Biological Activity of Natural Cleavage Products of Slit in the Developing *Drosophila* Heart

Biological Activity of Natural Cleavage Products of Slit in the Developing *Drosophila* Heart

By

Tanya Fariana Mahmood B.Sc. (Hons.)

A Thesis

Submitted to the School of Graduate Studies

In Parital Fulfilment of the Requirements

For the Degree

Master of Science

McMaster University

MASTER OF SCIENCE (2011) McMaster University
(Biology) Hamilton, Ontario

TITLE: Biological Activity of Natural Cleavage Products of Slit in the Developing *Drosophila* Heart

AUTHOR: Tanya Fariana Mahmood. B.Sc. (Hons) (University of Windsor).

SUPERVISOR: J. Roger Jacobs, Ph.D., Professor, McMaster University.

NUMBER OF PAGES vii, 68

ABSTRACT

The Slit morphogen is a secreted glycoprotein that is naturally cleaved into two fragments. The amino fragment (N-Slit) contains Leucine Rich Repeats (LRR) that are recognised by Robo receptors, and is sufficient to mediate attractive or repellent signalling in *Drosophila* tissues, for example, during growth cone guidance at the midline of the nervous system. The carboxy fragment (C-Slit) is composed of EGF repeats and a Laminin-like globular domain. Although C-Slit expression does not restore repellent signalling, it does rescue other morphogenetic defects in *slit* mutants.

Formation of the dorsal vessel (or heart) requires function of *slit* and *robo*. Slit is required for coordinated migration of heart cell precursors, cell polarisation and the formation of a lumen in the heart tube. We have characterised the morphogenetic activities of N- Slit and C-Slit during assembly of the heart.

Our laboratory has shown that Slit transgenes lacking the LRR region fail to rescue the mutant phenotype in the nervous system. However, *slit* trangenes lacking the LRR results in a partial rescue phenotype in the heart suggesting that C-Slit might have functional significance in the heart. Therefore, Slit function in heart vasculogenesis has different requirements compared to the nervous system. For example, Slit –Robo2 interaction may have an adhesive function in addition to a signalling function during vasculogenesis.

Our results indicate that C-Slit funtions as a heart morphogen. In *slit* mutants, over-expression of C-Slit results in a partial rescue phenotype with several features such as cell clumping, overlapping of cells and cells which are elongated. Together, these data suggest alternative functions for Slit during heart morphogenesis.

ACKNOWLEDGMENTS

First , I would like to thank Dr.Roger Jacobs for believing in me and allowing me to pursue my thesis in his lab. In times when I did not have faith on my data and my techniques, his continuous encouragement, support and advice kept me motivated. I would like to thank him for the opportunities he has given me to expand my knowledge through conferences. Dr. Jacobs has been the best mentor and teacher I have come across in my life and I will take away some great memories and a great appreciation for science from this lab.

I would like to thank all the people in my lab and my friends who have been there to console me about my confusing data and have let me cry on their shoulder.I will cherish the friends I have made in this lab forever. I want to thank Jessica Knox, Salima Chatur and Kendall Dutchak for the coffee sessions and the lunches where we laughed and cried together about our data and life. Lulu and Jessica Vanderploeg for encouraging me to be a good scientist. Mihaela for life lessons and all her help. Zhioli for being herself and always providing fun stories and laughter for us at the fly room. Katie Moyer for being my friend and helping me become a more organized scientist. Allison for being a wonderful friend and for her love and support. Leena for making the lab fun. Qamber and shahnaz for being wonderful lab members.

I would like to thank my parents. Without their sacrifices and love I would be nothing. I love and appreciate the way they have brought me up and the love they have shown me. I would like to thank both of my sisters for being my support system .They have loved and supported me and helped me become the person I am today. I would like to thank my Grandfather who taught me the value of education. Even though he is not here with us I know he is watching over me.

I would like to thank Dr. Campos for being in my committee and always providing me with good advice and moral support.

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Introduction

Congenital heart defects in infants due to irregular embryonic heart formation are one of the leading causes of infant deaths (Zaffran and Frasch, 2002). The study of organogenesis can be a useful tool in understanding the process of tube formation, a fundamental step in the development of organs such as the heart. One of the major events of organogenesis is proper cell specification and migration. The genetic pathways involved in such events are crucial in understanding organ formation.

During morphogenesis proper cell migration requires guidance cues. In the nervous system four known classes of guidance cues have been identified: Eprins, Netrins, Semaphorins and Slits. These ligands and their receptors have different roles in development of tissues. Ligand- receptor interaction results in either repulsion or attraction of cell or growth cone migration. The focus of my thesis is Slit, a guidance protein, and its involvement in the process of heart development.

1.1 *Drosophila* heart development

Heart formation is a significant developmental process during morphogenesis. In humans and vertebrates, disruptions of the normal developmental process of the heart can result in congenital heart defects (Tao et al., 2007). *Drosophila* cardiac tube morphogenesis has striking similarity to the formation of primary axial vessel of the

vertebrate heart (Hartenstein and Mandal et al., 2006). In both vertebrates and *Drosophila* the progenitors of the dorsal vessel are derived from the laterally positioned mesoderm. Additionally, morphogenesis requires similar pathways and transcriptional effectors in both (Tao et al., 2007). Therefore, *Drosophila* serves as an excellent model for studying formation of the heart and the genetic pathways related to abnormalities in heart defects.

The process of *Drosophila* heart formation can be divided into 3 major events: heart cell specification, cardioblast alignment and migration and lumen formation, depicted in Figure 1 (reviewed by Tao et al, 2007). During early stages of embryogenesis, maternal signals specify the mesoderm on the ventral part of the blastoderm. The mesoderm cell layer then invaginates and starts spreading laterally (reviewed by Tao et al., 2007). The process of mesodermal specification is initiated by the activation of the genes encoding the bHLH transcription factor Twist and the zinc-finger transcription factor Snail. This process is regulated by fibroblast growth factor (FGF) signalling pathway components such as Heartless, which in turn is involved in specifying further sub domains (reviewed by Tao et al, 2007). At stage 9 the lateral cells in the mesoderm receive signals from the overlying ectoderm instructing them to become the dorsal mesoderm. This allows the most lateral cells to be influenced by DPP expressing cells. DPP, a member of the TGF β superfamily, is secreted by the overlying ectoderm and is responsible for activating downstream transcription factors (reviewed by Tao et al., 2007). DPP specifies the dorsal mesoderm and concentrates the factor Tin to lateral

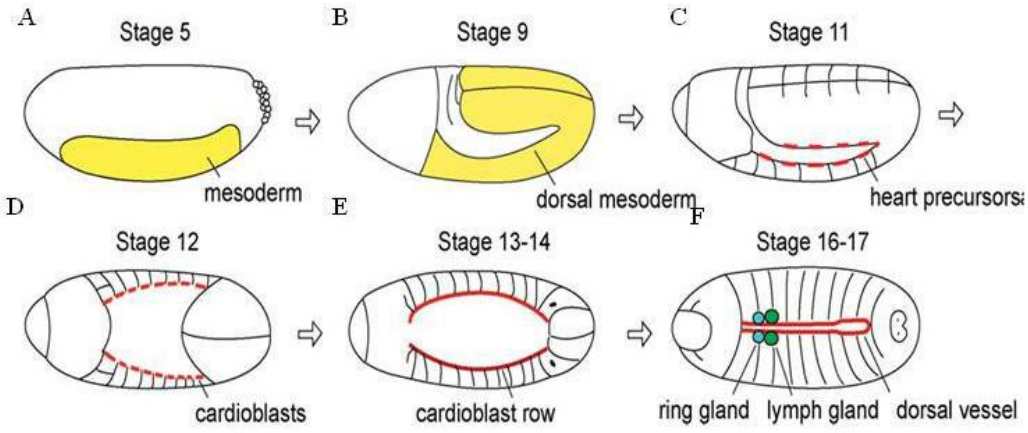

Figure 1-Stages of heart development during *Drosophila* morphogenesis.

The ventrally located mesoderm cell layer is shown in yellow. The dorsal mesoderm is derived from this layer. At stage 9 the lateral spreading of the mesoderm starts. At stage 11 the heart precursors are specified in the dorsal mesoderm (shown in red). Throughout stage 12-15 the cardioblasts migrate and align towards the midline. At stage 16 the dorsal vessel forms shown in red through the migration of the two rows of cardioblast towards the midline. The ring gland is (shown in blue) and the hematopoietic lymph gland is (shown in green). Adapted from (Tao & Schulz, 2007)

mesoderm, which is eventually specified as the dorsal mesoderm. Around stage 11 the dorsal mesoderm is further specified as the heart precursors which then aligns and forms a continuous row of cardioblasts on the either side of the embryo (reviewed by Tao et al., 2007). The cardioblasts migrate dorsally towards each other at stage 13 and by stage 16 they meet at the dorsal midline. At stage 17 the dorsal vessel is formed and initiates synchronized contractions (Reviewed by Tao et al., 2007).

The *Drosophila* organ of circulation is a linear tube called the dorsal vessel shown in Figure 2. The linear tube resembles the vertebrate heart at an early stage of development (Rugendorff et al., 1994). The dorsal vessel is derived from the dorsal-most mesoderm. The mature heart is a simple tubular organ consisting of three types of cell: cardioblast cells, pericardial cell and alary muscle. The cardioblasts, also called myocardial cells in the adult fly, form the tubular part of the dorsal vessel and are contractile (Rugendorff et al., 1994). The dorsal vessel is comprised of genetically distinct types of cells that appear in segments with six cardial cells in each hemisegment. Four out of the six cells express the homeobox gene *tinman* and the remaining two cells express the nuclear receptor gene *sevenup (*Bodmer et al., 1998, and Cripps et al., 2002). Tin transcription is restricted to the cells of the dorsal mesoderm that are fated to form the cardiac and visceral muscle lineages.

Pericardial cells are large cells that play a role in ultrafiltration and excretion of the haemolymph (Rugendorff et al., 1994). The alary muscles are responsible for suspending the dorsal vessel from the overlying body wall (Rugendorff et al., 1994). The

posterior segment of the dorsal vessel has a larger diameter and is referred to as the heart whereas the anterior segment is called the aorta.

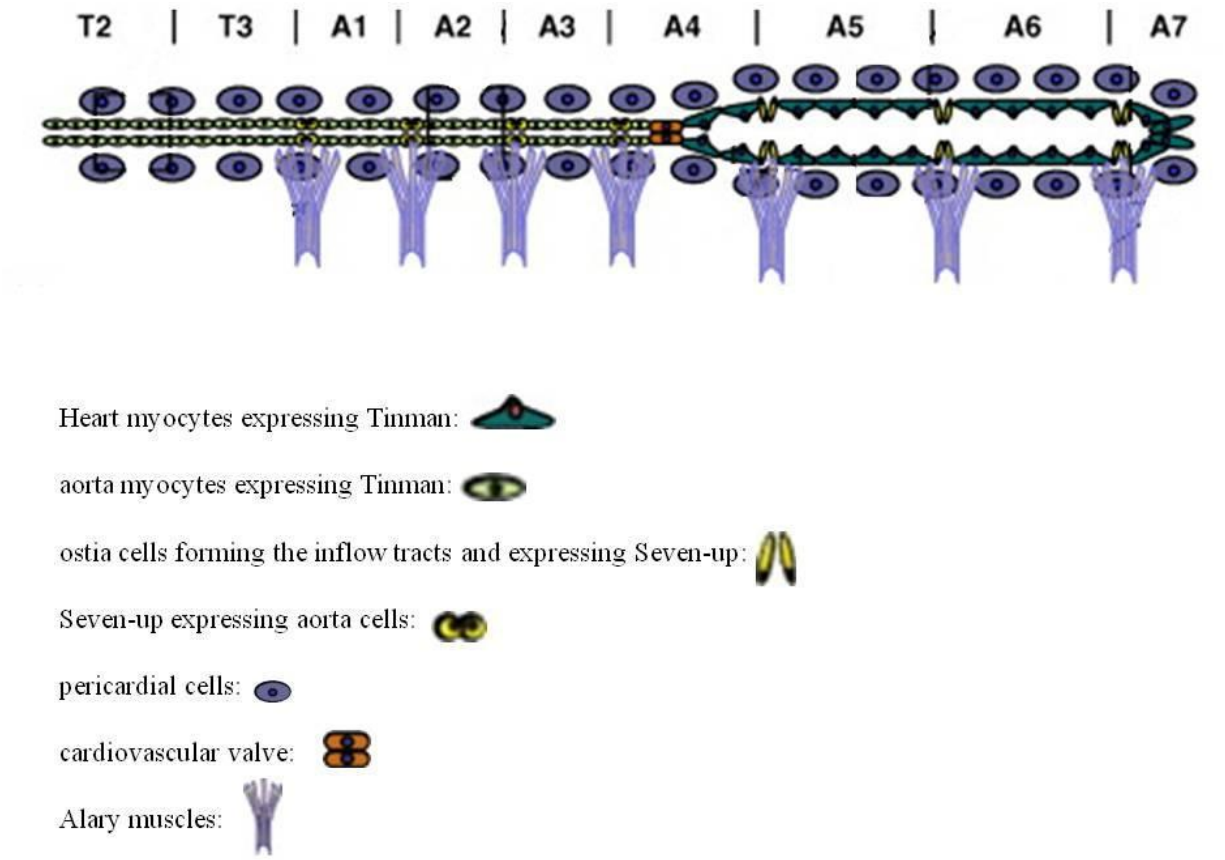

Figure 2-Structure of the dorsal vessel in *Drosophila.*

The dorsal vessel is composed of genetically distinct cell types. The hemisegments are composed of four large cardial cells and two sevenup cells. The heart has an anterior and posterior polarity. Adapted from (Medioni et al., 2009)

1.2 Structure of Slit

Slit is a 200kDa glycoprotein. It was originally studied for its role in the central nervous system (CNS). It is secreted by the midline glia cells of the *Drosophila* CNS and found at low levels in the surface of all CNS axons. (Rothberg et al., 1990; Simpson et al., 2000). Slit is a ligand for the Roundabout (Robo) family of receptors and it acts as a repellent cue during nervous system development (Brose et al., 1999; Simpson *et al.*, 2000) The *Drosophila* genome codes for a single Slit protein and three roundabout receptors : Robo, Robo2 and Robo3 (Rajagopalan et al., 2000). In contrast, mammalian genome encodes for three Slit proteins (Slit1-Slit3) and four Robo receptors (Robo1-Robo4) (Brose et al., 1999; Huminiecki et al., 2002).

Slit is composed of four domains: four Leucine Rich Repeat regions (LRR), seven epidermal growth factors (EGF) like repeats, a Laminin- like globular domain and a cysteine domain (Rothberg et al., 1990) shown in Figure 3. Slit has a cleavage site at the C-terminus which gives it the potential to generate three Slit products: N- Slit, C-Slit and Full-Slit (Rothberg et al., 1990). The amino fragment N-Slit contains the LRR and the C-Slit contains the EGF-repeats and Laminin like Globular domain (Rothberg et al., 1990).

The four LRR regions in the N-terminus of Slit are homologous to LRR regions found in proteins involved in protein-protein interaction (Howitt et al., 2004). LRR regions are connected by short linkers containing a cysteine residue that forms disulphide bridge with a cystein residue at the convex back of the previous domain (Howitt et al., 2004). Research from our laboratory and several others has shown that Robo binding requires an intact LRR domain but not the EGF-like repeats nor the Laminin like G-

domain (Battye et al., 2001). EGF repeats are known to play significant roles in controlling several cellular mechanisms such as cell proliferation and differentiation (Rothberg et al., 1990). The presence of both EGF repeat and LRR domain in Slit suggest that Slit may be involved in several other morphogenic events. The role of the G-domain and the cystein knot has not yet been characterized in Slit.

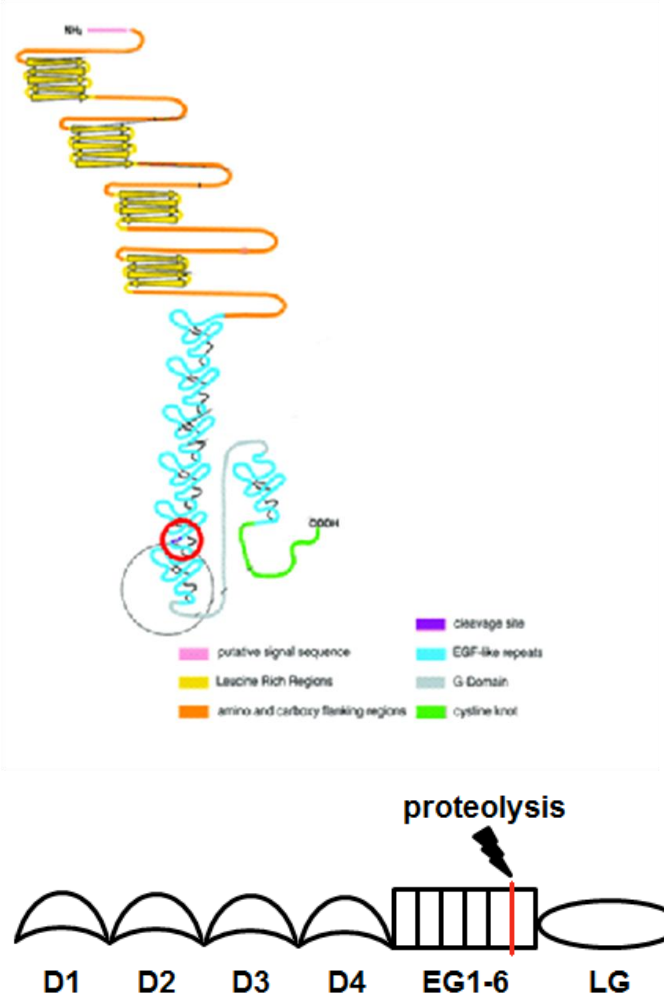

Figure 3-Structure of Slit in *Drosophila*.

Both top and bottom figures depict the domains of Slit. Slit has four domains: four leucine rich repeats (LRR) in the N-terminal shown in yellow, seven EGF-like repeats shown in blue, a globular G-domain shown in grey and a cysteine knot at the C-terminal shown in green. It has a natural proteolytic cleavage site shown in purple. Top figure adapted from (Battye et al., 2001). Bottom figure adapted from (Hohenester et al., 2006).

1.3 Function of Slit in the *Drosophila* nervous system

Slit was initially studied in the nervous system. Our laboratory and several others characterized Slit as a repellent guidance cue in the axon tracts of *Drosophila* CNS (Battye et al., 1999). Proper axon tract formation in the developing nervous system requires signalling between the midline and the growth cones. The midline consists of a set of specialized glial and neurons separating the two symmetrical halves of the CNS (Simpson et al., 2000). The midline glia is known to secrete several factors such as Slit and Netrin which are required for axon pathway and guidance (Simpson et al., 2000). Slit binds to Robo on axons and growth cones to specify the positions of axons in the longitudinal track (Battye et al., 2001). In addition, Slit is well-characterized as both repellent and attractant in the trachea and in the somatic muscles (Brose et al., 1999, Simpson et al., 2000).

As mentioned before, Slit is secreted by the midline glia and is a ligand for Robo receptors on the axons. This interaction between Slit and Robo is necessary for the repellent cues that prevent recrossing of the axons tracts (Simpson et al., 2000). In the CNS of *slit* mutants the axons remain in the midline causing a collapse in the scaffold like structure of CNS shown in Figure 4 (Battye et al., 1999; Kidd et al., 1999). In the CNS of wildtype embryos, axons that never cross the midline exhibit a high level of Robo (Simpson et al., 2000). However, in *robo* mutants the axons cross and recross several times (Simpson et al., 2000). Therefore, Robo is the receptor responsible for repelling the axons after crossing (Simpson et al., 2000).

In addition to characterizing Slit in the nervous system, our laboratory has used transgenes containing internal deletions to examine the domains responsible for Robo binding. The transgenes with deleted LRR region fail to rescue CNS phenotype of *slit* mutants (Battye et al., 2001). However, transgenes that have deleted EGF like repeats but have an intact LRR result in a partial rescue phenotype of the axon tracts (Battye et al., 2001). Therefore the LRR region in the N-Slit is responsible for Robo binding in the nervous system (Battye et al., 2001).

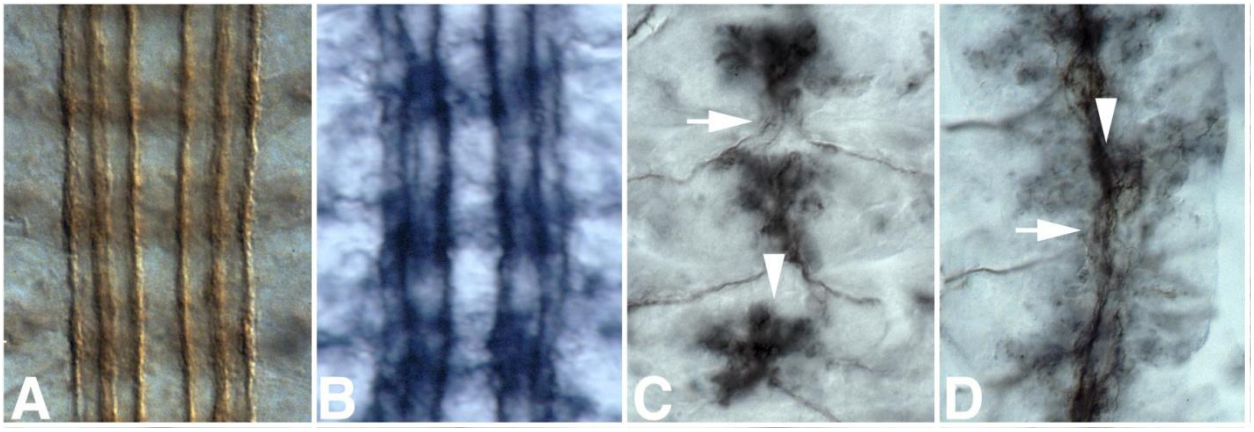

Figure 4- Midline guidance phenotype in wild type and *slit* mutant embryos.

Wild type embryos with bilateral sets of three axon fascicles, labeled with FasciclinII that run symmetrically along the length of nerve cord (A). In *sli*^{2}/+heterozygotes, axons deviate from the tracts but never cross the midline (B). In embryos deficient for *slit*, axon tracts fuse completely at the midline, however the phenotype is more severe due to deletion of segment 52A9-52D15 (C). In *sli*^{2} mutants, the axons fuse at the midline (D). These data demonstrate that *slit* is required for the lateral placement of axon tracts, by virtue of a repellent signal from the midline. Adapted from (Battye et al., 2001)

From previous studies in the nervous system it is understood that N- Slit and Full-Slit are required in the nervous system due to the presence of the LRR domains. However, the function of C-Slit in the CNS is not yet characterized. EGF repeats present in C-Slit are similar to EGF repeats found in Notch which is required for recognition of Notch by Delta (Rothberg et al., 1990; Leiber et al., 1992). In addition, the G-domain present on the α subunit of the Laminin protein is known to be involved in signalling through both integrin and non-integrin type receptors (Timpl et al.,2000; Ido et al., 2004). The presence of both of these domains in C-Slit suggests that it might be participating in signalling pathways involved in heart assembly such as the integrin signalling pathway.

Our laboratory has shown that Slit interacts with integrins in the nervous system (Stevens et al., 2002). Heterozygous *slit* mutants do not have any major defects in the axon tract formation. Similarly, heterozygous mutants for integrin have mild phenotypes in the nervous system such as wavy migration pattern of the axon (Stevens et al., 2002). However, transheterozygotes mutants of *slit* and integrins result in a more severe phenotype such as midline crossing and fusion of longitudinal axons suggesting that they do interact in the same or converging pathways (Stevens et al., 2002). It is possible that the laminin-like G domain of C-Slit signals through integrin in the nervous system. These findings prompted us to look into the role of Slit in the heart to determine if Slit acts in similar ways in the heart and the nervous system.

1.4 Function of Slit in the *Drosophila* heart assembly and lumen formation

The developing heart is unique as it expresses Slit and both of its receptors: Robo and Robo2 in the same cell (Qian et al., 2005). Slit mRNA is first detected at stage 12 in the lateral mesoderm, which eventually becomes elevated at stage13 in the cardial cells (MacMullin et al., 2006). Slit is uniformly distributed at the cardial cell cytoplasm in the early stages but becomes restricted to the lumen before meeting their contra-lateral cells around stage 16 (Santiago-Martinez et al., 2008, Qian, et al., 2005). Similarly, Robo protein is detected in the cardial cell cytoplasm in the early stages but concentrates apically at stage 16.

slit mutant hearts have several phenotypes such as gaps, blisters, overlapping cells and cell clumping (MacMullin et al., 2006). The breaks in the continuity of the cardial cell alignment at the dorsal midline are shown in Figure 5. These defects in the absence of Slit indicate that Slit is required for proper heart assembly. In addition to heart assembly, Slit is required for lumen formation as *slit* mutant hearts have no lumen or a reduced one (MacMullin et al, 2006). Over-expressing Slit in a *slit* mutant background results in ectopic or displaced lumen along with mislocalization of Robo (Santiago-Martinez et al., 2008). Therefore, Slit- Robo signalling is required for proper heart assembly and lumen formation.

Slit-Robo functions in cardiac tube formation by controlling proper migration, cohesion and alignment of two rows of CBs (Tao et al., 2007). Initially heart cells were assumed to have an apical-basal polarity however the examination of cell polarity

markers suggests otherwise. In wildtype embryos, Dlg and Alpha-Spectrin localize to the basolateral side of the cardial cells before cell contact is made during cell migration (Qian et al., 2005). Once contact is established the polarity is changed to apical-lateral. In *slit* mutants, the polarity of Dlg and Alpha-spectrin is compromised only when the cells contact at the midline (Qian et al., 2005). Therefore, Slit does not guide the cardioblast cells during cell migration but is required for switching the polarity from basal-lateral to apical-lateral once the cell contact is established (Qian et al., 2005).

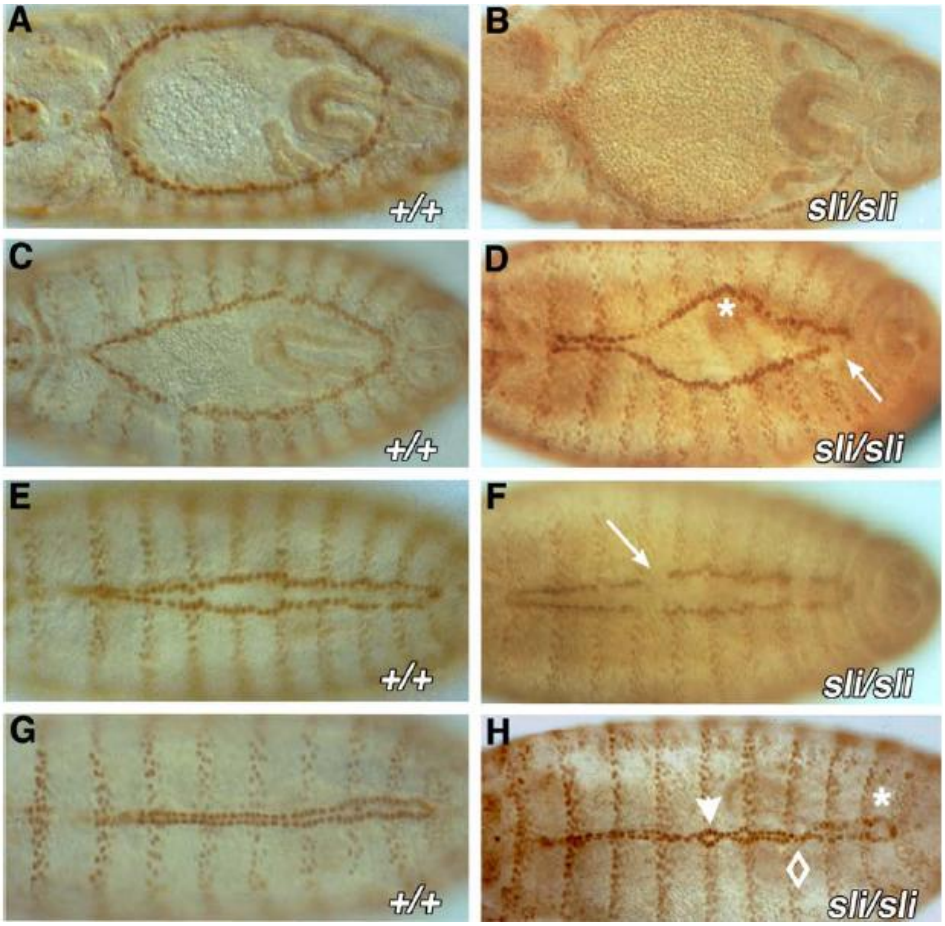

Figure 5-Heart assembly defects in *slit* Mutants.

The dorsal and medial migration of cardial cells shown in wild type and *slit* mutants. (A, C, E and G): wild type heart phenotype in stages 13, 14, 16 and 17 respectively. (B, D, F and H) shows sli^2 phenotype at stages 13, 14, 16 and 17 in the heart. Common phenotypes shown here include delayed migration (asterisk), gaps (arrows), and blisters in the heart (arrowhead), twists and midline crossing of the cardial cell nuclei (diamond). These data indicate that coordinated migration and alignment of cardioblasts requires *slit* function. Adapted from (MacMullin, 2006).

Heart tube assembly starts with the two rows of cardioblasts migrating towards the midline. The cardioblasts undergo a mesenchymal to epithelial transition before meeting at the midline. A recent study by Santiago-Martinez et al., (2008) examined the cardioblast cell migration and changes in the cell polarity markers to better understand the process of lumen formation. According to their data, during stage 16 the cardial cells change their shape and make contact at the dorsal and ventral ends leaving a luminal space (Santiago-Martinez et al., 2008). Around the same stage Slit becomes apically localized suggesting that Slit is involved in forming a lumen.

Robo is also involved in lumen formation. In *robo* mutant cadioblasts fail to appropriately adhere to contralateral partners and show defects in lumen formation (Santiago-Martínez et al., 2008). In addition, Slit does not concentrate at the apical domain of the cardioblasts in these *robo* mutants (Santiago-Martínez et al., 2008). Therefore, proper Robo localization is required for the polarized accumulation of Slit (Santiago-Martínez et al., 2008).

shg, which encodes DE-Cadherin, is required for lumen formation (Tepass et al., 1996, Umerua *et al.*, 1996). In *shg* mutants, cardioblasts fail to adhere to the contralateral partners and retain extracellular space between them (Santiago-Martínez et al., 2008). In contrast, in *robo* mutants the cardioblast adhere along the entire apical surface. Therefore, the study suggests that accumulation of Slit and Robo in the apical surface prevents E-cadherin mediated adhesion between contralateral cardial cells that would otherwise result in the formation of a lumen, depicted in Figure 7 (Santiago-Martínez et al., 2008).

In a separate study Medioni et al., (2008) proposed a mechanism of Slit-Robo signalling during lumen formation. According to the authors, cardioblasts initially have a pear like shape at the onset of dorsal closure (Medioni et al., 2008). As the cardioblasts come into contact they adopt a more crescent shape that allows the cells to join ventrally and close the tube. The cardioblasts shrink as they come into contact with each other to accommodate the large lumen, depicted in Figure 6 (Medioni et al., 2008). In *slit* mutants, cells do not change their shape during dorsal closure and keep their initial round shape. In addition, they do not detach from the overlying ectoderm. The cardioblasts are unable to contact at the ventral edges resulting in a lumen that is either absent or displaced ventrally (Medioni et al., 2008). Therefore, Slit is required for proper lumen formation.

As mentioned above, *Drosophila* cardioblasts do not have a classical apical-basal polarity (Qian et al., 2005). To accurately identify the domains that are involved in lumen formation, Medioni et al., (2008) classified the luminal domain that forms the lumen wall as the L domain, and the adherent domain, responsible for sealing the tube, as the J domain. The future L domain was shown to express classical basal domain components such as Dg, Slit and Trol surrounded by two J domains that express Arm, which is a cell-cell junction marker. In the absence of Slit-Robo pathway, the size of the L domain is reduced (Medioni et al., 2008). Therefore, activation of the Slit-Robo pathway controls the size of these two domains and prevents formation of E-Cadherin mediated adherent junction in the L domain (Medioni et al., 2008).

So far it is known that Slit- Robo signalling is involved in heart lumen formation through mechanisms mentioned above. In the nervous system Slit acts as a ligand for

Robo and interacts through LRR region of Slit to guide the axons. It is known that the LRR domain present in both N- Slit and Full-Slit is required for Slit-Robo signalling. Consequently, it is predicted that they will be able to rescue heart assembly in *slit* mutant embryos. Preliminary data from our laboratory show that in addition to N-Slit and Full-Slit, C-Slit is able to rescue heart morphogenesis. This result led to our interest in C-Slit and its potential of having biological activity during heart assembly.

The involvement of C-Slit has not been explored in the heart. The presence of EGF repeats and G-like domain in C-Slit indicate that there is possibility C-Slit is involved in heart formation by signalling through a Robo independent pathway. Based on our preliminary data we hypothesize that the C -Slit is involved in morphogenic events of *Drosophila* heart development.

Hypothesis.1: The expression of C-Slit in normal hearts will dominantly affect heart morphogenesis by preventing proper Slit-Robo signalling.

Hypothesis.2: The expression of C-Slit in *slit* mutant hearts will rescue the mutant phenotype by signalling through a Robo-Independent alternate pathway.

To test the hypothesis I have established four aims:

1. Overexpress Slit cleavage products in a wildtype background to visualize the effects in the heart cells.
2. Visualize the Robo and Slit distribution in embryos over-expressing Slit cleavage products.

3. Rescue *slit* mutant embryos by expressing the Slit cleavage products.
4. Visualize the Robo and Slit distribution in embryos expressing Slit cleavage products in a *slit* mutant background.

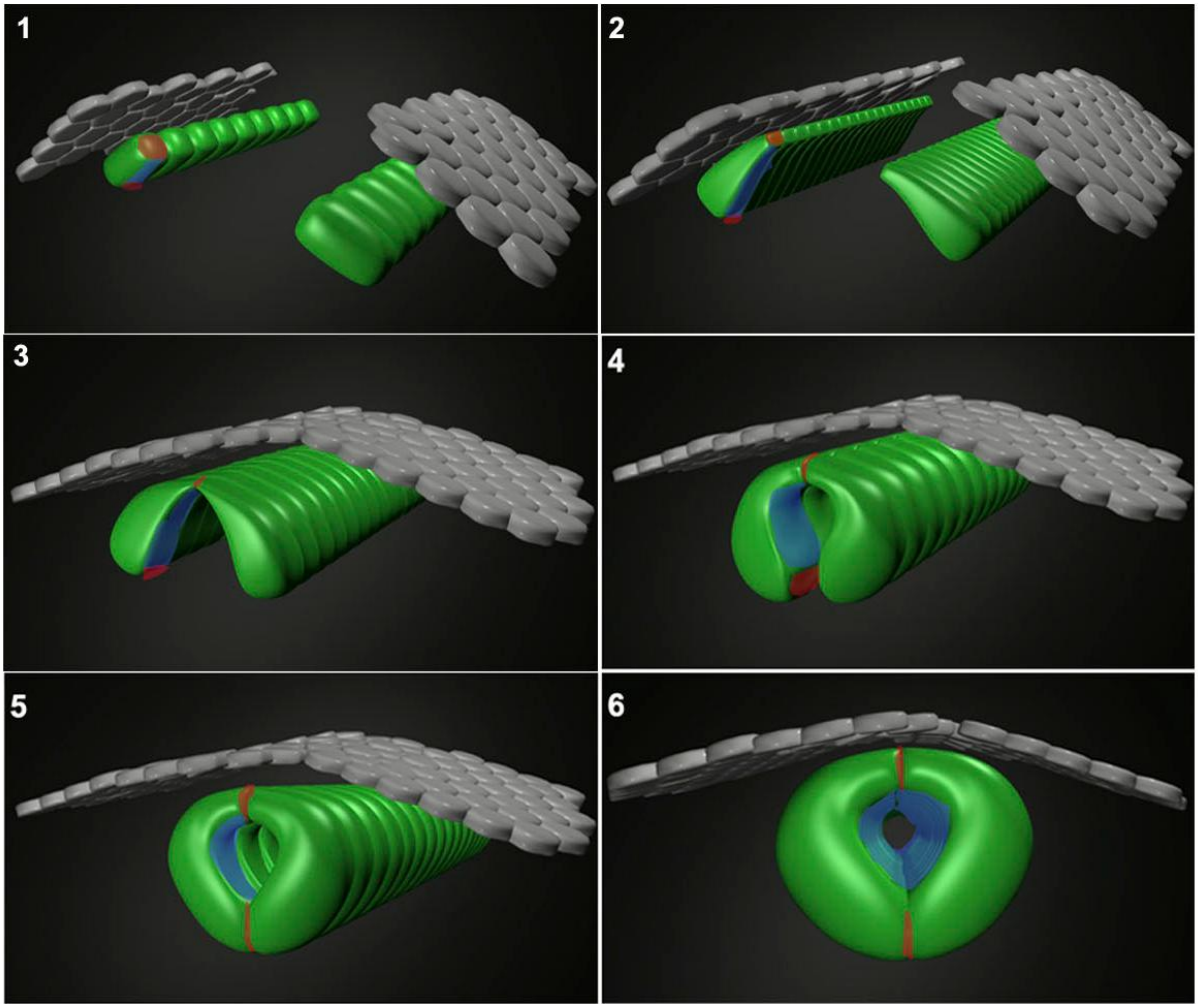

Figure 6-Model of cardiac tube formation in *Drosophila.*

The cardioblasts are shown in green, ectoderm in grey, Arm/DE-Cad in red, Slit-Robo in blue. The images (1-6) show the change in cell shape from round to a crescent morphology as the cardioblast migrate towards the midline. Slit and Robo are present in the lumen whereas the Arm/DE-Cad is at the dorsal and ventral ends sealing the dorsal tube. (Adapted from Medioni et al., 2008)

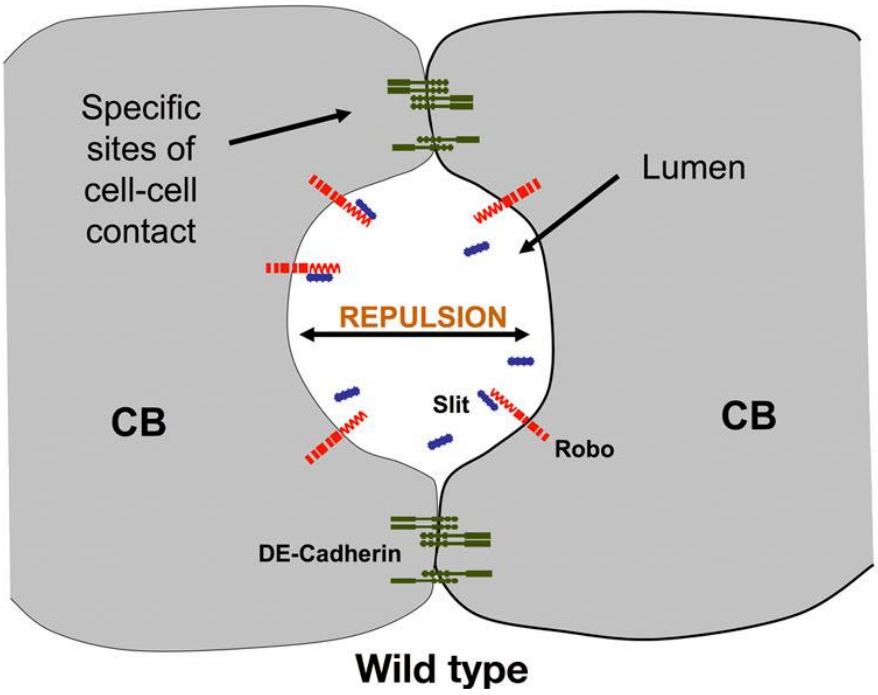

Figure 7-Model for lumen formation.

Slit-Robo concentrated at the lumen maintains separation of the cardioblasts forming a lumen. E-cadherins concentrate at the ventral and dorsal attachment sites allowing adhesion of the contralateral cells at specific sites. (Adapted from Santiago-Martínez et al., 2008)

Material and Methods:

2.1 *Drosophila* Stock

Drosophila stocks were maintained in vials 16 x 100 mm in a sugar-salt yeast agar medium at room temperature. The agar medium was prepared using sucrose, potassium sodium tartrate, potassium dibasic, sodium chloride, calcium chloride, magnesium chloride, ferric sulphate, yeast, agar and ddH20. The flies were allowed to lay eggs and changed every 15 days. Stocks were transferred to a new vial every 15 days and the adults were discarded after one week.

The mutant stains sli^2 and the muscle specific driver dMEFGAL4 were obtained from the Bloomington Stock center. The SVP-GAL4 stock obtained from R.Cripps. The UAS-FullSlit, UAS-NSlit and UAS-CSlit were made in our laboratory.

The Stock for rescues were recombined and made in our laboratory. The UAS stocks for rescues were sli^2/CyO_z; UAS-FullSlit/UAS-FullSlit, NSlit,sli^2/CyO_z and sli^2/ CyO_z;UAS-CSlit/UAS-CSlit. The GAL4 stocks used were sli^2/CyO_z; dMEFGAL4,B2-3-20/ TM3 and sli^2/CyO_z; svpGAL4, B2-3-20/TM3. All the stocks were tested for *slit* by complementation tests. The GAL4 stocks were tested by crossing the GAL4 stocks to UAS-CD8GFP. The GAL4 drove GFP in the cardioblasts and allowed us to test for the presence of the GAL4.

2.2 UAS-GAL4 system

The UAS-GAL4 system was used to overexpress the Slit cleavage products. The system as described by Brand and Perrimon (1993) is used to target the expression of

particular genes in the flies. The UAS "upstream activating sequence" is located upstream of the target gene. The GAL4 gene is required for the activation of the target gene that is under the control of a promoter expressed in a cell-tissue specific manner. The lines containing the UAS and GAL4 are maintained separately and crossed when it is required to overexpress or misexpress the gene in the organism.

I over-expressed the Slit cleavage products in the heart using a dMEFGAL4 driver. *Drosophila* Mef2 gene is expressed in all developing muscle cells and therefore highlights Slit expression by its cleavage products in all the muscle cells, including the cardioblasts. The dMEFGAL4 driver was recombined with a lacZ enhancer trap B2-3-20 (Bier et al., 1989, Hartenstein and Jan, 1992). The lacZ is expressed in various tissues including the dorsal vessel which allows for visualization of the cardial cells by immunohistochemical staining using anti-β –galactosidase antibody.

2.3 Embryo Collection and Fixation

Adult flies were crossed in 100mL beakers containing 30-35 females and 20-25 males. The beakers were capped using petri dishes containing apple juice agar and a drop of yeast and kept at two different temperatures for each cross, 29℃ and room temperature. The plates were changed twice a day in order to age the embryos to obtain them at different developmental stages. For room temperature collection, the plates collected in the evening were kept at 18℃ overnight and then moved to 4℃ in order to arrest their development for the plates collected in room temperature. The plates at 29° C were collected for 14-15 hours and then kept at 4° C to arrest their development. Prior to

fixation the plates then kept at room temperature in order for the repolymerisation of the microtubules.

For fixation the embryos were dechlorinated in 50% bleach in ddH20 for 5 minutes. The embryos were collected in nylon sieves and incubated in a fix consisting of 5ml heptane, 4.5 ml PBS and 0.5 ml formaldehyde. The embryos were fixed on a rotator for 25-27 minutes. The embryos were then washed with methanol cracking their vitelline membrane in the process. The embryos were then methanol-cracked, washed with methanol, and stored at 4°C for up to one week. When using anti-Robo as primary antibody the embryos were stained the same day as they were fixed.

2.4 Immunohistochemistry

To rehydrate the embryos, they were washed 5 times in PBT (0.1% Triton-x or Tween 20, 1XPBS) and rotated in PBT for 20-30 minutes.The embryos were treated blocked in 10% normal goat serum for 35 minutes to block antibody binding sites to increase its specificity. The primary antibody was then added. They included anti-β galactosidase chicken polyclonal (Knox, et al 2011) in a 1:150 dilution, C555.6D (mouse monoclocal provided by Developmental studies Hybridoma Bank) which recognizes Slit protein in a 1:30 dilution, 13C9 (mouse monoclocal provided by Developmental studies Hybridoma Bank) which recognizes Robo protein in a 1:30 dilution, anti-dmef2 (Rabbit polyclonal made by Jessica Vanderploeg) in 1/10,000 to 1/20,000 dilution and FasII (mouse monoclonal provided by Developmental studies Hybridoma Bank) in a 1:3 dilution. The embryos were incubated in the primary antibody overnight on an orbital

shaker at 4°C. To remove the unbound antibody, the embryos were washed using PBT every 30 minutes for 4 to 8 hours. The embryos then treated with NGS to re-blocked for 30 minutes and incubated with secondary antibody for 2 hours on a shaker at room temperature. The secondary antibodies used were: Goat anti chicken Bio (biotinylated antibody provided by Vector laboratories) in a dilution of 1:150, Goat anti Rabbit (biotinylated antibody provided by Vector laboratories) in a dilution of 1:150, Goat anti mouse alexa flouro 488 (Fluorescence antibody provided byMolecular Probes A11001) in a dilution of 1:150, Goat anti chicken alexa flouro 594 (Fluorescence antibody provided by Molecular Probes A11001) in a dilution of 1:150 and Goat anti Rabbit alexa flouro 597 (Fluorescence antibody provided by Molecular Probes A11001) in a dilution of 1:150.

The embryos stained with fluorescent antibody were covered in aluminium foil to protect from photobleaching. Following secondary antibody incubation, the embryos were washed 5 times and left overnight on a rotator at 4°C. The next day, the fluorescent stained embryos were washed 3 times and stored in 70% glycerol in 4° C. The double-labelled embryos were treated using the same techniques.

The biotinylated embryos were incubated for 1 hour in an ABC kit by Vector Laboratory (Vector Laboratory,PK-6100) in a solution of 2 µl of solution A and B and 100µl of PBT per embryo vial. Embryos were washed 5 times and incubated with 200µl PBT and 100µl 3,3-Diaminobenzidene tetra hydrochloride. 0.03% hydrogen peroxide was used to start the reaction and the embryos were reacted for 5-15 minutes depending on the progress of the reaction. The embryos were then washed 5 times and dehydrated in a

sequential ethanol wash of (50, 70, 90, 95 and 100 %) ethanol. Finally, embryos were stored in methyl salicylate at room temperature.

2.5 Mounting and Imaging

The embryos were stained with biotinylated antibody were mounted in a D.P.X neutral mounting medium and visualized using the Zeiss Microscope. The images were then obtained and viewed using: Image J, and Openlab. The fluorescent embryos were mounted on slides using a three coverslip method. Two coverslips were placed on a slide leaving gap between them. The embryos were mounted between the gap and covered with a third cover slip. The cover slips were then sealed with regular nail polish and left for 2 hours before being examined at the confocal microscope. The microscope used was a Leica SP5 microscope. The images obtained were then despeckled and the different channels were merged as single panels on adobe photoshop using 5-6 slices for each image that were about 1-1.5 µm thick.

2.6 Scoring of Heart Mutant Phenotype

The embryos were scored based on their severity using the scoring technique adapted from (MacMullin and Jacobs, 2006). The heart defects were categorized into 8 different phenotypes: gaps, twists, blisters, midline crossing, delayed closure, reduced lumen, and nuclei flattening as well as an "other" category. The other category included misplaced cells and elongated cells. For each genotype 20 embryos were used as a sample size at stage 17. For each category the embryos were given a score of 0 if no defects were found and a score of 1 in the presence of defects. Severity of each genotype was calculated by

quantifying the average number of defects per embryos in each category. Penetrance was assessed by calculating the number of embryos with two or more defects in the heart and dividing the number of embryos with more than one defect to the total number of embryos examined.

Statistical calculation was not performed during scoring of mutant embryos as these are scored using the ordinal system. Using parametric system would not be very conclusive for the data used in my thesis.

Results

3.0 Misexpression of Slit cleavage products result in CNS axon tract defects.

slit mutants show midline collapse of longitudinal axons in the *Drosophila* CNS (Battye *et al.,* 1999). Expressing a Full-Slit transgene in these mutants can partially rescue the phenotype. Proper axon tract formation in the *Drosophila* CNS requires an intact LRR region of the Slit protein to bind to Robo and provide proper chemo repellent cues to migrating axons (Battye et al.,1999). Therefore, the presence of N-Slit is a requirement in the *Drosophila* CNS for proper axon tract formation. The function of C- Slit in the nervous system however is still not clear. My first question was to address whether C- Slit has a role in the nervous system.

To perform the experiment, I misexpressed Slit cleavage products in the nervous system using elav-GAL4 driver, which expresses the GAL4 in all neurones. I performed immunohistochemistry on the embryos using fasciclin II antibody, which labels the bilateral sets of three fascicles that run along the nerve cord. The axon tracts were then examined for defects and compared to the wildtype nervous system. According to previous data from Battye et al., (2001), the presence of LRR domain in Full-Slit and N-Slit allows these domains to bind to Robo and maintain repellent axon guidance cues. Therefore, I would predict that misexpression of N-Slit and Full-Slit would result in improper binding of the misexpressed products to Robo on the axons tracts. This would

result in defects in axon tract formation. C-Slit does not have a Robo binding domain and I would expect the misexpression of C-Slit to have no affect on the axon tract formation.

The embryos misexpressing the Slit cleavage products showed minor deviation in the axon tract in three trangenes shown in Figure.3.0a. The deviations were minor with wavy appearance of the axons and defasciculation but there were no major fusions of the axon tracts. The number of embryos examined for each genotype was 20. The penetrance for defects such as deviation of the axon tracts and defasciculation in Full-Slit misexpressing embryos was 100%, N-Slit was 95% and C-Slit was 75%. The severity of the phenotypes ranged from minor to severe defects however the image showed in Figure 3.0a represents the average phenotype of the embryos. Therefore, misexpression of all three transgenes: Full-Slit, N-Slit and C-Slit directed by elavGAL4, is able to modify axon tract formation in the *Drosophila* CNS.

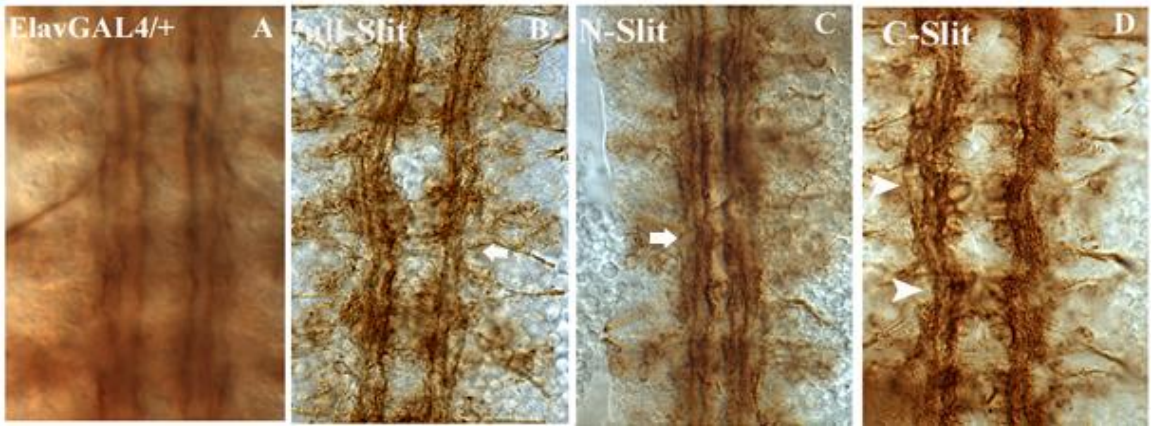

Figure.3.0a- Misexpression of the Slit cleavage products in the nervous system affects CNS axon tract formation. The Slit cleavage products were expressed in the nervous system using elavGAL4 driver. The embryos were labelled using FasII antibody that labels the fascicles. The wildtype nervous system is shown in (A). Full-Slit misexpression promotes defasciculation of axons with minor deviations of the axons (B) N-Slit misexpression results in defasciculation of the axons and medial deviation of axons shown in arrow (C) C-Slit misexpression (directed by *elav-GAL4*) results deviations of the axon fascicles shown by arrowhead (D).

3.1 Overexpression of Slit cleavage products results in Heart assembly defects.

As mentioned above, the LRR region is required for Slit-Robo binding to form proper axon tracts. However, the exact mechanism through which Slit-Robo signalling maintains heart formation is not clearly understood. From previous studies we know that Slit is required for proper assembly of the heart (MacMullin et al.,2006). *slit* mutants show a range of heart assembly defects during cardioblast alignment at the midline (MacMullin et al., 2006). The question I tried to address here is, whether overexpressing the Slit cleavage products in a wildtype background can affect heart assembly.

To assess the heart morphology, the cardial cells were visualized using a B2-3-20 enhancer trap (Bier et al., 1989). The Slit cleavage products UAS-Full-Slit, UAS-N-Slit and UAS-C-Slit were then overexpressed in a wild type background using the UAS-GAL4 system. I crossed the UAS-N-Slit, UAS-C-Slit and UAS-Full-Slit to two different GAL4 drivers in order to over-express the Slit cleavage products. The dMEF-GAL4 driver was used to express these products in the cardial cells. I performed the experiment using the dMEFGAL4 at two different temperatures: 23°C and 29°C. In a separate experiment I used the svp-GAL4 driver (explained below) to express the products in the svp cells.

3.1a Overexpression of the Slit cleavage products using dMEF-Gal4 driver in the heart.

Stage 16-17 embryos were assessed and and compared to wildtype embryos using the scoring system adapted from MacMullin et al., (2006) listed in Table.1.1. To avoid

any bias, the phenotypes of embryos for all three over-expression experiment were scored blind. The defects in the overexpression embryos ranged from large gaps to midline crossing as well as twists and blisters listed in Figure 3.1a. The severity and penetrance of the phenotypes in the overexpression embryos were higher than wildtype. Full-Slit and N-Slit had a higher penetrance and severity compared to C-Slit overexpression embryos, shown in Table 1.1. The most common phenotype in the C-Slit overexpression embryos was a change in morphology where the cells appeared to be more elongated. The elongated cell phenotype is listed under the category "other" in Table 1.1. N-Slit and FullSlit overexpression embryos also had presence of elongated cells but the penetrance was lower than in C-Slit.

The experiment performed at room temperature and 29°C had the same phenotypes. However, the experiments performed at 29°C showed delayed closure more frequently than embryos at 23°C.

3.1b. Overexpression of Slit Cleavage Products using SvpGAL4 driver.

The heart is composed of 10 segments: T2, T3 and A1-A8. Each segment from T3 to A7 has six pair of cardial cells. Two pair of cardioblasts in each of these segments express the Sevenup transcription factor (Bodmer et al., 1998, and Cripps et al., 2002) and the other four pairs express the Tinman transcription factor. T2 and A8 contain only two pairs of cardial cells.

To further observe effects of over-expression of Slit cleavage products in a subset of the heart, I used an svp-Gal4 driver. The Slit products were expressed only in the svp

cells which are surrounded by wildtype cells, unaffected by the svpGAL4 driver. This allowed us to examine whether the effects of overexpression driven in the svp cells is able to produce a cell autonomous phenotype in contrast to adjacent, unaffected cells.

The stage 16-17 embryos were scored and assessed using the scoring system adapted from MacMullin et al.,(2006) shown in Table.1.2. To avoid any bias, the phenotypes for the three crosses were scored blind. Overexpression of the Slit cleavage products resulted in various defects during heart formation shown in Figure 3.1b. The most common of the phenotypes was the formation of blisters in the area near the svp cells. The cells appear to be pinching at these intervals giving the cells an appearance of a blister. In addition, as observed previously, C-Slit overexpressing cells appear elongated at the areas where the cells pinch to form the blister, shown as in Figure 3.1b.

The penetrance and severity of the phenotype in overexpression embryos was higher than wildtype embryos. Similar to the previous data, severity of the phenotype in Full-Slit and N-Slit embryos was higher compared to C-Slit embryos.

Genotype	dMEF2-GAL4	UAS Slit Full/+ ; DmefGAL4	UAS Slit Cterm/+ ; dMEF2-GAL4	UAS Slit Nterm/+ ; dMEF2-fGAL4
Severity	1.25	2.05	1.85	2.2
Penetrance	0.35	0.85	0.6	0.8
Gaps	9	4	6	11
Twists	2	0	0	3
Blisters	2	7	6	1
Midline Crossing	5	4	6	7
Delayed Closure	2	5	2	5
Reduced Lumen	0	3	0	0
Nuclei Flattening	0	14	5	10
Other	5	4	12	7
Number of embryos	20	20	20	20

Table.1.1- Frequency of heart assembly defects in embryos overexpressing Full-Slit, NSlit and CSlit in a wildtype background using Dmef-GAL4 driver. The penetrance and severity of heart assembly defects is slightly higher in embryos overexpressing Full-Slit and N-Slit than C-Slit. However, C-Slit has the highest number of defects in the "others" category which is the elongated cell phenotype.

Genotype	svpGAL4	UAS Slit Full/+ ; svpGAL4	UAS Slit Cterm/+ ; svp-GAL4	UAS Slit Nterm/+ ; svp-GAL4	UASΔLRRSlit/+ ; svp-GAL4
Severity	1.1	2.55	2.4	2.55	2
Penetrance	0.3	0.9	0.85	0.9	0.75
Gaps	1	4	2	1	4
Twists	3	0	0	1	1
Blisters	6	14	14	15	14
Midline Crossing	2	1	3	7	3
Delayed Closure	3	7	6	5	2
Reduced Lumen	0	5	2	0	0
Nuclei Flattening	1	18	18	17	14
Other	6	2	3	5	2
Number of embryos	20	20	20	20	20

Table.1.2- Frequency of heart assembly defects in embryos overexpressing Full-Slit, NSlit and CSlit in a wildtype background using Svp-GAL4 driver. There is not a difference in penetrance and severity of phenotypes overexpressing Full-Slit, N-Slit and C-Slit. However, the most pronounced phenotype in all three genotype is the presence of blisters.

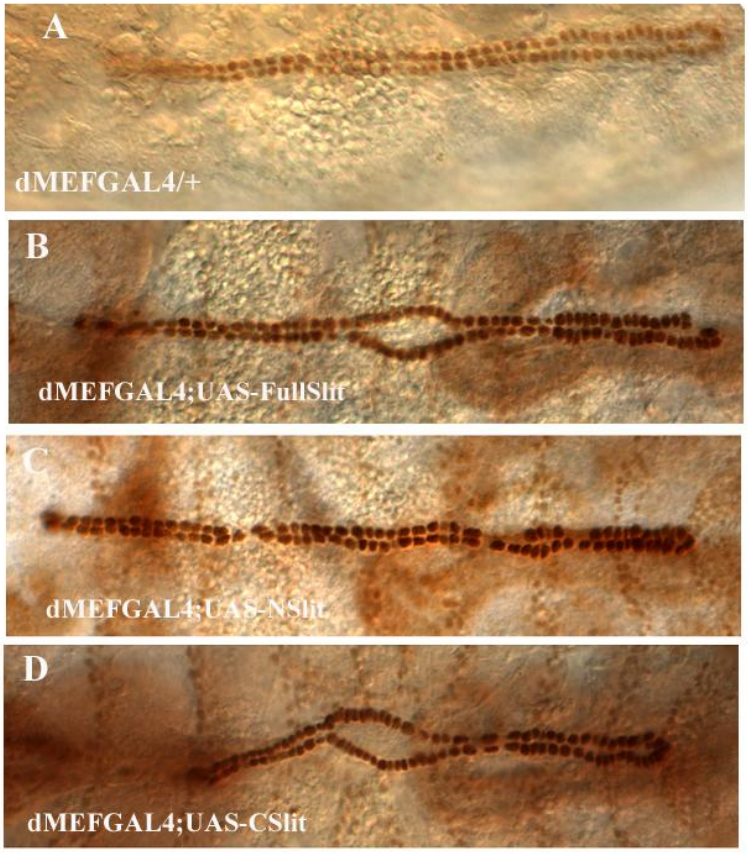

Figure 3.1a-Overexpression of Slit cleavage products in a wildtype background using dMEFGal4 driver. Cleavage products were expressed in the cardial cells using the dMEFGAL4 driver. The embryos were labelled using anti-βgalactosidase antibody to visualize the B2-3-20 enhancer trap. A wildtype embryo is shown in panel (A).Over-expression of Full-Slit generates an intermediate phenotype resulting in gaps shown by arrows(B).N-Slit over-expression in a wild type background results in gaps shown by arrows(C). In contrast, over-expression of C-Slit results in gaps shown in arrow and cells which are elongated along the length of the heart shown by arrowheads (D).

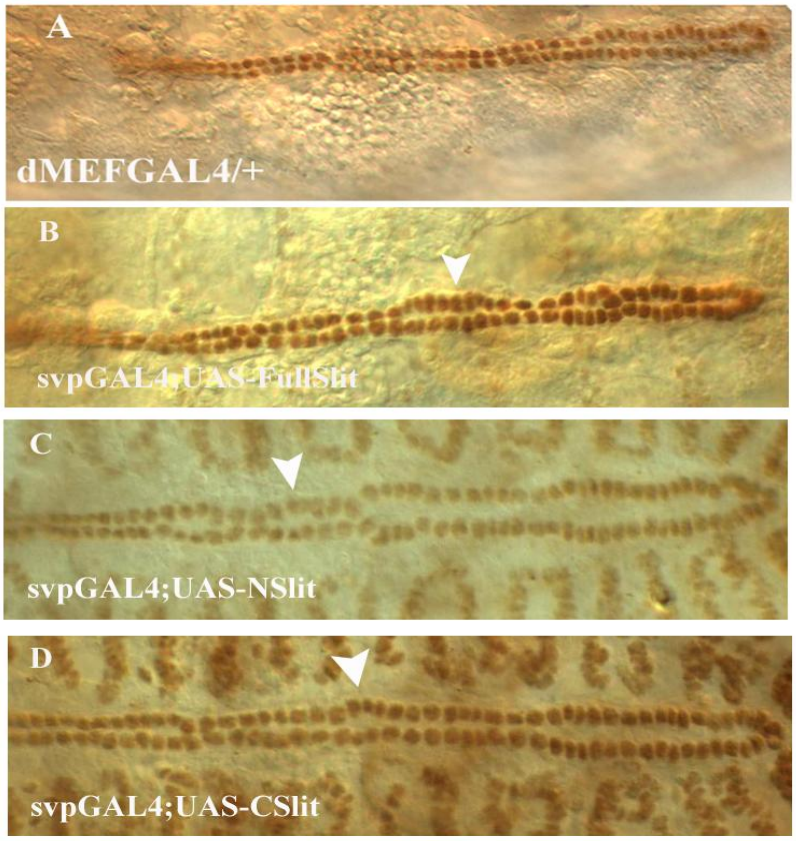

Figure 3.1b- Overexpression of Slit cleavage products in a wildtype background using the svp-Gal4 driver. The cleavage products were expressed in the svp cells using an svpGAL4 driver. The embryos were labelled with dMEF antibody that stains the cardial cells. A wildtype embryo is shown in Panel (A). The embryos expressing the Full-Slit have blisters along with midline crossing shown by arrowhead (B). The N-Slit expressing cells have multiple blisters along with improper midline crossing shown by arrowheads(C). The cells expressing C-Slit have blisters along with cells appearing elongated at the pinching sites shown by arrowheads (D).

3.2 Robo and Slit localization is not affected in embryos overexpressing the Slit cleavage products.

Heart assembly requires Slit-Robo signaling for proper heart formation (Mendioni et al., 2008 and Martinez et al 2008). *slit* mutants have defects in lumen formation and mislocalized Robo distribution (MacMullin et al., 2006, Qian et al., 2005). Therefore, our next goal was to address whether there were defects in Robo localization in the embryos overexpressing the Slit cleavage products.

To examine the Robo and Slit distribution in the embryos overexpressing the Slit cleavage products I performed immunohistochemistry on stage 16-17 embryos .The embryos were double labelled with the anti-βgalactosidase to detect the B2-3-20 cardial cell enhancer trap and monoclonal antibodies to detect Slit and Robo. The localization of Robo and Slit in the over-expression embryos was then compared to distribution pattern in wildtype embryos.

3.2a.Robo and Slit distribution in embryos overexpressing Slit cleavage products using a Dmef-Gal4 driver.

Slit and Robo apicalize and concentrate at the lumen around stage 16-17 in wildtype embryos (Qian et al., 2005; Mendioni et al., 2008; Martinez et al., 2008). In embryos overexpressing the Slit domains, the Robo localization and distribution pattern did not show any obvious mislocalization, as shown in Figure 3.2a. Similarly, the localization of Slit was not affected significantly in these embryos over-expressing the Slit domains. Slit levels were consistent in all three genotypes shown in Figure 3.2a'. The

“gain” option in the confocal microscopy adjusts voltage on the photomultiplier tube. Increasing the gain leads to having brighter imager therefore to maintain consistency of the Robo and Slit levels the embryos were imaged at the same gain.

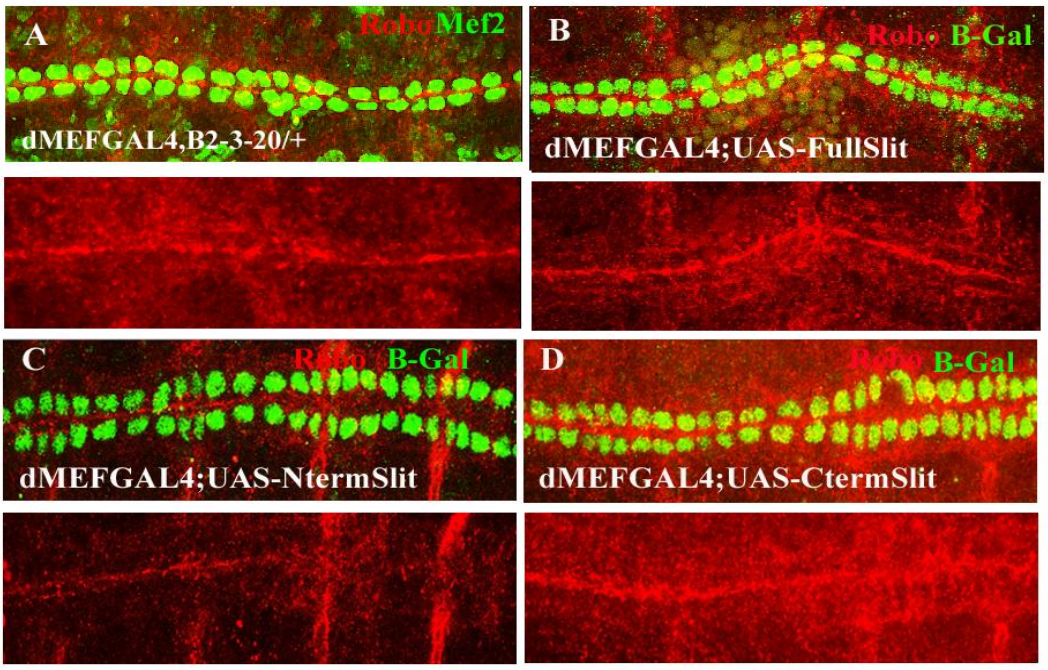

Figure3.2a-Robo distribution is not affected in the embryos overexpressing the Slit cleavage products in a wildtype background. The Slit cleavage products were overexpressed in the cardial cell using a dMEFGAL4 driver. The embryos were labelled using anti-βgalactosidase to visualize the cardial cells shown in the green and Robo antibody to visualize Robo shown in red. A wild type embryo is shown in panel (A). Robo localization is not affected in the embryos over-expressing Full-Slit shown in panel (B), N-Slit in panel (C) and C-Slit in panel (D) in a wildtype background. The samples were imaged at the same gain in confocal.

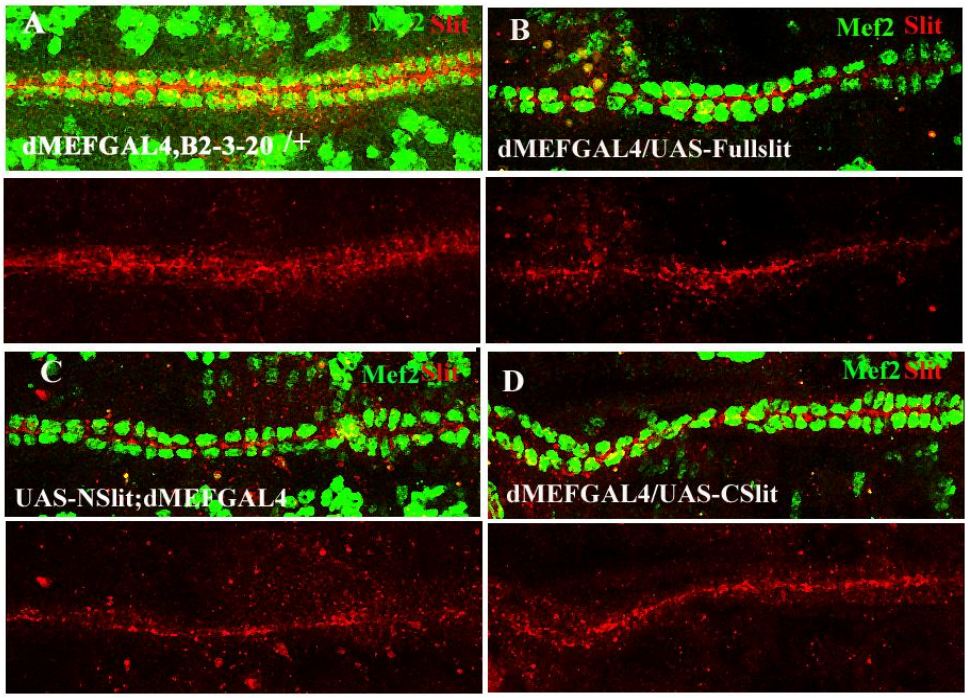

Figure 3.2a'-Slit distribution is not affected in the embryos overexpressing the Slit cleavage products in a wildtype background. Slit cleavage products were over-expressed in the cardial cells using a dMEFGAL4 driver. The embryos were labelled using anti-βgalactosidase to visualize the cardial cells shown in green and Slit antibody to visualize Slit shown in red. The wildtype embryo is shown in panel (A).Slit localization is not affected in the embryos over-expressing Full-Slit, shown in panel (B), N-Slit shown in panel(C) and C-Slit shown in panel (D) in a wildtype background The samples were imaged at the same gain in confocal.

3.2b.Robo and Slit distribution is embryos overexpressing Slit cleavage products using an svp-Gal4 driver.

To address the same question whether overexpressing Slit domain in the heart can alter Robo localization I used the svp-GAL4 driver in this experiment. This approach allowed me to visualize the affects of Robo distribution in a directed area, in contrast with the dMEFGAL4, which expresses the domains in all the cardial cells.

Stage 16-17 embryos were double labelled with Mef2 antibody to visualize the cardial cells and monoclonal antibodies to visualize Slit and Robo. Consistent with the previous data, the embryos did not have a significant change in the levels and localization of Robo shown in Figure 3.2b.

The Slit localization was wildtype in embryos expressing UAS-FullSlit and UAS-NSlit. However, the embryos expressing C-Slit appears to have less apicalization of Slit, as shown in Figure 3.2b'. I expected Slit labelling in the embryos overexpressing the Slit domains in the svp cells to show a higher level than the adjacent wildtype cell. However, it appears from the data that Slit is able to move to the neighbouring cell which is why we don't see any change in the Slit levels between svp and non-svp cells.

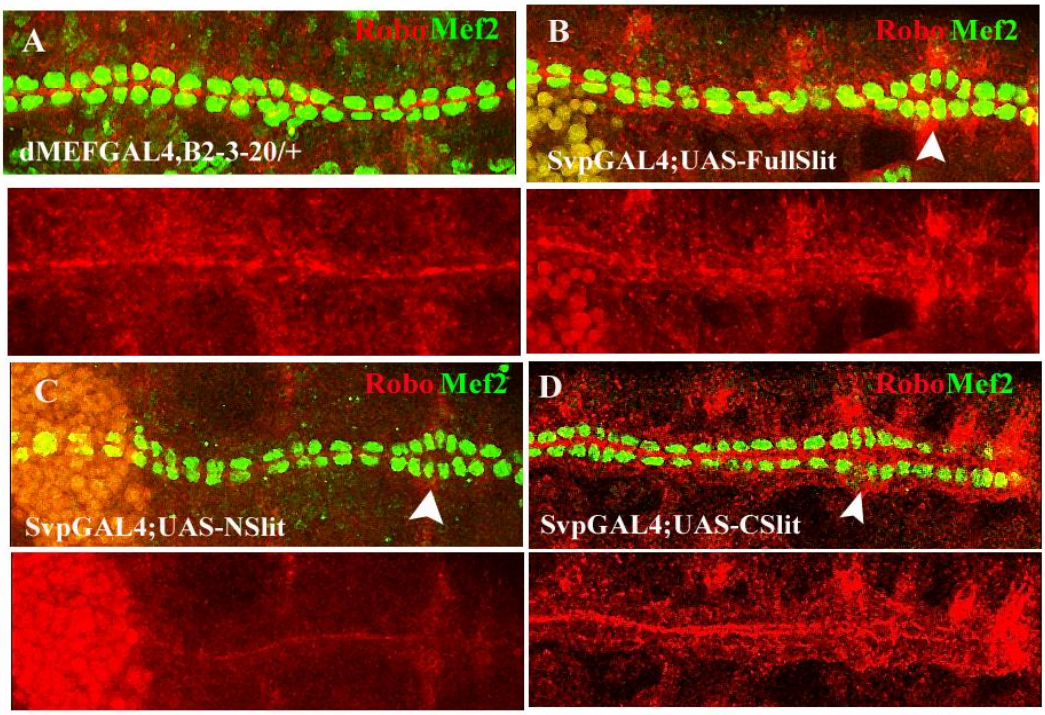

Figure 3.2b-Localization of Robo in embryos overexpressing the Slit cleavage products using the svpGal4 driver in a wildtype background. The Slit cleavage products were overexpressed in the cardial cells using a svpGAL4 driver. The embryos were labelled using anti-βgalactosidase to visualize the cardial cells (green channel) and Slit antibody (Red channel). Wildtype embryo is shown in panel (A). Robo localization is not affected in the embryos over-expressing Full-Slit, shown in (B), N-Slit in panel (C) and C-Slit in panel (D) in a wildtype background. However, blister formation can be seen with all three genotypes (shown by arrowheads). The samples were imaged at the same gain in confocal.

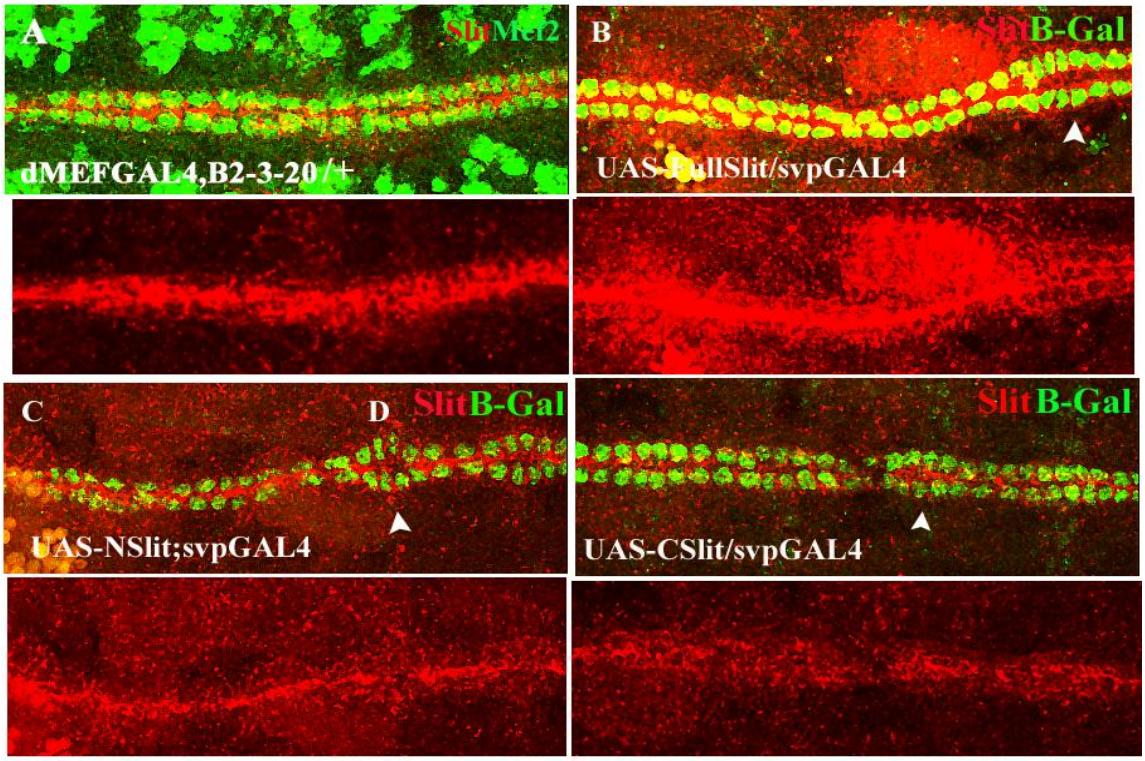

Figure 3.2b'-Localization of Slit in embryos overexpressing the Slit cleavage products using the svpGal4 driver in a wildtype background. The Slit cleavage products were overexpressed in the svp cells using a svpGAL4 driver. The embryos were labelled using anti-βgalactosidase to visualize the cardial cells (green channel) and Slit antibody (red channel). A wildtype embryo shown in panel (A). Slit localization is not affected in the embryos over-expressing Full-Slit, shown in panel (B) N-Slit shown in panel (C). However, the embryo expressing C-Slit shown in panel (D) appears to have less apicalization of Slit (arrow). The samples were imaged at the same gain in confocal. The blisters are shown by arrowheads.

3.3a. Expression of the Slit Cleavage products rescues the *slit* mutant phenotype.

A recent study by Martinez et al., (2008) has shown that expressing Full-Slit in *slit* mutants can rescue the mutant heart phenotypes, however it also results in the formation of multiple lumens. Full-Slit has all the domains of Slit therefore it is expected to partially rescue a *slit* mutant phenotype during heart assembly. If N-Slit and C-Slit have an actual function in the heart then expressing these domains in a *slit* mutant background should be able to partially rescue the heart. Our next question was to address whether in the absence of endogenous Slit, N-Slit and C-Slit can rescue heart assembly.

To generate the rescue embryos I crossed *sli^2/CyO_z; dMefGAL4;B2-3-20/TM3* stocks to *sli^2/CyO_z; UAS-Full-Slit/UAS-Full-Slil, sli^2/CyO_z; UAS-C-SlitIII/UAS-C-SlitIII and sli^2,N-Slit/ CyO_z*. From these crosses I selected embryos that were : *sli^2/ sli^2; dMefGAL4;B2-3-20/UAS-Full-Slit, sli^2,UAS-N-Slit; dMefGAL4;B2-3-20/+* and *sli^2/ sli^2; dMefGAL4;B2-3-20/UAS-C-SlitIII.* The *slit* mutant embryos were detected through the absence of lac_Z marker pattern in the stained embryos which were consequently *sli^2* homozygotes. The dMEFGAL4 driver was recombined with B2-3-20 lac_z enhancer trap, therefore the anti-β galactisidase antibody labelled the heart cells and muscles surrounding the embryos that express the dMEF transcription factor. This allowed me to recognize embryos that had the GAL4 and had the expression of the Slit cleavage products in a *slit* mutant background.

As controls I examined embryos that were dMEFGAL4/+ ,*sli^2/sli^2*as *slit* mutants and the UAS lines *sli^2/CyO_z; UAS-Full-Slit/UAS-Full-Slit, sli^2/CyO_z; UAS-C-SlitIII /UAS-*

C-SlitIII and sli^2*,N-Slit/* CyO_z shown in Figure 3.3a. I examined the UAS lines to ensure the embryos did not have any heart assembly defect phenotype due to the insertion of the UAS transgenes in the flies.

Expression of the Slit cleavage products in *slit* mutants resulted in rescued heart assembly in embryos that ranged from partial to complete rescue. The embryos had minor defects such as small gaps, delayed closure and midline crossing, but the penetrance of the defects were low. As expected, heart assembly in embryos rescued using Full-Slit were more successful compared to N-Slit and C-Slit, as shown in Figure 3.3a'.

The sample size of the embryos expressing the Slit products in a *slit* mutant background was really small due to difficulty in labelling the embryos. The embryos with rescued heart phenotype had very light labelling and it was difficult to image the cardioblasts. A large number of embryos did not label altogether. I examined 7-10 embryos for each genotype therefore I was not able to calculate the severity and penetrance of the embryos. However, the embryos collected for Full-Slit and C-Slit rescue was comparatively easier to locate and examine than hearts rescued using N-Slit.

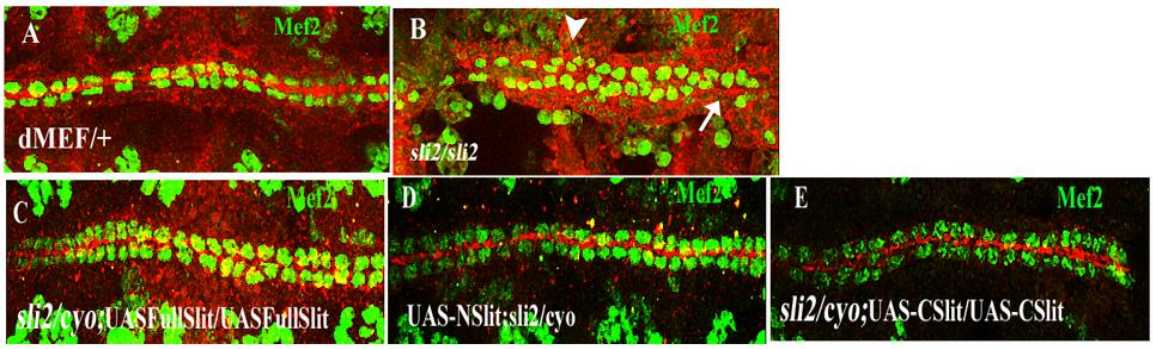

Figure 3.3a-Heart assembly in embryos used as controls for the rescue experiment.

Heart assembly is normal in wildtype embryo is shown in panel (A). *slit* homozygous mutant embryos have defects in heart assembly such as cell clumping (shown in arrowhead) and gaps (shown in arrow) in panel (B). Heart assembly is normal in embryos with one copy of *slit* and the UAS transgenes. Embryos with the genotype sli^2/CyO_z; UAS-Full-Slit/UAS-Full-Slit shown in panel (C), UAS-NSlit,sli^2/CyO shown in panel (D) and sli^2/CyO_z; UAS-C-Slit/UAS-C-Slit shown in panel (E).

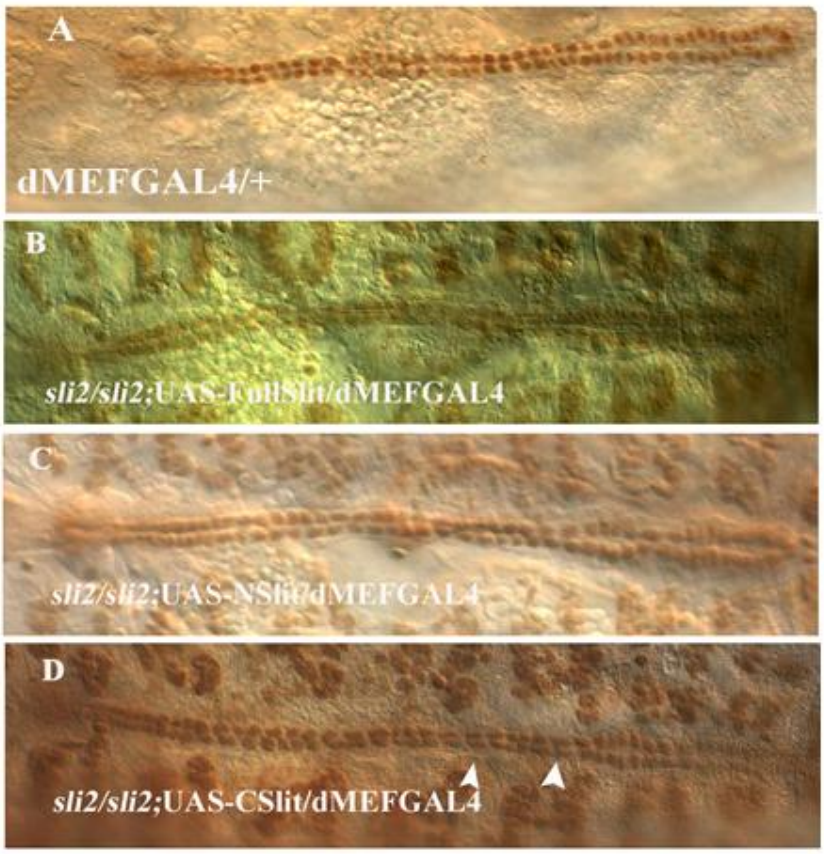

Figure 3.3a'- Expressing Slit cleavage products in the *slit* mutant background rescues heart assembly. Slit cleavage products were expressed in cardial cells using a dMEFGAL4 driver in a *slit* mutant background. The embryos were labelled using Mef2 antibody to visualize the cardial cells and anti-βgalactosidase to label lacZ from the marked balancer. A wildtype embryo is shown in panel (A). An embryo expressing Full-Slit is shown in panel (B) which shows a complete rescue in a *slit* mutant background. Embryos expressing N-Slit in a *slit* mutant background also show a complete rescue shown in panel (C). The C-Slit expression embryos were rescued however they had multiple elongated cell phenotypes shown by arrowheads (D).

3.3b. Robo distribution is affected in embryos rescued using the Slit cleavage products in *slit* mutants.

During the early stages of morphogenesis Slit and Robo are uniformly distributed on the cardioblast surface (Qian et al.,2005 ; Mendioni et al., 2008). However, Slit becomes more apicalized before the cardioblasts align at the dorsal midline. The previous section addressed whether heart assembly can be rescued in *slit* mutants using the expression of Slit cleavage products. Our next question was to address whether these rescued heart using the Slit cleavage products were able to localize Robo normally in the heart.

To generate embryos that would express the Slit cleavage products in a *slit* mutant background I crossed *sli^2/CyO_z; dMefGAL4;B2-3-20/TM3* stocks to *sli^2/CyO_z; UAS-Full-Slit/UAS-Full-Slit, sli^2/CyO_z; UAS-C-Slit/UAS-C-Slit and sli^2,N-Slit/ CyO_z*. I selected for embryos that were: *sli^2/ sli^2; dMefGAL4;B2-3-20/UAS-Full-Slit, sli^2,UAS-N-Slit; dMefGAL4;B2-3-20/+* and *sli^2/ sli^2; dMefGAL4;B2-3-20/UAS-C-SlitIII.* The *slit* mutant embryos were detected through the absence of lac_Z marker pattern in the stained embryos which were consequently *sli^2* homozygotes. The dMEFGAL4 driver was recombined with B2-3-20 lac_z enhancer trap. I used anti-β galactisidase antibody which labelled the heart cells expressing the dMEF transcription factor. This allowed me to recognize embryos that had the GAL4 and expressed the Slit cleavage products in a *slit* mutant background. I used monoclonal antibodies to visualize Slit and Robo. In addition, I confirmed that the embryos were in a *slit* mutant background by looking at the nervous system for the absence of Slit (data not shown).

Robo distribution and localization in the rescued hearts was compared to heart assembly in wildtype embryos and *slit* mutants shown in Figure 3.3b. In wildtype embryos Robo is localized apically between the two rows of cardioblasts in the heart. In *slit* mutants, Robo distribution is mislocalized basally and lateral.

The *slit* mutant hearts rescued using Full-Slit show mislocalization of Robo, similar to that of *slit* mutants. Robo is localized basally and laterally as opposed to an apical distribution in wildtype. In comparison, N-Slit rescued embryos show a wildtype Robo localization. C-Slit rescued embryos show slight mislocalization of Robo with Robo located mostly in the apical surface with some mislocalization in the basal and lateral surface.

3.3c. *slit* mutants rescued using the Slit products have rescued Slit distribution.

Slit antibody is able to recognize the region of EGF2-EGF6 (Battye et al., 200). The epitope is located only in Full-Slit and C-Slit therefore it should be able to recognize these domains only. However, one of the limitations of my data is that Slit antibody is able to detect all three transgenes shown in Figure 3.3c. In addition, the Slit localization appears wildtype in these embryos expressing Full-Slit, N-Slit and C-Slit in a *slit* mutant background.

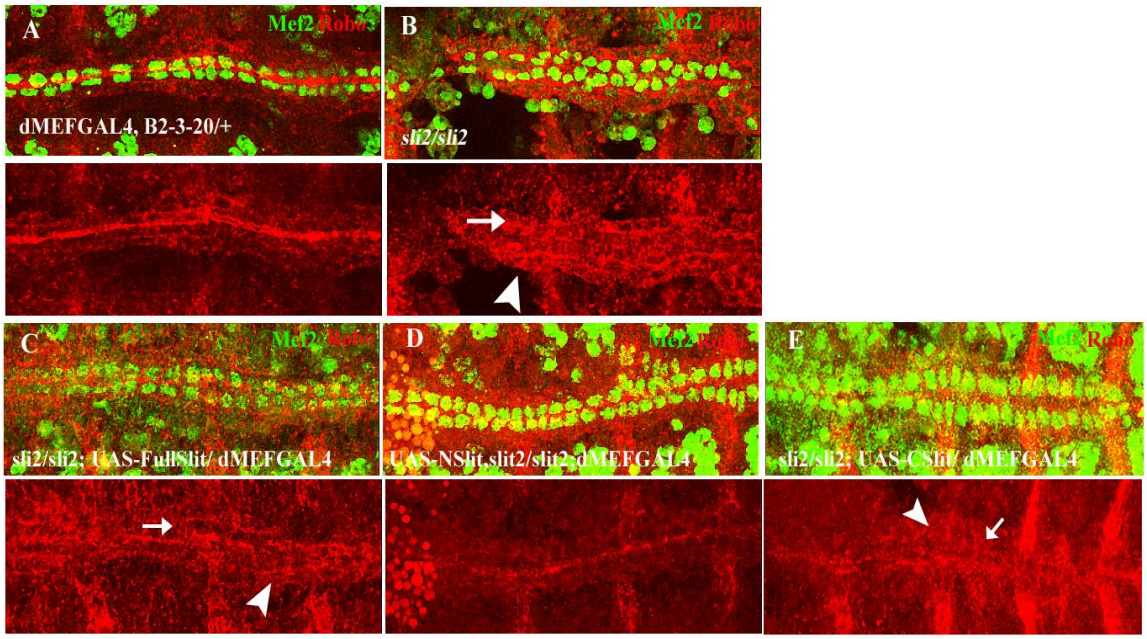

Figure 3.3b-Robo localization in embryos expressing the Slit cleavage products in the *slit* mutant background. The Slit cleavage products were expressed in a *slit* mutant background using a dMEFGAL4 driver. The cardial cells are shown in green and Robo localization is shown in red. The wildtype Robo localization is shown in panel (A). In *slit* mutants (B), Robo is mislocalized in the basal (shown in arrowheads) and lateral surface (arrow). Robo localization in embryos expressing Full-Slit in a *slit* mutant background shows similar patterns to *slit* mutants. Robo appears to be mislocalized in the basal (shown in arrowheads) and lateral surface (shown in arrow) in panel (C). Robo localization is closer to wildtype in embryos expressing N-Slit in a *slit* mutant background shown in panel (D). However, Robo localization in embryos expressing C-Slit in a *slit* mutant background shows some basal (shown in arrowheads) and lateral (shown in arrow) mislocalization shown in panel (E). The samples were imaged at the same gain in confocal.

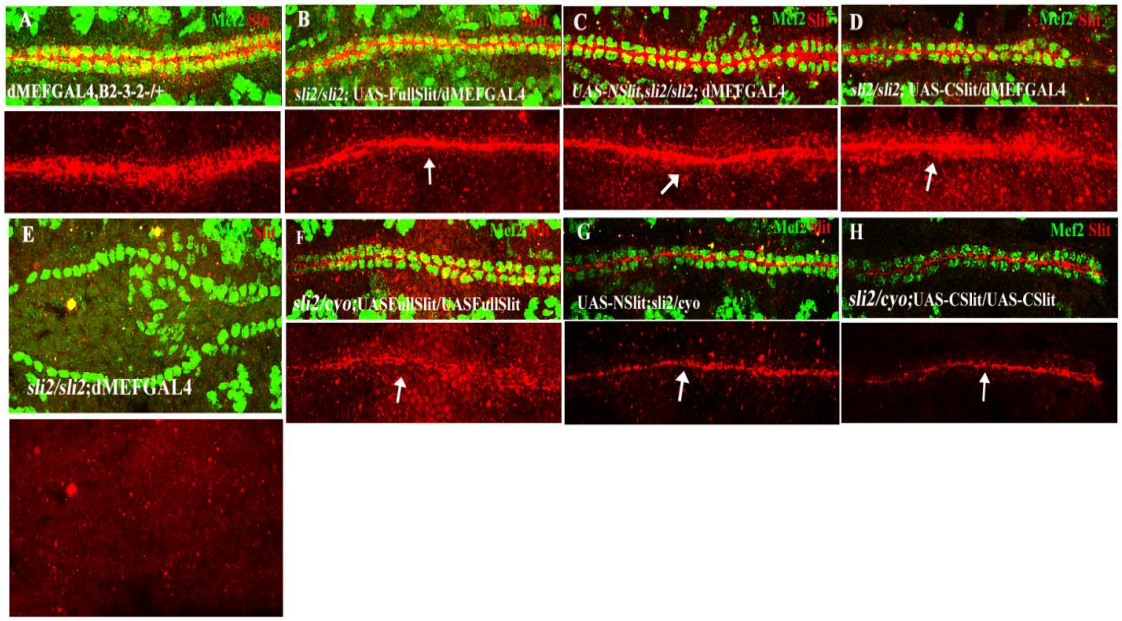

Figure 3.3c-Slit localization in embryos expressing the Slit cleavage products in the *slit* mutant background. The Slit cleavage products were expressed in a *slit* mutant background using a dMEFGAL4 driver. The cardial cells are shown in green and Slit localization shown in red. Wildtype Robo localization is shown in panel (A). The localization of Slit appears to be similar to wildtype in embryos expressing Full-Slit (B), N-Slit (C) and C-Slit (D) shown in arrow. A *slit* mutant embryo is shown in panel (E). Panel (F) (G) and (H) show embryos expressing the UAS transgenes in *slit* heterozygotes. The Slit labelling appears to be absent in *slit* mutants and the UAS-Slit embryos have a reduced Slit labelling due to the presence of only one functional copy of *slit* (shown in arrowheads). The samples were imaged at the same gain in confocal.

3.3d. *slit* mutants rescued using the Slit products driven by svpGAL4 driver have rescued Robo distribution.

Expressing Slit cleavage products using a dMEFGAL4 driver in the cardial cells in a *slit* mutant background can rescue the heart. The objective was to examine Slit distribution in embryos that express the Slit cleavage products in the svp cells only in a *slit* mutant background. From the data above it was seen that Slit domains are able to rescue the heart cells and Slit localization. Therefore, expressing the Slit domains only in the svp cells should result in a pattern of Slit localization that has the rescue phenotype only in the svp cells surrounded by the cells that have a *slit* mutant phenotype.

As previously mentioned, I performed immunohistochemistry on embryos staged 16-17 and labelled these with anti-βgalactosidase, anti-Mef2 and anti-Slit antibody. The localization of Slit in embryos expressing Full-Slit and N-Slit in svp cells in a *slit* mutant background appears normal. The pattern of Slit distribution appeared reduced in intervals in embryos expressing C-Slit, consistent with the presence of svp cells in these embryos. This data however needs to be replicated several times.

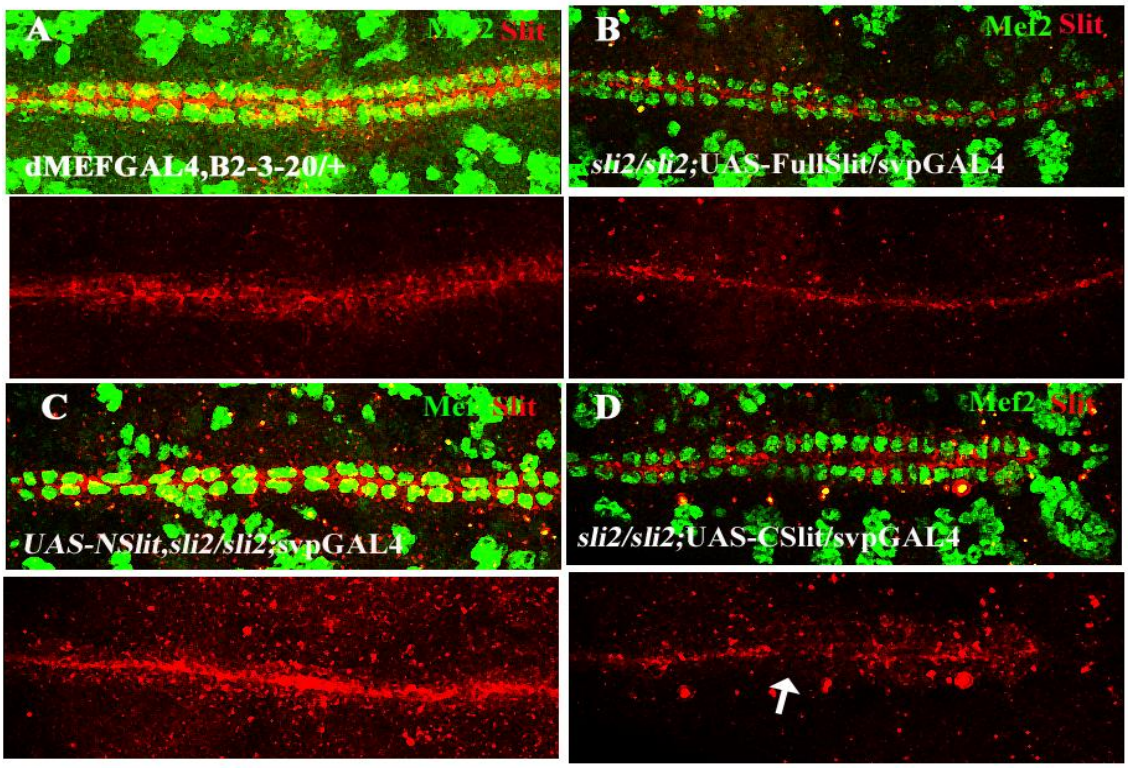

Figure 3.3d-Slit localization in embryos expressing the Slit cleavage products in SVP cells in a *slit* mutant background. The Slit cleavage products were expressed in a *slit* mutant background using an svpGAL4 driver that expresses the products only in the svp cells. The cardial cells are shown in green and Slit localization shown in red. Wildtype Slit localization is shown in panel (A). The Slit localization in the hearts of embryos rescued using Full-Slit (B) and N-Slit (C) appears to be normal. However the C-Slit rescue embryos had altered Slit expression (D). Slit is expressed in intervals in embryos expressing C-Slit similar to a pattern of the svp cells localization. Reduced Slit in C-Slit embryos shown by arrow.

DISCUSSION

The *Drosophila* heart is formed when two rows of cardioblasts migrate to meet at the dorsal midline and fuse to form the heart tube. This event is triggered by various signalling pathways. Slit, which is secreted by the cardioblasts, binds to Robo on the same cell leading to Slit-Robo signalling required for proper heart assembly. (MacMullin et al., 2006).

The proteolytic cleavage site in Slit indicates that it has the potential to generate three Slit fragments: N-Slit, C-Slit and Full-Slit. Among the fragments, N-Slit and Full-Slit contain an intact LRR which is known to be required for Slit-Robo signalling in the nervous system. The model in the heart for Slit-Robo signalling might be different from the nervous system. N-Slit and Full-Slit are predicted to function through binding to the Robo receptor in the heart. However, C-Slit, lacking the LRR domain, has the potential to function through alternate pathways due to presence of the EGF repeats and G-domain. The main focus of my thesis is to understand the biological function of C-Slit during heart formation.

4.1 The Slit cleavage products play a role in heart assembly.

According to the model in nervous system, endogenous Slit binds to Robo resulting in Slit-Robo signalling which is responsible for axon guidance. An intact LRR is required in the nervous system for proper Robo binding. Furthermore, previous studies

from our lab have reported that transgenes with deleted EGF repeats and G domain were more potent than Full-Slit in rescuing axon guidance in *slit* mutants (Battye et al.,2001).

To assess the requirement of Slit cleavage product in the heart, I over expressed the Slit domains in a wild type background. Based on previous data, we predicted that if the Slit cleavage products are in fact involved in heart assembly then interference from over-expression would lead to the Slit domains acting in a dominant negative manner. Slit products N-Slit and Full-Slit would be produced in excess due to the GAL4 and without any feedback mechanism excess protein would not be sequestered. This would compete with endogenous Slit for binding to Robo and result in excessive and less spatially restricted signalling, eventually leading to heart defects.

In accordance with our prediction, I observed heart assembly defects outlined in Table1.1 with all three Slit cleavage products. The over expression of the Slit domains in the heart produced heart assembly defects such as delayed closure, twists, gaps, blisters and misplaced cells. This indicates that all three Slit cleavage products play a role in the heart.

One possible explanation for the heart defects due to the overexpression of Full-Slit is that amplified signalling interferes with the endogenous Slit from binding to Robo. The excess product is not sequestered and might also bind to other complexes and prevent the signalling pathways from performing its function. However, a threshold of Slit-Robo binding is probably required for heart assembly. Some endogenous Slit might still be able to bind to Robo, which is why we don't see a complete disruption of heart formation.

I performed the over expression experiment using the dMEFGAL4 driver at room temperature and 29°C. A large number of embryos collected at 29°C were unfertilized therefore it was difficult to stain and collect embryos at stage 16-17. However, the dominant phenotype in the embryos collected and staged at 16-17 was delayed closure. At higher temperature the GAL4 is more effective which leads to an abundance of over expressed products. It is possible that due to the presence of excess production of the Slit domains they can more successfully compete with endogenous Slit to bind to Robo. In addition, the saturated Slit products can bind to other complexes required for heart assembly leading to a more pronounced heart defect.

To confirm the results achieved with dMEFGAL4, I repeated the over expression experiments using a svpGAL4 driver. The svpGAL4 driver expresses the Slit domains only in the svp cells. These embryos had heart assembly defects outlined in Table 1.2. The dominant phenotype was the presence of blisters.

As mentioned before, the svpGAL4 driver overexpresses the Slit domains in the svp cells. The overexpressed Slit domains bind to Robo in these cells leading to an aberrant signalling. This lack of proper Slit-Robo signalling leads to heart defects in certain cardioblasts. The blisters are possibly the result of defects in the affected contralateral cardioblasts. It is not clear whether the defects are limited to the svp cells. To further investigate if the presence of blisters correlates with svp cells, these embryos can be stained using an SVP antibody. However, our first prediction was confirmed that all three Slit domains are involved in heart assembly.

4.2 C-terminal Slit has a function during heart assembly.

The C-terminal region of the Slit protein contains EGF repeats, the laminin like G domain and a cysteine knot. The C-terminal domain has not been characterized yet and it is not known whether they play a role in Slit signalling. However, C-Slit the domains present in C-Slit has the potential to be signal through several pathways. To examine whether C-Slit has role in the heart I examined the phenotypes produced by the over expression of C-Slit in the cardioblasts.

The embryos with C-Slit overexpression using a dMEFGAL4 driver show irregularities in the heart assembly listed in table 1.1. The C-Slit over expression hearts have gaps, blisters, twists and most notably elongation of cells. In wild type embryos, the cardioblast nuclei are round, therefore the presence of these elongated cells signify an alteration of the morphology in these hearts. The over-expression of C-Slit may have disrupted Slit-Robo signalling, causing these heart assembly defects.

The change in morphology in the cells over-expressing C-Slit indicates that C-Slit might be involved in events such as ECM detection and cytoskeleton arrangement. One explanation, for this change in cell morphology is that in the absence of Robo binding, C-terminal Slit cannot be sequestered in these cells. Therefore it is activated and is free to initiate its own signalling through an alternative pathway.

A likely candidate for C-Slit alternate signalling pathway is the integrin pathway. In *Drosophila*, one of the 5 α integrins and one of 2 β integrins are necessary for heart development. The dimer of one α and one β integrin binds to laminin and localizes to the

sites where cells migrate and invaginate (Stark et al.,1997). Therefore, it is possible that when C-Slit is activated it is able to signal through the G-domain, in a manner similar to Laminin to the integrin pathway, resulting in a change in cell morphology.

My next aim was to examine whether over-expression of C-Slit can cause mislocalization of Robo in the heart. In addition to C-Slit, I looked at Robo localization in N-Slit and Full-Slit overexpression embryos. The hearts of embryos overexpressing Full-Slit, N-Slit and C-Slit were able to localize Robo properly in the lumen at stage 16-17. It is possible that a threshold of endogenous Slit and Robo binding is enough to localize Robo properly to the apical surface. The embryos were all imaged at the same gain. However, a more detailed quantitative analysis of the levels of Robo in these embryos is required to reach a proper conclusion.

4.3 Slit cleavage products can rescue the heart assembly in the absence of endogenous Slit.

slit mutant embryos have heart assembly defects and fail to localize Robo in the apical lumen. Therefore, my next step was to examine if Slit cleavage products can rescue heart defects in *slit* mutants.

According to our prediction, in *slit* mutants the expression of Full-Slit should be able to rescue the heart due to the presence of all Slit domains. N-Slit contains the LRR domain which is required for Robo binding and is also expected to rescue the heart. As predicted, heart assembly is rescued in embryos expressing both Full-Slit and N-Slit in a *slit* mutant background. However, the ability of C-Slit is to rescue the heart was of

interest to my thesis. C-Slit does not contain the Robo binding domain yet was able to rescue the heart. This further strengthens my hypothesis that C-Slit has biological activity in the heart. In the absence of Slit, C-Slit might be activating an alternate pathway and acting thorough it to rescue the heart. This theory is backed up by the presence of elongated cells in these rescue embryos which further confirms that C-Slit is indeed involved in pathways related to ECM and cytoskeleton remodelling.

In wildtype embryos, Robo is localized to the apical lumen at stage 16-17. The proper localization of Slit and Robo is necessary for proper heart lumen formation. In the absence of Slit, Robo is mislocalized leading to heart defects. Therefore, I examined whether the rescued hearts also had rescued Robo localization.

According to Kramer et al., (2008), expression of Full-Slit in *slit* mutants results in a rescued heart phenotype. However, Robo accumulates ectopically at the basal surface and Slit localizes basally and on the pericardial cells around stage 16 in these embryos (Kramer et al., 2008). I noticed a similar pattern of Robo localization in embryos rescued using Full-Slit where it was mislocalized in the basal and lateral surface along with apical localization. It is possible that Slit expression level is higher in embryos expressing Full-Slit which leads to ectopic localization of Robo. In addition, Robo localization in N-Slit rescue was normal with no lateral or basal deposition. Robo localization in C-Slit rescue embryos had some lateral and basal accumulation of Robo.

According to Battye et al., (2001), N-Slit is more potent in rescuing the nervous system compared to Full-Slit. It is possible that the truncated protein is more stable or has

a higher level of Slit expression in the cells. This could be a possible explanation for the difference in Robo localization in hearts of embryos rescued using N-Slit and Full-Slit .

C-Slit rescue embryos had a normal heart assembly phenotype but Robo was mislocalized in the heart cells. It is possible that due to the lack of LRR region in C-Slit rescued embryos Robo cannot bind to Slit. Robo is not being sequestered in these embryos leading to the accumulation of Robo in the basal and lateral surface of the cell.

A major caveat in my experiment was the presence of Slit labelling in the embryos rescued using N-Slit. The anti-Slit used in my experiment is a monoclonal antibody. It has a single epitope situated in a region present in the EGF5-EGF7 region. This region in only present in Full-Slit and C-Slit, therefore the Slit antibody is able to recognize these two domains. Our lab (Dr. Mihaela Georgscu), has previously performed western blot with the monoclonal antibody Slit in order to examine the sizes of protein recognized by the anti- Slit antibody. Slit antibody is able to recognize C-Slit and not the N-Slit in the western blot shown in Figure 4.1.

The presence of Slit labelling in embryos which express N-Slit in the absence of Slit was unexpected. The stock was tested by complementation test several times to eliminate any contamination. It is possible that Slit antibody is able to somehow recognize the N-Slit. The sequence of N-Slit is shown in Figure 4.2. To eliminate any the possibility of a problem with the stock the flies containing the N-Slit transgene should be sequenced. This would eliminate doubts about the integrity of the N-Slit stock.

FUTURE WORK:

The purpose of my thesis was to determine whether C-Slit has biological activity in the developing *Drosophila* heart. Over-expression of C-terminal Slit leads to heart defects. In addition, expressing C-Slit in *slit* mutants can rescue heart assembly. Together, these data suggest that C-Slit indeed has biological activity in the heart. However, the nature of C-Slit signalling is not known.

C-Slit contains EGF repeats and Laminin like G domain , both of which are functionally significant. The presence of these domains makes C-Slit a likely candidate for signalling through ECM ligands and integrin receptors. Scab, an integrin protein, has the potential to be involved in the C-Slit signalling pathway. Our lab has previously shown that embryos with one functional copy of Slit and one functional copy of Scab result in heart assembly defects (MacMullin et al., 2006). If C-Slit is indeed able to signal through the integrin protein Scab then expressing C-Slit in the transheterozygotes of *slit* and *scab* should be able to rescue the mutant phenotype.

For further experiments we can use immunohistochemistry to examine whether in the absence of C-Slit integrin is able to localize correctly in the heart. If C-Slit is signalling through integrins then absence of C-Slit might lead to mislocalization of integrins. A more molecular approach would be to perform a pull-down assay and examine whether C-Slit can physically interact and bind to integrin proteins.

C-terminal Slit can be studied in detail by looking at different proteins involved in the cell adhesion pathways. In addition, other parallel pathways can be studied to examine the contribution of Slit in the formation of the developing Drosophila heart.

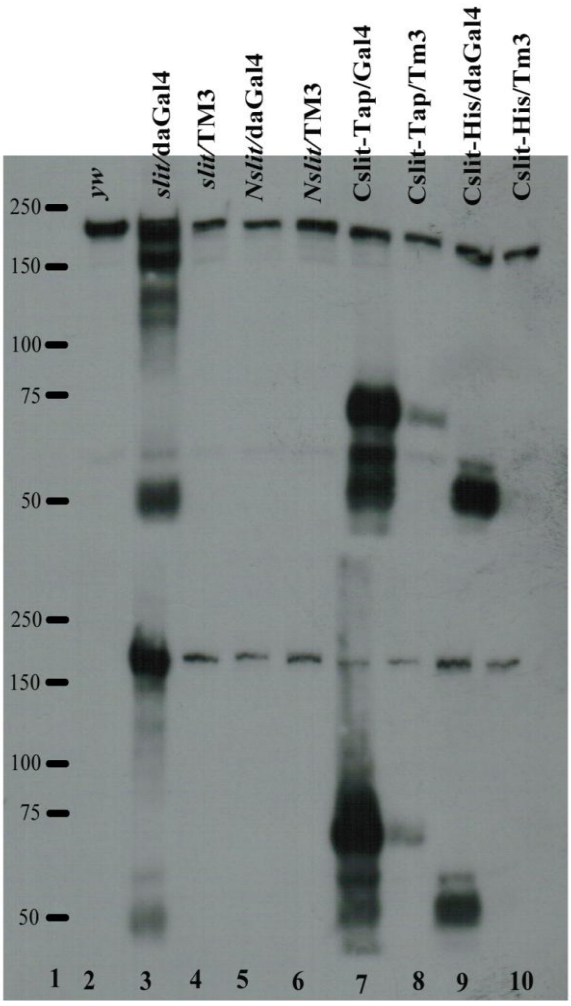

Figure.4.1-Western blot using the monoclonal Slit antibody. The anti-Slit antibody is only able to recognize Slit (200kDa) and C-terminal Slit (predicted to be about 55-60kDa). C-Slit is tap tagged in lane 7 and 8 and his tagged in lane 9 and 10. Slit antibody detects Slit in Lane 3 and 4. Lane7 and 8 shows the presence of tap tagged C-Slit detected by Slit antibody. Slit antibody does not detect N-Slit (140kDa) in lane 5 and 6. Therefore, Slit antibody is only able to detected Full-Slit and C-Slit.

> n-slit from 1 - 3397

ATGGCCGCGCCGTCCAGGACGACGTTGATGCCACCACCCTTCCGGCTCCAGCTGCGGCTACTGATACTACCCATCCTGCTACTCCTGC
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GGC ACC AAC TGC ACG GAC AAT ATT GAC GAC TGC CAG AAC CAC ATG TGC CAG AAC GGT GGA ACG TGC GTG GAC GGC
ATC AAC GAC TAC CAA TGC CGC TGT CCA GAC GAC TAT ACG GGC AAG TAC TGT GAA GGC CAC AAC ATG ATC TCG ATG ATG
TAT CCA CAG ACG TCG CCT TGT CAA AAC CAC GAG

ATT GGT ACC ATG GCC GCG CCG TCC AGG ACG AC

Mt primer

CTC CAA GTG AAT CAT GTC AGT GCA ACT AAA GGG GGG ATC TAG ATC

45 bp 69 C

Nrev primer

5-3 r c

GGT ACC TTT GTC ATC ATC ATC TTT GTA ATC AGG CGA AGT CTG TGG AT

ATC CAC AGA CTT CGC CT G ATT ACA AAG ATG ATG ATG ACA AA G GTA CC (kpnI)

48 bp 68 C

Figure 4.2: Sequence of N-terminal Slit used to contruct the UAS-NSlit stock.

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