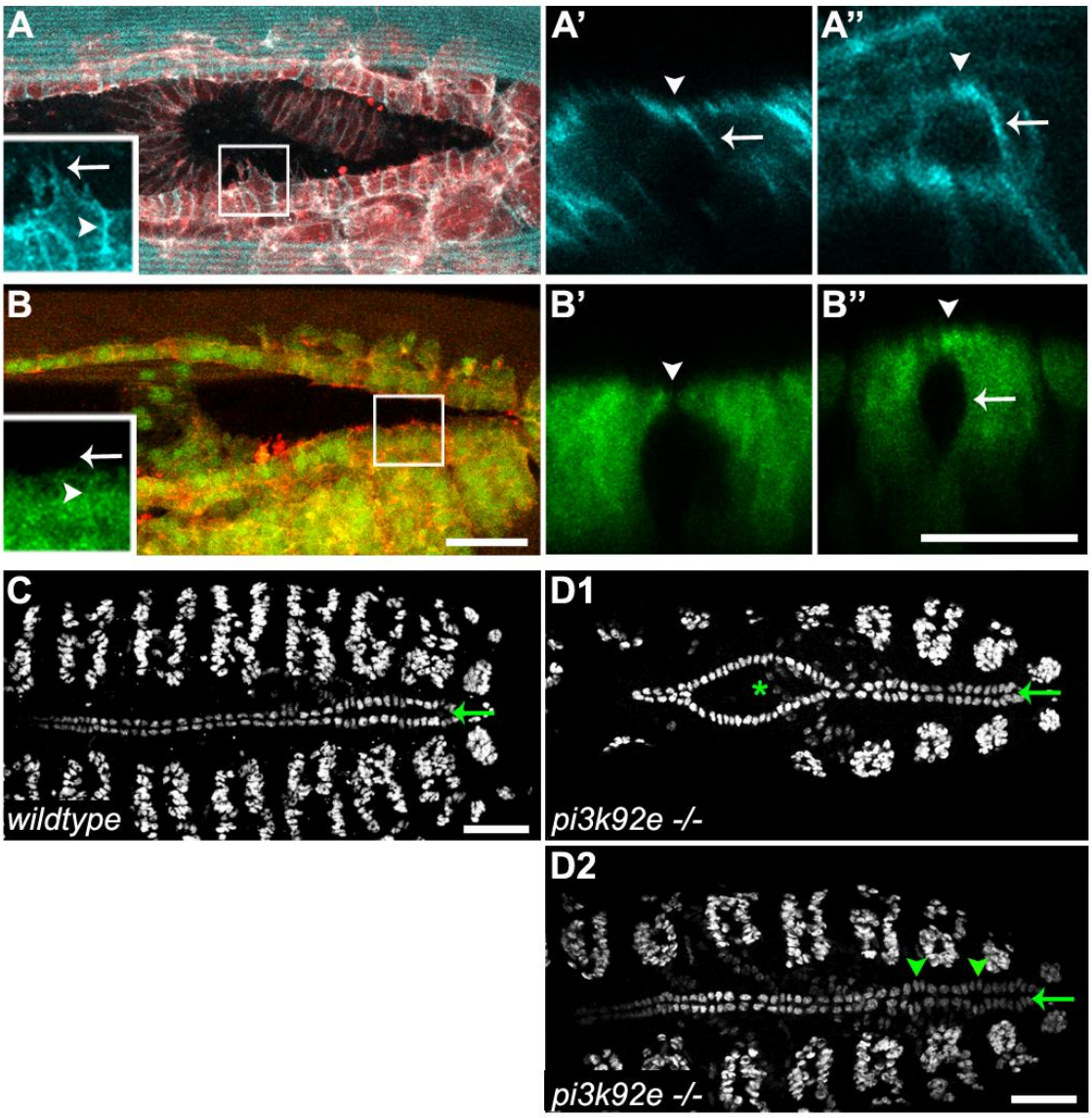
arrowhead). Accumulation was also observed at the filopodia and lamellopodial edges (A arrow, Movie A.1). PIP_3 localised at the lateral domains and its accumulation at the apical protrusive domain is absent (B, Movie A.2). At stage 16, PIP_2 accumulates at the contact site (A' arrowhead) and luminal domain (A' arrow). PIP_3 at this stage accumulation at the basal or apical membrane was not detected (B' arrowhead). At stage 17, PIP_2 localized at both the dorsal-ventral contact points (A'' arrowhead) and at the luminal domain (A'' arrow). A slight accumulation of PIP_3 was observed at the apical adhesion domains at stage 17 (B'' arrowhead), however luminal localisation was not noted (B'' arrow). Next, we analysed heart morphology in *pi3k* mutants by labelling embryos with a heart specific marker Mef2. In the absence of PI3K, conversion of PIP_2 to PIP_3 does not occur. In wildtype stage 17 hearts, CBs align at the midline and form a medial luminal space (C arrow). In *pi3k* stage 17 mutant hearts, CBs display delayed migration (D1 asterisk). Upon reaching the midline, CBs align at the midline however a reduced lumen forms (D2 arrow). Blisters in the heart are also commonly noted (D2 arrowheads).

A.3 Discussion

In vertebrate the role of PIP_2 in regulating actin cytoskeletal regulation has been demonstrated by several studies (Ling et al., 2006; Thapa and Anderson, 2012; Tsujita and Itoh, 2015). Presence of PIP_2 in the cytoplasmic protrusions suggests that PIP_2 might be required for the formation of filopodia and lamellopodia during CBs CCM. Additionally, PIP_2 might be crucial for maintaining the lateral and basal adhesion. Our localisation study did not determine a possible role for PIP_3 during migratory stages. Nevertheless, PIP_3, at low levels might be required to maintain basolateral adhesions

during migration and apical junctions during lumen formation. In this scenario, low level of PI3K activity might convert PIP_2 to PIP_3 at the basolateral domain but not at the apical domain during migration and hence promote migration. Accordingly, we observe mild cardiac developmental defects in the absence of PI3K. Further analysis must be conducted to address the role of these molecules during heart development.

Figure A.1. Localisation of PIP_2 and PIP_3 in the CBs. (A-B’’’) Images of live embryos expressing PIP_2 (A) and PIP_3 (B) and Actin (*UAS-Moesin-mCherry*) markers under the control of *mef2*-GAL4 driver. (A-A’’) PIP_2 localisation is reported by UAS-*Phospholipase C-ΔPH*-CFP in the CBs. (A) PIP_2 accumulation is observed at the basolateral (arrowhead) and apical domain. Localization at the filopodia and lamellopodial edges is also noted (arrow). (A’) Cross-sectional images of CBs reveal localization of PIP_2 at the luminal domain (arrow). (A’’) At stage 17, PIP_2 localizes at the apical junctions (arrowhead) and at the luminal domain (arrow). (B-B”) PIP_3 localisation is reported by UAS-*Grph1*-GFP in the CBs. (B) Low levels of PIP_3 were noted at the lateral regions of the CBs (arrowhead), however most of the signal was present in the cytoplasmic or nuclear. PIP_3 did not accumulate in apical outgrowths (arrow). (B’) Cross-sectional images of CBs reveal the cytoplasmic localization of PIP_3 in the CBs. (B’’) Accumulation of PIP_3 is noted at the dorsal attachment sites of stage 17 hearts (arrowhead) but luminal accumulation is not observed (arrow). (C) In wildtype embryos, CBs uniformly align at the midline and form a medial luminal space at the posterior heart proper. (D1) Delayed migration is observed embryos mutant for *pi3k* (asterisk). (D2) The luminal space at the posterior heart proper is reduced in *pi3k* mutants (arrow). Formation of blisters is also observed in the heart (arrowheads). (A,B,C,D1,D2) Scale bar is 25 μm. (A’-A’’,B’-B’’) Scale bar is 10 μm.


A
A'
A''
B
B'
B''
C
wildtype
D1
pi3k92e -/-
D2
pi3k92e -/-

Slit/Robo signalling in the ostial precursor cells of the heart is required for overall heart development

APPENDIX B

Appendix B

Knocking down Slit and Robo signalling in all of the CBs using the *mef2*GAL4 cells disrupts lumen formation across the heart. However, the specific role of Slit and Robo signalling in the Svp expressing ostial cells is not known. To test the role of Slit and Robo signalling, we downregulation Slit and Robo levels in the ostial progenitor by expressing RNAi construct specific to these proteins. We drove the expression using a svp-GAL4 construct which is specifically active in the Svp cells (Supp. Movie X). We performed time lapse imaging and immunolabelling to determine whether both tinman and seven-up expressing CBs are able to extend outgrowths and form a lumen when Slit and Robo levels are depleted in seven-up cells.

The following experiments reveal an essential role for Slit/Robo signalling specifically in the seven-up cells. Seven-up cells fail to form any medial contact when Slit and Robo levels are depleted and do not form luminal domain which localizes luminal marker Dg and Integrin. Additionally, adjacent tinman cells failed to form outgrowths and a medial lumen. Localization of luminal markers Dg and Integrin was disrupted in tinman cells as well. Unlike seven-up cells, tinman cells contacted each other at the midline but the localisation of adhesion marker Dlg was observed all along the apical membrane, suggesting that the luminal domain failed to form. We propose that upon downregulation of Slit/Robo in seven-up cells, normal functions of all the CBs are disrupted. Therefore, Slit/Robo signalling in the ostial cells non-autonomously regulates heart development by signalling to the adjacent CBs.

Figure B.1. Depletion of Slit and Robo in the ostial progenitor result in lumen formation defects in both seven-up and tinman expressing CBs. Frontal (A,B,C) and cross sectional (A'-A'', B'-B'', C'-C'') images of hearts double labelled with αDg (green) and αDlg (red) antibodies. In wildtype embryo, a medial luminal encompassed by Dg localization is observed between tinman cells (A, A''arrow). Accumulation of Dg was noted between contralateral seven-up cells (A' arrow). Dlg localization was observed at the dorsal and ventral junctional domains (A', A'' arrowhead). When Slit RNAi was expressed, gaps between contralateral partners were detected between seven-up cells (B, B' arrow). Tinman cells, migrated to the midline and did not form a luminal domain (B arrow) or formed a reduced sized lumen (B'' arrow). Dlg localisation was extended across the entire apical domain in seven-up cells, however, in tinman cells a larger than normal apical adhesion domain was noted (B', B'' arrowhead). When Robo RNAi was expressed, seven-up cells failed to contact contralateral partner cells (B, B' arrow). Tinman cells failed to form a luminal domain (C' arrow). Dlg localisation at entire apical domain was observed between both seven-up and tinman cells (C', C'' arrowheads). Formation of lateral luminal pockets localising Dg in seven-up and tinman cells were also observed in both Slit and Robo depletion in seven-up cells (purple arrowheads). Seven-up cells are labelled with a white circle. Micron scale is 10 μm.

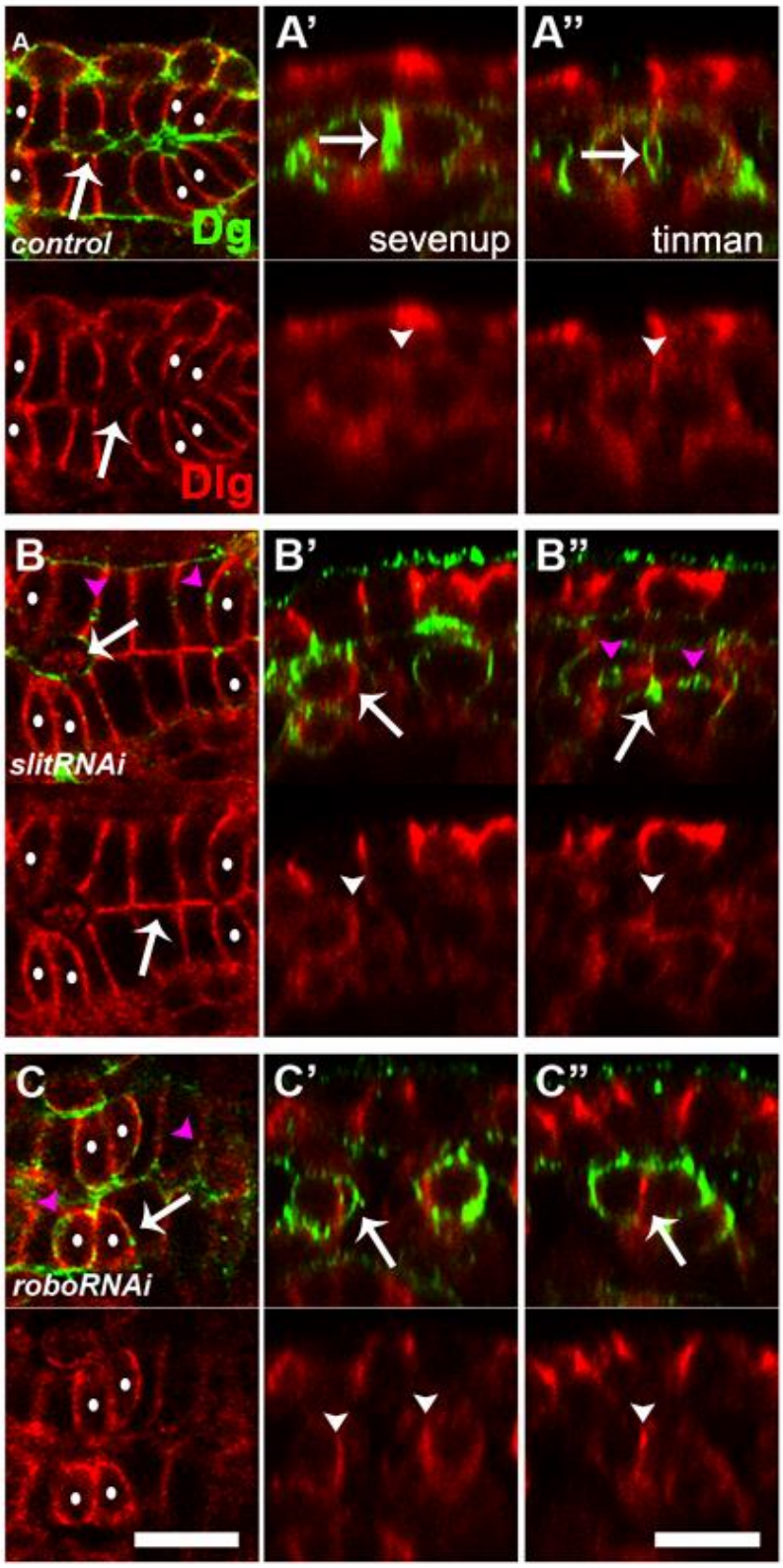

A
control
Dg
Dlg
A'
sevenup
A''
tinman
B
slitRNAi
B'
B''
C
roboRNAi
C'
C''

Figure B.2. Expression of Slit and Robo in the ostial cells non autonomously affects Integrin localization in the CBs. Frontal (A,B,C) and cross-sectional (A', B' C') images of embryos labelled with α-βPS (red) and α-Mef2 (green) antibody. In wildtype emrbyos, βPS localises to the basal (A arrowhead) and luminal domains (A, A' arrow) of the CBs. Upon Slit downregulation in the seven-up cells, βPS localisation is disrupted across the whole heart and luminal accumulation is absent (B, B' arrow). Upon Robo downregulation in the seven-up cells, βPS localisation is also disrupted across the whole heart and luminal presence is not observed (C, C' arrow).

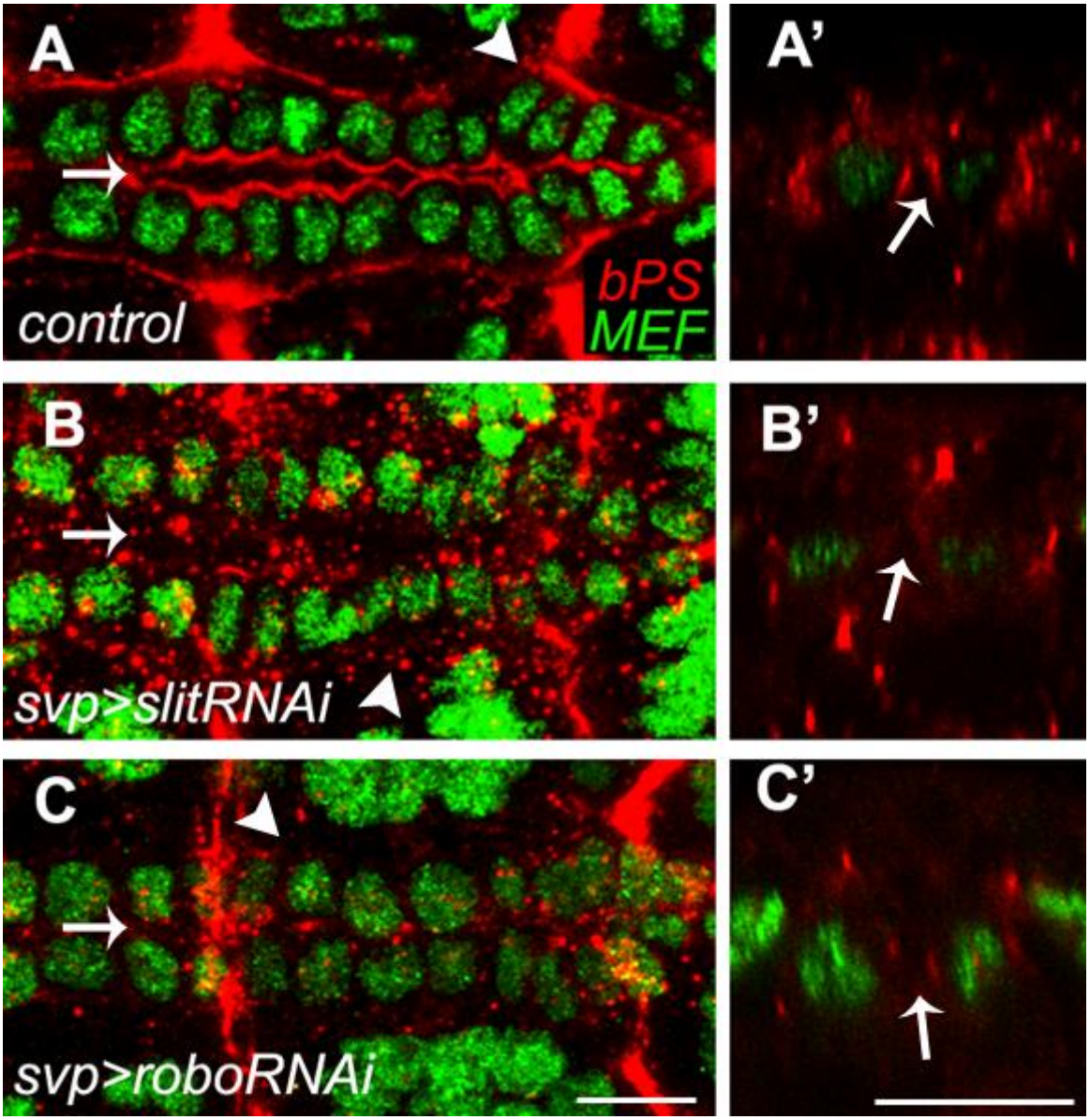
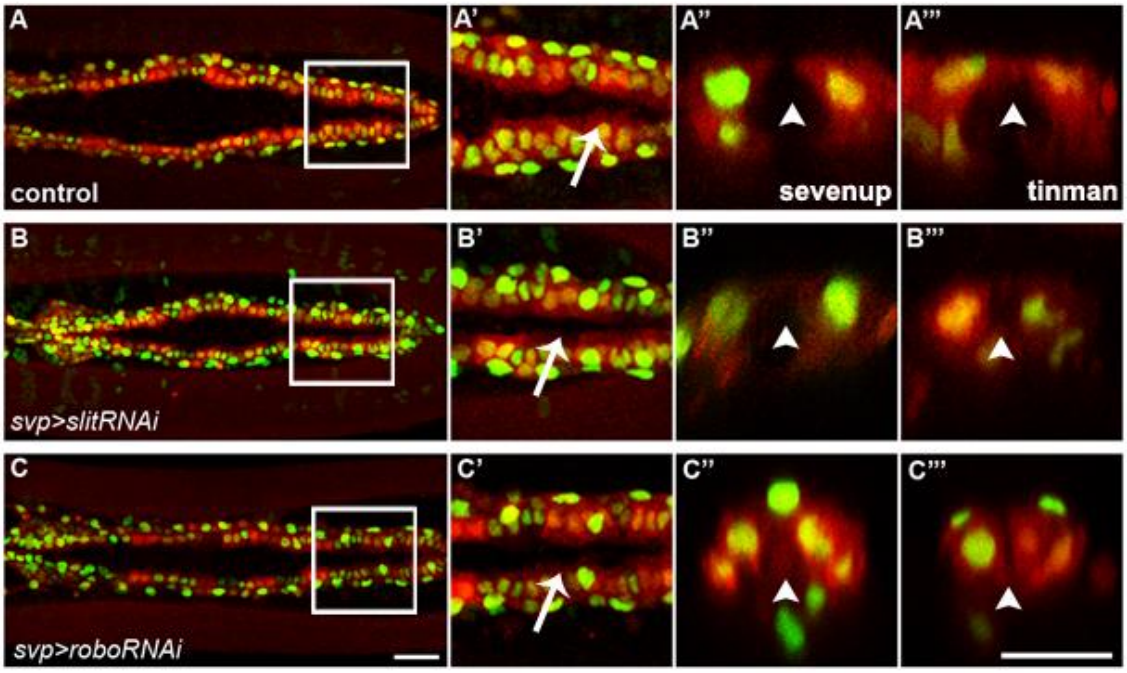

A
control
bPS
MEF
A'
B
svp>slitRNAi
B'
C
svp>roboRNAi
C'

Figure B.3. Expression of Slit and Robo in the ostial cells affects outgrowth formation in all CBs. Frontal (A-A', B-B', C-C') and cross sectional (A''-A''', B''-B''', C''-C''') images of live embryos expressing *hand>moesin*-mCherry and *tup*-GFP constructs. In wildtype hearts, CBs extend protrusions towards the contralateral partners (A, A' arrow). During both Slit (B,B') and Robo RNAi (C, C') expression in the seven-up cells, the entire LE of the CBs is relatively inactive (arrows). In wildtype embryos, both seven-up and tinman cells extend apical protrusion towards the contralateral partner cells (A'', A''' arrowheads). When Slit RNAi is expressed in the seven-up cells, CBs fail to form protrusion in both seven-up and tinman cells (B'', B''' arrowheads). When Robo RNAi is expressed in the seven-up cells, CBs fail to form protrusion in both seven-up and tinman cells (C'', C''' arrowheads).

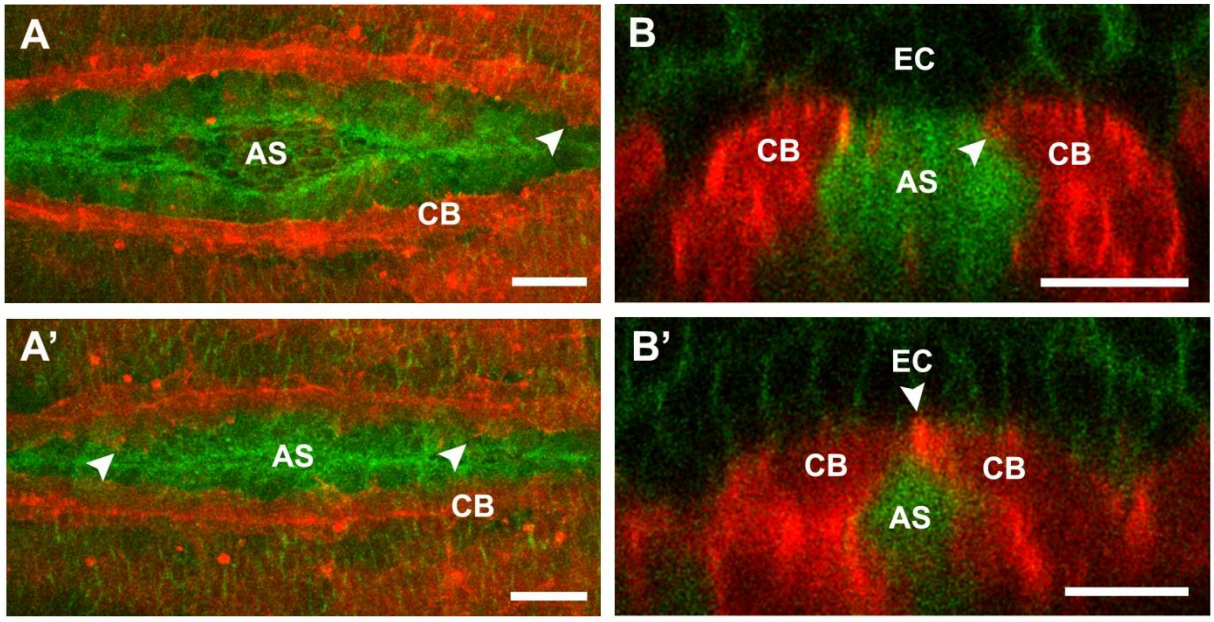

A
A'
A''
A'''
control
sevenup
tinman
B
B'
B''
B'''
svp>slitRNAi
C
C'
C''
C'''
svp>roboRNAi

Cardioblasts and the Amnioserosa Perimeter cells physically interact during migratory stages of heart development

APPENDIX C

Appendix C:

Figure C.1. Amnioserosa perimeter cells physically interact with Cardioblasts. (A-B') Embryos expressing Moesin-mCherry under the control of dmefGAL4, basigin-GFP and *yet1*-GFP. *basigin*-GFP localizes to the plasma membrane whereas *yet1*-GFP is a cytoplasmic marker and labels the AS perimeter cells. (A-A') Dorsal view of timelapse images demonstrating the contiguous interaction between AS and CBs apical domain (arrowhead). (B) CB extend cytoplasmic extensions over the internalizing AS (arrowhead). Yet1 cells are the last cells to lose contact with the EC. CB physically interact with AS, specifically Yet1 cells. (B') Once the contact between Yet1 cells and EC is lost, CBs contact their contralateral partners to form the dorsal apical adhesion which is stabilized by the recruitment of Actin(arrowhead). AS – Amnioserosa, EC – Dorsal Ectoderm, CB – Cardioblast. (A,A') Micron scale is 25 μm. (B,B') Micron scale is 10 μm.

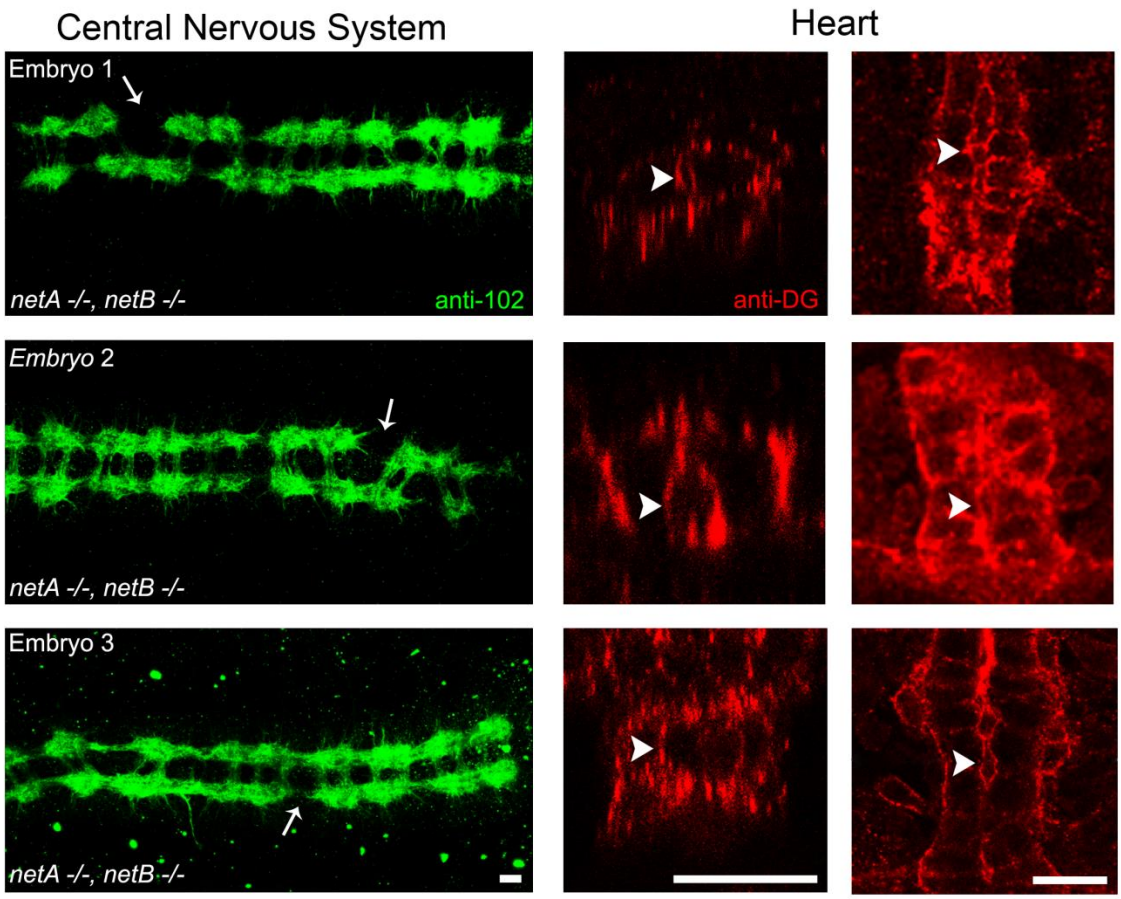

A
AS
CB
B
EC
CB
AS
CB
A'
AS
CB
B'
EC
CB
CB
AS

Netrin is not essentially required for lumen formation in the heart

APPENDIX D

Appendix D:

Figure D.1. Netrin is required for formation of a continuous lumen bot not essential for lumen formation. To determine whether Net is required for lumen formation, immunolabelling was carried out in *netA -/-, netB-/-* mutant embryos. Net is required for the formation of the ladder like structure of the commissures during CNS formation (Mitchell et al., 1996). To confirm the genotype, mutant embryos were labelled with α102 antibody which labels the CNS of the heart and αDg antibody which labels the luminal surface of the CBs. In three independent embryos which demonstrated a classic *netA -/-, netB-/-* mutant phenotypes (Fig D.1 arrows), hearts were found to contain lumens (arrowheads). Even though apical luminal pockets were observed, a continuous lumen along the entire length of the heart failed to form. These result refute the previously established by role of Net by Albrecht et al., which claims that Net is essential for lumen formation (Albrecht et al., 2011).


Central Nervous System
Heart
Embryo 1
netA -/-, netB -/-
anti-102
anti-DG
Embryo 2
netA -/-, netB -/-
Embryo 3
netA -/-, netB -/-

Time-lapse movies

APPENDIX E

The following time-lapse videos are supplemental to the still images presented in this thesis:

Movie 3.1 – *moesin*-mCherry and *tup*-GFP expression in *wildtype*

Movie 3.2 – *moesin*-mCherry and *tup*-GFP expression in *slit -/-*

Movie 3.3 – *moesin*-mCherry and *tup*-GFP expression in *robo,lea -/-*

Movie 3.4 – *moesin*-mCherry and *tup*-GFP expression in *netA -/-, netB -/-*

Movie 3.5 – *moesin*-mCherry and *tup*-GFP expression in *fra -/-*

Movie 3.6 – *moesin*-mCherry and *tup*-GFP expression in *unc5 -/-*

Movie 3.7 – EMT of CBs in *slit -/-*

Movie 3.8 – EMT of CB in *robo -/-, lea -/-*

Movie 3.9 – EMT of CB in *fra -/-*

Movie 4.1 – *vkg*-GFP expression in *wildtype*

Movie 4.2 – *vkg*-GFP expression in *mmp1 -/-*

Movie 4.3 – *vkg*-GFP expression in *mmp2 -/-*

Movie 4.4 – *vkg*-GFP expression in *mmp1 -/-, mmp2 -/-*

Movie 4.5 – *moesin*-mCherry and *tup*-GFP expression in *mmp1 -/-*

Movie 4.6 - *moesin*-mCherry and *tup*-GFP expression in *mmp2 -/-*

Movie 4.7 - *moesin*-mCherry and *tup*-GFP expression in *mmp1 -/-, mmp2-/-*

Movie 4.8 – *moesin*-mCherry and *tup*-GFP expression in *mef2>mmp2*-RNAi

Movie A1 – *phospholipase C Δ-PH*-CFP expression in *wildtype*

Movie A2 – *gph1*-GFP expression in *wildtype*

Movie C1 – *basigin*-GFP, *yet-1-GFP* and *moesin*-mCherry expression in *wildtype*