The Rab5 GTPase is required for lumen formation in the embryonic *Drosophila* heart

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A Thesis

Submitted to the School of Graduate Studies in Partial Fulfillment of the Requirements

For the Degree Master of Science

McMaster University, Master of Science (2019) Hamilton, Ontario (Biology)

Title: The Rab5 GTPase is required for lumen formation in the embryonic *Drosophila* heart

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Number of Pages: i-101

Abstract

Tube formation, or tubulogenesis, is an elaborate form of epithelial morphogenesis that includes processes such as cell migration and cell shape changes. The embryonic *Drosophila* heart, or dorsal vessel, is an excellent model of tubulogenesis and more specifically the signaling mechanisms required for cell migration and lumen formation. Similar to vertebrate heart formation, *Drosophila* heart tubulogenesis begins with the collective migration of cardioblasts that meet at the midline and adhere at specialised junctions, enclosing a lumen between them. Roundabout, and its ligand Slit, are required to restrict cell-to-cell adhesions to the junctional domains of contralateral cardioblasts, as well as maintain the integrity of the lumen. The localisation patterns of Robo, and other luminal cell surface receptors important for lumen formation are significantly modified throughout heart formation. Initial receptor expression is broadly distributed over the cardioblast surface. Receptors are then relocalised to specific cell surface domains by late embryonic development. The mechanisms by which Robo and other cell surface receptors are localised have yet to be determined. Endocytosis is a promising mechanism by which cell surface receptors are targeted and trafficked to cell surface domains. Specifically, vesicular trafficking proteins, such as Rab GTPases, are molecular switches that regulate endocytic events. Here, we investigated the roles of Rab5, Rab11, and Sec6 during heart formation. Of these, only Rab5, a regulator of the early endosome, was required for lumen formation. Particularly, gain of function, loss of function, and overexpression of *rab5* resulted in reduced lumen phenotype, characterised by lumen pockets rather than a continuous lumen along the anterior-posterior axis. Perturbed Rab5 function also resulted in the mislocalisation of Robo at the basal domain. Live imaging

showed that expression of *rab5* dominant negative, constitutively active, and overexpression constructs did not perturb apical membrane motility of migrating cardioblasts in the developing heart.

Acknowledgements

I would like to thank my supervisor Dr. Roger Jacobs for his encouragement and guidance over the past two years. Thank you for your advice when things did not go as planned and your continual support. Thank you for pushing me to be the best student I could be.

Thank you to my committee member Dr. Juliet Daniel for your feedback at committee meetings. I would like to thank Dr. Deda Gillespie for being part of my committee for the defense.

I would like to thank all past and present Jacobs' lab members. Thank you to Chris and Duygu for the advice and encouragement when I joined the lab. Courtney, thank you for all the early morning and last minute flips. Plate collections would have been a lot more difficult without you. Thank you to Mae and Runyang for all the help with embryo collections and immunolabelling.

Rachel and Danielle, thank you for your continuous support and friendship throughout this process. Rachel, thank you for all the adventures to Donut Monster or wherever we decided to go that day. Danielle, thank you for all the hangouts on various patios around Hamilton and your encouraging words. Thank you both for plate flips and confocal troubleshooting.

Lastly, I would like to thank my family and friends for their support and encouraging words throughout graduate school. Thank you to my mom, Linda, for providing constant support and cooking my favourite meals when you would come to visit. To my loving husband, Shishir, thank you for your unwavering love and constant reassurance that I could do this. I couldn't have done this without you.

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List of Abbreviations

CB	Cardioblast
CA	Constitutively active
CE	Convergent Extension
Dlg	Discs-Large
Dg	Dystroglycan
DN	Dominant Negative
Dsh	Dishevelled
ECM	Extracellular Matrix
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial mesenchymal transition
Fz	Frizzled
GAP	GTPase activating protein
GEF	Guanine nucleotide exchange Factor
GDF	GTP-dissociation factor
GDI	Guanine dissociation inhibitor
HGF	Hepatocyte growth factor
MDCK	Madin-Darby canine Kidney
MET	Mesenchymal epithelial transition
MT	Malpighian tubule
Net	Netrin
PCP	Planar Cell Polarity
PCRs	Photoreceptor Cells
RabGGTase	Rab geranylgeranyltransferase
Rbsn	Rabenosyn
REP	Rab escort protein
RILP	Rab7 Interacting Lysosomal Protein
RNAi	RNA interference
Robo	Roundabout
RTK	Receptor Tyrosine Kinase
TGN	Trans-Golgi Network
Wg	Wingless
WT	Wildtype

Introduction

1.1 Tubulogenesis

Tubes are very common structures found in a variety of settings. For example, houses are filled with tubes such as ducts and pipes. All tubes in the house are relatively similar; however, they perform a variety of functions such as transporting clean water, waste and exhaust to their destinations. Without these tubes, the house would not be habitable because there would be no water supply or waste removal systems. Tubes also play analogous roles in living organisms as they are found within all metazoan species. For instance, the kidney contains many tiny tubes that function to absorb salts and secrete waste products, and the gastrointestinal system comprises tubes that act as conduits for nutrients and waste (Abrams *et al*., 2003; Choubey and Roy, 2017; Zegers *et al*., 2003). These tubes, as well as all others found in the body are highly intricate structures and require complex epithelial structuring to form properly (reviewed in Hogan and Kolodziej, 2002; reviewed in Zegers *et al*., 2003).

Tubes are made of epithelial tissues which often perform specialized functions such as secretion and absorption. Although the development of each kind of tubular structure is very different, the molecular players underlying the formation of a tube, or tubulogenesis, are highly conserved. Tubulogenesis can be considered a unique form of epithelial morphogenesis. A defining feature of epithelial cells is that they have apical-basal polarity. Cell polarity occurs when a cell is spatially divided into domains with specified organisation and function (reviewed in Hogan and Kolodziej, 2002; reviewed in Zegers *et al*., 2003). Tubulogenesis is highly dependent upon cell polarisation. An example of the effects of improper polarisation of major membrane proteins such as

transporters and signalling receptors is observed in polycystic kidney disease, which results in the formation of cysts instead of tubules. In this case, failure to localise polarity proteins results in increased diameter of the tubule and ultimately leads to organ failure (Wilson, 2011; Saxena *et al*., 2014). The following describes the importance of cell polarity during tubulogenesis.

1.2 Cell polarity and tubulogenesis

Processes required for tubulogenesis can include cell migration, cell shape change, growth, adhesion and others, leading to the formation of an enclosed lumen, known as lumenogenesis. To coordinate these tasks, each compartment of a cell must be organised into molecularly distinct cytoplasmic and surface domains (reviewed in Nelson, 2003). Polarised epithelial cells contain apical, basal, and lateral domains (Figure 1.1). The cell signalling pathways, receptors, growth factors, and adhesion molecules that localise to each domain have a specific function in the developmental plan of tubulogenesis (reviewed in Hogan and Kolodziej, 2002; reviewed in Zegers *et al*., 2003). The mechanisms involved in establishing and maintaining cell polarity are highly conserved across tissues in invertebrates and vertebrates (reviewed in Tyler, 2003). For example, the *Drosophila* gene *bazooka* was discovered due to its effect on cell polarity. Bazooka localises to the apical membrane of epithelial cells in the ectoderm where it is responsible for embryonic patterning. It was noted that the loss of Bazooka caused changes in embryonic patterning resulting from a loss of apical-basal polarity (reviewed by Ohno, Goulas and Hirose in Ebnet, 2015; Wieschaus *et al*., 1984). Bazooka was also discovered to be a scaffolding protein that assembles and maintains adherens junctions in the subapical domain (Laprise and Tepass, 2011). *Par* genes were discovered when a

comparable sequence to Bazooka was revealed in *C. elegans*. *Par* genes are responsible for differentiating cell types and establishing the polarity axis needed to produce anterior-posterior polarity of the embryo (Kempheus *et al*., 1988; Kempheus, 2000, reviewed in Ohno, Goulas and Hirose in Ebnet, 2015). Both Bazooka and the Par proteins also serve as markers for polarity in epithelial cells. While cell polarity is important for all epithelial tissues, it is especially important for epithelial cells involved in tubulogenesis (Ebnet, 2015; Bryant and Mostov, 2008). The following describes two models of tube formation and the importance of cell polarity in each.

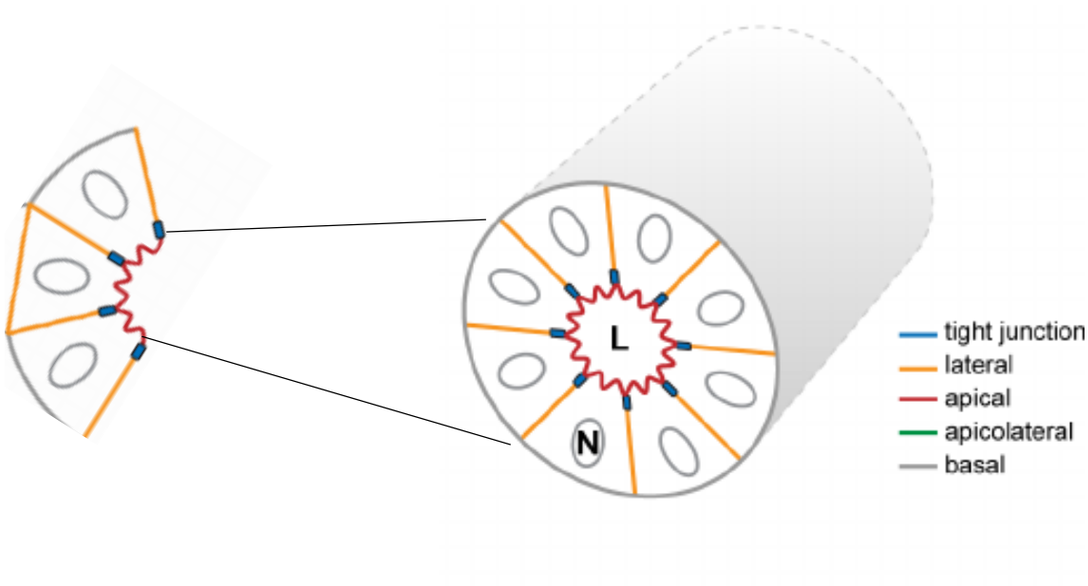


Figure 1.1: Schematic of a tube. Tubular organs are comprised of epithelial cells with apical-basal polarity and cell-to-cell adhesion (Adapted from Slim *et al*., 2013).

A mammalian model of cell polarity and tube formation is the Madin-Darby canine kidney (MDCK) cell line. This cell line has been the most commonly used vertebrate cell model in revealing the roles of polarity and membrane trafficking proteins during lumen formation. MDCK cells form hollow spheres called cysts that are composed of a single layer of polarised epithelial cells. When these cysts are introduced to Hepatocyte Growth Factor (HGF), they generate branches of tubules in a similar fashion to branching tubulogenesis of epithelial organs such as the vascular system. To form the tubules, cells produce an extension, then divide and migrate away from the cyst. They then form chains of cells that become cords that are 2-3 cells thick and eventually form a large lumen between the cords. These coordinated events rely on the establishment and maintenance of cell polarity (reviewed in Zeger *et al*., 2003).

A simpler model of tubulogenesis is *Drosophila* Malpighian tubule (MT), which functions similarly to mammalian kidneys. Formation of the tubules begins by budding from the hindgut, with one pair growing towards the anterior end and the other pair towards the posterior end. At the beginning of embryogenesis, they are comprised of 6 to 10 cells enclosing a lumen. However, through the processes of elongation and cell differentiation, they become long thin tubules containing a hollow lumen between two rows of cells. For cells to begin to bud from the hindgut, Wingless (Wg) signalling is required to induce cell specification for tubule development (reviewed in Jung *et al*., 2005). Two types of cells are required for the MTs to perform their excretion functions: Principal cells, involved in urine production, and Stellate cells, which contain channels for water and ion flow. Once they are part of the tubule, the Stellate cells assume epithelial characteristics and develop apical-basal polarity after establishing adherens

junctions with neighbouring Principal cells. Therefore, apical-basal polarity is crucial to tubulogenesis of MTs (reviewed in Jung *et al*., 2005).

1.2.1 Signalling and cell polarity

There are a variety of signalling pathways that are involved in tubulogenesis which may include Wnt, Receptor Tyrosine Kinases (RTKs), and Slit signalling (reviewed in Hogan and Kolodziej, 2002). Specifically, Wnt signalling functions in events such as cell specification, cell polarity, neural patterning, and organogenesis during development (reviewed in Mlodzik, 2002). Wnt signalling engages in 3 distinct pathways: the canonical Wnt pathway, the non-canonical planar cell polarity (PCP) pathway, and non-canonical calcium pathway (reviewed in Huelsken and Behrens, 2002; reviewed in Komiya and Habas, 2008). PCP is the development of polarity across the apical surface of an epithelium. An example of PCP can be found in the wing of *Drosophila*. Each cell in the wing produces one actin based hair, known as a trichome, that points distally, which is attributed to cytoskeletal reorganisation as a result of Wnt signalling (Mlodzik, 2002; Strutt, 2001). Frizzled (Fz) and subsequently Dishevelled (Dsh), two major polarity receptors in the Wnt pathway, are activated. This activates Rac and JNK signalling to induce cytoskeletal rearrangements to establish PCP in epithelial cells (reviewed in Komiya and Habas, 2008). Loss of Fz results in arbitrary arrangement of trichomes on the surface of the epithelial cells. Increased levels of the receptor have been found on the distal edges of wing cells, indicating that uneven localisation of Fz may be the mechanism by which Fz controls polarised trichome formation (Strutt, 2001).

Another example of PCP can be found in MTs. In MTs, the RTK, Epidermal Growth Factor Receptor (EGFR) is required for PCP during convergent extension.

Convergent extension (CE) is the constricting of a cell in one axis to elongate the cell in the perpendicular axis. To achieve CE, cells must undergo cell intercalation which involves lamellipodial growth so that cells can move over one another and modification of cell-to-cell junctions (Saxena *et al.*, 2014). In MTs, pairs of cells located at the tip of each tubule emit polarisation signals via the EGF ligand, Spitz. Spitz induces cell rearrangement via Myosin II accumulation at the proximal basal portion of the cell. Removal of the tip cells or disruption of EGFR resulted in tubule defects including failure to elongate. PCP occurred independently of known PCP pathways like Wnt signalling (Saxena *et al.*, 2014). Moreover, the establishment of cell polarity is crucial to system development as demonstrated by the *Drosophila* wing and MTs.

1.3 Epithelial Mesenchymal Transition

Although cell polarity is important for tube formation and function, some developmental processes require the partial loss of cell polarity. Epithelial mesenchymal transition (EMT) is a reversible process where polarised epithelial cells transition to a mesenchymal state, losing their apical-basal polarity, and cell-to-cell adhesions. As a result of these changes, the cell is now capable of mesenchymal behaviours which include increased migration ability, invasiveness and over production of ECM constituents (Kalluri and Weinberg, 2009). EMT is required for malignant tumor progression. For metastasis to occur, tumour cells must migrate from the initial tumour site and invade other tissue types (Moreno-Bueno, Portillo and Cano, 2008). While EMT is mostly known for its role in tumour progression, it is also crucial for embryogenesis and organogenesis, specifically gastrulation, neural crest migration, and heart formation (Leroy and Mostov, 2007). However, a full EMT is not always required for some

developmental processes. Partial EMT is also possible whereby cells do not completely transition to a mesenchymal state. MDCK cells go through a partial EMT during the first stage of tubulogenesis when they produce extensions toward the cyst from their basal membrane. During this stage, cells maintain some apical-basal polarity, however, a few of the cells then begin to migrate away from the wall losing their apical-basal polarity entirely. However, their cell-to-cell adhesions remain intact and they do not fully transition to mesenchymal cells. These cells will eventually regain apical-basal polarity, becoming epithelial cells and forming tubules containing lumens (Leroy and Mostov, 2007; reviewed in Zegers *et al*., 2003).

The reverse process of EMT is known as Mesenchymal-Epithelial Transition (MET). During MDCK cyst and *Drosophila* MT development, METs are mandatory for tube formation. In the MDCK branching cyst model, the chains of cells, formed by cells that migrated away from the cyst, must regain epithelial characteristics by completing a MET (Leroy and Mostov, 2007). In *Drosophila* MTs, Stellate cells undergo MET when they are integrated into the MTs (Jung *et al*., 2005). In both cases, the process of MET is critical to final system formation because cells need mesenchymal features to migrate, however, post-migration cells must become fully epithelial to form a functional system.

1.4 *Drosophila* dorsal vessel as a model of tubulogenesis

An excellent model for studying tube formation is the embryonic heart, the dorsal vessel, of *Drosophila Melanogaster*. *Drosophila* heart formation is genetically similar to early vertebrate heart formation, and shares many of the same regulatory networks and molecular players as the mammalian heart (reviewed in Bier and Bodmer, 2004). Many genetic techniques have been developed to manipulate gene expression in *Drosophila*

such as the Gal4/UAS system and RNA interference (RNAi; Brand and Perrimon, 1993, Fire *et al*., 2008) As well, the underlying mechanisms of tube formation are highly conserved across tube types and species. For example, Wnt signalling is required for heart formation and intestinal formation in invertebrates and vertebrates (reviewed in Ahmad, 2017; reviewed in Perochon *et al*., 2018). Fewer genes and cells are responsible for heart formation in *Drosophila* making it simpler to study molecular players (reviewed in Davies, 2002). Additionally, the *Drosophila* embryonic heart is visible without dissection, allowing for live observation of heart development (reviewed in Bier and Bodmer, 2004).

The *Drosophila* embryonic heart is a tubular organ that begins developing at stage 11 of embryogenesis. At stage 17, the embryonic heart is fully developed and begins to beat. The *Drosophila* heart consists of two rows of polarised cardioblasts (CBs), enclosing a lumen (reviewed in Tao and Schulz, 2007). Cardiac progenitors and CBs make several transitions before assuming their place in the fully formed heart tube. Beginning with gastrulation, differentiated cardiac mesodermal cells make a full EMT in order to migrate from the dorsal most mesoderm. CBs will then migrate in a partial mesenchymal-epithelial state towards the dorsal midline (Haack *et al*., 2014; reviewed in Tao and Schulz, 2007). During this migration phase, the cells undergo several shape changes and formation of filopodial and lamellipodial outgrowths (Figure 1.2; Vanderploeg, MacMullin, and Jacobs, 2012). Once they reach the midline, they adhere first dorsally and then ventrally to their contralateral partners. At this point, the CBs complete a MET, becoming fully polarised cardiac cells, forming the dorsal vessel (reviewed in Tao and Schulz, 2007).

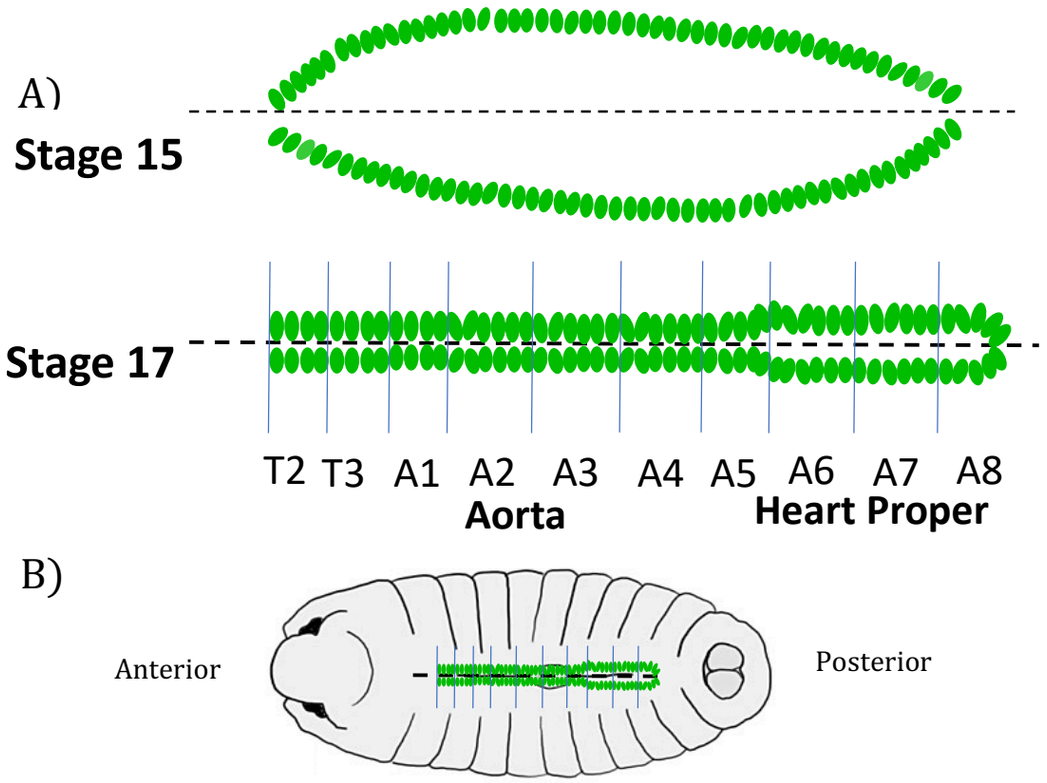


Figure 1.2: *Drosophila* embryonic heart development. Dorsal view of tubulogenesis of *Drosophila* heart at stage 15 and stage 17. Two rows of contralateral CBs migrate towards each other, forming a lumen (A)**.** Schematic of dorsal position of the dorsal vessel in an embryo (B). T is thoracic segment and A is abdominal segment. (B was adapted from Gorfinkiel, n.d.)

1.5 Embryonic *Drosophila* cardiogenesis

The embryonic *Drosophila* heart is comprised of two contralateral rows of 52 CBs, epithelial-like precursors of cardiomyocytes, that are flanked by pericardial cells, which have a detoxifying function (reviewed in Hughes and Jacobs, 2017; Medioni *et al*., 2008; Rotstein and Paululat, 2016). The embryonic heart begins to develop at stage 11 when cells originating from the mesoderm begin to migrate dorsally and medially towards each other during an ectodermal event called dorsal closure (reviewed in Hogan and Kolodziej., 2002). During embryogenesis, the amnioserosa, a transient epithelium, covers the dorsal opening produced by ectodermal germ band retraction. The amnioserosa begins a developmentally programmed disassembly and apoptosis allowing space for the leading edges of ectoderm and underlying two rows of mesodermal CBs to collectively migrate dorsally and meet at the midline (Rugendorff and Hartenstein, 1994; Haack et. al, 2014; Vanderploeg, 2014). During their migration phase, the apical surface of CBs has both a non-motile pre-luminal domain and a motile domain known as the Leading Edge (LE; reviewed in Medioni *et al*., 2009). This organisation of the apical domain is similar to PCP in that CBs are polarised across the dorsal to ventral axis of the apical domain. Before meeting their contralateral partners at the midline, CBs make a series of shape changes (Figure 1.3). Multiple signalling pathways work together to promote extension of filopodial and lamellipodial protrusions at the LE, required for contralateral CBs to make appropriate contact and attachments at the junctional domains (Raza and Jacobs, 2016). Once the CBs meet at the midline, they first adhere to their contralateral neighbours via adhesion proteins such as Cadherins at their dorsal most

point. They then adopt a crescent shape and adhere via junctional proteins at their ventral most point, forming a lumen between them. By stage 17, the heart is fully formed, with both an aorta and a heart proper (reviewed in Medioni *et al*., 2009; reviewed in Rotstein and Paululat, 2016).

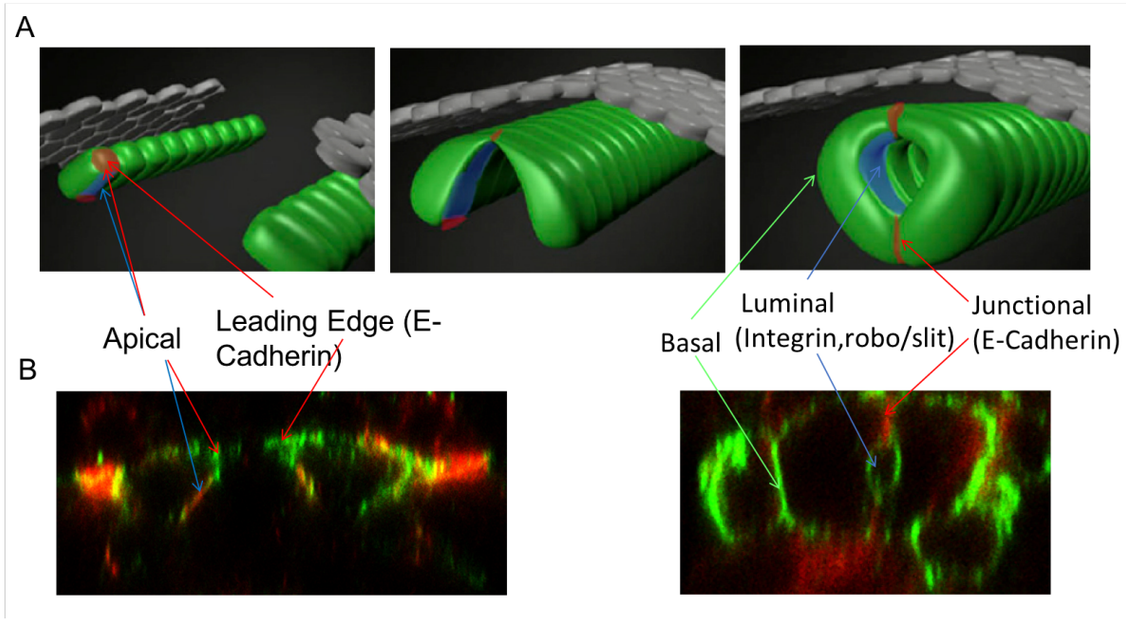


Figure 1.3: Cross sectional schematic of developing heart from stage 16 to late stage 17. Two rows of contralateral cardioblasts (CBs) migrate towards each other and make contact at discrete sites of adhesion first dorsally and then ventrally, eventually enclosing a lumen (A). Cross sectional view of migrating CBs and resulting lumen formation (B). CBs establish spatially and molecularly distinct domains (Leading Edge, Luminal, Basal and Junctional). Green is Dystroglycan and red is Discs Large. (Adapted from Medioni *et al*, 2008 and Vanderploeg, 2014)

1.5.1 Forming a lumen

To form a lumen, cells must align properly with ipsilateral and contralateral neighbours, create specific sites of adhesion, and form a functional lumen between contralateral cells. Cell surface proteins that are required to generate these processes will be discussed in further detail. However, their presence alone within the cell is insufficient to coordinate lumen formation. The localisation of these factors is also critical (reviewed in Medioni *et al*., 2009). For example, Vanderploeg, MacMullin and Jacobs (2012) discovered that localisation of Integrin at the luminal domain of migrating CBs is needed to establish the luminal domain as well as the basal domain. Without Integrin, a luminal domain fails to form. Another cell surface receptor, Roundabout (Robo), along with its ligand Slit are required to localise to the luminal domain of CBs to restrict Cadherins, adhesive molecules, to the junctional domain. Furthermore, Robo localisation depends upon Integrin. Mislocalisation or absence of Robo results in rounded CBs, that have not made any shape changes, and have formed adhesions along their luminal domain as a result of accumulation of Cadherin (Kidd *et al*, 1998; Medioni *et al*., 2008; Vanderploeg, MacMullin, and Jacobs, 2012). These examples demonstrate the importance of proper localisation of cell signalling receptors during tubulogenesis of the heart. Though these signalling mechanisms of lumen formation are well characterised, the regulators responsible for their localisation are unknown. Understanding both upstream and downstream organisers of these localisation patterns is vital to understanding lumenogenesis and tubulogenesis as a whole.

1.6 Major cell surface receptors involved in cardiac tubulogenesis

1.6.1 Integrins and Cadherins

Integrins are surface receptors that localise to the apical non-motile luminal domain, although they are involved in establishing cellular polarity and LE motility (Vanderploeg, MacMullin, and Jacobs, 2012). They interact with the Extracellular Matrix (ECM) and convey signals to the cell from the ECM through inside-out and outside-in signalling via their ligands (Laminins and Collagens; Vanderploeg, MacMullin, and Jacobs, 2012). Cadherins are junctional proteins that localise to the motile LE domain where they will form adhesions when migrating CBs meet at the midline. They are responsible for adhering to ipsilateral and contralateral cells (Haag *et al*., 1999; Santiago-Martínez *et al*., 2008). Other cell signalling pathways such as Slit and Netrin signalling interact with Cadherins and Integrins to establish boundaries of the Integrin based lumen and Cadherin based cell junctions (Santiago Martínez *et al*., 2008; Raza and Jacobs, 2016). The intracellular mechanisms that establish these discrete domains are not well understood and the regulators of these processes have yet to be elucidated.

1.6.2 Dystroglycan and Discs Large

Besides Integrin and Cadherin, other cell surface proteins are essential to lumenogenesis as well. Dystroglycan (Dg) is a cell surface receptor that localises to the luminal domain and is required for lumen formation. It interacts with ECM components such as Laminins and Perlacan similarly to Integrin. Antibodies to Dg are commonly used as a luminal marker (Medioni *et al*., 2008; Vanderploeg, MacMullin and Jacobs, 2012). Discs-Large (Dlg) localises with Cadherin and is required for junctional integrity.

It is part of the Scribble complex and is used as a marker of the baso-lateral domain (Firestein and Rongo, 2001; Laprise and Rivard, 2004).

1.6.3 Slit signalling

Slit is the ligand for the receptor Robo which is essential for the maintenance of the lumen. Slit and Robo are localised to the luminal domain, however, this is dependent upon Integrin (MacMullin and Jacobs, 2006; Santiago-Martínez *et al*., 2008). Slit and Robo signalling modulate cell shape changes, formation and maintenance of robust LE activity and localisation of Cadherin (Kidd *et al*, 1998; Medioni *et al*., 2008; Vanderploeg, MacMullin, and Jacobs, 2012). In Slit and Robo/Robo2 mutants, the CBs maintain their round shape instead of extending a LE, a step necessary to form a tube that contains a luminal space (Medioni *et al*., 2008). Slit signalling is also required for restricting Cadherin to the LE/junctional domains. In the absence of Robo, the entire apical surface is fused through Cadherin adhesion as a consequence of Cadherin aggregation at the luminal domain (Santiago-Martínez *et al*., 2008). During earlier stages (stage 14 to 16) of cardiogenesis, Robo localises apically, basally, and laterally in the migrating CBs, however, by stage 17, Robo is almost completely restricted to the luminal domain (Figure 1.4, Vanderploeg, MacMullin, and Jacobs, 2012; Vanderploeg, 2014). It is unknown what mechanisms establish this luminal localisation pattern of Robo. However, in an axon migration model, it was shown that in a Slit-dependent manner, Robo must be internalised and trafficked to the early and late endosomes to be activated (Chance and Bashaw, 2015). Endocytic trafficking may be responsible for localisation of Robo to the luminal domain from stage 15 to stage 17 during heart formation. More

insight into the link between endocytosis and receptor activation and trafficking needs to be gained.

1.6.4 Netrin signalling

Slit and Netrin signalling work in conjunction to activate LE activity (Raza and Jacobs, 2016). Net is the ligand for the receptors Frazzled (Fra) and Uncoordinated5 (Unc5). Unc5 and Fra regulate luminal identity and LE activity, respectively (Albrecht *et al*., 2010; Macabenta *et al*., 2013; Raza and Jacobs, 2016). Although Unc5 and Fra both respond to Net and localise in areas where Net is most concentrated, they have complementary localisation patterns and signals. During the migration stage of heart formation, Fra localises to the LE to transduce chemo-attractive Net signals that induce growth of protrusions, which facilitates the attachment of contralateral cells at discrete attachment regions when they meet at the midline (Albrecht *et al*., 2010; Macabenta *et al*., 2013, Quinn and Wadsworth, 2008; Raza and Jacobs, 2016). In the absence of Fra, a lumen fails to form as a result of midline attachment defects, which can be attributed to a decrease in LE activity. A robust LE is required in order for appropriate contacts to be made with contralateral CBs (Macabenta *et al*., 2013). Some CBs also become fully mesenchymal in *fra* mutants and begin to migrate away from the rows of CBs demonstrating a tumor suppressive role of Fra (Raza, 2015, Raza and Jacobs, 2016). In mammals, deletion of Fra (known as Deleted in Colorectal Cancer (DCC)) also produces a similar migration phenotype. Loss of DCC function in a Mesencephalic Dopaminergic Neurons in mice resulted in abnormal single cell migration of progenitors, which is a feature of many cancer cells (Xu *et al*., 2010; reviewed in Friedl and Gilmour, 2009)

In the *Drosophila* heart, Fra localises to the dorsal LE during early stage 16. By late stage 16, Fra accumulates at the ventral most point of the cell. Finally at stage 17, Fra is localised to sites of cell to cell contact (Macabenta *et al*., 2013). The mechanism by which these precise localisation patterns of Fra is achieved is not yet understood. Similar to the mechanism by which Robo is activated and localised, endocytosis may also be potentially responsible for these patterns of Fra localisation.

While Fra produces chemo-attractant signals, Unc5 produces chemo-repellent signals when bound to Net. Its localises to the luminal domain, accumulating here by stage 16 (Albrecht *et al*., 2010; Macabenta *et al*., 2013, Quinn and Wadsworth, 2008; Raza and Jacobs, 2016). Through induction of cytoskeletal remodelling, Net/Unc5 signalling causes the contralateral rows of migrating CBs to repel each other, in order to maintain the integrity of the lumen. In the absence of Unc5, CBs fail to repel their contralateral partners resulting in the loss of a luminal space (Albrecht *et al*., 2010; Macabenta *et al*., 2013). Current models suggest that Fra may be acting upstream of Unc5, as *fra* and *unc5* double mutants resemble a *fra* mutant phenotype, with CBs failing to appropriately attach at the midline (Macabenta *et al*., 2013). As with Fra, the mechanism by which Unc5 localises and accumulates at the luminal domain is unknown.

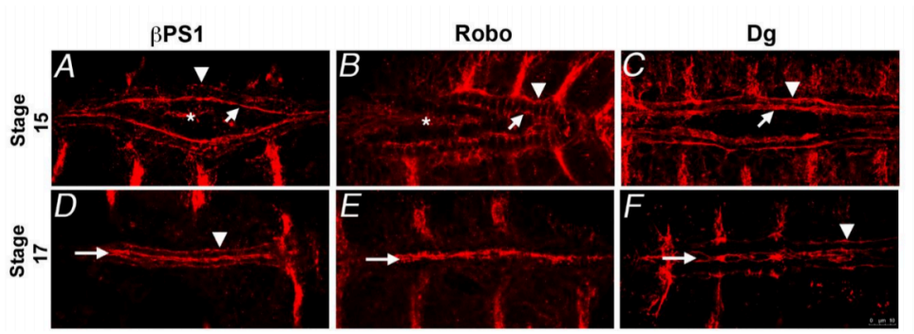


Figure 1.4: Localisation of cell surface receptors is modified from stage 15 to stage 17. Arrows indicate apical localisation. Arrowheads indicate basal localisation. (Adapted from Vanderploeg, 2014).

1.7 Endocytosis as a regulator of cell polarity

Cell polarisation is crucial to tubulogenesis of the *Drosophila* heart and without it a non-functional organ is formed. Key signalling receptors, like Robo, Fra, and Unc5 involved in lumenogenesis of the embryonic *Drosophila* heart have been well characterised, however, regulators and their effectors of these mechanisms have yet to be examined. It is clear that cell receptor localisation is modified throughout heart formation as localisation of Robo, βPS Integrin, and Dg significantly changes throughout heart formation (Vanderploeg, MacMullin and Jacobs, 2012). It is unknown how Robo and these other receptors are targeted and trafficked to their final destinations, which proves to be a clear gap in the understanding of mechanisms of cell polarisation during tubulogenesis of the heart.

Intracellular vesicle trafficking is the transport of either newly synthesized proteins from the Trans-Golgi Network (TGN) or recycled proteins from the cell membrane inside or outside of the cell. There are two main types of intracellular vesicle trafficking: exocytosis, inside-out transport, and endocytosis, outside-in transport (reviewed in Tokarev, Alfonso, and Segev, 2009). The process of endocytosis is a potential mechanism employed during cell polarisation whereby membrane proteins are removed from a membrane and trafficked to another membrane. During endocytosis, cell surface receptors are sorted into budding vesicles. The endocytosed receptors are then either recycled back to the cell membrane or trafficked to lysosomes where they are degraded (reviewed in Stenmark, 2009). Trafficking of receptors can also facilitate their activation which is required to transmit their signal (Chance and Bashaw, 2015). Consequently, endocytosis could play a major role in the establishment of cell polarity

and receptor activation during tubulogenesis (reviewed in Das and Guo, 2011). More specifically, protein players involved in endocytosis, including Rab GTPases and their effectors, may play a vital role in the localisation of CB cell surface signalling receptors (reviewed in Stenmark, 2009).

To establish cytoplasmic and surface domains, proteins need to be transported appropriately. It has been shown in yeast and mammalian cell models that Rab GTPases function as molecular switches that activate and deactivate effectors of intracellular trafficking (reviewed in Stenmark, 2009; Zhang *et al*., 2007; Morrison *et al*., 2008; Desclozeaux *et al*., 2008). Rab GTPases have been implicated in the activation of CDC42 during lumen formation of MDCK cells (Bryant *et al*., 2010). During *Drosophila* heart development, CDC42 is one of the proteins responsible for cytoskeletal remodelling, which is a requirement for cellular shape changes and filopodial and lamellipodial outgrowth. This suggests that Rab GTPases may also have a role in *Drosophila* heart development. Rab GTPases in *Drosophila* border cell migration, a process in oogenesis similar to CB migration, have been implicated as having a regulatory role in oogenesis (Jékely *et al*., 2005). In a *Drosophila* MT model of tubulogenesis, the Rab GTPase, Rab11, was associated with the localisation of Dlg and Bazooka, polarity proteins required for MT formation, as well as F-actin localisation, which is crucial to cytoskeletal rearrangement (Choubey and Roy, 2017). Taking these into consideration, Rab GTPases will be the major focus of my research because they may also be responsible for the targeting and trafficking of key protein players such as Robo, Integrin, and Fra in our model of *Drosophila* embryonic lumenogenesis. The following section describes Rab GTPase functions and explore their potential role in tubulogenesis.

1.7.1 Rab GTPases

As mentioned previously, intracellular trafficking is very important for localisation of signalling and adhesion proteins involved in the process of lumenogenesis and overall cell function. Rab GTPases have been implicated as organisers of this process as they are involved in regulating cargo sequestration, vesicular trafficking, vesicle budding, and membrane fusion (Zhang *et al*., 2007; Morrison *et al*., 2008; Desclozeaux *et al*., 2008; reviewed in Stenmark, 2009). Rab GTPases are members of the Ras superfamily of GTPases. There are as many as 70 human Rab proteins, while *Drosophila* possesses 31 Rab proteins (Zhang *et al*., 2007). Certain Rab GTPases localise to specific subcellular areas where they regulate specificity of vesicular trafficking, ultimately leading to the maintenance of membrane identity. Rab GTPases (Rabs 5, 11, 8, 22, 4, 35 and others) have specified roles in the endosome (Figure 1.5). They are associated with either the early, late, and/or recycling endosome (reviewed in Zhen and Stenmark, 2015). The early endosome removes proteins from the plasma membrane, while the late endosome trafficks proteins and fuses with the lysosome, which degrades the proteins. The recycling endosome functions to transport proteins from the early endosome to the plasma membrane (reviewed in Stenmark, 2009). The first Rab GTPase to be discovered was Sec4p in a yeast model where it functions as a secretory vesicle during exocytosis (Saleminen and Novick, 1987; Zhang *et al*., 2007). The Rab GTPases were subsequently discovered in a rat brain and officially termed Rab (Ras-like proteins from rat brains; Touchot, Chardin, and Tavitian, 1987; reviewed in Pfeffer, 2017; Zhang *et al*., 2007), and were numbered in the order that they were sequenced (reviewed in Pfeffer, 2017). In the past, Rabs have largely been studied in yeast and mammalian models, however in recent

years, *Drosophila* researchers have begun to recognize the importance of Rab GTPases and their role in *Drosophila* development (Zhang *et al*., 2007). For example, Rab5, a known regulator of the early endosome, was discovered to modulate the Wnt signalling pathway in a *Drosophila* cell culture model (Seto and Bellen, 2006; Zhang *et al*., 2007).

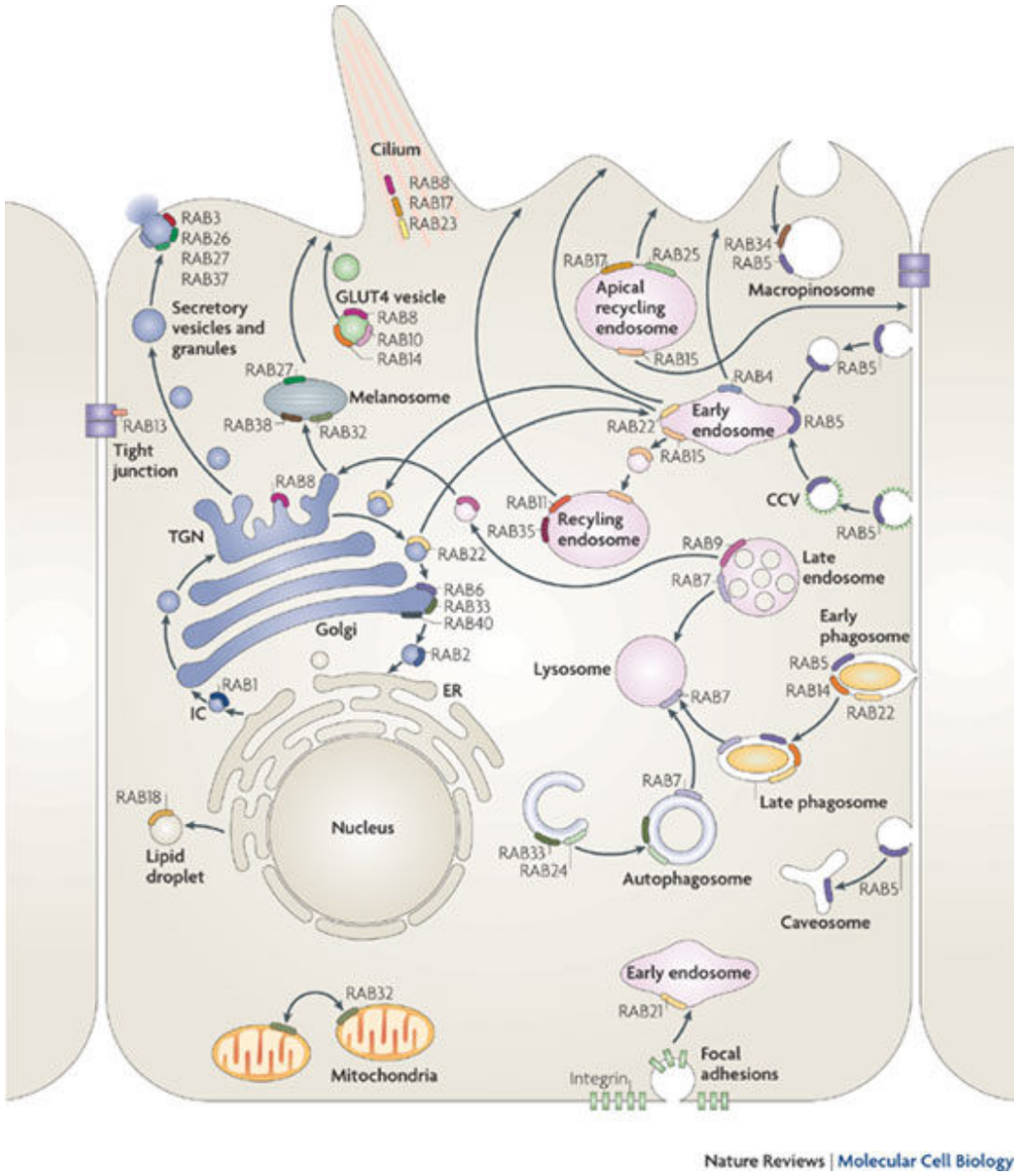


Figure 1.5: A summary of Rab GTPase interactions and involvement in the endocytic pathway (Excerpt from Stenmark, 2009 with permission from Springer Nature).

In endocytic pathways, Rab GTPases act as molecular switches that are molecularly switched “on” or “off”. As such, they can be found in two forms: GTP bound form being their “on” (active) state and GDP bound form being their “off” (inactive) state (Figure 1.6). When activated, the function of Rab GTPases during endocytosis is to recruit downstream effectors such as motors and tethers that enable endocytic events including cargo sequestration and membrane fusion (Goody, Müller and Wu, 2017; reviewed in Pfeffer, 2017; reviewed in Stenmark, 2009). Rab GTPases are excellent targets to determine the role of endocytosis in development due to the switch mechanism by which they function. Manipulating Rab GTPase function by locking the Rab in their active or inactive state provides a means to observe the role of endocytosis in a given model (Weigandt *et al*., 2015).

Rabs are activated and inactivated through a series of reactions. When a Rab is introduced to a Rab geranylgeranyltransferase (RabGGTase) by a Rab escort protein (REP), the Rab’s C-terminus is prenylated on one or two cysteine residues, which facilitates their tight association with a membrane (reviewed in Pfeffer, 2017). Activation of the Rab occurs through the removal of GDP and attachment of GTP, which is facilitated by a Rab-specific Guanine nucleotide exchange factor (GEF). GTP is present in high concentrations to ensure GTP attaches to the Rab (reviewed in Stenmark, 2009; Zhang *et al*., 2007; reviewed in Zhen and Stenmark, 2015). Active GTP bound form of a Rab has highly specific conformations necessary for binding to their effectors which includes but is not limited to sorting adaptors, tethering factors, kinases, phosphatases and motors (reviewed in Pfeffer, 2013; reviewed in Stenmark, 2009).These effectors facilitate events such as sorting, membrane fusion, motility, and tethering. Once the

membranes have fused and the endocytic event has occurred, the Rab is inactivated through hydrolysis where by GTP activating proteins (GAPs) catalyze the reaction. Guanine dissociation inhibitor (GDI) then removes the Rab from the membrane and it remains bound to the GDI until it is removed by a GTP-dissociation factor (GDF) (reviewed in Stenmark, 2009, Molendijk *et al.*, 2004).

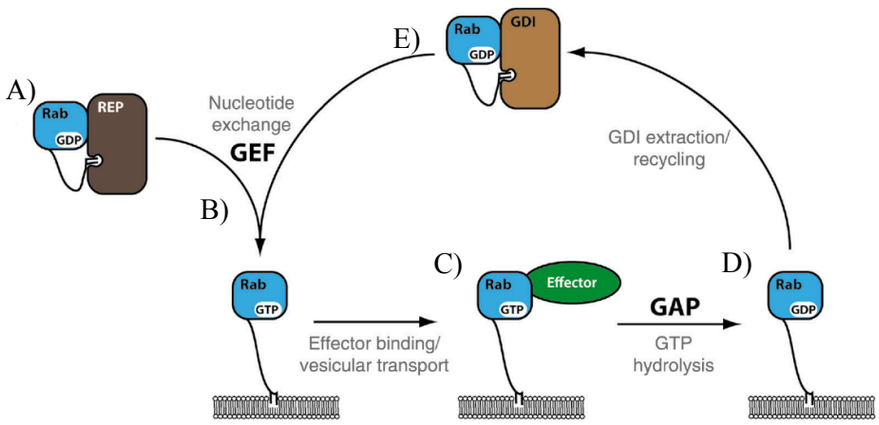


Figure 1.6: Rab GTPases act as molecular switches to activate downstream effectors. A prenylated Rab bound to a Rab escort protein (REP; A) Rab is transported to a membrane and activated through the addition of GTP catalyzed by a Guanine nucleotide exchange factor (GEF; B). GTP bound Rab binds to a membrane and subsequently to an effector to initiate endocytic events such as membrane fusion (C). Rab is deactivated through a hydrolysis reaction catalyzed by a GTP activating protein (GAP) whereby GTP is exchanged for GDP (D). Guanine disassociation factor binds to the inactivated Rab to remove it from the membrane (E; Adapted from Goody, Müller and Wu, 2017).

Rabs are localised to specific cellular compartments, however, multiple Rabs can be localised in the same compartments where they form their own microdomain (Molendijk *et al*., 2004; Pfeffer and Aivazian, 2004). GDIs are required to deliver Rabs to their specific compartments, however, GEFs have also been shown to be involved in targeting Rabs to the appropriate cellular compartments and membranes. Blümer *et al*. (2013), showed that mislocalisation of the Rab5 GEF, Rabex-5, to mitochondria resulted in the mislocalisation of Rab5 by its GDI (reviewed in Pfeffer, 2013). Together, GDIs and GEFs localise Rabs to their compartments.

The coordination of different Rabs in the endocytic pathway is required for the recycling and trafficking of proteins to particular membranes. To facilitate this coordination, Rab effectors can bind two Rabs at the same time to organise the next step in the trafficking pathway. Rabs can also recruit the GEF for the following Rab in the cycle and the following Rab can recruit the GAP for the previous Rab (Figure 1.7; reviewed in Pfeffer, 2017). For example, GTP bound Rab4 can bind to Rabex-5 and Rab5 can bind to Rab4 effectors such as Rabenosyn-5 (De Renzis, Sönnichsen, and Zerial, 2002; Frittoli *et al*., 2014; reviewed in Markgraf, Peplowska, and Ungermann, 2007). Thus, effectors, GAPs, and GEFs are essential in coordinating trafficking events.

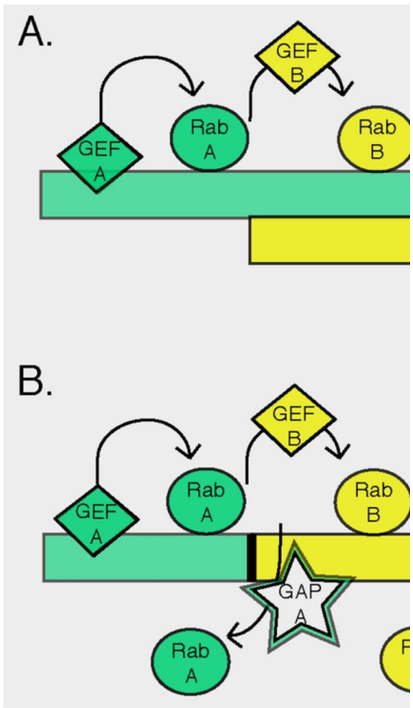


Figure 1.7: Coordination of endocytic events RabA recruits RabB's GEF to facilitate a "Rab GTPase cascade" and coordination of endocytic events (A). RabB recruits RabA's GAP to inactivate RabA (B; Adapted from Pfeffer, 2017).

1.7.2 Rab GTPases in *Drosophila*

There are three main compartmental groups of Rab GTPases in a cell. The first includes Rab5, the second Rab4 and Rab5, and the third Rab4 and Rab1l (Sonnichsen *et al*., 2000; reviewed in Stenmark, 2009). Zhang *et al*. (2007) observed that Rab4 transgenics (Rab4 dominant negative) did not have evident defects in *Drosophila*. The overlap in function of Rab4 with Rab5 and Rab11 may explain the lack of defects and indicates that Rab5 and Rab11 might have more significant roles in endosomal trafficking (Zhang *et al*., 2007). Based on this evidence, and that Rab5 and Rab11 are the most abundant and important Rab GTPases, I focused on the regulatory role of Rab5 and Rab11 in our *Drosophila* model. Their phenotypes observed in other *Drosophila* tissues also make them candidates for this regulatory role in the heart. The following describes Rab5 and Rab11 trafficking roles in other tissues.

Rab5 is a component of the early endosome necessary for endosome to vesicle fusion (Zhang *et al*., 2007). Rab5 has been reported to be a regulator of an important developmental pathway in *Drosophila*. Morrison *et al*, discovered the Rab5 effector Rabenosyn (Rbsn) in a *Drosophila* eye imaginal disc tissue model. They found that Rab5 regulated early endosomal fusion through Rbsn-dependent recruitment of VPS45, which has been identified in yeast as necessary for Golgi-to-lysosome traffic. In Rbsn mutants, Notch remained in the cell periphery instead of being internalized by early endosomes. This mutant phenotype is very similar to the phenotype of *rab5* mutants (Morrison *et al*., 2008).

In various models of metastasis, Rab5 has been implicated in having an effect on cell motility and invasion as well as in regulation of cytoskeletal constituents. In a HeLa

cell model, elevated levels of Rab5a were observed. Knockdown of Rab5a resulted in a reduction in cell motility, downregulation of integrin-interacting proteins (integrin ß-1, Fak, p-Fak, p-paxillin, paxillin, vinculin) and reduced filopodial and lamellipodial protrusions. A reduction in GTP bound CDC42, RhoA and Rac1 was also observed (Liu *et al*., 2011). In a *Drosophila* imaginal epithelium model, mutations in *rab5* and *vsp25* resulted in the formation of polyploid giant cells, which are required for tumor progression and metastasis. These are known as neo-plastic tumor suppressors (Cong, Ohsawa and Igaki, 2018). This evidence shows that Rab5 is required for cell motility, protrusion formation, and regulation of integrin-interacting proteins.

In a mammalian neuronal model, suppressing Rab5 through the use of a dominant negative resulted in decreased activity of the JNK pathway. Rab11 was not found to have the same effect. This decrease in Rab5-mediated endocytosis resulted in increased cell adhesion, which was attributed to an increase of N-Cadherin at the cell surface, specifically in the neuronal body, and led to impaired neuronal migration (Kawauchi *et al*., 2010). Similarly, a constitutively active form of Rab5 lead to reorganisation of the cytoskeleton in a fibroblast cell model. Cytoskeletal reorganisation stimulates the formation of lamellipodia and leads to an increase in cell motility. This demonstrates the importance of Rab5 in cell migration (Spaargaren and Bos, 1999).

Rab11 is a component of the recycling endosome responsible for protein transport and localisation (reviewed in Stenmark, 2009; Zhang *et al*., 2007). In Choubey and Roy's *Drosophila* MT model, Rab11 was to be found to be a regulator of lumenogenesis. Knockdown of Rab11 in primordial cells of the MTs resulted in shorter tubules, mislocalisation of Dlg and Bazooka as well as ion transports, and cytoskeletal

disorganisation (Choubey and Roy, 2017). Desclozeaux *et al*. (2008) also discovered that E-Cadherin mislocalises in Rab11-inactivated epithelial biogenesis model of lumen formation. Based on these findings, interruption of the endocytic pathway via Rab11 causes disruption of cell polarity.

Another candidate for regulation of tubulogenesis is the exocyst complex. The exocyst consists of 8 subunits that interact with Rab GTPases and are involved in specifying the destination and fusion of vesicles. More specifically, in epithelial cells, it is responsible for trafficking of vesicles from the recycling endosome to the plasma membrane for secretion (Beronja *et al*., 2005). The exocyst is called the Sec6/Sec8 complex in mammalian models. It localises to the TGN and to the tight junctions (Langevin *et al*., 2005; reviewed in Lipschutz and Mostov, 2002). In MDCK cell models of tubulogenesis, components of the exocyst have been associated with cell polarity. During tubulogenesis, MDCK cells require the proper localisation of Cadherin for cell-to-cell adhesion. Grindstaff *et al*. (1998) demonstrated that the Sec6/Sec8 complex directs vesicles containing baso-lateral adhesion proteins such as Cadherin to regions of cell-to-cell adhesion in MDCK cells. In *Drosophila*, Langevin *et al*. (2005) demonstrated that Sec5, Sec6, and Sec15 interact with the recycling endosome, specifically Rab11, and mediate Cadherin localisation in a similar way as in MDCK cells. In our cardiac lumenogenesis model such regions of cell-to-cell contact would be the LE and the junctional domains. Considering the evidence, the exocyst may have a role in localisation of polarity proteins within CBs.

Members of the exocyst have been shown to have overlapping functions. Beronja *et al*. (2005) saw that Sec6 specifically accumulated at adherens junctions in their

Drosophila photoreceptor cells (PCRs) model. In late PCRs, Sec5, Sec6, and Sec8 accumulated in the subdomain of apical membrane. Sec5 was shown to form a complex with Rab11 but Sec6 is required to interact with Sec5. This indicates that the exocyst may be a Rab11 effector (Beronja *et al*., 2005). In a *Drosophila* nervous system and ovarian model, Sec5, Sec6, and Sec8 appeared to work in conjunction to localise proteins. Sec5 localisation relied upon Sec6 function and Sec8 mislocalised in both Sec5 and Sec6 mutants (Murthy *et al*., 2005). Based on these findings, we will be focussing on Sec6. The integrated function of the exocyst allows us to knockdown any exocyst member to observe its effects on lumenogenesis.

1.7.3 Rab GTPases and regulation of tubulogenesis of the *Drosophila* embryonic heart

Rab GTPases have been observed to interact with proteins that are similar to signalling receptors required for heart formation. In *Drosophila* border cell migration models, Rab5 and Rab11 have been associated with proteins also important for cardiac lumenogenesis. Rab5-dependent endocytosis is necessary for Rac1 to be activated by motogenic stimuli (Palamidessi *et al*., 2008; reviewed in Stenmark,2009). With respect to the heart, Fra/Net signalling triggers Rac1 and CDC42 to cause cytoskeletal rearrangements that induce cell shape changes and protrusion growth. Fra/Net signalling could be referred to as motogenic stimuli in our heart model (Swope *et al*., 2014).

Endocytic trafficking has also been shown to contribute to Slit/Robo signalling in an axonal guidance model in *Drosophila*. Dominant negative expression of Rab5 and Rab7, but not Rab11, resulted in axonal crossing defects. Trafficking of Robo to the early and late endosome positively regulated Robo-mediated midline repulsion. This model revealed a positive feedback mechanism by which Slit-dependent endocytosis of Robo

activates the receptor (Chance and Bashaw, 2015)**.** More work needs to be done to investigate the regulatory role of Rab GTPases in lumenogenesis of the heart and specifically in the transport and localisation of cell surface receptors.

As previously noted, the regulators of cell receptor localisation involved in tubulogenesis of the *Drosophila* heart are not well understood, which leaves a major gap in our understanding of tube development. Based on results from other research groups, Rab GTPases could potentially be the modulators of the protein localisation, cytoskeletal rearrangement and growth involved in tubulogenesis, and more specifically lumen formation (Choubey and Roy, 2017; Desclozeaux *et al*., 2008).

1.7.4 Targeting Rab GTPases

My goal is to examine the effects of dominant negative (DN), constitutively active (CA) and overexpression forms of Rab GTPases as well as mRNA knockdowns on embryonic cardiac lumenogenesis in *Drosophila melanogaster*. Specifically, I aim to discover which proteins perform a regulatory role in the localisation of key signalling and adhesion proteins such as Cadherin, Integrin, Robo, Fra, and Unc5, that are required for lumenogenesis (Figure 1.8).

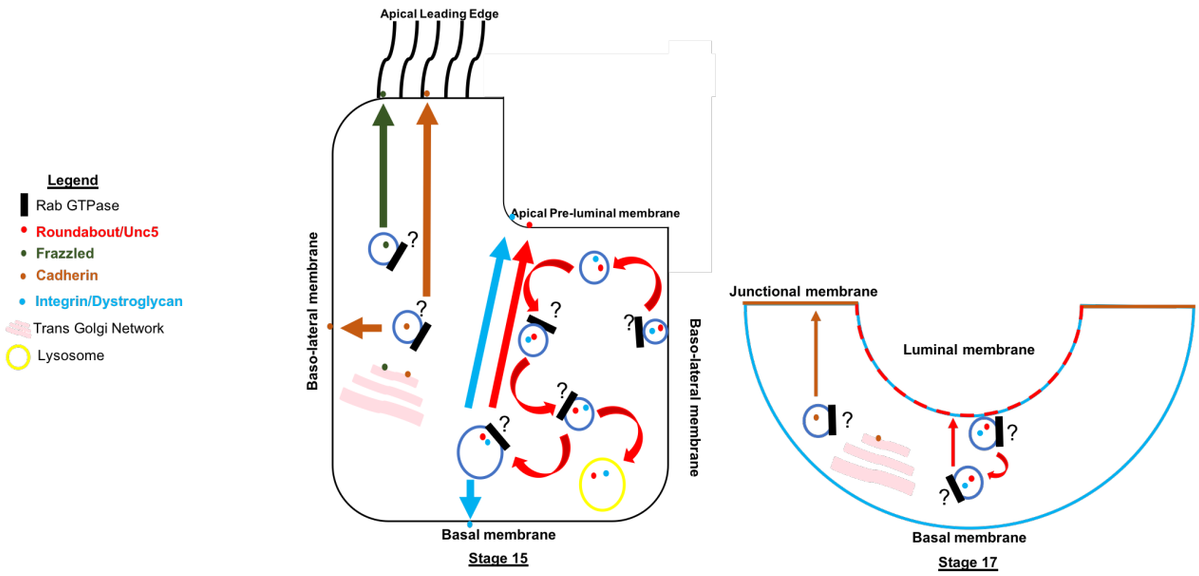


Figure 1.8: Proposed schematic of receptor trafficking in the cardioblast. Cell surface receptor localisation is modified from stage 15 to stage 17 of heart development. It is hypothesized that Rab5, Rab11, and Sec6 are responsible for targeting and trafficking these receptors. It is proposed that luminal receptors (Integrin, Dystroglycan, Robo, Unc5, (blue and red)) are initially targeted to the baso-lateral membrane (from the TGN– not shown) and endocytosed from there and retargeted to the apical pre-luminal domain from stage 15 onwards. Integrin and Dystroglycan are also trafficked to the basal domain (light blue). At stage 15, newly synthesized Cadherin (brown) and Frazzled (green) are trafficked from the TGN to the apical leading edge, which will form the future junctional domain. In the stage 17 schematic, cell membranes are colour coded with the dominant transmembrane proteins. Cell surface receptors are retained in their sub-apical domains (adhesive or luminal) and newly synthesized receptors are trafficked to the cell surface from the TGN.

1.8 Objectives/Hypotheses

Our objective was to examine the role of Rab GTPases in the establishment of the molecularly distinct cytoplasmic and surface domains of CBs. Our first goal was to conduct a gene survey using Rab5, Rab11, and Sec6 knockdowns. We hypothesized that Rab GTPases, Rab5 and Rab11, are significant regulators of the process of lumenogenesis through trafficking cell surface signalling receptors and adhesion proteins to specific cell surface domains. The proposed mechanism by which Rab5 regulates lumenogenesis is that Rab5 targets CB cell signalling receptors and adhesion proteins (Robo, Unc5, Fra, Integrin, Cadherin, etc.) at the baso-lateral cell membrane to the early endosome for recycling back to the apical membrane. The proposed mechanism of Rab11 is that it modulates CB polarity protein localisation through the recycling endosome, trafficking protein to luminal and LE domains of the plasma membranes. Furthermore, Sec6 was proposed to mediate specified localisation of cell-to-cell adhesion proteins (Cadherin) in CBs. Through these mechanisms, we hypothesized that Rab5, Rab11 and Sec6 activate and regulate growth and attachment, CB migration, cytoskeletal rearrangement and protein localisation, resulting in the formation of a lumen. Based on our results of the gene survey, we continued to further our investigation of Rab5. We hypothesized that expression of both dominant negative and constitutively active forms of Rab5 will cause the mislocalisation of Robo, a surface receptor required for repulsive signalling at the lumen. I aimed to quantify changes in migration velocity, LE activity, cell alignment, lumen size and localisation of polarity proteins in Rab5 transgenic embryos using confocal microscopy.

2. Methods

2.1 Fly maintenance

Fly stocks were maintained on standard medium at room temperature. Flies were transferred to new food every 14 days. All crosses were incubated at 24.5°C unless otherwise noted.

2.2 Fly stocks

Below is a table of fly stocks used in this thesis.

Table 2.1: List of fly stocks used in this thesis.

Stock	Name in This Thesis	Source	Stock #
y^1, w^{118}	yw	Bloomington Stock Center	6598
dmef-Gal4/TM3		Bloomington Stock Center	50742
tail-up-GFP		Robert Schulz	
UAS-*moesin*-mCherry		Thomas Millard	
dmef-Gal4, *tail-up*-GFP/TM3		made in this lab	
dmef-Gal4, *tail-up*-GFP, UAS-*moesin*-mCherry/TM3		made in this lab	
UAS-*dicer*; dmef-Gal4		Bloomington Stock Center	25756
UAS-*daughterless* Gal4/TM6		Bloomington Stock Center	55851
UAS-$rab5^{DN1}$-YFP	$rab5^{DN1}$	Bloomington Stock Center	9771
UAS-$rab5^{DN2}$-YFP/TM3	$rab5^{DN2}$	Bloomington Stock Center	9772
UAS-$rab5^{CA1}$-YFP/TM3	$rab5^{CA1}$	Bloomington Stock Center	9773
UAS-$rab5^{CA2}$-YFP	$rab5^{CA2}$	Bloomington Stock Center	9774
UAS-$rab5^{WT}$-YFP	$rab5^{WT}$	Bloomington Stock Center	24616
UAS-*rab5* RNAi		Vienna Drosophila Resource Center	103945
UAS-$rab11^{DN1}$-YFP		Bloomington Stock Center	23261
UAS-*rab11* RNAi		Vienna Drosophila Resource Center	108382
UAS-*sec6* RNAi		Vienna Drosophila Resource Center	22079

2.3 Embryo collection for fixation

Approximately 60-80 virgin females (UAS line) and 10-20 males (Gal4 driver line) were crossed at 24.5°C. Embryos were collected on apple juice agar plates with a small smear of yeast paste. Plates were collected two or three times a day every 4 hours. Plates were incubated at 18°C or 24.5°C for a total of 20-24 hours (collection and incubation). Plates were placed in 4°C refrigerator for no more than 72 hours prior to fixation. RNAi crosses were collected at 24.5°C for 8 hours and incubated at 29°C for 8-10 hours for a total of 16-18 hours.

2.4 Fixation

Plates were removed from 4°C refrigerator and incubated at room temperature for 90 minutes. Embryos were incubated in 50% bleach for 5 minutes for dechorination. Bleach and embryos were poured through a sieve to collect the embryos. Distilled water was used to wash the embryos in the sieve. Sieve was then dried and embryos were placed in a scintillation vial containing fixative (1 ml formaldehyde (37% Caledon 5300-1 or 4% formaldehyde made in the lab by heating 4% paraformaldehyde solution), 1 ml 10X phosphate buffer solution (PBS), 8ml dH_2O, 4.5ml Heptane). Embryos were rotated for 20 minutes. The bottom layer of fixative was removed using a glass pipette. Methanol was added aggressively and the vial was shaken for about 20 seconds to remove the vitelline membrane. Embryos sank to the bottom of the scintillation vial and removed using a disposable plastic pipette and placed in a glass vial. Embryos were washed with methanol three times each time being removed and placed into a new vial. Embryos were washed three times in the final vial followed by immunolabelling.

2.5 Immunolabelling

Immunolabelling protocol was adapted from Patel, 1994. Following fixation, methanol was then replaced by PBS + 0.03% TritonX (PBT). When using anti-Robo as a primary antibody, PBS+0.01% Tween was used instead. Embryos were then washed five times with PBT, before being placed on the rotator for 20 minutes. A final wash was then performed. As much PBT was removed as possible and 100 μL of PBT and 10 μL of Normal goat serum was added. Vial was placed on an orbital shaker for 30 minutes of blocking. Primary antibodies were then added and embryos were incubated overnight on the orbital shaker at 4°C. The next day embryos were washed 5 times with PBT and then placed on the rotator for 4-8 hours with washes every 0.5-1 hour. As much PBT was removed as possible and 100 μL of PBT and 10 μL of Normal goat serum was added. Embryos were placed on the orbital shaker for 35 minutes of blocking. Secondary antibodies were added and incubated on the orbital shaker for 2 hours. Embryo were then washed 5 times with PBT and placed on the rotator overnight at 4°C. In the morning, PBT was replaced with 50% glycerol. Once the embryos settled to the bottom of the vial, 50% glycerol was replaced by 70% glycerol.

Table 2.2: List of primary antibodies used for immunolabelling.

Antibody	Dilution	Host Animal	Source	Catalogue #
α-Dystroglycan	1:100	Rabbit	This lab	
α-Discs Large	1:30	Mouse MAb	C. Goodman	
α-Roundabout	1:30	Mouse MAb	C. Goodman	
α-dMEF	1:100	Rabbit	This lab	
α-GFP	1:100	Chicken	Cedarlane	ab13970

Table 2.3: List of secondary antibodies from Goat used for immunolabelling.

Antibody	Dilution	Source	Catalogue #
Alexa 488 α-Ch	≤ 1:150	Invitrogen	A11039
Alexa 488 α-Rb	1:150	Invitrogen	A11034
Alexa 546 α-Rb	1:150	Invitrogen	A11010
Alexa 647 α-M	1:150	Thermofisher	A21235

2.6 Mounting fixed samples

Embryos were mounted on a glass slide. Two 22mmX22mm No. 1.0 cover slips were fixed in place on each edge of the slide with a drop of glycerol underneath. Approximately 40 µl of 70% glycerol solution containing embryos was placed between the two coverslips. A 22mmX22mm No. 1.5 cover slip was placed on top of the embryos.

2.7 Imaging of fixed tissue

Cross sectional (XZ) images of the embryonic heart were obtained with a Leica SP5 confocal microscope using 63X objective (NA = 1.4) at 100 Hz with a 1 AU (60 µm) pinhole. Frontal (XY) view stacks were also obtained using the 63X objective (NA = 1.4) at 200 Hz. Approximately 20 to 30 step stacks were obtained with each slice 1 µm apart. Images were processed using Las X and ImageJ.

2.8 Embryo collection for live imaging

Approximately 20-30 virgins (UAS stock) and 10-15 males (Gal4 stock) were crossed and allowed to lay at 24.5°C (or 29°C for RNAis) on apple juice agar plates with a smear of yeast paste for approximately 14-24 hours. Stage 15 embryos were selected for live imaging.

2.9 Live imaging

The hanging drop method (Reed *et al*., 2009) was used to mount live embryos for time lapse and still imaging using a Leica SP5 confocal microscope. The nucleic marker tup-GFP was used to observe cardioblasts and UAS-moesin-mCherry was used to observe the cell membrane actin and filopodial outgrowths. All images were obtained using the 63X objective at 200 Hz or 400Hz with 1.5 AU (143 μm) pinhole. Still images, approximately 20-30 step stacks (each step 1 μm apart) were acquired every five minutes to determine migration velocity and leading edge activity. For time lapse videos, each stack was obtained within 1 minute for a total of 30 minutes.

2.10 Assessment of lumen phenotypes

Cross sectional images of lumens in fixed samples were assessed based on lumen phenotype (0-no lumen, 1-reduced lumen, 2-lumen). All samples were assessed by two independent researchers to account for experimenter bias.

2.11 Analysis of fluorescence distribution

Thin rectangular transects were drawn across the cross sectional images of the. Fluorescence intensity across the width of the heart was plotted to compare the difference in protein expression at the luminal domain and the basal domain the CBs. All images were processed using ImageJ.

2.12 Live imaging analysis

Assuming that both LE migrate at the same speed, measurements between CB in the A5-A6 segment on each LE were obtained and used to calculated the migration velocity of the two LE. Measurements were taken at d_0 (stage 15) and d_1 (30 minutes later). Migration velocity was calculated by subtracting d_1 from d_0 divided by time (30

minutes) which was then divided by two. A T-test was used to test for significant difference.

Active LE was measured at stage 15. The percent of active LE was calculated by dividing the sum of active LE by total sum of LE. A T-test was used to test for significant difference.

2.13 Embryo imaging protocol using a Leica SP5 confocal microscope

Place mounted embryos on the upright Leica microscope securing the slide under the clips. On the microscope panel display, select the Fluorescence tab and the desired fluorescence filter for viewing the labelled samples. Select the 10X objective in the Microscopes tab on the panel display. View the embryos through the eyepiece. Using the x, y, and z axis joystick on the computer table, find a suitable embryo for imaging. Switch to 63X objective (NA = 1.4) by pressing 63X on the microscope display. When the stage lowers, place a drop of Leica Type F immersion oil on to the cover slip above the region containing the selected embryo. Press 63X again to raise the stage. Press fine focus on the joystick and adjust the z axis knob so that the embryo's heart is clearly visible. Open Las AF on the computer, click the Configuration tab in Las AF, select the required lasers, and set the total laser power to 29% (40% for live imaging). Click the Acquire tab. In the Acquisition Mode window, set Acquisition Mode to xyz for frontal view images and xzy for cross sections. Select sequential scan in the Acquisition Mode window if imaging samples labelled with multiple fluorophores. Sequential scanning mode scans each channel individually to avoid bleedthrough. Choose 1024 X 512 format and 400 Hz speed in XY window. When imaging, the speed should be adjusted to 200 Hz for XZ images and 100 Hz for XY images. In the Beam Path Settings window, load the

GFP settings (or desired saved settings) from the drop down menu. Press live at the bottom left of the screen to view the embryo on the screen. Rotate the sample using the Scan Field Rotation knob on the control bar or the Rotation slider in the XY window. Set a stack using the Z-position knob on the control bar. Select the begin tab in the Z stack window. Black tab indicates that the z position will change when the physical control bar knob is turned. Adjust the z position knob to the desired position. Click the begin tab again. The tab is now orange indicating the beginning of the stack has been selected. Repeat the same sequence to set the end of the stack. Select step size and input 1 micrometer. Step size should be not be less than double the lateral resolution ($r_{lateral}$ = 0.4.λ /NA where λ is emitted wavelength) otherwise oversampling will occur resulting in photobleaching of the sample. Stack should be approximately 20 - 30 steps. For sequential scanning, load previously saved settings (fixed images: scan1: Alexa 488 - 488 laser line set to 15%, scan 2: Alexa 546 – 543 laser line set to 15% , scan3: Alexa 647 - 633 laser line set to 33%; live imaging: scan1: GFP - 488 laser line set to 7% and scan 2: mCherry – 594 laser line set to 75%-100%). Scans are set to between lines when live imaging and between frames when imaging fixed samples. Activate live mode at the bottom left of the screen and adjust the gain and offset for each channel using the knobs on the control bar. Select the saturation look up table at the top left of the image screen to set the appropriate gain. Blue indicates saturation and green indicates no signal. Set the gain so that image is just below white. Offset should be set to 0%. Set the pinhole for each scan using the pinhole knob on the control bar or by typing the pinhole value in the XY window. A pinhole value of 1 Airy Unit is the minimum requirement for optimal Z axis resolution. A higher pinhole value will result in loss of Z axis resolution. The

equation 1 AU = (0.61 X emitted λ X total magnification)/NA calculates the pinhole diameter in nm for 1 AU. On the control bar, zoom should be set to 1 unless acquiring cross sections or to observe smaller objects. Activate live mode at the bottom left of the screen and select start at the bottom right of the screen to acquire a stack. To take a cross sectional image, rotate the heart vertically using the Scan Field Rotation knob on the control bar or the Rotation slider in XY window. Select xzy in the Acquisition Mode window and drag the cross section line to the area of the heart where a cross section is desired. Press capture image at the bottom right of the screen to acquire the cross sectional image.

3. Results

3.1 Screen of Rab GTPase candidates and subsequent focus on Rab5

To determine which Rab GTPases function during tubulogenesis, I conducted an investigation of the requirement for Rab5, Rab11, and Sec6 during heart lumenogenesis. The screen was performed by driving the expression of RNAi against each candidate with *dmef*-Gal4, as this was a potent, yet selective driver for the earlier stages of CB migration and lumenogenesis. Cross sectional images of hearts in Rab knockdowns were obtained from fixed and live samples. Fixed samples were labelled with anti-Dg and anti-Dlg. In wildtype, Dg is localised at the luminal domain and minimally at the basal domain. Dlg is localised to sites of cell-to-cell adhesion (Medioni *et al*., 2008; Firestein and Rongo, 2001). To visualise CBs in live embryos, endogenous tail-up-GFP marked nuclei and moesin-mCherry, an Actin cytoskeleton marker, was driven by *dmef*-Gal4. Preliminary findings suggested that knockdowns of Rab5, Rab11 and Sec6 did not produce a significant mutant phenotype (Supplemental Figure 6.1 and 6.2), however, knockdowns of Rab5 and Rab11 had collapsed luminal spaces in some areas along the heart tube. However, cross sectional images of these knockdowns were captured along the aorta, which has a smaller luminal diameter and can appear reduced compared to the heart proper. As confirmation of the function of the RNAi constructs, a viability test was conducted with *daughterless*-Gal4 and *dmef*-Gal4. Each RNAi (*rab5, rab11, sec6*) cross resulted in no adult progeny indicating that knockdown of these proteins is lethal. RNAi does not result in complete gene knockdown, thus, a subsequent investigation of *rab11* DN and *rab5* DN was conducted to determine whether our results were due to incomplete knockdown. Unfortunately, genetic tools such as DNs or CAs are not available for Sec6,

therefore, further investigation of the role of Sec6 in lumen formation was not possible. Expression of *rab11* DN did not perturb lumen formation resulting in wildtype luminal spaces (Figure 3.1). Conversely, *rab5* DN expression resulted in a reduced lumen phenotype, characterised by pockets of reduced lumen rather than a continuous lumen along the A-P axis (Figure 3.2).

Migration behaviour in Rab5, Rab1l, and Sec6 knockdowns was observed as LE activity is representative of cell polarisation, which is required for lumen formation. Live still images of the heart were obtained at stage 15 to observe filopodial and lamellipodial protrusions. We anticipated that production of a robust LE would be affected by *rab5* knockdown because the localisation of cell signalling receptors (Integrin, Robo, Fra) that required for LE activity would be disrupted (Raza and Jacobs, 2016). Unexpectedly, filopodial and lamellipodial formation was not affected by knockdown of Rab5, Rab11, or Sec6 (Supplemental Figure 6.3). The results of this preliminary investigation lead to the more in depth exploration of the involvement of Rab5 in lumenogenesis as it was the only candidate that produced a notable phenotype.

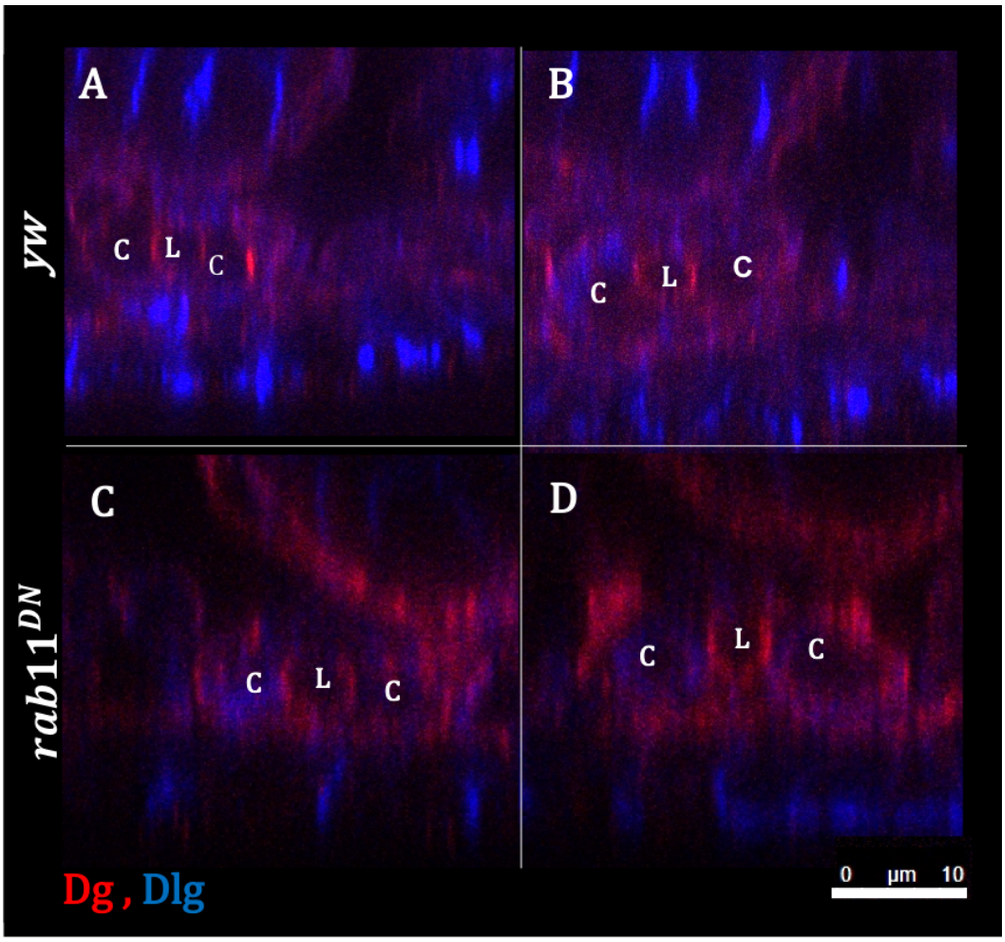

A
B
C
D
yw
rab11DN
C L C
C L C
C L C
C L C
Dg , Dlg
0 µm 10

Figure 3.1: Rab11 DN expression does not perturb lumen formation. Cross sectional images of contralateral cardioblasts taken along the A-P axis at stage 17 in 1 embryo per genotype. Wildtype luminal spaces (n=7; A,B) *rab11* DN (n=3; C,D) *dmef*-Gal4 driver was used to drive UAS constructs. Dystroglycan (Dg) in red and Discs Large (Dlg) in blue. C, cardioblast; L, lumen. Scale 10 μm.

3.2 Both Rab5 gain of function and Rab5 loss of function results in a reduced lumen phenotype.

The role of Rab5 during lumenogenesis was further investigated by employing two *rab5* DN and CA lines, each with the same DN or CA construct inserted into different chromosomes. The location of a construct on a chromosome can affect expression levels so multiple inserts were utilized. The expression of *rab5* DN, CA as well as *rab5* overexpression resulted in a reduced lumen (Figure 3.2 and 3.3). When using $rab5^{DN2}/TM3$ driven by *dmef*-Gal4, the presence of the transgene could not be confirmed since we could not confirm if the progeny contained the balancer chromosome or $rab5^{DN2}$. Thus, the presumed reduced lumen phenotype was confirmed using a homozygous DN insertion ($rab5^{DN1}$). Severity and penetrance of the reduced lumen phenotype in all transgenic lines was notably variable. Markedly, Dg expression appeared wildtype in all transgenics (*rab5* DNs, *rab5* CAs, and *rab5* WT overexpression). However, further quantitative analysis of Dg expression showed increased expression internally in *rab5* DN samples (Figure 3.6 G,H,I). Normally Dlg can be observed concentrated at the junctional domains of CBs in wildtype. However, this pattern was highly variable in all samples, including *yw* wildtype samples. Frontal views of the heart were, therefore, used to confirm a wildtype Dlg expression pattern in all transgenics (refer to Figure 3.10) .

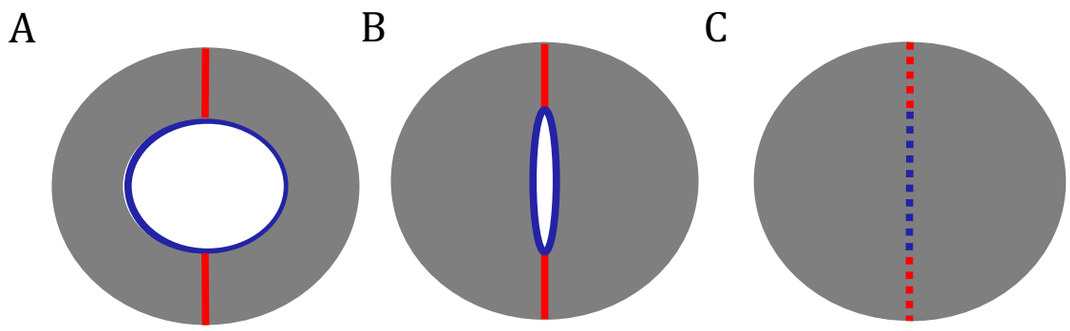

A
B
C

Figure 3.2: Schematic of luminal spaces between contralateral cardioblasts. Wildtype luminal space (A). Reduced Lumen (B). No luminal space (C). Dashed lines indicate that clearly defined molecularly distinct domains will not always occur in these cases. When two or more lumen types are present in the same embryo, it is termed reduced lumen. Red indicates junctional domain. Blue indicates luminal domain.

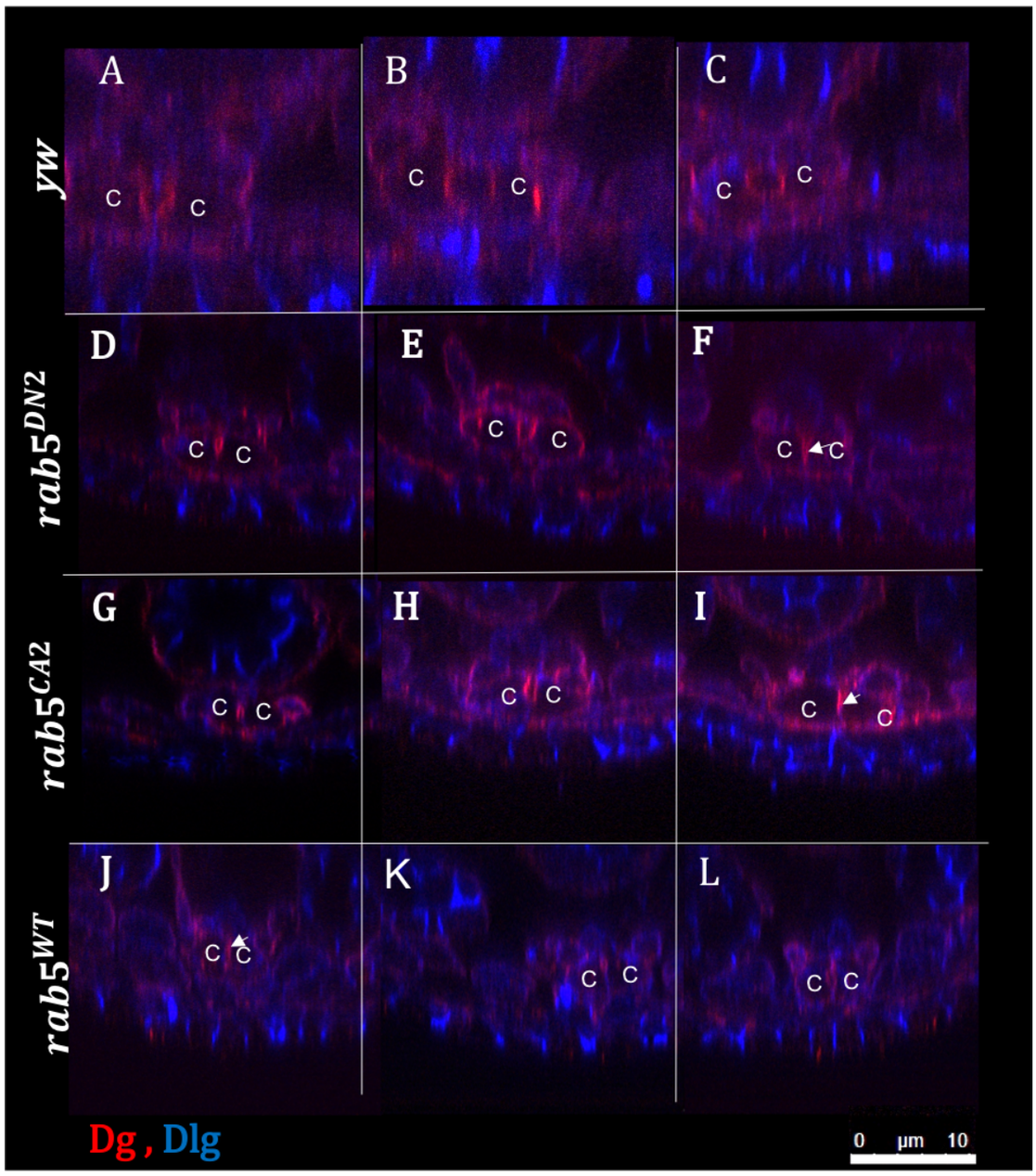

A
B
C
D
E
F
G
H
I
J
K
L
yw
rab5DN2
rab5CA2
rab5WT
Dg , Dlg
0 µm 10

Figure 3.3: Expression of *rab5* DN, CA, and overexpression results in a reduced lumen phenotype. Cross sectional images of contralateral cardioblasts taken between segments A5 and A8 at stage 17 along the A-P axis in 1 embryo per genotype. Wildtype luminal spaces (n=7; A,B,C). *rab5* DN. Presence of the DN transgene could not be confirmed (n=10; D,E,F). *rab5* CA (n=6; G,H,I) *rab5* overexpression (n=6; J, K, L). Cardioblasts demonstrate abnormally formed lumens compared to wildtype (E,F,H,I). Cardioblasts appear to have failed to attach at dorsal junctional domain (F,H,I). Arrows indicate absences of a luminal space (F,I). All crossed to *dmef*-Gal4 driver. C, cardioblast. Scale 10 μm.

3.3 Quantitative analysis of lumen phenotypes

Embryos were scored for their lumen phenotype (0 – no lumen, 1- reduced lumen, 2 – Continuous Lumen). To account for experimenter bias, a blind assessment of the lumens was conducted by another researcher. Statistical analysis of the assessments showed that the two assessments are positively correlated (R = 0.628, Pearson Correlation Coefficient) indicating that the researchers' assessments were generally robust to experimenter bias. I conducted a Chi-squared test to determine whether the occurrence of a continuous lumen phenotype in rab5 DN, CA, and overexpression samples was significantly different compared to wildtype (Figure 3.4). The results indicate that Rab5 is required for lumen formation (DN1 P=0.0530; DN2 P=0.03; CA1 P=0.0503; CA2 P =0.0284; overexpression P=0.3039 (n.s.)).

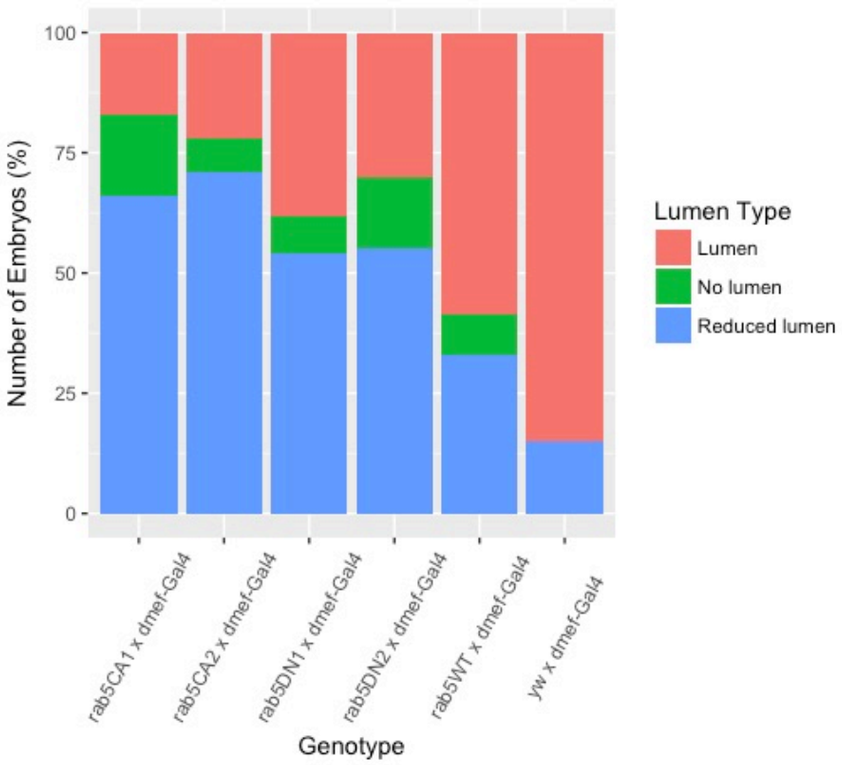

100
75
50
25
0
Number of Embryos (%)
rab5CA1 x dmef-Gal4
rab5CA2 x dmef-Gal4
rab5DN1 x dmef-Gal4
rab5DN2 x dmef-Gal4
rab5WT x dmef-Gal4
yw x dmef-Gal4
Genotype
Lumen Type
Lumen
No lumen
Reduced lumen

Figure 3.4: Quantification of lumen phenotypes. Expression of *rab5* DN, CA, and overexpression results in a reduced lumen phenotype or absence of a lumen. The occurrence of a lumen phenotype is statistically different between wildtype and rab5 DN and CA, but not overexpression (P = DN1 0.0468, DN2 0.0254, CA1 0.0393, CA2 0.0226). Values presented are a percentage of the total number of embryos within each genotype. Each percentage was obtained by taking the average of two independent assessments.

3.4 Robo mislocalises baso-laterally and internally in *rab5* DN and CA but not in *rab5* overexpression.

During heart development, Robo localises basally, laterally, and apically during early stages of heart formation. By stage 17, however, Robo is almost exclusively localised to the luminal domain (Vanderploeg, 2014). Robo localisation was examined in *rab5* DN, CA and WT overexpression samples. I expected that Robo would be mislocalised in Rab5 transgenics as a result of disruption to internalisation and trafficking of Robo. In *yw* individuals, Robo localised almost exclusively to the luminal domain, as expected. In frontal view images, Robo mislocalised internally and baso-laterally in *rab5* DN and CA samples (Figure 3.5), however, Robo expression appeared wildtype in *rab5* overexpression frontal view images. To identify $rab5^{DN2}$ individuals in these experiments, anti-GFP was used to label YFP tagged Rab5 transgene.

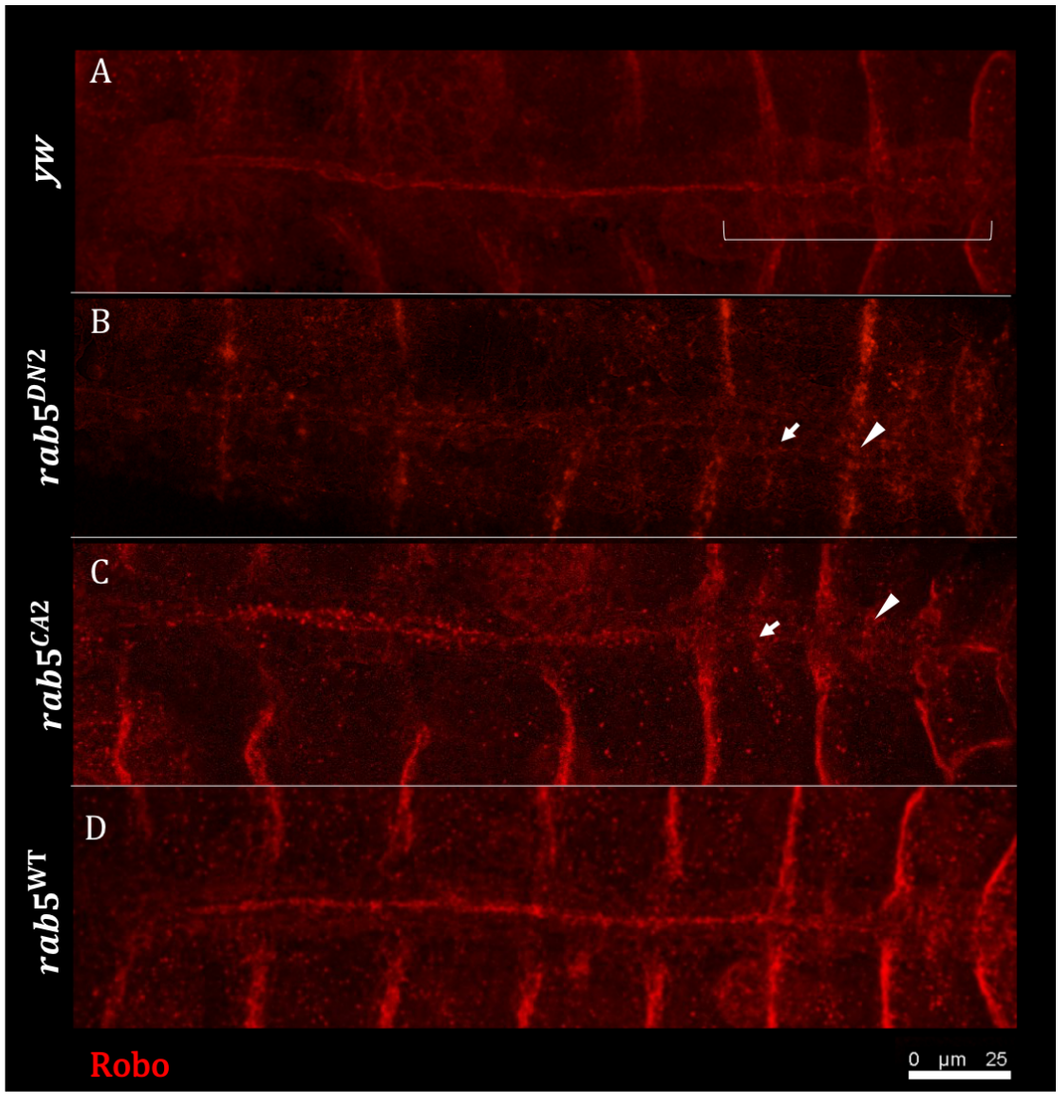

yw
A
rab5DN2
B
rab5CA2
C
rab5WT
D
Robo
0 µm 25

Figure 3.5: Robo expression is reduced at the luminal domain and mislocalises baso-laterally and internally in *rab5* DN and CA. Robo localises at the luminal domain at stage 17 in *yw* wildtype embryos. (n = 7; A) Abnormal accumulation of Robo baso-laterally (arrow) and internally (arrowhead) in $rab5^{DN2}$ (n = 1) and $rab5^{DN2}$ (n = 6; B,C) Overexpression of *rab5* appears similar to *yw* wildtype (n=14 ; D). All crossed to *dmef*-Gal4 driver. Single parentheses indicates heart proper. Frontal view, Anterior to the left. Scale 25 μm.

3.4.1 Analysis of Robo distribution

To corroborate aberrant Robo expression pattern in frontal views, Robo expression was quantified in cross sectional images. Rectangular transects were drawn across the width of the heart tube and the grey scale values were then plotted. Plots also graphically depicted the reduced lumen phenotype in rab5 DN, CA, and overexpression samples (Figure 3.6). Regrettably, I was unable to obtain cross sections of $rab5^{DN2}$ due to technical difficulties with embryo collection and immunolabelling. For this genotype, Dg expression for these samples as Dg is also localised luminally in wildtype (Figure 3.6 G,H,I). Plots demonstrated a reduction in lumen size and an increase in basally and internally localised Robo or Dg expression.

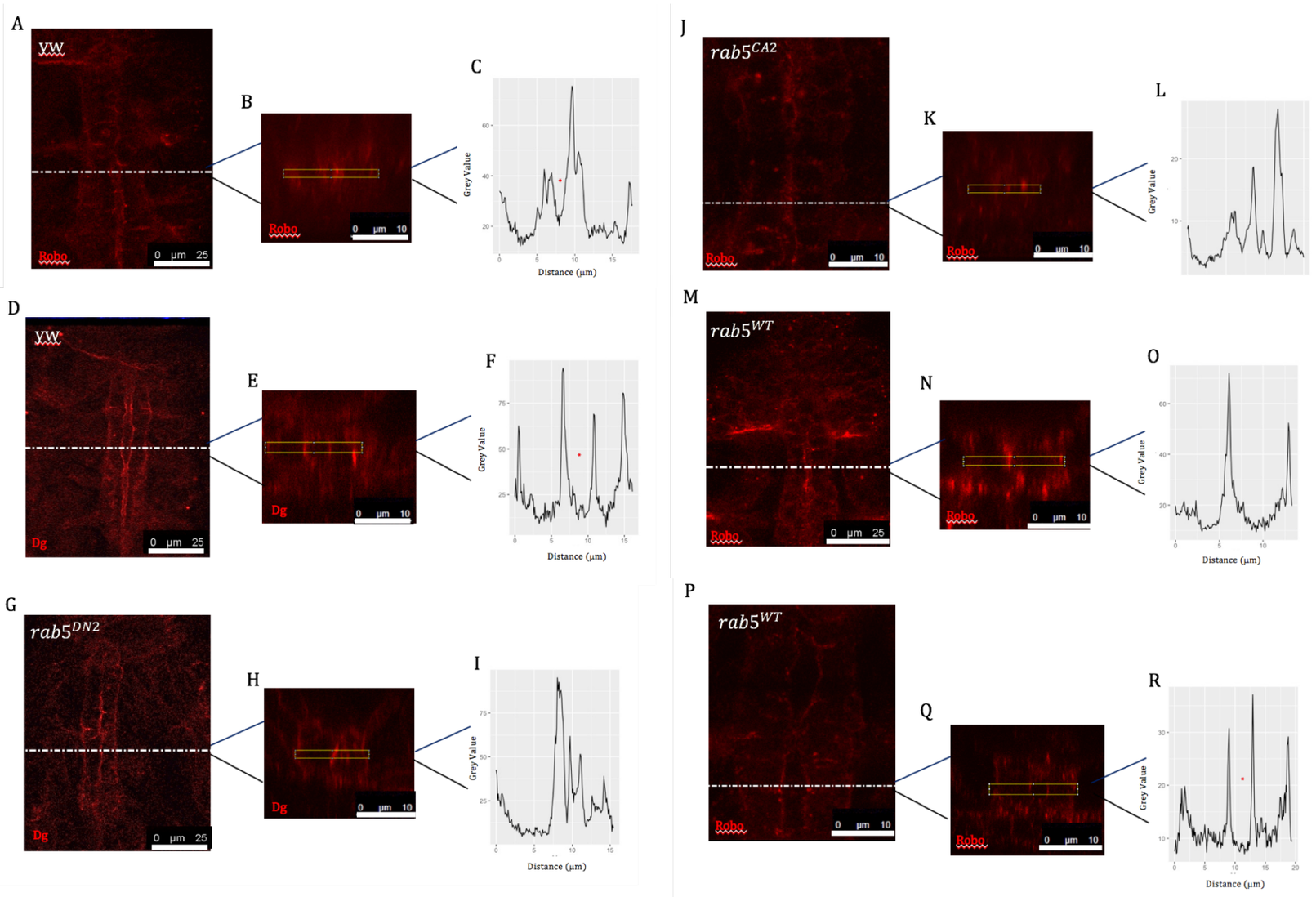

A
yw
Robo
0 µm 25
B
Robo
0 µm 10
C
Grey Value
Distance (µm)
D
yw
Dg
0 µm 25
E
Dg
0 µm 10
F
Grey Value
Distance (µm)
G
rab5DN2
Dg
0 µm 25
H
Dg
0 µm 10
I
Grey Value
Distance (µm)
J
rab5CA2
Robo
0 µm 10
K
Robo
0 µm 10
L
Grey Value
M
rab5WT
Robo
0 µm 25
N
Robo
0 µm 10
O
Grey Value
Distance (µm)
P
rab5WT
Robo
0 µm 10
Q
Robo
0 µm 10
R
Grey Value
Distance (µm)

Figure 3.6: Robo distribution in cross sections of the heart. Frontal view of the heart proper. Scale 10 μm or 25 μm (A, D, G, J, M, P). Cross section of the heart proper with a rectangular transect drawn across the width of the tube. Scale 10 μm (B, E, H, K, N, Q). Quantification of Robo or Dg expression within the transect. Red asterisks indicates location of the lumen (C, F, I, L, O, R). Wildtype (A,B,C, D, E, F). *rab5* DN. Dg expression for this sample was quantified due to technical difficulties with embryo collection and immunolabelling (G,H,I). *rab5* CA (J,K,L). *rab5* overexpression (M, N, O, P, Q, R)

3.5 Rab5 and Robo do not colocalise in CBs in stage 15/16 or stage 17 embryos.

Next, I examined whether Rab5 and Robo colocalise in Stage 15/16 and 17. During this period of time, Robo's localisation changes from the basal, lateral, and apical membranes to exclusively the apical membrane, which necessitates a mechanism to reorganise protein distribution. In our model, we expected that Rab5 would be involved in targeting to the early endosome from the baso-lateral membrane for subsequent trafficking of the receptor to the apical membrane. This means that Rab5 and Robo should colocalise between Stage 15 and Stage 17. However, although Robo and Rab5 appeared punctate, Robo and Rab5 localisation did not appear to overlap (Figure 3.7). The punctate presentation of Robo expression suggests that it has been internalized at the basal membrane.

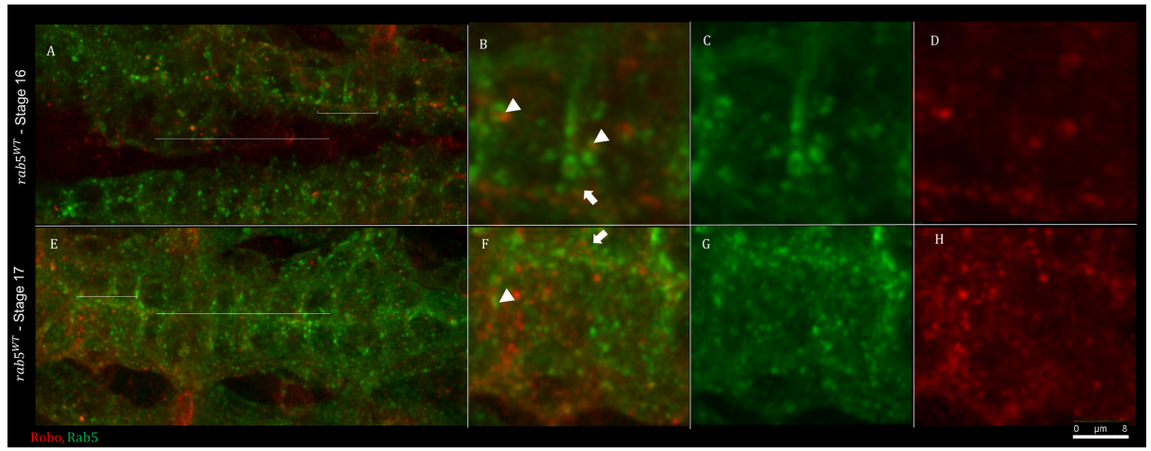

rab5WT - Stage 16
rab5WT - Stage 17
A
B
C
D
E
F
G
H
Robo, Rab5
0 µm 8

Figure 3.7 : Rab5 and Robo do not colocalise during heart formation. Frontal views of the heart proper depicting Robo and Rab5 localisation (A,E). Zoom on two cells in the heart proper showing both Robo and Rab5 expression. Robo and Rab5 colocalise at a very small subset of puncta. Arrowheads show areas of colocalisation (B,F). Zoom on two cells in the heart proper showing only Rab5 expression (C,G). Zoom on two cells in the heart proper showing only Robo expression (D,H). All crossed to *dmef*-Gal4. Single parentheses indicates zoom on two cells. Line indicates midline. Arrows indicate apical. Arrowheads indicate colocalisation. Frontal view, 4X zoom. Scale 8 μm.

3.6 Gain of function and loss of function of *rab5* fail to perturb filopodial and lamellipodial formation.

Live images of the developing heart were captured to visualise LE activity, an indicator of cell polarisation. To monitor CB migration behaviours, UAS-*moesin*-mCherry was used to visualise the muscle cell membrane and tail-up-GFP to visualise the nuclei. Wildtype CBs produce a robust LE during their active migratory phase beginning at Stage 15. The number of filopodial and lamellipodial protrusions increase as the distance from the midline decreases. A robust LE is required for maintaining forward migration towards the midline (Raza and Jacobs, 2016). Surface receptors such as Integrin, Robo, and Fra mediate growth of protrusions and overall CB motility. It was expected that Rab5 disruption would affect the endocytosis and trafficking of aforementioned receptors, leading to decreased motility and protrusion formation. Surprisingly, expression of DN, CA, nor overexpression forms of *rab5* did not perturb filopodial or lamellipodial activity (Figure 3.8). It should be noted that the presence of two UAS transgenes (UAS-*moesin*-mChery and UAS-*rab5*) in these experiment could reduce the level of Rab5 transgene expression relative to previous experiments. As well, the presence of the $rab5^{DN2}$ transgene could not be confirmed in these experiments.

Migratory behaviours of Rab5 DN samples were quantified to determine whether migration velocity and LE activity differed from *yw* wildtype samples. On average, 37% of the LE was active in *rab5* DN samples (DN1: n = 9 LEs, DN2: n = 6 LEs), which is similar to wildtype (42%, n = 8 LEs; Figure 3.11A). Migration velocity was also quantified for *rab5* DN samples. Prior to quantification, it appeared as if $rab5^{DN2}$ resulted in delayed migration to the midline (Figure 3.10) because CBs were farther from the midline than its wildtype counterparts 45 minutes after stage 15. This

suggested that *rab5* DN individuals have decreased migration velocity. Nevertheless, migration velocity of rab5 DN individuals was not significantly different (DN1: P=0.3928; DN2: P=0.8702) from wildtype (Figure 3.11B). Delayed migration to the midline may instead reflect slower development prior to stage 15. The integrity of the epidermis was also analyzed in fixed *rab5* individuals because migrating CBs follow the overlying ectoderm during early dorsal closure. Our observed delayed heart closure phenotype may have been a result of delayed epidermal dorsal closure. To determine if this was the case, Dlg expression in the overlying epidermis was examined because Dlg localises to sites of adhesion in the epidermis. In fixed $rab5^{DN2}$ samples with presumed transgene expression, epidermis appeared similar to wildtype, suggesting that dorsal closure was normal (Figure 3.9).

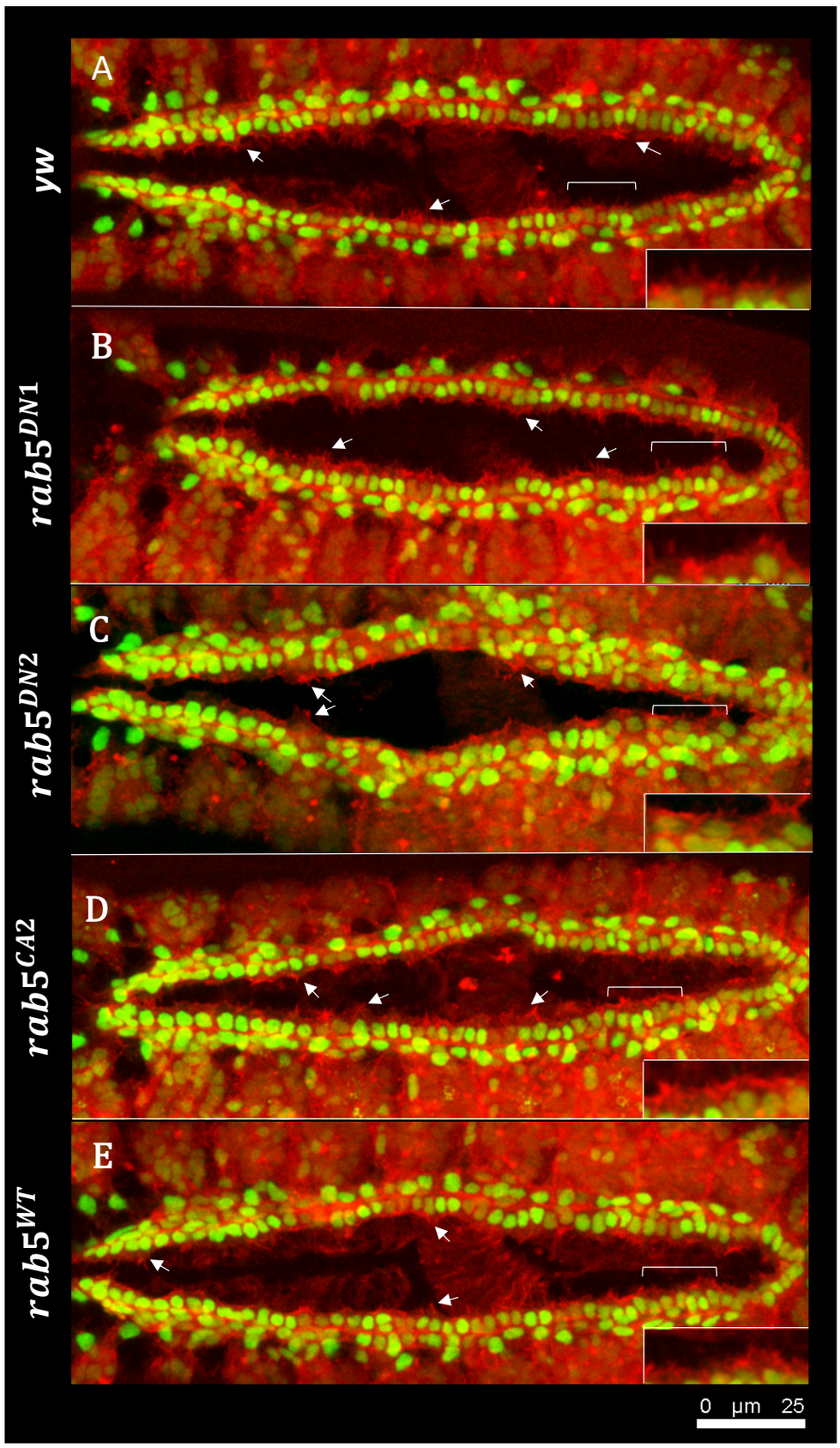

A
yw
B
rab5DN1
C
rab5DN2
D
rab5CA2
E
rab5WT
0 µm 25

Figure 3.8: Loss of function, gain of function and overexpression of *rab5* does not perturb filopodia and lamellipodia activity. Live still images of the developing heart at stage 15. Wildtype protrusions (n=5; A) *rab5* DN (DN1: n=7; DN2: n=4; B,C) *rab5* CA (n=2; D) *rab5* overexpression (n=5; E) Insets are zoomed to show protrusions. Arrows indicate protrusions and single parentheses indicate where enlarged inset image was obtained (A, B,C, D). Red indicates Moesin, a cytoplasmic linker to the Actin cytoskeleton, and green indicates Tail-Up, a CB nuclear marker. All crosses were completed using *dmef*-Gal4 to drive UAS – *moesin* - mCherry and transgene. Frontal view, Anterior to the left. Scale 25 μm.

0 minutes
45 minutes
yw
rab5DN2
0 μm 25
*

Figure 3.9: Loss of function of Rab5 results in delayed dorsal vessel closure. Live images of dorsal vessel closure at stage 17. Wildtype dorsal vessel closure (A) Expression of *rab5* DN results in delayed migration to the midline, n = 5/10. The presence of the *rab5* DN could not be confirmed (B) Green is tup-GFP and red is moesin-mCherry. Asterisk marks the space resulting from delayed dorsal vessel closure. All crossed to *dmef*-Gal4. Frontal view, Anterior to the left. Scale 25 μm.

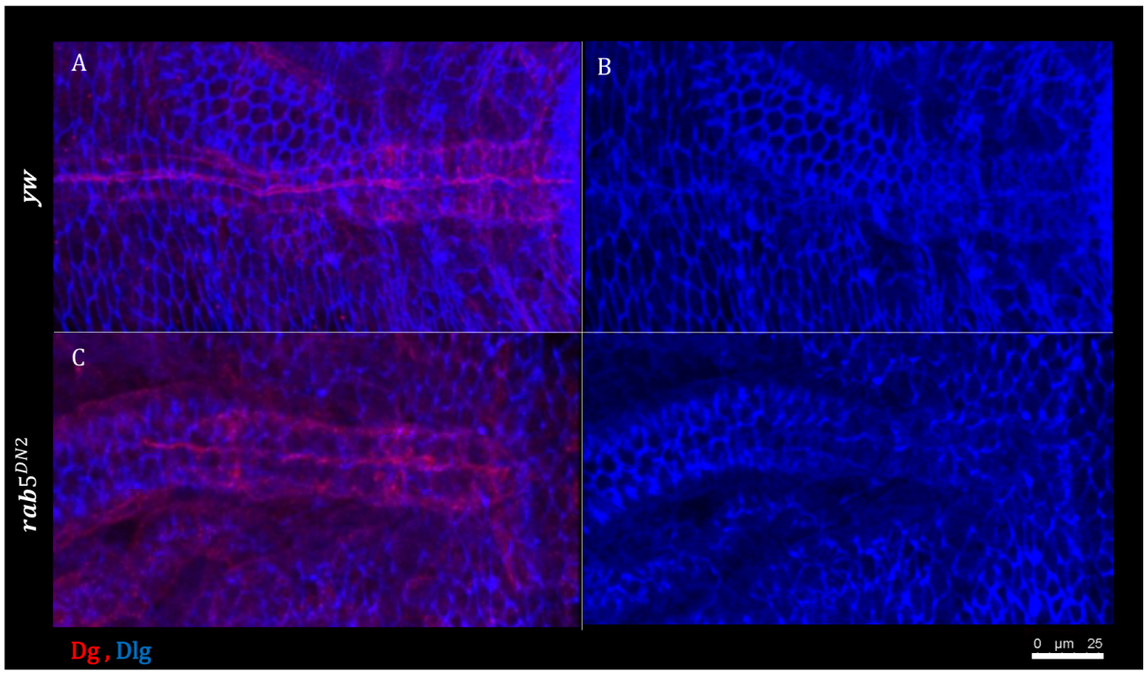
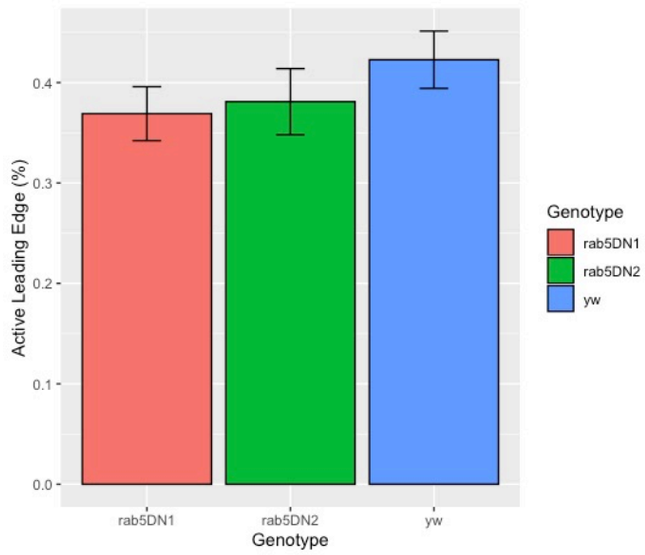

A
B
C
yw
rab5DN2
Dg , Dlg
0 µm 25

Figure 3.10: Loss of function of Rab5 does not affect ectodermal dorsal closure. Wildtype (A,B) *rab5* DN (C,D) Dlg expression shows that the ectoderm has been formed properly above the heart (B,D). All crossed to *dmef*-Gal4. Frontal view, Anterior to the left. Scale 25 μm.

A) Leading Edge Activity



B) Migration velocity

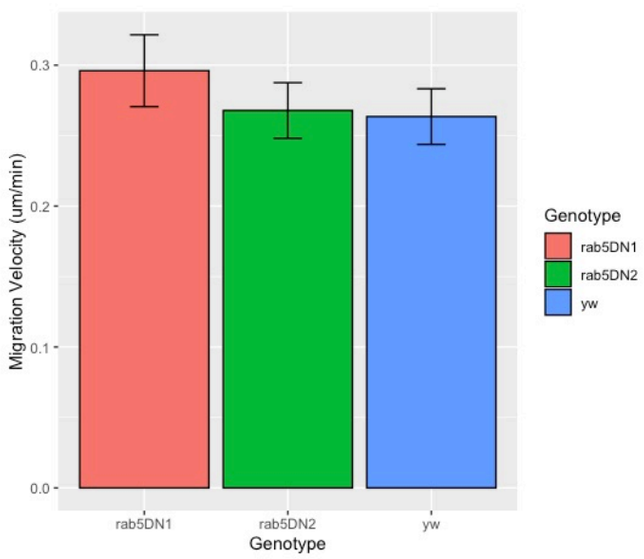


Figure 3.11: Quantification of migratory behaviour of cardioblasts in *rab5* DN samples. The amount of active LE is not significantly different (DN1: 0.2398; DN2: P=0.1552) from wildtype in embryos expressing *rab5* DN (DN1: n = 9 LEs, DN2: n = 6 LEs, yw: n = 8 LEs ; A) Migration velocity is not significantly different (DN1: P=0.3928; DN2: P=0.8702) from wildtype in embryos expressing *rab5* DN (DN1: n = 3; DN2: n = 5; yw: n = 5; B).

4. Discussion

Intracellular trafficking, and more specifically endocytosis, are critical for regulation of protein localisation within a cell. These mechanisms are especially important during epithelial morphogenesis, which necessitates re-arrangement of proteins to specific cellular domains (Jewett and Prekeris, 2018). In the heart, Robo, Integrin, and Dg are luminally localised receptors, but do not localise to the lumen until stage 17 (Vanderploeg, MacMullin and Jacobs, 2012). The mechanism behind the change in their localisation patterns is not well understood, but the endocytic pathway is an ideal candidate for trafficking and targeting of cell surface receptors during tubulogenesis. Here, I investigated the role of Rab GTPases during heart formation as they are major coordinators of endocytosis. Specifically, I monitored the effects of *rab5*, *rab11*, or *sec6* perturbation on heart lumen size and formation.

4.1 Rab11 and Sec6 are not required for lumen formation

My gene survey of *rab5*, *rab11*, and *sec6* revealed that neither *rab11* nor *sec6* knockdown perturbed lumen formation, and resulted in wildtype luminal spaces. Rab11 localises to the recycling endosome, which is important for trafficking newly synthesized or recycled proteins to the cell surface (reviewed in Stenmark, 2009). It was anticipated that *rab11* knockdown would reduce lumen size because Rab11 has been implicated as a regulator of Cadherin localisation, cytoskeletal rearrangement, and lumen formation in other tubulogenesis models (Choubey and Roy, 2017; Desclozeaux *et al*, 2008). Therefore, it was unexpected that knockdown of *rab11* results in wildtype luminal spaces in the heart.

Additionally, it was surprising that *sec6* knockdown did not affect lumen formation. Sec6 is one of the 8 subunits in the exocyst complex, which partners with the recycling endosome to traffic newly synthesized or recycled proteins to the baso-lateral domain of a cell (Beronja *et al*., 2005). Sec6 has been implicated in Cadherin trafficking to sites of adhesion at the baso-lateral membrane of epithelial cells (Langevin *et al*., 2005; Grindstaff *et al*., 1998). Thus, disruption of sites of adhesion should affect lumen formation. Unfortunately, adhesion markers in *sec6* knockdowns were not monitored in this study as only live images with cell membrane and nucleic markers were obtained. In the future, Dlg distribution in *sec6* knockdowns will need to be evaluated to determine whether cell adhesions are disrupted. This subtle phenotype may have been overlooked in this study and bears revisiting.

Taken together, knockdown of *rab11* or *sec6* is not sufficient to disturb lumen formation in the heart. However, low sample sizes and incomplete RNAi knockdown may have lead to overlooking subtle phenotypes . Nevertheless, expression of *rab11* DN in the developing heart also resulted in wildtype luminal size, which was consistent with the results obtained using RNAi knockdown. This supports the conclusion that *rab11* is not required for lumen formation in the heart. Unfortunately, other genetic tools such as DNs and CAs are not available for Sec6, thus, the *sec6* knockdown could not be further validated.

4.2 Rab5 is required for lumen formation

The following investigations focused on whether Rab5 plays a role in trafficking cell surface receptors involved in lumen formation. Rab5 localises to the early/sorting endosome where it is required for cargo sequestration and endosomal fusion (reviewed in Stenmark, 2009). Evaluation of *rab5* DN, CA, and overexpression constructs all resulted in a reduced lumen phenotype, characterised by a discontinuous lumen and/or absence of a lumen along the A-P axis, that varied in severity across and within all genotypes. A subset of samples observed had no lumen. The occurrence of a continuous lumen in *rab5* DN and CA, but not *rab5* overexpression, samples were significantly different from wildtype. Although it was not a significant portion of the individuals examined, reduced lumen phenotype did occur in some *rab5* overexpression samples.

Cell attachment defects were also observed in a subset of *rab5* DN and CA samples. This could indicate an issue with Cadherin localisation, as Cadherin is required for cell to cell adhesion, however Dlg, a Cadherin adhesion complex protein, localised appropriately to sites of adhesion. This suggests that Cadherin localisation was not disrupted by perturbing Rab5 function. Collectively, these results provide multiple lines of evidence that Rab5 is required for lumen formation in the heart.

4.2.1 Robo localisation in *rab5* transgenics

Rab5 function in lumen formation was further analyzed by investigating the localisation of Robo, a key luminal signalling receptor. Based on previous studies, it was possible that mislocalisation of Slit/Robo signalling could be the cause of the reduced lumen phenotype (MacMullin and Jacobs, 2006; Vanderploeg, MacMullin and Jacobs,

2012). Expression of *rab5* DN and CA constructs resulted in mislocalisation of Robo to the internal domain and baso-lateral cell membrane. In the heart, Robo localisation changes dramatically throughout development. Robo localises apically, laterally and basally at earlier stages, however, by stage 17 Robo is almost entirely localised at the luminal domain (Vanderploeg, MacMullin and Jacobs, 2012). Our findings suggest that Rab5 is required for internalisation of Robo so that it can be subsequently trafficked to the luminal domain. To follow up on this result, I investigated whether Rab5 and Robo colocalise between stages 15 and 17 during which time Robo localisation is altered.

Examining stage 15/16 and stage 17 embryos that expressed a YFP-tagged Rab5, Robo was found to be in many puncta near the apical and baso-lateral membrane suggesting that it has been endocytosed at the basal membrane. Colocalisation analysis revealed that Robo and Rab5 colocalise in a very small subset of puncta. Although Zhang *et al*., 2007, reported that overexpression of Rab5 produced expression patterns similar to an endogenous Rab5, it is possible that localisation patterns of overexpressed YFP-tagged Rab5 is not representative of endogenous Rab5 distribution in the heart. Consequently, it would be beneficial to investigate whether endogenous Rab5 detected by an antibody in wildtype samples shows a similar lack of association with Robo.

Disruption of Robo localisation patterns could be a consequence of disrupting Integrin localisation. During heart development, Integrins are the first proteins to become localised to the luminal domain. Moreover, proper localisation of Integrin is required to localise Slit/Robo signalling (Vanderploeg, MacMullin and Jacobs, 2012). Rab5 has been shown to be involved in trafficking Integrins in other models. For example, Rab5-mediated Integrin trafficking has been connected to cell motility and tumour invasion

(Liu *et al*., 2011; Torres and Stupack, 2011). Therefore, it will be worthwhile to track movement of Integrin in our model.

4.2.2 Migratory behaviours of *rab5* DN

Migratory behaviours of CBs were observed to determine whether Rab5 is required for the production of a robust LE. Specifically, I assessed filopodial and lamellipodial protrusion formation. Filopodia and lamellipodia are Actin protrusions that are required for migration and make the first contact when migrating CBs meet their contralateral partners. Filopodia explore and respond to environmental cues whereas lamellipodia physically propel the cell forward (Mejillano *et al*., 2004). Proper attachment of CBs and successful lumen formation necessitates a robust LE. Integrin, Robo, Fra, and Unc5 are all required for the production of robust LE activity as well as lumen formation (Raza and Jacobs, 2016; Albrecht *et al*., 2010; Macabenta *et al*., 2013). Surprisingly, our data suggested that filopodia and lamellipodia formation and overall LE activity is not perturbed in *rab5* knockdown and *rab5* DN, CA, or overexpression samples. Previously, Rab5 has been implicated in filopodia and lamellipodia formation in cell models (Spaaragen and Bo, 1999; Liu *et al*., 2011). Therefore, it is unexpected that Rab5 DN and CA expressing CBs produced an active LE but subsequently failed to form a continuous lumen because LE activity is indicative of cell polarisation (Raza and Jacobs, 2016).

Despite this result, it may be possible for LE production to proceed without culminating in lumen formation. Slit and Net signalling have been shown to work in conjunction to produce an active LE. Specifically, overexpression of Fra rescued LE activity in *robo* mutants, however, lumen formation did not occur (Raza, 2015).

Additionally, the strength of the Gal4 driver used in this experiment could have reduced the effects of the Rab5 transgenes because of the demands on one transcription factor to activate two UAS-promoter transgenes. To address this concern, the use of an endogenously labelled membrane marker would be beneficial in follow up experiments to corroborate the observations in this study.

4.3 Addressing genetic considerations with Rab GTPases

The endocytic pathway contains considerable molecular redundancy, thus, other Rabs or compensatory mechanisms may be able to offset disruption of Rab function in early developmental processes. Candidates for redundancy in the endocytic pathways are revealed in synapses of *Drosophila* neurons where Rab11 localises at the recycling endosome along with 7 other Rabs. This indicates possible redundancy or interaction (Harris and Littleton, 2011). Considering this, compensation mechanisms may have ameliorated the effects of *rab11* or *sec6* knockdown in our model.

Additionally, it is important to consider the effect Rab GTPase transgene expression (DN, CA and overexpression) has on other Rabs in the endocytic pathway (Fukuda *et al*., 2008; Homma *et al*., 2019). For example, Rab5 can interact and bind to Rab4 effectors (Markgraf, Peplowska, and Ungermann, 2007). If a Rab5 DN binds to a Rab4 effector, the interaction could result in disrupting Rab4-mediated steps in the pathway. This results in observation of the interaction between Rab5 and Rab4 effectors but not the action of Rab5 itself. Additionally, perturbing Rab function through transgene expression may also not be sufficient to observe any negative effects due to redundancy and compensatory effects. This highly complex cascade has multiple levels of interaction

between proteins (Rabs, GEFs, GAPs and effectors), making the observation of the effects of one misexpressed protein difficult to interpret.

RNAi does not induce a complete knockout of the gene product of the targeted mRNA sequence. As a result, low levels of protein may be sufficient for the system to be functional. As such, complete knockouts of Rab GTPases have become increasingly useful when observing the roles of Rab GTPases. Similarly to RNAi, knockouts do not result in aberrant effects on other Rab GTPases as observed with DNs and CAs. Homma *et al*. (2019) produced complete knockout lines for each Rab GTPase in MDCK cells to circumvent these concerns. Using these lines, they determined that knockout of Rab11 is the only Rab GTPase required for formation of a single lumen in MDCK cell cysts. However, Bryant *et al*. (2010) showed that Rab8 and Rab11 produced a multiple lumen phenotype while employing RNAi. Although knockout techniques may not be possible in our model due to lethality, Rab GTPase knockout techniques are a valuable tool to observe the effects of the target gene without inadvertently affecting other Rab GTPases.

4.3.1 More Rab GTPase candidates

This study focused on Rabs known to have an effect on cell polarisation in other models. Other Rab GTPases should also be investigated to determine whether they have an organisational role in heart tubulogenesis.

As mentioned previously, Rab5 localises to the early endosome where it is responsible for targeting proteins to the early endosome. Vesicles then bud off from the early endosome, which are either destined to be recycled back to the membrane through the recycling endosome, or trafficked through the late endosome to the lysosome for degradation (reviewed in Stenmark, 2009; Zhang *et al*., 2007). Therefore, in our model, Robo could be contained within late or recycling endosomes that bud off from the early endosome. However, it is unknown which Rab GTPase is responsible for trafficking Robo from the early endosome. There are two Rab GTPases that interact with Rab5 and could be responsible for this role: Rab4 and Rab7. Rab4 localises to the early endosomal compartment where it is required for the fast recycling of proteins to the cell membrane. Rab7 localises to the late endosome where it is responsible for mediating the lysosomal degradation pathway (reviewed in Stenmark, 2009). The following will describe the roles of Rab4 and Rab7 in intracellular trafficking in other models and their potential role in heart tubulogenesis.

As mentioned previously, Rab4 is involved in "fast recycling", which trafficks proteins directly from the early endosome back to the cell membrane. In HeLa and breast cancer cell models, Rab5a was essential for the formation of invadosomes, and Rab4-mediated recycling of ß3-Integrin and MT1-MMP was also required for the formation of invadosomes, though, Rab7, Rab8, and Rab11 were not required for invadosome

formation (Frittoli *et al*., 2014). In a Xenopus Retinal Growth Cone embryo model, Rab5 and Rab4 were both shown to be important for retinal ganglion cell axon extension. In growth cones expressing *rab5* CA, Rab4 was mislocalised and expression was reduced in the axon and growth cone. *rab4* DN expression resulted in reduction in axon elongation and shorter axons, indicating Rab4 dependent recycling is necessary for axon extension. Rab4 CA and Rab7 DN did not have an effect on axon extension, indicating that axon reduction is solely impaired by obstructing the "fast" recycling pathway (Falk *et al*., 2014). These observations suggest Rab4 may be involved in recycling proteins such as Integrins to regulate cell migration during *Drosophila* heart formation.

Rab5 recruits Rab7 to the early/sorting endosome. Once activated, Rab7 recruits its effectors and mediates protein degradation through interaction with the late endosome and lysosomes. The Rab7 effector, Rab7 Interacting Lysosomal Protein (RILP), is required for transport of cargo from the late endosome to lysosomes for degradation. In a breast cancer cell model, lysosomal degradation of EGFR was required to prevent activation of motogenic stimuli necessary for tumor cell migration. Therefore, Rab7 and RILP are required for targeting and trafficking EGFR to the lysosome for degradation to prevent tumor cell migration (Runkle *et al*., 2012). In a *Drosophila* axon development model, Rab7 and Rab5 mediate Slit-depend Robo signalling through endosomal trafficking. Through a positive feedback mechanism, Slit binds to Robo to initiate the internalisation of Robo, which is required to activate Robo. Expression of Rab7 DN or Rab5 DN resulted in neuronal ectopic crossing defects (Chance and Bashaw, 2015). Taken together, Rab7 could be responsible for regulating cell receptor signalling during heart formation.

4.4 Significance and future directions

Cell polarisation requires the localisation of proteins to specific cell surface domains (Jewett and Prekeris, 2018). Intracellular vesicular trafficking is an important mechanism for transporting proteins within a cell. Until now, the role of intracellular trafficking, and more specifically endocytosis, has not been investigated in the *Drosophila* heart. Here we showed that Rab5, an important regulator of the early endosome, is required for proper lumen formation in the *Drosophila* heart. Specifically, we determined that Rab5 is essential for proper lumen size and Robo localisation, however, dispensable to the production of filopodial and lamellipodial protrusions and cell migration in the heart. To further our findings, the localisation pattern of other cell surface receptors, such as Integrins, Fra, and Unc5, need to be investigated to fully understand the role of Rab5 in heart formation. As mentioned above, the role of other Rab GTPases also needs to be investigated especially in light of the fact that Robo appeared in discrete puncta in the heart. Robo is likely contained within vesicles, which was suspected to be regulated by Rab5. However, Rab5 and Robo expression did not overlap, and it has yet to be determined which Rab GTPase is regulating the vesicles containing Robo. Although our initial investigations revealed that Rab11 and Sec6 did not perturb lumen formation, continued exploration employing other genetic tools in our model or other models may shed light on their possible role during heart formation, as well.

Rab GTPases, and specifically Rab5, are critical to lumen formation of the *Drosophila* heart. Furthering our understanding of the role of other Rab GTPases during tube formation will reveal the mechanisms by which cell surface proteins are re-

organised during tubulogenesis. Rab GTPases may prove to be targets for potential interventions during abnormal organ development (polycystic kidney disease) as a result of aberrant cell polarity.

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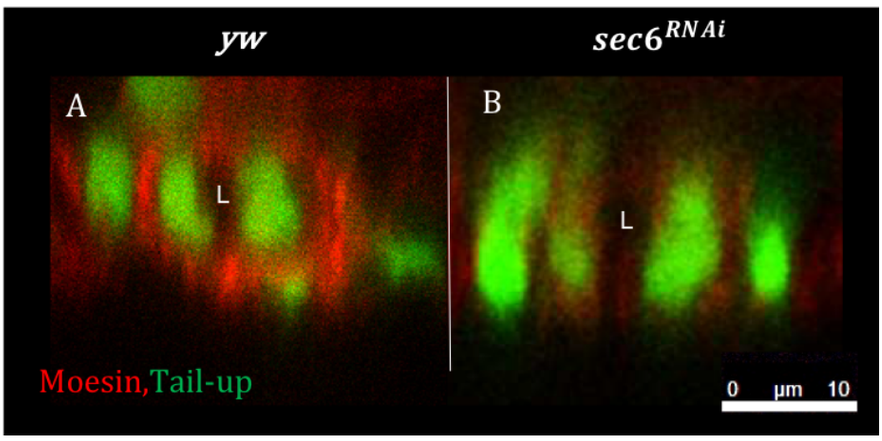
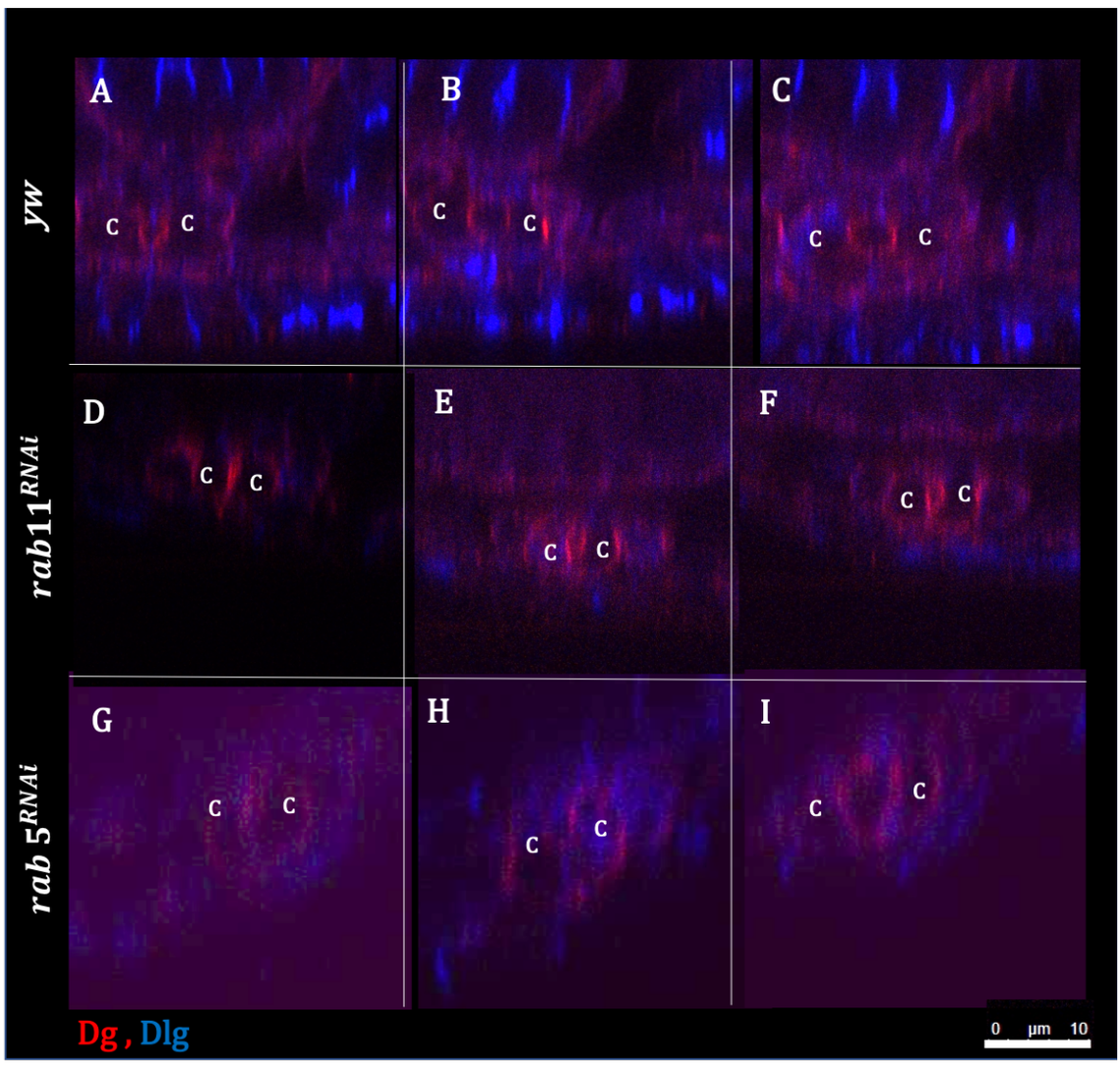
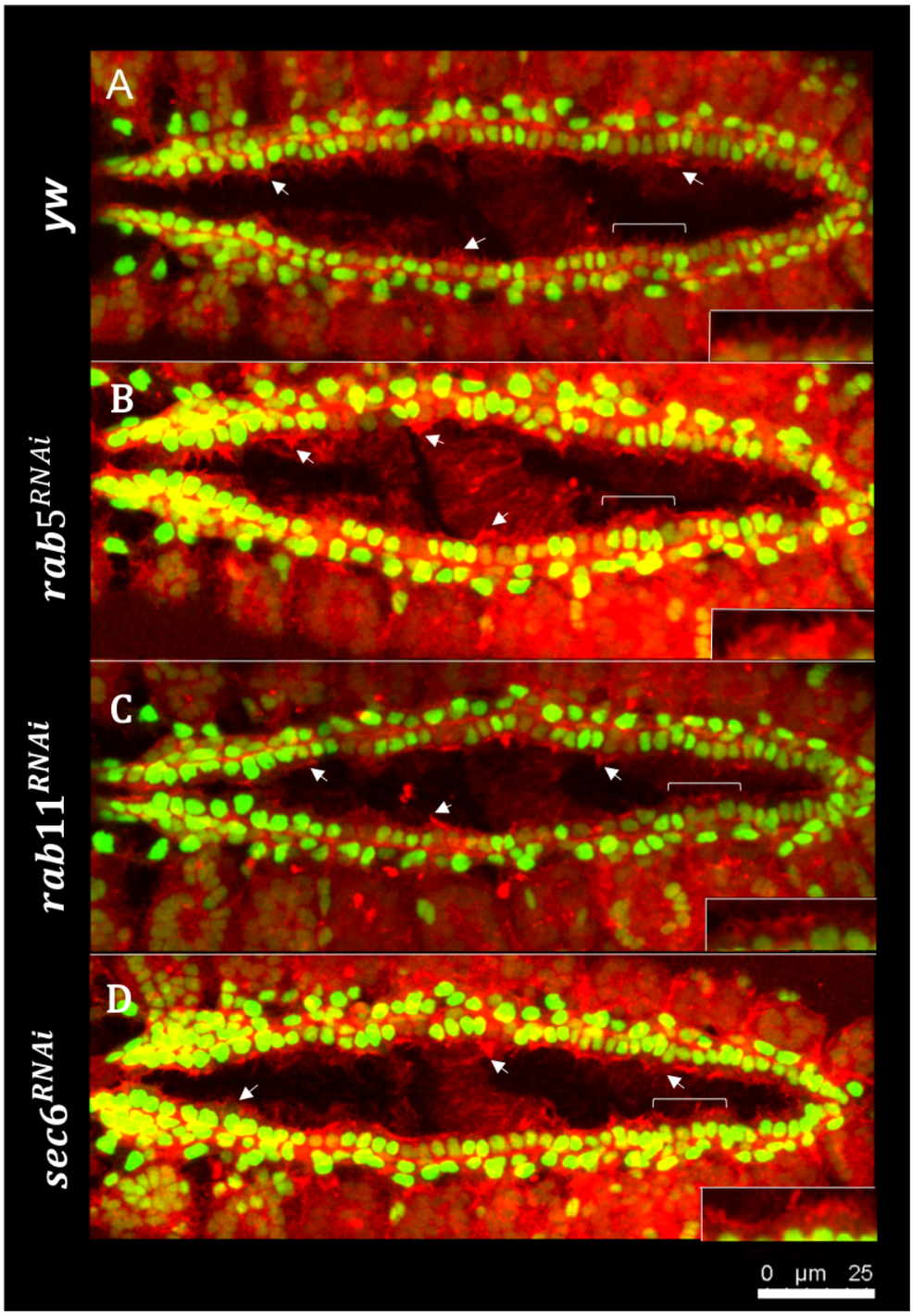
6. Supplemental Figures



Supplemental Figure 6.1: Knockdown of *sec6* does not affect lumen formation. Cross sectional images of contralateral cardioblasts at stage 17. Wildtype luminal space (A) *sec6* RNAi (B) Red indicates Moesin, a cytoplasmic linker to the Actin cytoskeleton, and green indicates Tail-Up, a CB nuclear marker. All crossed to *dmef*-Gal4 driver. L, Lumen. Scale 10 μm.


yw
rab11RNAi
rab5RNAi
A
B
C
D
E
F
G
H
I
c
Dg , Dlg
0 µm 10

Supplemental Figure 6.2: Cross sectional images of contralateral cardioblasts at stage 17. Wildtype luminal space (A, B) *rab5* RNAi (C, D) *rab11* RNAi (E,F). Cross sections were taken along the aorta. Red indicates moesin, a cytoplasmic linker to the Actin cytoskeleton, and green indicates tail-up, a CB nuclear marker. All crossed to *dmef*-Gal4 driver. C, cardioblast. Scale 10 μm.


yw
A
rab5RNAi
B
rab11RNAi
C
sec6RNAi
D
0 µm 25

Supplemental Figure 6.3: Knockdown of *rab5*, *rab11*, and *sec6* does not perturb filopodia and lamellipodia activity. Arrows indicate filopodial protrusions and singe parentheses indicate inset of zoom on filopodial protrusions (A, B, C, D). All embryos at stage 15. Red indicates Moesin and green indicates Tail-up. All crosses were completed using *dmef*-Gal4. Frontal view, Anterior to the left. Scale 25 μm.