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MUMMY REGULATES SLIT-ROBO SIGNALING TO MEDIATE AXON GUIDANCE IN *DROSOPHILA* EMBRYONIC NERVE CORD

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MUMMY REGULATES SLIT-ROBO SIGNALING TO MEDIATE AXON GUIDANCE IN *DROSOPHILA* EMBRYONIC NERVE CORD

by

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Dedication

I would like to dedicate this to my husband, Mr. Nitin Gregory and my parents for their unwavering support.

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During human brain development, precise connections have to be established to maintain the structural and functional integrity of the nervous system. Mutations in axon guidance molecules have been reported to cause various human nervous system disorders. The Slit-Robo signaling pathway is a well-studied evolutionarily conserved axon guidance mechanism. Slit is a chemo-repellant secreted by midline glial cells and binds to specific receptor Roundabout to mediate axonal repulsion. However, molecules involved in finer regulation of this pathway are unknown. I used the *Drosophila* embryonic nervous system as an experimental model to study the axon guidance mechanism during nervous system development.

Previous work from the lab has demonstrated the transport of Slit from the midline to the longitudinal axon tracts. A Slit transport mutant allele mmy^{slm} was discovered, revealing a novel role for the Mummy protein in regulating Slit-Robo signaling pathway during development.

Mummy regulates Slit post-translationally during nerve cord development. Lack of functional Mummy significantly reduces the transport of Slit from the midline to the longitudinal tracts and exhibits axon guidance defects. In mmy^{slm}, the longitudinal axon

tracts are closer to the midline and are not discrete suggesting that the lateral specification of the longitudinal tracts is affected. The guidance defects only manifest later in development since early patterning of the nerve cord is regulated by maternally deposited *mmy* mRNA. This signifies the importance of Mummy protein since a continuous supply of functional Mummy is required for proper patterning of the nerve cord. Mummy also regulates glycosylation of the Slit protein. We have demonstrated that glycosylation of Slit is not essential for binding to its receptor Roundabout. Glycosylation seems to play an important role in regulating the cleavage of Slit protein.

Mummy also regulates the Slit specific Robo receptor protein levels post-translationally. Absence of Mummy leads to significant down-regulation of Robo and Robo3 receptor proteins. Transport of Slit is independent of Robo-receptor protein suggesting that Slit may bind to a transporter molecule and transport it to the axon tracts. This study emphasizes the importance of Mummy in regulating the Slit-Robo signaling pathway to generate a functional nervous system during development.

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List of Abbreviations

AC	Anterior commissure
Dock	Dreadlocks
DCC	Deleted in Colorectal Cancer
Fas II	Fasiculin II
Fra	Frazzled
GAP	GTPase Activating Protein
GEF	Guanine nucleotide Exchange Factor
HGPPS	Horizontal Gaze Palsy with Progressive Scoliosis
HSPG	Heparin Sulphate Proteoglycan
LT	Longitudinal tracts
Mmy	Mummy
Net	Netrin
Pak	p21 Activated Serine Threonine Kinase
PC	Posterior commissure
PNS	Peripheral nervous system
Robo	Roundabout
Sos	Son of sevenless
VNC	Ventral Nerve Cord

MUMMY REGULATES SLIT-ROBO SIGNALING TO MEDIATE AXON GUIDANCE IN DEVELOPING DROSOPHILA NERVE CORD

Chapter 1: Mummy regulates both Slit and Robo to mediate axon guidance in the developing *Drosophila* nerve cord.

ABSTRACT

The nervous system is a highly complex structure and requires precise wiring of neuronal connections for it to function properly. During development, axon guidance is one of the critical events that help in the formation and maintenance of a highly intricate pattern of connections ensuring a functional nervous system. This is also important in the adults with brain injury where recovery requires re-wiring of the neuronal connections.

For this work, I have focused on the Slit-Robo signaling pathway during development. Slit is a chemo-repellant secreted by the midline glial cells, and interacts with its specific receptors Robo, Robo2 and Robo3 found on axon growth cones. We have previously reported the presence of Slit in the longitudinal axon tracts and in the midline, where it is secreted. The functional significance of Slit in the longitudinal tracts is not known and we have tried to address that in this work. We discovered a mutant in which transport of Slit from the midline to the axonal tracts is significantly reduced. On mapping the mutation, it was found to encode Mummy, the only predicted UDP-*N*-acetylglucosamine diphosphorylase in *Drosophila.* The mutant, mmy^{slm} also exhibits significant axon guidance defects in the ventral nerve cord. Analysis of the mmy^{slm} nerve cord phenotype reveals that the longitudinal axon tracts are located closer to the midline

when compared to that of the wild type along with multiple midline crossings. Also, the lateral positioning of the longitudinal tracts is disrupted.

We found that Mummy regulates Slit to mediate Slit transport and axon guidance mechanisms in developing embryonic nerve cord. Mummy protein is directly involved in Slit glycosylation in wild type embryos. Lack of functional Mummy in mmy^{slm} leads to non-glycosylated Slit, which does not get transported into the longitudinal axon tracts. On characterizing the non-glycosylated Slit, we found that lack of glycosylation does not affect its binding efficiency to its receptor, Roundabout (Robo). However, it was found that glycosylation affects the cleavage of Slit protein. In mmy^{slm}, the Slit cleavage products are significantly reduced when compared to that of wild type. This data suggests that binding of Slit to Robo does not necessarily activate axonal repulsion. Also, mmy^{slm} exhibits reduction of Slit cleavage products proposing a functional role for Slit protein cleavage in axon guidance.

Mummy also regulates Robo protein levels in wild type embryos. I have shown that Robo is down-regulated in mmy^{slm}. I have also observed down-regulation of Robo2 and Robo3 in mmy^{slm}. It was found that transport of Slit to the longitudinal axon tracts is a *robo/robo2/robo3* independent process. Morever, ectopic expression of Robo in mmy^{slm} does not rescue the axon guidance defects nor the transport of Slit. These data suggest that Mummy might regulate the Robo proteins through an un-indentified molecule, possibly by regulating its function through glycosylation. This study utilizes the *Drosophila* ventral nerve cord as a model system for studying the novel role of Mummy protein in the Slit-Robo signaling pathway during embryonic development. It is especially important since it will give further insight towards translational approaches for various axon guidance diseases and axon regeneration.

INTRODUCTION

The human brain has an extremely complicated neuronal wiring mechanism which accounts for its remarkable information processing ability. Each neuron makes connections with over a thousand target cells. There is an intricate mechanism which precisely connects every neuron, facilitating the proper functioning of the nervous system. Failure to make the correct synaptic connections in the brain is reported to cause a number of common neurological disorders ranging from Autism to Schizophrenia (Lewis and Lieberman, 2000; Eastwood et al., 2003; Anitha et al., 2008; Lin et al., 2009; Chen et al., 2011; Narayanan et al., 2011; Suda et al., 2011).

NEURON STRUCTURE

A neuron is an electrically excitable cell that can receive, transmit and process information. Electrochemical impulses are the mode of communication between neurons. Neurons differ based on their location and function. Each part of the neuron has an important role in the communication of information, but morphologically they have three distinct regions with specific functions (Figure 1.1):

1. Cell body: The cell body (soma) (Figure 1.1) is the region of the neuron where all the metabolic processes are carried out. The soma does not play an active role in transmitting neuronal signals. It includes the nucleus, where the genetic material is stored. It also includes the endoplasmic reticulum which synthesizes the proteins required by the cell; the golgi apparatus which packages products created by the cells and secretes them out of the cell.
2. Dendrites: The cell body gives out several short processes known as dendrites (Figure 1.1). They are required for receiving the incoming electrical information from other cells.

3. Axon: The axon (Figure 1.1) is a long process extending from the cell body. It transmits information received by the dendrites. It is the main conducting unit and conducts signals to other neurons. It can transmit signals through distances from 0.1mm to about 3m.

AXON GROWTH CONES:

Elongating axons end in protuberances called ***growth cones.*** Growth cones have two distinct functions: sensory activity, where it responds to directional cues from the environment and a motor activity, by which it leads to axonal elongation or retraction (Huber et al., 2003a; Dent et al., 2011). Structurally, the growth cone has three distinct regions: a central core that is rich in microtubules, mitochondria and a variety of other organelles; filopodia, which are thin extensions projecting from the body; and lamellipodia, which are motile and give the growth cone its characteristic ruffled appearance.

Filopodia are largely responsible for the sensory activity of the growth cone. They are rod-like, actin-rich, membrane-limited structures which are highly motile. The filopodial membrane contains receptors which can sense directional cues for the axon. The filopodia extend in length and sample the environment first, before the central core. Their motility allows them to extensively discern the environment and their flexibility allows them to navigate the neuron along the right path. The receptors on the filopodial membrane encounter axon guidance cues which stimulate the growth cone to advance, retract, or turn (Quinn and Wadsworth, 2008).

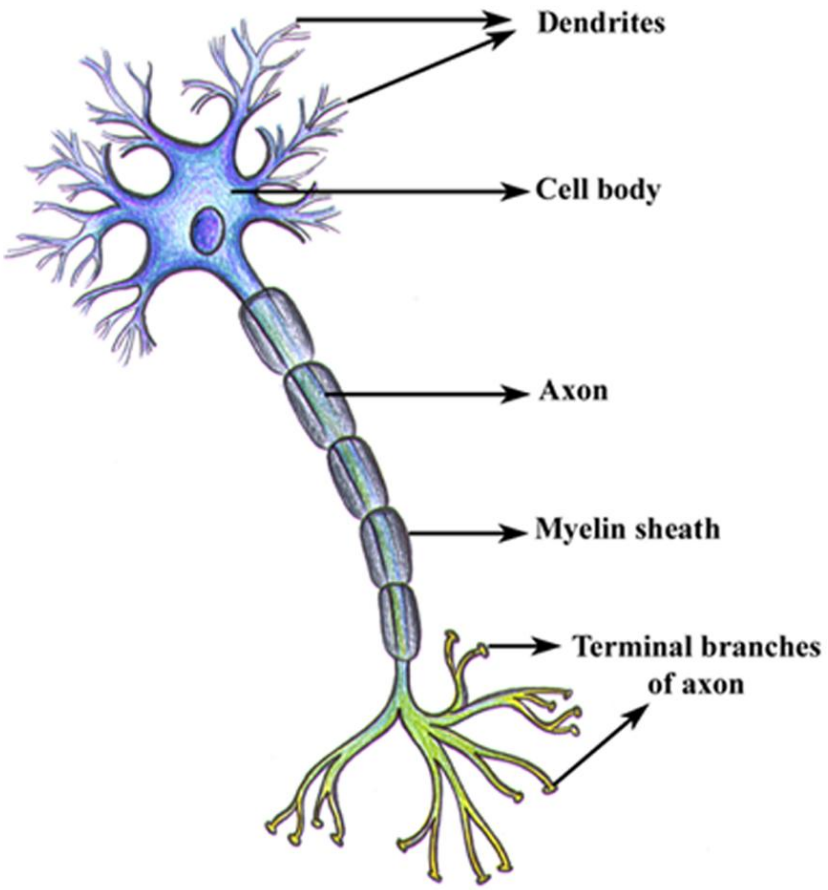

Figure 1.1: Structure of a Neuron. Pictorial representation of a neuron depicting the Cell body, Dendrites and Axon. Growth cones are represented at the tip of the axons.

AXON GUIDANCE DURING DEVELOPMENT

The basic function of the brain depends on how the neuronal connections are made. This usually begins during embryonic stage and continues till post natal development. It is highly regulated by specific developmental events which are in turn determined by definite molecular mechanisms. During embryogenesis, each differentiating neuron sends out axonal outgrowths with growth cones at the tip. The growth cones respond to the environment around it and navigate towards their preferred

target. This navigation is highly directed and precise. The growth cones perform this highly specific navigation by interacting with various molecular guidance cues in their surroundings. These diffused guidance molecules may be involved in short range or long range signaling and may function to evoke chemo-attractive or chemo-repulsive response in the axons (Varela-Echavarria and Guthrie, 1997).

CHARACTERISTICS OF AXONAL GROWTH:

The fact that the neuronal connections are so precise is surprising when taking into consideration the large distances axons must elongate to reach their targets. There are various mechanisms by which the human nervous system simplifies this complex process. To start with, the axons grow in a segmented manner in their search for a target site. Each segment (fragment) is focused on reaching a specialized intermediate site, which then gives further guidance information to reach the next intermediate target site and so on (Bate, 1976; Ho and Goodman, 1982; Keshishian and Bentley, 1983; Klose and Bentley, 1989; Klambt et al., 1991; Silver, 1993; Tear et al., 1993; Colamarino and Tessier-Lavigne, 1995b; Tessier-Lavigne and Goodman, 1996). The second important feature is that this process occurs in a stepwise manner. In the beginning, the axons grow in an environment which is free of other axons which is when guidance cues are most important. Later during development, the axonal projections occur in an environment which already has a large number of axonal projections. Many axons that develop later in development are known to follow pre-existing axonal tracts, which makes this complex process simpler (Tessier-Lavigne and Goodman, 1996).

GUIDANCE CUES AND MECHANISMS:

It has been proposed that long range chemo-attractants are secreted by the target cells which in turn attract the axons towards it (Cajal, 1892). This was supported by various in vitro experiments (Lumsden and Davies, 1983; Heffner et al., 1990; Fitzgerald et al., 1993). Later, it was shown that certain tissues secrete long range chemo-repellants which causes the neurons to grow away from them (Colamarino and Tessier-Lavigne, 1995a; Tamada et al., 1995). Short range axon guidance occurs by contact mediated mechanisms. It may be attractive or repulsive and could be a mechanism for guiding the axons through a specific path of permissive substrates. Therefore, it is accepted that there are four different guidance mechanisms: attractive long term guidance, attractive short term guidance, repulsive long term guidance and repulsive short term guidance (McKenna and Raper, 1988; Baier and Bonhoeffer, 1994). Based on these four mechanisms, there are a number of diffusible and non-diffusible factors which influence axonal paths. Studies have shown that these molecules and pathways are highly conserved through evolution.

AXON GUIDANCE DEFECTS

The molecules and the regulatory mechanisms that affect axon growth and guidance have been studied extensively in *Drosophila melanogaster, Caenorhabditis elegans, Xenopus laevis, Mus musculus* (Harris and Holt, 1990; Wadsworth and Hedgecock, 1992; Chien and Harris, 1994; Ogura et al., 1994; Otsuka et al., 1995; Culotti and Kolodkin, 1996; Zallen et al., 1999; Feinstein et al., 2004; Erskine and Herrera, 2007; Atkinson-Leadbeater et al., 2010; Gordon et al., 2010) . Diagnosis of axon growth and guidance defects has been difficult due to the limited sensitivity and specificity of the

tools. However, a number of human disorders are known to be a result of axon guidance defects which include:

CORPUS CALLOSUM DYSGENESIS

Corpus callosum is a thick band of neural fibres that connect the cortical regions of the left and the right brain hemispheres (Aboitiz et al., 2003). Evidence suggests that weakening of corpus callosum in aging adults contributes to their declining cognitive function (Zahr et al., 2009; Voineskos et al., 2012) where as thickening of the corpus callosum is correlated to intelligence (Hutchinson et al., 2009; Luders et al., 2011) and problem solving abilities (van Eimeren et al., 2008).

Corpus Callosum Dysgenesis is the condition where the corpus callosum is malformed or incompletely formed. Corpus Callosum Dysgenesis result from a number of inherited nervous system defects including primary axon proliferation, axon growth and guidance, and midline glial development (Engle, 2010). The disease presents differently in different patients. It has been reported that, in some cases, the topography of the nervous system is maintained which suggests that the axons are responsive to guidance cues even though they don't cross the midline (Utsunomiya et al., 2006). The molecular mechanism and the genes involved in this nervous system disorder have not been completely elucidated.

HORIZONTAL GAZE PALSY WITH PROGRESSIVE SCOLIOSIS (HGPPS)

Horizontal Gaze Palsy with Progressive Scoliosis is a genetic disorder which presents in patients with restricted horizontal gaze and scoliosis (Engle, 2010).It is an autosomal recessive trait due to defect in ROBO3 gene The phenotype has been reported

in multiple patients with different ROBO3 mutations, all of which result in complete loss of ROBO3 function (Jen et al., 2004).

In reference to these human diseases, there is a significant gap in understanding the mechanism of function. In this work, we hope to bridge this gap and attain new targets for chemotherapeutic approach.

DROSOPHILA AS A MODEL ORGANISM

The common fruit fly, *Drosophila melanogaster* is a small invertebrate insect that feeds on soft and rotting fruits. It is one of the most extensively studied organisms in biology. The organism is unique in that it has a number of developmental and cellular processes which are similar to humans and other vertebrates even though they diverged from a common ancestor around 700 million years ago (Adams et al., 2000). Other than these factors, *Drosophila* is inexpensive and easy to maintain. It has a short life span (~30 days in 29° C). This is advantageous in that a number of generations can be obtained in a period of few months. The short generation time is especially helpful when performing various genetic manipulations *in vivo.* They also produce a large number of offspring at a time and thus there is no dearth of research material. Furthermore, the *Drosophila* genome has been completely sequenced. Research in *Drosophila* has resulted in a number of well characterized genetic techniques (Jeibmann and Paulus, 2009). Time and tissue specific inducible promoters are available which gives more control on the over-expression model when compared to other model organisms. All of these characteristics make *Drosophila melanogaster* an ideal model organism to study neurodegenerative diseases like Parkinson Disease (Auluck et al., 2002; Deng et al., 2008), Alzheimer's disease (Crowther et al., 2006; Crowther et al., 2008; Jeibmann and Paulus, 2009; Bonner and Boulianne, 2011; Chakraborty et al., 2011) Spino-bulbar muscular atrophy (Katsuno

et al., 2002; Takeyama et al., 2002; Chevalier-Larsen et al., 2004; Pandey et al., 2007); neuromuscular diseases (Lloyd and Taylor, 2010) like Spinal Muscular dystrophy(Chan et al., 2003; Rajendra et al., 2007; Cauchi et al., 2008; Chang et al., 2008); cardiac diseases (Bier and Bodmer, 2004; Ocorr et al., 2007; Piazza and Wessells, 2011) among others.

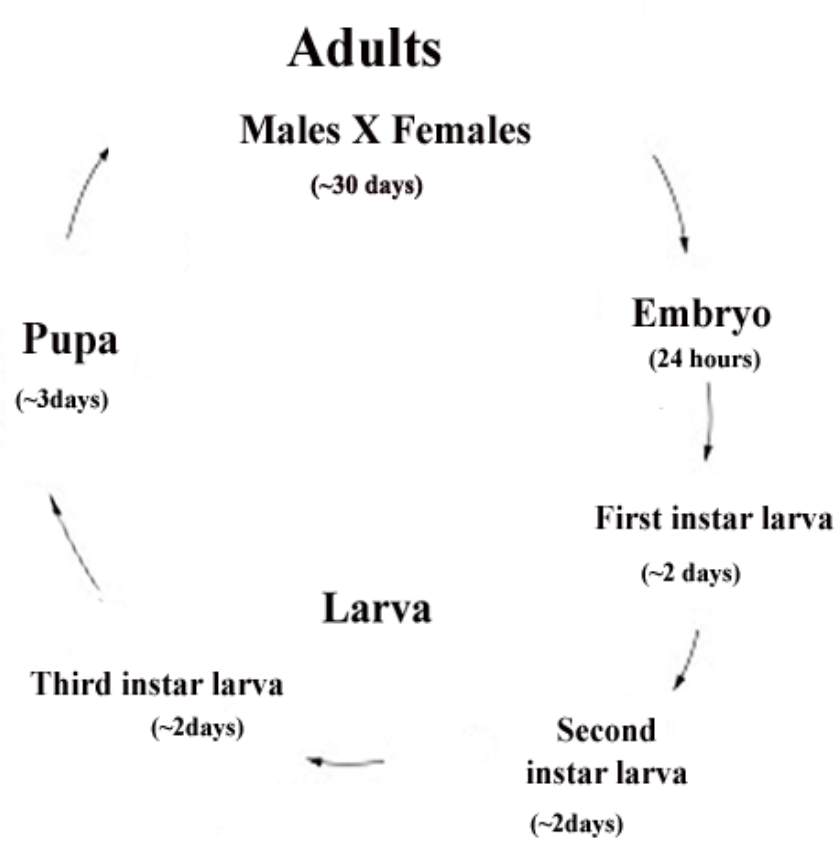

Figure 1.2: Life cycle of Drosophila Melanogaster. The different stages in Drosophila life cycle include embryo (~1day), the larval stage (~6days), the pupa stage (~3days) and the adult fly. All the four different stages of the Drosophila life cycle have been utilized as model system to study various vertebrate systems.

DROSOPHILA CNS MODEL TO STUDY AXON GUIDANCE

Organisms with bilateral symmetry are ideal to study axon guidance mechanism, since they exhibit both attractive and repulsive axon guidance mechanisms. In the developing Drosophila embryonic system, the ectoderm gives rise to the neurogenic precursor cells: the ventral region gives rise to the neuroblasts, and from the pro-cephalic region emerges the brain (Hartenstein, 1993). The fully developed Drosophila embryonic nervous system is divided into a) the ventral nerve cord (VNC), b) the peripheral nervous system (PNS) and c) the brain proper.

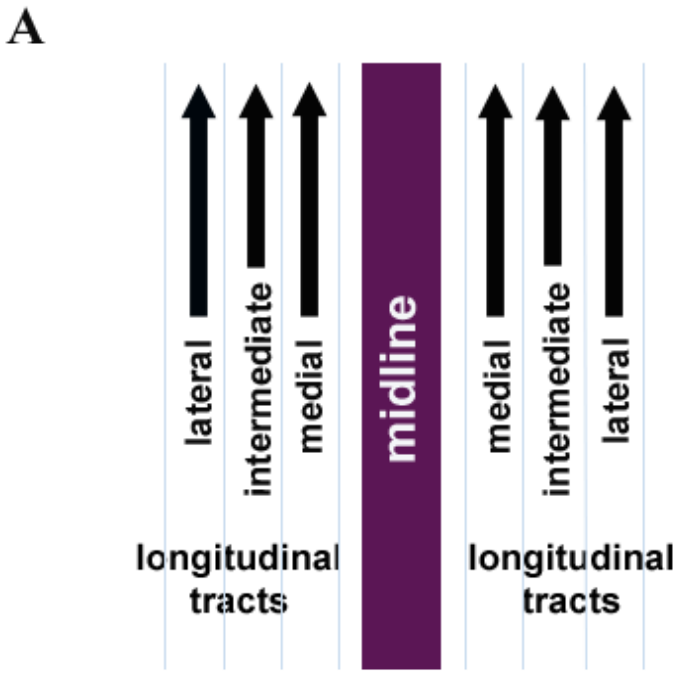

Figure1.3: Drosophila ventral nerve cord in wild type. Diagrammatic representation of the ventral nerve cord with central midline glial cells and longitudinal axon tracts on either side. The tracts are divided into medial, intermediate and lateral based on their proximity to the midline.

The embryonic ventral nerve cord is an ideal model to study the different molecules involved in proper guidance of growing axons. Drosophila ventral nerve cord has a central midline and has about 20 longitudinal axon tracts arranged on either side on either side of the midline. Based on its position from the midline, the longitudinal axon tracts are divided in to three sets of tracts: medial (located closest to the midline), intermediate and lateral (located furthest away from the midline). The midline cell population extends along the ventral surface of the embryo. In vertebrates, the developing spinal cord has a subset of specialized cells that give out chemotropic cues called the floor plate (Cajal, 1892). In Drosophila, the midline cells are analogus to the floor plate (Goodman et al 1991, Goodman et al 1993). The midline glial cells provide molecular cues for the neural axons.. The midline presents a repulsive environment to prevent the ipsilaterally projecting axons from crossing the midline (Guthrie, 1997). Several interneurons project their axons to the contralateral side by crossing the midline, which now presents as an attractive environment. After exiting the midline, these axons execute an orthogonal turn and join the longitudinal axon tracts (Colamarino and Tessier-Lavigne, 1995b; Kaprielian et al., 2001). These axons first require chemo-attractive cues to cross the midline, and chemo-repulsive cues to maintain their paths along the longitudinal tract along the midline (Kaprielian et al., 2001). Both the longitudinal axon tracts, which lie parallel to the midline and the commissural axon tracts which never cross the midline, grow in the same environment and are guided by the similar molecular mechanisms. The complexity of the signaling pathway is evident as both types of axons receive the same combination of attractive and repulsive cues, but choose different paths (Serafini et al., 1996; Zou et al., 2000; Charron et al., 2003; Long et al., 2004; Sabatier et al., 2004). Drosophila has a complex signaling pathway to guide the axons to the correct neuronal network and thus is a great model to study both repulsive and attractive growth cone navigation along with their relevant signaling pathways. Our research group utilizes

the Drosophila embryonic nerve cord as a model to study axon guidance signaling pathways.

Stage	Time	Developmental events
1- 4	0:00 - 2:10 h	Cleavage
5	2:10 - 2:50 h	Blastoderm
6 - 7	2:50 - 3:10 h	Gastrulation
8 - 11	3:10 - 7:20 h	Germ band elongation
12 - 13	7:20 - 10:20 h	Germ band retraction
14 - 15	10:20 - 13:00 h	Head involution and dorsal closure
16 - 17	13:00 - 22:00 h	Differentiation

Table 1: **Time Table of embryogenesis:** The table depicts the different developmental events that occur in the developing embryo [Adapted from flymove/university of Muenster].

We use “hours of development” rather than the more conventional “stage” to denote specific developmental time points. Table 1 is the conversion table for the different stages, the time of development and the different developmental events that

occur at that time. We adopted this method since different stages have different durations ranging from 20 minutes to several hours.

During development, the neurons send out axonal processes, which establish synaptic contact with other distant cells (neurons, muscles, glia). The synaptic contact helps in transfer of information which is crucial for the functioning of the nervous system (Sanchez-Soriano et al. 2007). For precise wiring of the neuronal networks, the growing axons have axonal processes which are responsive to guidance cues from the environment. These axonal processes have structures known as growth cones at the tip, which have receptors for the various chemo attractive and chemo repulsive ligands which guide the axons via conserved pathways (Tessier-Lavigne, 1994; Goodman, 1996; Tessier-Lavigne and Goodman, 1996; Harris and Holt, 1999; Seeger and Beattie, 1999; Van Vactor and Flanagan, 1999; Yu and Bargmann, 2001; Dickson, 2002; Huber et al., 2003b). Depending on the environmental cues, pathways regulating the cytoskeleton are activated. However, it is still unclear how the receptors choose between the attractive and repulsive cues. This interaction guides the growth cones towards or away from specific sites or regions.

The central role of midline in axon guidance was first observed by the Spanish neurobiologist, Santiago Ramon y Cajal. He proposed the existence of a chemotropic mechanism, where the commissural neurons are guided across the midline by chemo-attractive cues secreted by the floor plate (Cajal, 1892). Diffusible chemo-attractants are released by target cells to attract axons to them, while diffusible chemo-repellents are released by non-target cells to generate areas that axons avoid (Keynes and Cook, 1995). In Drosophila, Netrin and Slit signaling pathways act as the two main and opposing signaling pathways that specify the positioning of the longitudinal axonal tracts along the midline of the ventral nerve cord (Kidd et al., 1999). Netrin (Net) and its receptor Frazzled (Fra) mainly function as an attractant while Slit and its receptor Roundabout (Robo) function as an axonal repellant (Engle, 2010). The growth cones interact with

these molecular cues at the midline where it determines they determine whether to cross the midline or not. In *Drosophila*, the midline also appears to control the different growth cone properties including the number of functional receptors.

Netrin-Frazzled signaling pathway

One family of axon guidance cues is Netrin, a laminin-like protein ~70-80kDa. It is a secreted protein conserved through flies, worms and vertebrates (Hedgecock et al., 1990; Kennedy et al., 1994; Serafini et al., 1994; Shirasaki et al., 1995; Harris et al., 1996; Mitchell et al., 1996b; Deiner and Sretavan, 1999). It has an important axon guidance function which is conserved through the bilateral symmetric vertebrates and also has a critical role in attracting the axons towards the ventral midline of a developing CNS (Mitchell et al., 1996b). *Drosophila* has two analogs- Netrin A and Netrin B (Harris et al., 1996). They have been reported to be both chemo attractive as well as chemo repulsive (Hedgecock et al., 1990; Ishii et al., 1992; Chan et al., 1996; Wadsworth et al., 1996). In *Drosophila*, Netrins guide axons through their interaction with two different receptors- DCC (Deleted in Colorectal Cancer) family receptor Frazzled (Fra) (Figure 1.4) functioning as a chemo-attractant to guide the CNS commissural neurons and specific motor neurons (Harris et al., 1996; Keino-Masu et al., 1996; Kolodziej et al., 1996; Mitchell et al., 1996a; Winberg et al., 1998; Hong et al., 1999) and Unc5, functioning as a chemo-repellant to guide the motor neurons to exit the CNS (Keleman and Dickson, 2001). However, this repulsion is independent of the classical repulsive mechanism mediated by Slit in the midline.

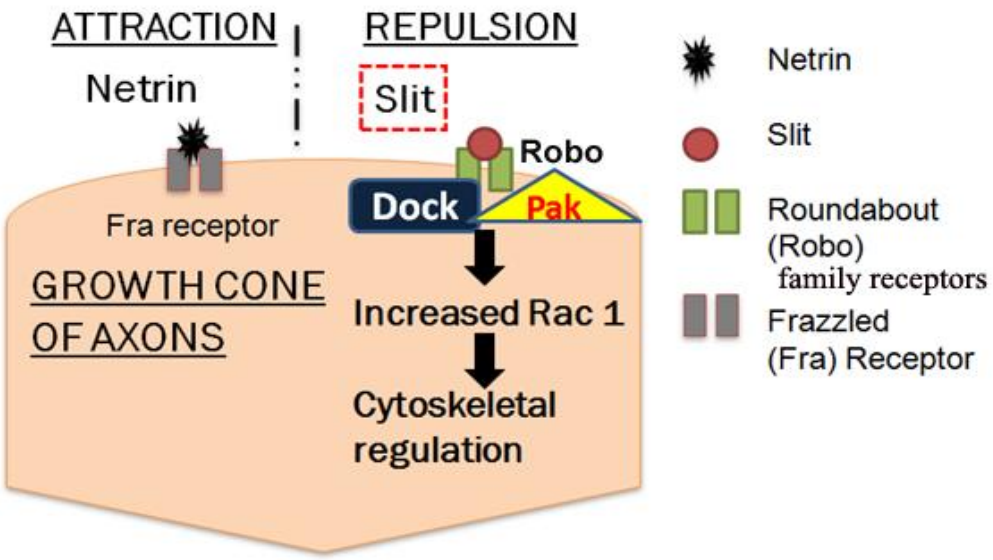

Figure 1.4: Schematic Representation of the two major axon guidance pathways in developing Drosophila ventral nerve cord: Binding of Netrin to Frazzled receptor on the growth cones activates axonal growth towards the target whereas Slit-Robo interaction leads to repulsion of the axons from the target.

Slit-Robo signaling pathway

Slit-Robo signaling pathway plays a very important role in axon guidance during development as well in regulation of cell migration, cell death and angiogenesis. It has also been recently reported to have important roles in the reproductive system (Dickinson and Duncan, 2010; Liao et al., 2010), cancer (Wang et al., 2003; Wang et al., 2008; Yang et al., 2010), angiogenesis (Bicknell and Harris, 2004; Fujiwara et al., 2006; Chen et al., 2010) among others.

During development, the growing axons are guided by the various guidance cues it encounters either by cellular contact or diffusion in the extracellular matrix, to reach its target. Slit bind to Robo receptor on the growth cones (Figure 1.4) and functions

primarily a chemo-repellant pathway and prevents the axon tracts from crossing the midline (Brose et al., 1999; Kidd et al., 1999; Li et al., 1999; Nguyen Ba-Charvet et al., 1999; Wang et al., 1999; Bashaw et al., 2000; Rajagopalan et al., 2000a; Simpson et al., 2000). *Drosophila* has a single Slit and three Robo isoforms.

SLIT AND ITS RECEPTORS

Slit protein: Slit is a 200kDa glycoprotein secreted by the midline glial cells. The first identified role of the *sli* gene was in patterning of the larval cuticle (Anderson and Nusslein-Volhard, 1984). Subsequently, it was discovered that Slit was released by the midline glial cells (Kidd et al., 1999) and its role in patterning the nervous system elucidated (Brose et al., 1999; Rajagopalan et al., 2000a).

The *sli* gene has mammalian homologs. In humans, there are three Slit homologs- *sli 1, sli2* and *sli3 (Itoh et al., 1998; Little et al., 2002).* Each slit gene encodes a putative secreted protein, which contains conserved protein-protein interaction domains (Rothberg and Artavanis-Tsakonas, 1992) including Leucine-rich repeats (Hohenester, 2008) and epidermal growth factor like repeats, similar to *Drosophila* Slit. *sli1* mRNA is found exclusively in the brain, *sli2* mRNA is present in the spinal cord while *sli3* mRNA is exclusively found in the thyroid. (Itoh et al., 1998; Brose et al., 1999)

Nusslein-Volhard et al first isolated mutants in the *sli* gene (Nusslein-Volhard et al., 1985), and its role in axon guidance was uncovered in 1999 by Kidd et al (Brose et al., 1999; Kidd et al., 1999) where they demonstrated the role of Slit in preventing the commissural axons from re-crossing the midline. Studies were also done in the vertebrate system where the repulsive function of Slit is conserved in motor neurons (Brose et al., 1999) and olfactory bulb axons (Li et al., 1999; Nguyen Ba-Charvet et al., 1999).

Structure of Drosophila Slit protein: Slit protein has a unique structure with different motifs that are required for its various functions. The amino terminus of the protein has four regions containing tandem repeats of Leucine Rich Region (LRR) with

flanking sequences on either side which are conserved (flank-LRR-flank). This is followed by two regions of Epidermal Growth Factor (EGF-like) repeats, a laminin G-like domain and a cystine knot domain (Figure 1.5) (Rothberg et al., 1988; Rothberg et al., 1990).

Processing of Slit molecule: Human Slit and *Drosophila* Slit have been found to be proteolytically cleaved by an unknown protease into a large N terminal fragment (~140kDa) and a smaller C terminal fragment (~60kDa).

Proteolytic processing of Slit was first reported by Brose et al (Brose et al., 1999), where they detected two fragments; one of 140kDa (N-terminal fragment) and the other of ~55-60 kDa (C-terminal fragment). The function of Slit processing is still unknown. Vertebrate studies have shown that cleavage of the Slit protein yields an active N-terminal fragment (Wang et al., 1999). It is thought that Slit cleavage is not fully required for Slit-mediated repulsion of the midline. This was based on the fact that ectopic expression of the non-cleavable Slit rescues the *sli* loss-of-function phenotype of midline repulsion (Coleman et al., 2010). Even though midline repulsion is rescued, the longitudinal axon tracts still remain collapsed which strongly suggests that Slit cleavage products may be required for fine-tuning of the axon guidance mechanism by Slit-Robo signaling pathway, which underlines the importance of this work.

Robo receptors: Robo receptors are single-pass transmembrane receptors found on the growth cones of developing axons. They are highly specific to Slit and have an important role during axon guidance. The Robo family of proteins was first identified in a screen for genes that regulated the midline crossing of the commissural axon tracts (Seeger et al., 1993; Kidd et al., 1998a). Four Robo proteins (Robo1, Robo2, Robo3/Rig1, vascular specific Robo4/magic Roundabout have been established in mammals (Kidd et al., 1998a; Huminiecki et al., 2002; Park et al., 2003)

Robo proteins belong to the immunoglobulin superfamily and have five Ig domains followed by three Fibronectin type III repeats (FNIII), a transmembrane portion, and a long intracellular tail containing up to four conserved cytoplasmic motifs (Figure 1.5). Robo2 and Robo3 are unique by the fact that they lack CC2 and CC3 (Conserved Cytoplasmic Domain motifs) which have been reported to be required to prevent midline crossings (Bashaw et al., 2000). Also, no catalytic domains have been observed in Robo2 and Robo 3 proteins (Chedotal, 2007).

There are three different Robo proteins in *Drosophila: robo, robo2* and *robo3*. Although they specifically bind to Slit ligand, they have different expression patterns in the longitudinal axon tracts. The medial axon tracts only express Robo, while the intermediate axon tracts express both Robo and Robo3. The lateral axon tracts express all three Robo proteins during early development but are differentially localized in the midline, by stage 16 or 13 to 22 hours of development (Rajagopalan et al., 2000b).

Heparan Sulphate Proteoglycans (HSPGs)

Heparan sulphate proteoglycans (HSPGs) are proteins with a proteoglycan core that is attached to heparin sulphate chains. They either exist as membrane bound proteins, or may be secreted. The roles of HSPGs have been extensively studied and can be classified broadly into cell adhesion regulatory molecules and signaling molecules (co-receptors) (Lee and Chien, 2004; Johnson et al., 2006; Van Vactor et al., 2006). In context of the Slit-Robo signaling pathway, they have been reported to stabilize the Slit homodimer by binding of heparin sulfate with the D4 domain of Slit (Figure 1.5) (Seiradake et al., 2009). In *Drosophila*, Robo and Slit have been reported to bind to an HSPG called Syndecan (Sdc) and affect Slit-Robo mediated repulsion (Steigemann et al., 2004; Johnson et al., 2006).

SLIT SIGNALING PATHWAY

Both Slit and Robo are multi-domain proteins. It has been reported that leucine rich repeat (LRR) of Slit and the immunoglobin-like (IG) domain of Robo are required for Slit-Robo binding (Nguyen Ba-Charvet et al., 1999; Battye et al., 2001; Chen et al., 2001). Studies have shown that all three Robo proteins compete for the same D2 site (on the Leucine Rich Repeat) on Slit protein (Howitt et al., 2004) whereas the Robo proteins bind to Slit through their Ig1 and Ig2 domains (Liu et al., 2004).

The Slit protein binds to its receptor Robo on the growth cones, activating downstream pathways (Figure.1.5). GTPase Activating Proteins (GAP) like CrossGAP/Vilse (Lundström et al., 2004; Hu et al., 2005) and Slit-RoboGAPs (SrGAPs) or Guanine nucleotide Exchange Factors (GEF) like Son of sevenless (SOS) (Yang and Bashaw, 2006) regulate the small GTPases of the Rho family which in turn control cytoskeletal rearrangement. It is known that Slit stimulation recruits a complex of SH3-SH2 adaptor protein Dreadlocks (Dock) and p21 activated Serine-theronine-kinase(Pak) to the Robo receptor and triggers an increase in Rac1 activity, which is an important cytoskeleton regulatory protein (Figure 1.5) (Wong et al., 2001; Fritz and VanBerkum, 2002; Fan et al., 2003; Matsuura et al., 2004). However, some axons do cross the midline and this is accounted for by expression of lower levels of Robo on their growth cones, which is regulated by Commissureless (Kidd et al., 1998b; Rajagopalan et al., 2000a; Keleman et al., 2002).

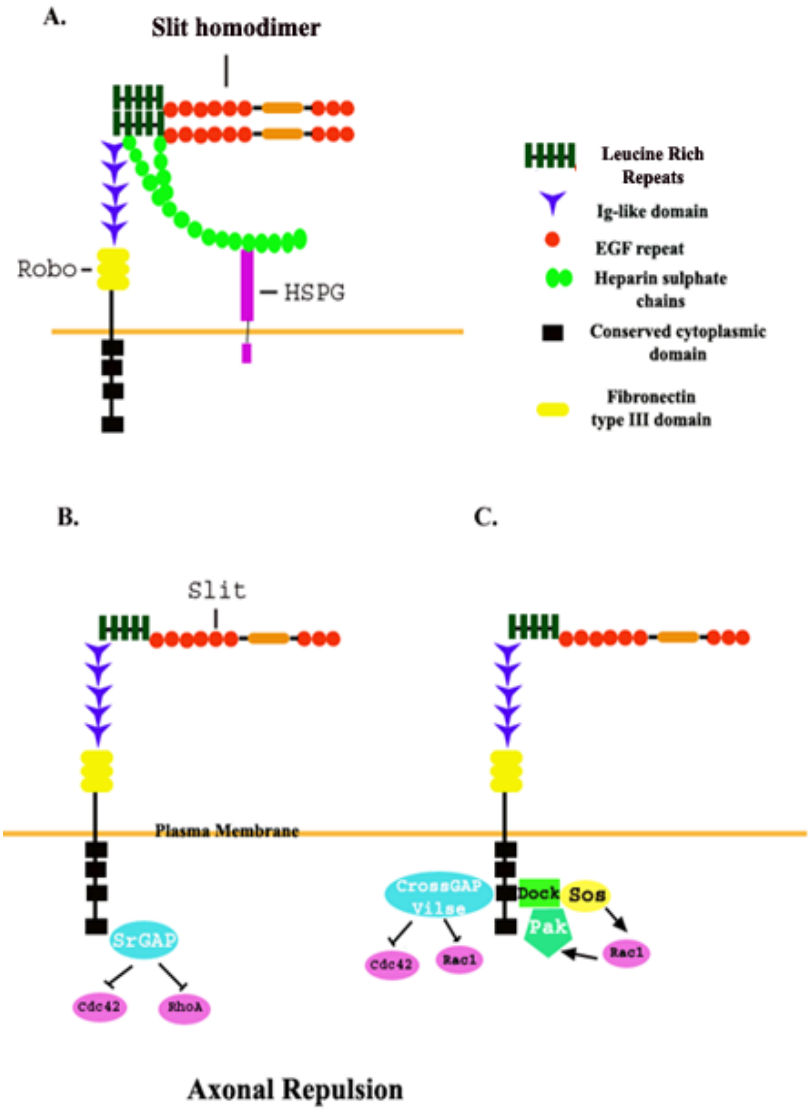

Fig. 1.5: Schematic of Slit Robo signaling: A) Slit binds to IG domain of the Robo receptor through the D2 domain. Heparin Sulphate proteoglycans (HSPG's) stabilize the Slit homeodimer while binding forming a ternary complex. B) On binding of Slit with Robo, the Slit-RoboGAP1 (srGAP1) binds to the CC3 domain of Robo and inactivates RhoA, Cdc42 mediating axonal repulsion. C)Slit-Robo interaction facilitate the binding of Vilse/CrossGAP to CC2 domain of Robo inhibiting Rac1, Cdc42. Recruitment of the GEF protein Son of Sevenless (Sos) through the binding of Dreadlock (Dock) to CC2-3 domain of Robo activates Rac1and p21 mediated kinase activity which controls repulsion and cytoskeletal dynamics.

The Slit signaling pathway has been implicated in preventing the axonal tracts from projecting into the midline (Rothberg et al., 1990; Brose et al., 1999; Kidd et al., 1999; Simpson et al., 2000; Long et al., 2004; Engle, 2010). The Slit signaling pathway is also required for the proper positioning of the longitudinal tracts from the midline. The specific expression of the different *robo* genes in different tracts is responsible for the lateral positioning from the midline (Rajagopalan et al., 2000b). It was hypothesized by Rajagopalan et al, that Slit is released in a gradient manner from the midline glial cells, which interacts with the Robo receptors on the axonal tracts and specifies their lateral positioning. In this scenario, Robo receptors on the axonal tracts at the furthest end would interact with the lowest level of Slit, the intermediate tracts would interact with a slightly higher level of Slit, and the medial tracts would interact with the highest level of Slit (Rajagopalan et al., 2000b). The over-expression of Slit protein at the midline did not alter the lateral positioning of the axonal tracts (Kidd et al., 1999). However, a gradient of Slit has not been observed from the midline**.** We have repeatedly observed and reported the presence of the Slit in the longitudinal tracts (Bhat et al., 2007), which I believe contributes to the lateral positioning of the longitudinal axon tracts. However, there is a significant gap in our understanding of the different steps between movement of Slit from midline to its interaction with Robo receptors on the growth cones.

We are ideally suited to study this problem since we have discovered the mutant mmy^{slm} in which the Slit protein is not transported out of the midline. This phenotype also involves an absence of Slit protein in the connectives and the commissures. Further, the axon tracts were found to be disrupted and present closer to the midline when compared to wildtype. These observations make this phenotype a suitable model to study the functional significance of Slit movement in the longitudinal axon tracts. Mummy has a central role in glycosylation (Araújo et al., 2005; Schimmelpfeng et al., 2006; Tonning et al., 2006), so I will give a background on Post translational modifications of proteins.

Post translational Modifications of Proteins

The primary structure of a protein obtained from the human genome is not sufficient to decipher the various different functions. After translation, the resultant product is an unfolded polypeptide which is not functionally active. Usually need to attain a specific molecular structure occur by folding for the protein to carry out its function. Protein post translational modification may include cleavage of the protein, or addition of functional groups or molecules to newly synthesized proteins to aid in folding or for its biological regulatory function. They may occur in different cellular compartments and usually include enzymatic modification of the nascent protein. The modifications may include phosphorylation, glycosylation, ubiquitination, nitrosylation, methylation, acetylation, lipidation and proteolysis (Seo and Lee, 2004). These modifications determine the tertiary and quaternary structure of the protein.

GLYCOSYLATION AS A POST TRANSLATIONAL MODIFICATION:

Several eukaryotic proteins have sugar molecules (oligosaccharides) covalently attached to specific amino acids. These post translational additions are essential for protein folding, trafficking, biological activity, half-life, and solubility (Stanley, 1992; Varki, 1993; Hounsell et al., 1996). Protein glycosylation may be classified into four different groups depending on where the oligosaccharides are attached to the protein chain. They are O-linked glycosylation; N-linked glycosylation, C-mannosylation and glycophosphatidylinositol (GPI) anchor attachments.

Since Mummy has been implicated in N-linked glycosylation of Drosophila proteins (Eisenhaber et al., 2003; Schimmelpfeng et al., 2006), I shall discuss N-linked glycosylation here.

***N-linked glycosylation*:**

A protein is classified as N-glycosylated when the sugar moieties are attached to the amino group of an asaparagine. This post translational modification is thought to occur in the endoplasmic reticulum and has significant effect on protein folding. The consensus site for N-glycosylation is Asn-X-Ser/Thr/Cys (Gavel and Heijne, 1990; Miletich and Broze, 1990) . It should be noted here that the consensus site alone is not sufficient for glycosylation, but it is a pre-requisite for N-linked glycosylation.

MUMMY

Mummy is a metabolic enzyme required for housekeeping activity in the developing embryo (Araújo et al., 2005; Schimmelpfeng et al., 2006; Tonning et al., 2006). The *mmy* gene encodes a protein with significant homology to the eukaryotic UDP-*N*-acetylglucosamine phosphorylases and is the only predicted *Drosophila melanogaster* UDP-*N*-acetylglucosamine diphosphorylase. This enzyme is required for the synthesis of UDP-*N*- acetylglucosamine, the activated form of N-acetylglucosamine, which is the key precursor necessary for N- and O-linked glycosylation (Illustration 1.1) (Araújo et al., 2005). It is also a known intermediate for chitin and glycan synthesis. It has been reported that functional Mmy protein is required for the formation of glycosylphosphatidylinositol (GPI) linker that anchors many cell surface molecules to the plasma membrane. *Mmy* has an analogous gene is humans, *UAP-1* (UDP- N-Acetylglucosamine Pyrophosphorylase 1) which is primarily produced in the human sperm (Diekman and Goldberg, 1994; Mio et al., 1998).

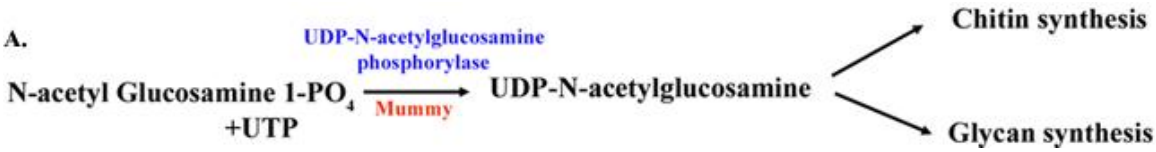

Illustration. 1.1:Mummy has a central role in the Glycosylation pathway: Mummy is a central enzyme required for chitin and glycan synthesis and has a reported role in glycosylation.

MATERNAL DEPOSITION:

Oogenesis is a process by which the Drosophila female gametes are formed, developed and matured. Similar to other organisms, the female fly loads the developing oocyte with mRNA and proteins that are necessary for its fertilization and embryo survival until zygotic transcription begins. This is usually around 2 hours into development. We have reported that there is significant maternal contribution of *sli* mRNA, but not protein in embryos (Bhat et al 2007). *Mmy* is a maternally deposited gene since high levels of transcript are seen in freshly laid eggs (Araújo et al., 2005; Tonning et al., 2006).

Based on the literature review and our preliminary observations, *I hypothesize that Mummy has an important regulatory role in the Slit-Robo pathway. We will analyze how Mummy regulates the transport of Slit from midline to the longitudinal tracts and elucidate the mechanism by which mutation in mmy leads to an axon guidance defect in the Drosophila ventral nerve cord.*

MATERIALS AND METHODS

Mutant strains and genetics:

The flies were maintained and crossed at 22°C and 28° C (as indicated) using standard *Drosophila* maintenance techniques. We generated various mutant combinations using genetic approaches. The following alleles were utilized through the course of the project: *w1118;mmy^{1}/Cyo twi-GFP*, *w1118;mmy^{slm}/Cyo twi-GFP*,*w1118;mmy^{slm}/Cyo kr-GFP;w1118;sli^{2}/Cyo twi-GFP*, *w1118;sli Df/Cyo twi-GFP*, *w1118;mmy df/Cyo twi-GFP* (from Bloomington), *w1118;robo 1df/Cyo twi-GFP(BL#27358)*, *w1118;robo 2;robo3 df/Cyo twi-GFP (BL#*24629), *w1118;*UAS-*robo*/UAS-*robo* (Insertion on X chromosome), *w1118;Gla/Cyo-twiGFP,* and *OR/R.*

We also generated a new set of fly lines with UAS-*mmy* transgene in different chromosomes, where Mummy protein can be over-expressed with specific driver of interest.

The gene of interest was over-expressed ectopically using elav gal 4 (pan neural inducible Gal 4).

To identify homozygous mutant embryos, we used *w1118;Gla/Cyo twi-GFP* and picked the GFP negative homozygous embryos. Embryos were staged as described by Weischaus and Nusslein-Volhard (Wieschaus, 1986)

Whole mount immunohistochemistry

Embryo whole mount Immunohistochemistry was performed using the standard techniques with slight alterations based on the antibody used. The embryos were collected for a specified amount of time, aged and dechorionated with 50% bleach for about 5 minutes. The embryos were washed under tap water and fixed using n-heptane and formaldehyde. Two fixing solutions were used based on the antibody specifications:

E-fix (0.8 mL Common fixation buffer: 0.1 M PIPES 2 mM EGTA, 1mM MgSO4, pH= 6.9-7.0, 1ml n-heptane for 30seconds, 0.2 ml formaldehyde for 22 minutes) used for anti-Robo (1:5,DHSB), anti-Robo2 (Santa Cruz Biotechnologies,1:20), anti-Robo3(14C9,DHSB); K-fix (0.8 mL n-heptane and 0.8mL formaldehyde for 6 minutes) for anti-BP102 (DHSB,1:10) anti-Sli-C (DHSB, 1:25), anti-Sli N (see (Bhat et al., 2007), anti-Fasiculin II (DHSB, 1:5). Alkaline Phosphatase or DAB-conjugated secondary antibodies were used.

The vitelline membrane was "popped off" by rapid shaking in the methanol-heptane interface.

The fixed embryos are treated with 30% hydrogen peroxide solution for 15 minutes to inactivate the endogenous peroxidase enzymes. The treated embryos are rehydrated, and permeabilized in PBST (PBS, 0.05% TritonX). The embryos are blocked in the blocking solution (PBST, 5% NGS, 1% BSA) for 2 hours at room temperature. After blocking, they are incubated in primary antibody overnight at room temperature, washed and treated with secondary antibody for 2 hours at room temperature. In case of HRP conjugated secondary antibodies, the embryos are treated with 0.5mg/ml DAB (3,3'-diaminobenzidine) in PBSTx for 15 minutes. The color reaction is developed by adding 3% hydrogen peroxide solution in ~3-5 minutes. This reaction can be halted by washing the embryos with PBSTx thrice before suspending them in 70% glycerol in PBS. The embryos are then mounted between coverslips and observed under ZIESS Axioplan2 microscope.

Images were prepared using Adobe Photoshop.

βgalactosidase staining

βgalactosidase staining is used to determine the area of expression of driver fly lines by detecting the yeast activator transcription activator protein GAL4. The embryos are collected over 24 hours, dechorionated with 50% bleach solution and rinsed with

water. The embryos are transferred into a depression slide and then fixed with 0.5ml heptane in a depression slide for 15 minutes at room temperature till they turn slightly yellow. The depression slide is covered with a glass slide to prevent evaporation of heptane. After fixing, excess heptane is removed by blotting it with filter paper. The embryos are suspended in PBS and transferred into a micro centrifuge tube. The PBS is removed and the embryos are suspended in 300 µL of staining solution without X-gal at room temperature for 5 minutes. The staining solution without X-gal is removed and replaced with staining solution containing X-gal. The embryos are incubated from 2hr at room temperature. The staining solution is removed and the embryos are rinsed by vortexing once in 70% ethanol and then in 100% ethanol. The embryos are stored in 90% glycerol at 4°C and observed under the microscope.

Generation of transgenic fly lines:

Gold standard full length cDNA for *mmy* gene was obtained from Berkely Drosophila Genome Project (LD24639). The *mmy* cDNA was initially inserted in a POT2 vector. The multiple cloning sites of pot2 vector include sites for EcoRI(5') and XhoI(3'). The mmy cDNA doesnot have Xho I or EcoRI restriction sites in the coding sequence. These enzymes were used to digest and isolate the mmy cDNA from pot2 vector. The resulting mmy cDNA has a sticky EcorI end (5') and XhoI end (3').

In *Drosophila*, transgenic lines are created using pUAST *P*-element vector. The pUAST vector is also digested with EcoRI/XhoI (NEB) to create compatible ends. A ligation process was performed to insert the *mmy* cDNA into the pUAST vector. The resulting DNA was transformed in to colonies, and the ampicillin resistant colonies were checked for inserts of the corresponding base pair units. The directionality of the pUAST-*mmy* obtained was double checked by digesting it with BglII/EcoRI(NEB). The enzyme digestion was analyzed for the correct molecular size DNA fragments to ensure that *mmy*

cDNA was inserted in the right direction. The colony is grown in LB media, plasmid DNA isolated and then and sent in for embryonic injection to generated transgenic fly lines. We obtained UAS-*mmy* transgenic fly lines with insertions in 2nd, 3rd and X chromosome.

Generation of triple *robo* mutants

One of the major advantages of using Drosophila as a model system is the flexibility in genetic manipulation. The Bloomington Drosophila Stock center had two fly lines: one deficient for *robo* gene, and the other deficient for *robo2 and robo3* genes. However, all the three genes are on the same chromosome. So we crossed *robo* Df flies to *robo2,3*Df flies and screened the female progeny for recombinants. Recombined fly lines were few since all the three Robo proteins play an important roles and it is genetically disadvantageous to have an organism null for all three *robo* genes. Finally, two separate recombinant fly lines were generated and propagated on the second chromosome; they were reconfirmed by examining the phenotype and staining for Robo proteins.

Western blotting experiments

For western blot analysis, proteins were extracted into lysis buffer (homozygous mutant embryos were recognized by lack of GFP expression), separated by electrophoresis, and protein levels detected using anti-Sli N (see Bhat, 1:50000) , anti-Sli-C (DHSB, 1:100), anti-Robo (DHSB, 1: 40) and detected with HRP conjugated anti-Rabbit (1:20000), HRP conjugated anti-Mouse (1:20000). Anti-Tubulin antibody (Abcam, 1:4000) is used as a loading control.

DESIGN

For western blot analysis, 30 embryos were collected (homozygous mutant embryos were recognized by lack of GFP expression) under the microscope, homogenized in 37.5 µL lysis buffer (0.15 M NaCl, 0.02 M Tris pH=7.5, 0.001M EDTA, 0.001 M MgCl2, 1% Triton-X-100 and PIC) and kept on ice for 10 minutes. The lysed protein is centrifuged for 5 minutes at 13,000 rpm, the supernatant is collected and diluted with 12.5 µL 4X Laemelli sample buffer. The protein sample is boiled in water for 10 minutes and kept in 4°C for 10 minutes. Equal amount of lysed protein 20 µL (15 embryos per lane) was loaded on to a 4-12% SDS-PAGE gel. The separated proteins are transferred into a Nitrocellulose (NT) membrane (Whatman). The efficiency of transfer was determined by Ponceau S staining. The membrane is blocked in 5% milk (5% milk in 1% BSA for anti-Robo) for 2 hours at room temperature, primary antibodies (anti-Slit N 1: 50000, generated in the lab; anti-Sli C 1:100, DHSB; anti-Robo 1:40,DHSB) were incubated overnight for 4°C and washed with PBST (PBS+0.02% Tween20). The Nitrocellulose membrane is then incubated with HRP-conjugated secondary antibodies (anti-Rabbit 1:20000, anti-mouse 1:20000; anti-mouse 1:20000 respectively) for 2 hours at room temperature and washed with the wash buffer. Proteins levels are detected by the chemi-luminiscent ECL reaction (Thermo Scientific). The signal obtained was scanned and intensities are analyzed using the software AlphaEaseFC.

Co-immunoprecipitation

Immunoprecipitation experiments were conducted to determine the ability of unglycosylated Slit to bind to Robo. Since there is a reduction of Robo protein levels in mmy^{slm}, we sought to normalize the Robo protein levels in both samples to evaluate the binding ability. The protein reduction in mmy^{slm} was determined by quantification of protein levels from the Western Blots and scaled up.

Wild type (200 embryos) and *mmy*^{slm} (500 embryos) were aged at room temperature (~12-14 hours), selected under the microscope and homogenized in 37.5µL of ice-cold lysis buffer 50mM HEPES (pH 7.2), 100mM NaCl, 1mM $MgCl_2$, 1mM $CaCl_2$, and 1% NP-40 (Banerjee et al., 2010). The lysates were incubated on ice for 30 minutes, before centrifuging at 15,000X g for 30 minutes at 4°C. 30 µL of the supernatant was used as starting material for each IP reaction using the Catch and Release v2.0 Reverse Immunoprecipitation System (Millipore #17500). The columns were washed with 1X Wash buffer thrice, before incubating the protein sample overnight with anti-Slit (1: 20) at 4°C. The columns were washed three times with 1X Wash buffer, before elution in 60 µL of PBS-based elution buffer. The proteins were then resolved on SDS-PAGE and immunoblotted with anti-Robo.

Gal4-UAS system

The Gal4-UAS system in *Drosophila* is a well-characterized technique to study ectopic expression of a specific gene product. It contains two parts: the Gal4 driver, which encodes a yeast transcription activator Gal4; the **U**pstream **A**ctivator **S**equence (UAS), a promoter region that is specific to Gal4. Gal4 binds to the UAS sequence attached to the gene of interest and activates the gene. Since Gal4 is not a *Drosophila* protein, its expression is controlled by a native driver gene (Brand and Perrimon, 1993). Depending on the specificity of the driver, Gal4 can be expressed in a particular set of cells. In this subset of cells, the Gal4 binds to the UAS promoter and activates the gene of interest, thus generating over-expression model system. The specificity of over-expressing the gene product in relevant cells helps focus the research question and minimize any back ground effect.

For ectopic expression of a specific protein, flies carrying specific UAS driver are crossed to transgenic flies (with a specific UAS-gene insert). The progeny will have both

the Gal4 driver as well as the UAS responder. Thus, Gal4 driver can drive the expression of a specific gene of interest in a specific pattern generating a over-expression model.

All the UAS-Gal4 crosses are performed at 26 °C.

Polymerase Chain Reaction

Embryos from the mutants were collected and dechorionated in 50% bleach. The embryos were washed with water. Around 100 homozygous embryos were collected (GFP negative) and lysed in lysis buffer (0.15 M NaCl, 0.02 M Tris pH=7.5, 0.001M EDTA, 0.001 M MgCl2, 1% Triton-X-100 and PIC). The DNA is cleaned by phenol: chloroform and precipitated with ethanol. It is re-suspended in TE buffer (0.1M Tris, 0.01M EDTA pH-7.4). Primers for *mmy* gene was designed based on the gene sequence and synthesized by Sigma-Aldrich. These primers were used for the PCR reaction to dissect the site of mutation. The reaction conditions were as follows: 95°C 3 minutes, 95°C 45 seconds, 58°C 30 seconds, 72°C 1 minute 30 cycles, 72°C 10 minutes. Forward and Reverse primers for each set of PCR reactions are denoted below.

Primer set 1- 5’ AATCAACAGCCGCACGCA 3’
5’ AGGTGCCACAGTCCAAGAAACG 3’

Primer set 2- 5’ TTATGGCAGGCGGACAAG 3’
5’ AGTGGAGCCGTCAGACAAC 3’

Primer set 3- 5’ AGGGCAAGCTAATCTCCATTG 3’
5’ GCCTTCCACATTGCTGCTG 3’

Primer set 4- 5’ TATCGGCTACTGCGTGCA 3’
5’ CAGCAAACGGAACCTCCA 3’

Primer set 5- 5’ GAGGAATCTATCGGGCTATGA 3’
5’ TCCATCGCCTGCACAAGAAGT 3’

Primer set 6- 5’ TTCGAGTTCGCCCAGAAGTTC 3’
5’ GATGGACGGATAGTAGTCG 3’

Real Time Polymerase Chain Reaction

Wild type and *mmy*^{slm} embryos were collected and aged (12-14 hours). They were dechorionated in 50 % bleach and washed with water. 150 wildtype and 150 homozygous mutant embryos were selected under the microscope and total RNA extracted using the RNaqueous Kit (Ambion). The resulting RNA extract was DNase treated and quantified using Nanodrop Spectrophotometer (Nanodrop Technologies) and analyzed on RNA Nanochip using Agilent 2100 Bioanalyzer (Agilent Technologies). cDNA was synthesized using 1 µg of total RNA in a 20 µL reaction using the Taqman Reverse Transcription Reagents Kit (ABI). Reaction conditions were as follows: 25°C, 10 minutes, 48°C, 30 minutes and 95°C, 5 minutes. Primers were designed and synthesized by the Molecular Genomic Core facility at UTMB. Real-time PCR were done using 1.0 µL of cDNA in a total volume of 20 µL using the Faststart Universal SYBR green Master Mix (Roche, #04913850001). RpL32 was used as endogenous control. All PCR assays were performed in the ABI Prism 7500 Sequence Detection System under the conditions: 50°C, 2 min, 95°C, 10 min, 40 cycles of 95°C, 15 sec and 60°C, 1 min.

Primers used: *robo:* Forward: 5-CAGCATTAGTCTTCGTTGGGC-3,
Reverse: 5-AATCCAACCAGTTTGCAGATTC-3

The qRT-PCR was done on three seperate embryo collections for each genotype and in triplicates for each collection.

Glycosidase treatment

Two different glycosidase enzymes were used to confirm glycosylation of Slit:

- Protein deglycosylation mix (P6039S, New England Biolabs) which consists of PNGaseF, *O*-Glycosidase, Neuraminidase, β1-4Galactosidase, βNacetylglucosaminidase. It removes both N-linked and O-linked oligosaccharides in the glycoprotein mixture.

- PNGase F(P0704S, New England Biolabs) which is specifically removes N-linked oligosaccharides from the glycoprotein.

PROTEIN DEGLYCOSYLATION :

About 30 wild type and homozygous mutant embryos were selected under the microscope and aged for 12-15 hours. The embryos were lysed in 40µl extraction buffer (0.15 M NaCl, 0.02 M Tris pH=7.5, 0.001M EDTA, 0.001 M MgCl2, 1% Triton-X-100, PIC) and kept on ice for 15 minutes. The lysed protein is centrifuged for 5 minutes at 13,000 rpm, the supernatant is collected About 18 µL of the supernatant was used for the deglycosylation reaction. To this, 2 µL of 10X Glycoprotein Denaturing Buffer (0.5% SDS, 40 mM DTT) was added to make a 20 µL total reaction volume. This reaction mixture was denatured by heating at 100 C for 10 minutes. The denatured glycoprotein is chilled on ice and centrifuged for 10 second. To the denatured glycoprotein, 5µl 10X G7 Reaction Buffer (50 mM sodium phosphate, pH 7.5 @ 25°C), 5µl 10% NP40 and 15µl water was added. Mix gently and add 5µl Deglycosylation Enzyme Cocktail (mixture of glycerol free PNGase F, *O*-Glycosidase, Neuraminidase, β1-4 Galactosidase, β-N-acetylglucosaminidase). The reaction mixture was incubated at 37° C overnight. After incubation the sample is diluted with 12.5 µL 4X Laemelli sample buffer. The protein sample is boiled in water for 10minutes and kept in 4C for 10 minutes. Equal amount of protein sample was loaded on to a 4-12% SDS-PAGE gel. The molecular weight of the treated protein sample was analyzed and compared to non-treated samples by assessing the mobility shift on SDS-PAGE gels.

PNGASEF:

Wild type and *mmy*^{slm} were collected and aged for 12-15 hours. The homozygous mutant embryos were selected under the microscope based on GFP expression. 40 embryos were lysed in 40µl of extraction buffer (0.15 M NaCl, 0.02 M Tris pH=7.5, 0.001M EDTA, 0.001 M MgCl2, 1% Triton-X-100, PIC) and kept on ice for 15 minutes. The lysed protein sample is centrifuges at 13,000 rpm for 5 minutes and the supernatant is collected. 9µl of the protein sample is mixed with 1µl of the 10X Glycoprotein denaturing buffer and incubated in boiling water bath for 10 minutes. 2µl of 10X G7 reaction buffer, 2µl of 10% NP-40, 5µl of autoclaved and distilled water, and 1µl of PNGase F is added to the denatured protein sample. The reaction mixture is incubated at 37° C for 1 hour. After incubation, the protein sample was prepared for Western Blotting by diluting with 8µL 4X Laemelli sample buffer. It is boiled in water for 10 minutes and cooled on ice. For untreated controls, the extracted protein sample is diluted with 10µL 4X Laemelli sample buffer and incubated in boiling water bath for 10 minutes. Equal amounts of PNGase F treated and non-treated samples are loaded on to 4-12% SDS-PAGE gel and separated. The molecular weight before and after treatment were analyzed based on mobility shift.

RESULTS 1A: GLYCOSYLATION OF SLIT BY MUMMY REGULATES SLIT TRANSLOCATION, PROCESSING AND SLIT-MEDIATED AXON REPULSION

Mummy regulates axon guidance by interacting with Slit in the developing *Drosophila* ventral nerve cord

Drosophila ventral nerve cord is equivalent to the spinal cord in vertebrates. The Bhat lab utilizes this model to study the different axon guidance signaling pathways and their regulatory mechanisms. The *Drosophila* embryonic nervous system is comprised of the brain and the ventral nerve cord. The nerve cord is comprised of a central midline and longitudinal axon tracts arranged spatially on either side of the midline. ~20 longitudinal tracts are specifically arranged on either side of the midline. However, the mechanism of this specific positioning has not been completely elucidated.

During development, the growing axons have to navigate through long distances to reach their target cells. To facilitate this movement, they have structures known as growth cones at the tip. The growth cones interact with the different chemotropic factors released from the midline (guidance molecules), which guide it along the correct path. The two major axon guidance signaling mechanisms are the Slit/Robo and the Netrin/Frazzled signaling pathway. In this project, we focus on the Slit-Robo signaling pathway.

Slit is a chemo-repellent and expressed in the midline glial cells (Kidd et al., 1999). We have previously reported that although Slit is not transcribed outside the midline, Slit protein is present in the connectives and the commissures. (Bhat et al., 2007). It has been proposed that Slit diffuses as a gradient out of the midline to interact with the different Robo receptors to specify the lateral axonal tract (Rajagopalan et al., 2000b). However, no other investigators including our lab have been able to detect such a

gradient emanating from the midline. More importantly, over expression of *sli* in the midline did not alter the positioning of the longitudinal tracts (Bhat et al., 2007). If the Slit gradient model were true, the expected result would be a further separation between the tracts as result of increased Slit in the midline. Also, ectopic expression of Slit in front of growth cones of Robo-expressing neurons did not alter the projections of these neurons (Bhat, 2005). All of the above data suggests that Slit moves out of the midline, into the longitudinal connective and commissural tracts.

This led to a major unanswered question: How does Slit get transported out of the midline in to the axonal tracts and what is functional significance of Slit transport?

GENETIC SCREEN TO UNCOVER SLIT TRANSPORT MUTANTS

Our lab was interested in tackling the question of Slit transport from the midline to the axonal tracts. So we conducted a genetic screen to identify molecules which are required of movement of Slit from the midline to the longitudinal axon tracts.

Towards this, we obtained "deficiency" fly lines from the Bloomington Stock center, each of which contain a deletion of a contiguous region of a *Drosophila* chromosome. The deletions are mapped to a specific region on the chromosome. We acquired different over-lapping deficiency fly lines, such that when combined, the deficiency region covered the entire chromosome. Thus we could screen for specific regions on the chromosome that may have the gene which are required for movement of Slit from the midline to the longitudinal axon tracts. The embryonic nerve cord of these fly lines was analyzed by immunostaining with an antibody against Slit protein.

Our mutant of interest, *slim (slm)* was first uncovered in a fly line which was deficient for the chromosomal region 2L: 34E2-35B5 (Grewal R and Bhat KM, unpublished work). The mutant was named "slim" emphasizing the slimness of the nerve cord in the mutant when compared to that of the wildtype. On analysis of Slit protein by immunostaining, we observed that in *slm*, there is a significant reduction in movement of Slit from the midline to the longitudinal axon tracts (Figure 1.6). To analyze the gene involved in Slit movement, we first had to map the mutation. The mutant fly line had a deficiency region mapped to the second chromosome. On further analysis, we found that this region has 86 genes. Logically, one of the 86 genes should have been responsible for the axon guidance defects in the fly line. We tried to locate the mutation by crossing the mutant fly lines to fly lines deficient for shorter regions for all the 86 different genes. We expected that homozygous embryos from one of these crosses would yield the mutant phenotype, and help in determining the location of the gene. However, none of the 86 genes showed lack of complementation to the phenotype. This suggested that the mutated gene may not be in the deficiency region, but may have come from the genetic back ground.

We knew that the mutation is on the second chromosome since the line was propagated with a second chromosome balancer. To identify the region of the gene of interest in the second chromosome, we obtained fly lines with gene deficiencies all through the second chromosome, and crossed it to the mutant fly line of interest. For this screen, we first narrowed down the region by determining if the deficiency lines complemented our mutant line. After that, the embryos from smaller deficiency lines in that region were stained to confirm the phenotype.

We found that the deficiency line BL# 24378 (Bloomington stock center Indiana University) which is deficient in the region 2L: 26D7-26E3 failed to complement our mutant fly line. This data proved that the mutated gene came from the genetic

background and not contributed by any of the genes present in the chromosomal region 2L: 34E2-35B5 (the fly line where the phenotype was first observed).The chromosomal region 2L: 26D7-26E3 contains several genes. We refined the area further by using different deficiency lines to eliminate different blocks of the chromosome.

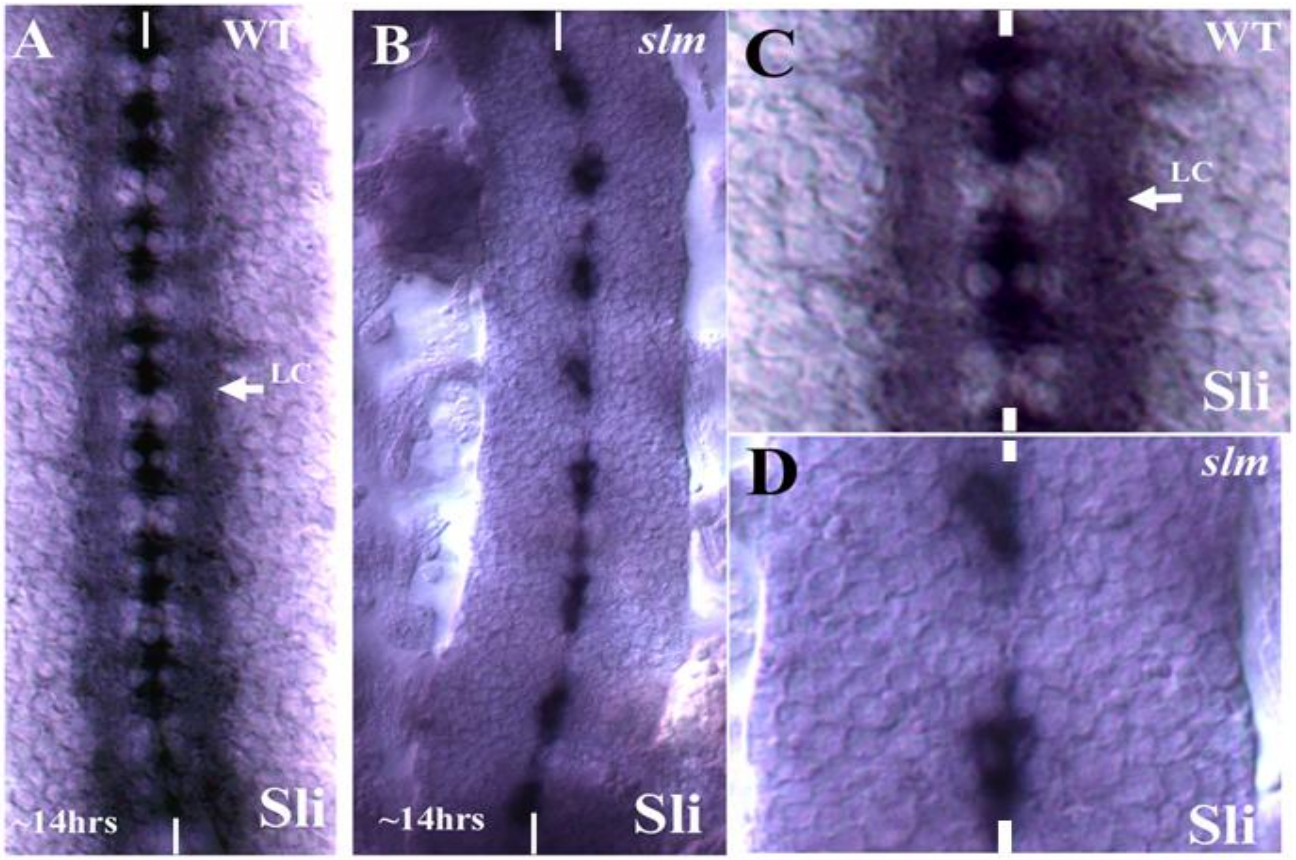

Figure 1.6. **Transport of Slit is disrupted in** *slm* **mutants**. Embryos are stained with Slit antibody. Anterior end is up, midline is marked by vertical lines. LC- Longitudinal connective (indicated by arrows), *(*A) Wild type embryo stained with Slit antibody. Slit protein is found in high levels at the midline, as well as at lower levels in the longitudinal connectives and commissural tracts (B) *slm* homozygous embryo stained with anti-Slit. High levels of Slit are present in the midline, however Slit is not detected in the axon tracts. (C and D) Magnified images of the Slit staining in wild type and *slm* embryos. Absence of Slit in the longitudinal axon tracts shows that transport of Slit may be disrupted in *slm* mutants.

On analyzing the data, we finally hit upon 2 genes of interest: 1. *sec61 alpha*, and 2: *mmy.* To finally resolve the gene, we crossed the mutant fly line of interest to mutant alleles of *sec61 alpha* and *mmy* (for each of the genes in the deficiency region). We found that mmy^{1} mutant (previously isolated by Nusslein-Volhard and obtained from the Bloomington Drosophila Stock Center) failed to complement. Thus we finally narrowed down our mutation of interest to that in *mmy* gene (Jayasinghe R.,Bhat KM; unpublished work). The mutation was found to be allelic to *mmy* and was renamed as mmy^{slm}. We sequenced the allele to find a premature stop codon that yields a 75 amino acid peptide instead of functional Mummy (Figure 1.7).

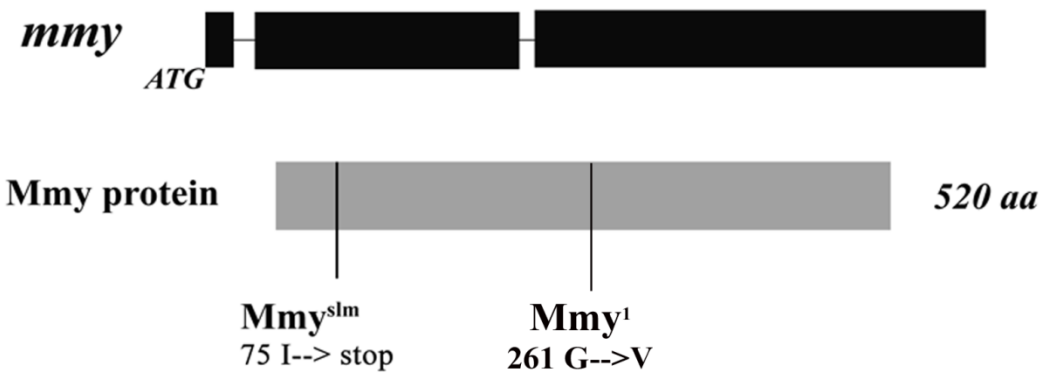

Figure 1.7: Line drawing depicting *mmy* gene and Mmy protein with molecular lesions in mmy^{slm}.

MMY^{SLM} EXHIBITS AXON GUIDANCE DEFECTS IN THE EMBRYONIC NERVE CORD

After discovering that mmy^{slm} exhibits significant decrease in Slit transport, we wanted to examine if mmy^{slm} exhibits an axon guidance phenotype in the embryonic ventral nerve cord. We analyzed the nerve cord of the *mmy* mutant embryos using anti-

FasII and anti-BP102 (Figure 1.8 I and 1.8 II). Anti-Fas II stain the longitudinal axon tracts into three distinct bundles- Medial, Intermediate and Lateral axon tracts in wild type embryonic nerve cord. Anti-BP102 staining is carried out to examine the commissural architecture of the ventral nerve cord. They are established techniques of studying the structure of the ventral nerve cord.

From both the stainings it was observed that in *mmy* mutant embryos, the axon tracts are disrupted and located closer to the midline when compared to that of the wild type embryos (Figure 1.8 I and II). The longitudinal axon tracts in *slm* is not distinct (Figure 1.8 I C and D) which suggests that the lateral specification of the longitudinal axon tracts are also affected due to loss of functional Mummy. We thus decided to name the phenotype *mmy* ^{slm}.

We also analyzed the embryonic nerve cord of *mmy*^{slm} mutant during development. Relevant figures are presented below. It was quite interesting to note that the phenotype of *mmy*^{slm} embryonic nerve cord is similar to that of the wild type till ~10 hours of development. This suggests that the initial pattern of axon projection is not affected in the *mmy*^{slm}, and that the effect on the phenotype only occurs later in development.

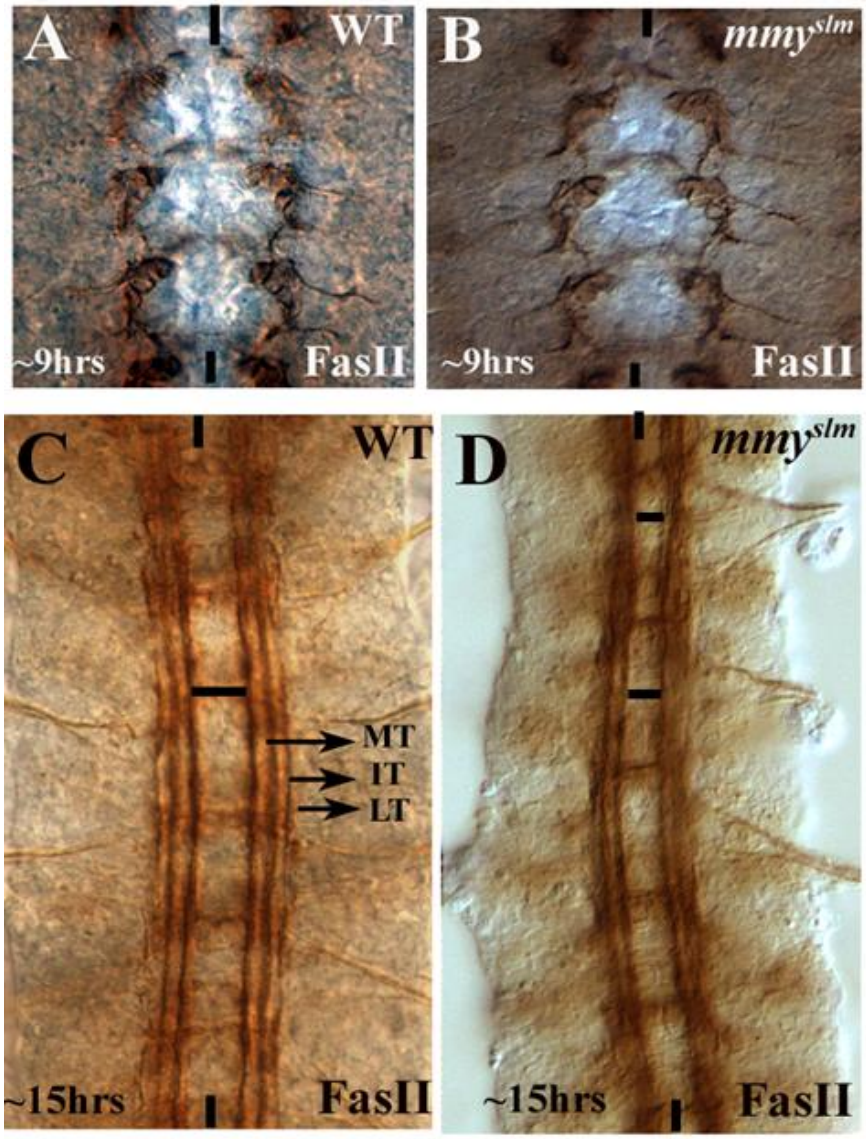

Figure 1.8 I. Axon tracts are disrupted and located closer to the midline in *mmy^{slm}*. Panels A-D embryos stained with Fas II antibody, MT- Medial tract, IT- Intermediate tracts, LT- Lateral tract. AC- Midline is marked by vertical lines, anterior end is up. Black bars denote the distance between the tracts. Panel A: Wild type embryo, 9 hours old, where the pioneering axons are beginning to grow, either upwards or downwards, along the midline. Panel C: Wild type embryo, 15 hrs old, where proposer longitudinal axon tracts are well-set on either side of the midline. Panel B: 9 hr old *mmy^{slm}* embryo, where the pioneering tracts are properly projected as in wild type. This indicates that the initial axon projection pattern is not affected in the mutant. Panel D: 15 hr old mutant embryo, where the axon tracts are set much closer to the midline, with tracts crossing the midline.

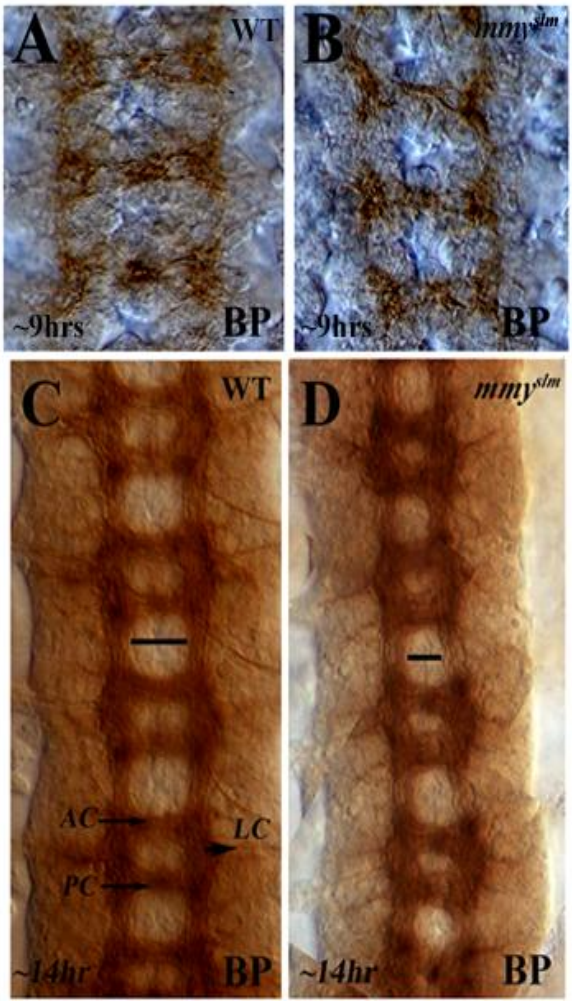

Figure 1.8 II: Axon tracts are disrupted and located closer to the midline in *mmy*^{slm}: Panel A-D, Embryos stained with BP102 antibody, AC- Anterior commissure, PC- Posterior commissure, LC- Longitudinal connective. Midline is marked by vertical lines, anterior end is up. Black bars denote the distance between the tracts. Panels A,C: Wild type embryos showing the newly forming commissural tracts in a young (A) and older stage embryo (C). Panels B,D: Commissural tracts in a young mutant embryo (B) showing nearly normal commissural tracts, and in an older stage mutant embryo (D) with disrupted commissures.

MMY^{SLM} DOES NOT SHOW REDUCTION IN SLIT PROTEIN LEVELS, BUT SHOWS A REDUCED MOLECULAR WEIGHT.

As discussed in the introduction, in *sli* mutants the axon tracts collapse completely in to the midline due to the loss of the chemo-repellant Slit activity. *mmy^{slm}* embryos have axon tracts located closer to the midline, but not completely collapsed into the midline. A possible lack of Slit transport as well as the axon guidance defects suggests Mummy regulates Slit-Robo signaling pathway.

On examining *mmy^{slm}* embryos stained with anti-Slit (Figure 1.6), it was observed that there was mis-localization of Slit (absence of Slit in the longitudinal connectives rather than reduction in protein levels. We re-confirmed this by analyzing the Slit protein levels in *mmy^{slm}* by western blotting. This suggests that, while Slit protein levels are not affected there might be loss of Slit activity leading to impaired Slit transport to the longitudinal tracts. This might be due to loss of post-translational modifications on Slit, like addition of sugar moieties which may be required for the movement of Slit from the midline into the longitudinal and commissural axonal tracts.

On analysis of Slit protein in *mmy^{slm}* by Western Blotting technique, it was found that the Slit protein in *mmy^{slm}* migrated with a higher mobility when compared to Slit protein from wild type embryos (Figure 1.9). This suggests that Slit may be post translationally modified in wildtype, which is lost in *mmy^{slm}* due to loss of functional Mummy. However in *mmy^{slm}*, Slit is present in un-glycosylated state which presents as the lower molecular weight on the membrane.

We also analyzed the molecular weight of Slit in a previously isolated *mmy* allele: *mmy^{1}*. Slit protein migrated as a lower molecular weight protein in *mmy^{1}* (Figure 1.9) as well, suggesting that the effect on Slit is not due to genetic background.

Since *mmy* has a known role in the glycosylation pathway, it is probable that the post translational modification is glycosylation (Schimmelpfeng et al., 2006).

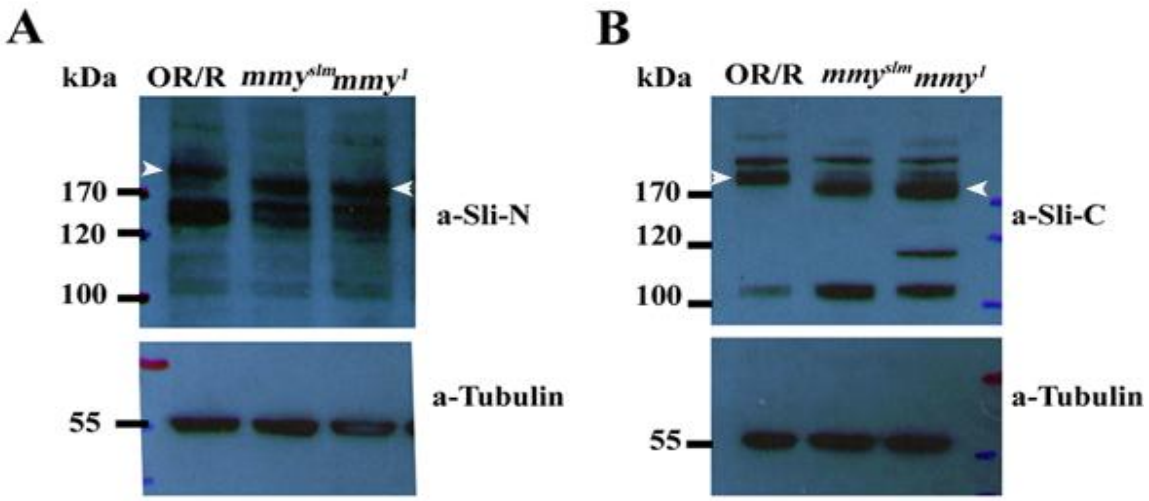

Figure 1.9: Slit exhibits lower molecular weight in *mmy^{slm}* and *mmy^{1}* A) Western blot with OR/R (wildtype), *mmy^{slm}* and *mmy^{1}* probed with Slit antibody against N-terminus of Slit protein. B) A) Western blot with OR/R (wildtype), *mmy^{slm}* and *mmy^{1}* probed with Slit antibody against C-terminus of Slit protein. Arrowheads indicate Slit full length protein which migrates differently in *mmy^{slm}*,*mmy^{1}* when compared to wild type.

SLIT PROTEIN SEQUENCE HAS THIRTEEN PUTATIVE GLYCOSYLATION SITES

Mummy is the only known glycosyl transferase enzyme in *Drosophila* and plays an important role in glycosylation of various proteins during development (Araújo et al., 2005). It is quite logical that Slit may be one of the potential target proteins that is mediated by Mummy activity since Mummy has a known role in N-linked glycosylation (Schimmelpfeng et al., 2006). So, the Slit protein sequence was analyzed for putative N-linked glycosylation sites using a glycosylation site predicting software (NetNglyc 1.0). It was found that Slit protein has thirteen putative glycosylation sites (Fig 1.10.)

As previously described, Slit undergoes proteolytic cleavage. To determine if glycosylation may affect Slit proteolysis, we examined if the predicted glycosylation sites were on or near the cleavage site. We found that site 9 was quite near the cleavage site

(Fig. 1.10) and that it also has a strong affinity for glycosylation based on Slit protein sequence analysis using NetNglyc 1.0.

Figure 1.10: Slit protein has thirteen putative glycosylation sites: Line drawing of Slit protein with glycosylation sites denoted by black bars. Sites with stong affinity for glycosylation are starred. The red arrow indicates the proteolytic cleavage site.

MMY MATERNAL DEPOSITION

In situ hybridisation studies show that embryos have a significant amount of maternal *mmy* mRNA, which is sufficent for basic cellular needs during early embryogenesis (Tonning et al. 2005). Since the axon guidance phenotype does not appear until 10 hours of development, it is possible that the maternal mummy is responsible for the maintenance of the CNS until zygotic *mmy* transcription begins. It is reported that zygotic *mmy* transcription begins at ~ stage 11 or 7 hours, 20 minutes of development (Refer Table 1) in tracheal cells (Tonning et al., 2006). The expression of mutant Mmy protein at that stage may be the reason for the late onset of axon guidance phenotype in mmy^{slm}. We analyzed that by examining the molecular weight of Slit in early embryos (~7-9 hours, when maternal *mmy* was prevalent) and compared it to the molecular weight of Slit in later stage embryos (12-14hours) (Figure 1.11). We found that in early stage (7-9 hours), the molecular weight of Slit in mmy^{slm} remains unchanged and comparable to wildtype Slit (Figure 1.11). This explains our observation that the phenotype of mmy^{slm} is similar to that of wildtype until 10 hours of development. However at later stage (12-

14hours of development), the molecular mass of Slit is reduced when compared to the wildtype i.e, Slit likely becomes non-glycosylated. This correlates with the observation that the axon guidance phenotype only manifests at a later stage of development (Figure 1.8 I and II).

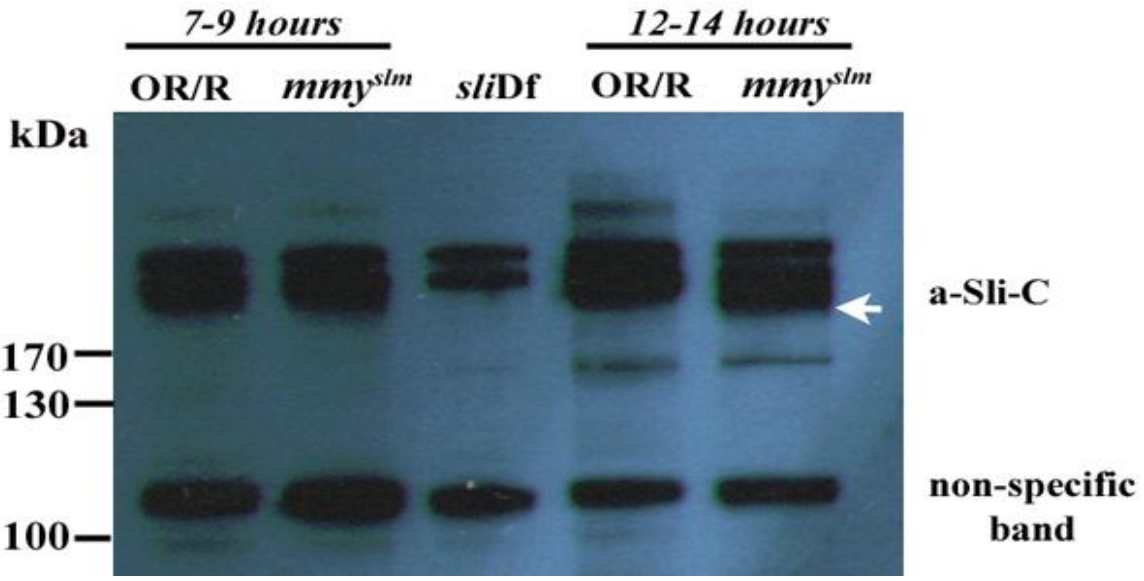

Figure 1.11: Maternal functional Mummy likely glycosylates Slit until ~9 hours of development: Proteins extracted from OR/R and *mmy*^{slm} embryos collected at 7-9 hours and 12-14 hours were probed with anti-Sli-C. sliDf represents the flyline where *sli* gene is deleted. At 7-9 hours, Slit protein has the same mobility in both wildtype and *mmy*^{slm} embryos. However, at 12-14 hours, the mobility of Slit protein is reduced in *mmy*^{slm} embryos when compared to the wildtype (denoted by the arrow). This suggests that maternal Mummy glycosylates Slit until ~9 hours after which mutant zygotic Mummy is expressed, which leads to non-glycosylated Slit. The Slit protein specific band is completely absent in *sli*Df (12-14 hours). The non-specific bands serve as the loading control.

The role of maternally deposited Mmy in glycosylating Slit in the early stage of development could be addressed by generating germ line clones, whose progeny would

not have any maternal Mmy. However, it has been reported by Tonning et al, that the germ line clones show an arrested development and thus do not survive (Tonning et al., 2006). This is expected since Mummy is the only known glycosyl transferase, and would be an essential gene with other roles in early development.

SLIT IS GLYCOSYLATED IN WILD TYPE AND LACK OF FUNCTIONAL MUMMY LEADS TO NON-GLYCOSYLATED SLIT IN MMY MUTANT.

It was observed that Slit protein in mmy^{slm} moves with a faster mobility during electrophoresis than Slit protein isolated from wild type embryos. Since Mummy has a known role in the glycosylation pathway, it was expected that in wild type, Slit protein may be present in glycosylated form. In mmy^{slm}, the loss of functional Mummy might prevent Slit glycosylation.

To conclusively prove that Slit is glycosylated, we treated the wild type Slit (from wildtype protein extract) with two glycosidase enzymes: 1) Protein deglycosylation mix: contains a mix of both N- and O- glycosidase enzymes; 2) PNGase F: only containing N glycosidase enzyme. The glycosidase treated wild type Slit protein was analyzed using western blotting and compared to untreated wild type Slit and untreated Slit protein from mmy^{slm}. Interestingly, the molecular mass of glycosidase treated wild type Slit was found to have a lower mobility than untreated wild type Slit (Figure 1.12). Also, the lower molecular mass of glycosidase treated Slit is comparable to that of Slit protein in mmy^{slm} (Figure 1.12). These results confirm that Slit undergoes post-translational N-linked glycosylation. Also, this data further reaffirms that Slit glycosylation is disrupted in mmy^{slm} and emphasizes the role of *mmy* in the development of the central nervous system.

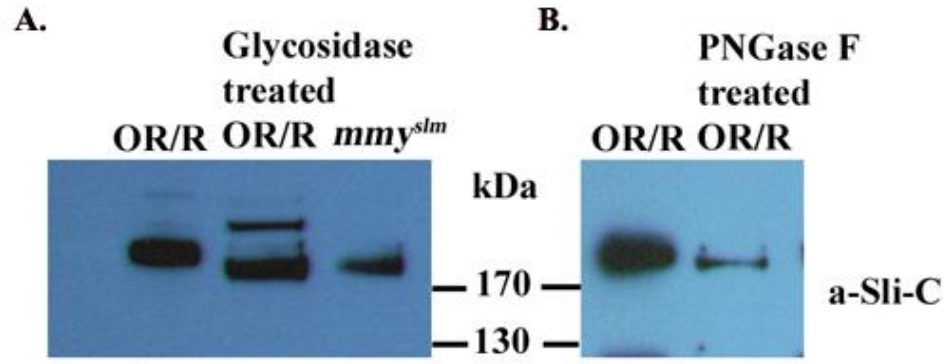

Figure 1.12: Slit is N-glycosylated in wild type embyros: *Panel A* is a western blot with wild type; Glycosidase treated wild type extract and mmy^{slm} (all samples aged for 12-14 hours) and probed with anti-Sli-C. On glycosidase treatement, the molecular weight of Slit is reduced and comparable to that of Slit in mmy^{slm}. A higher molecular weight band was also observed, which is likely due to the addition of external glycosidase enzyme. Panel B shows western blot is wild type and wildtype sample treated with PNGaseF; probed with anti-Sli-C. There is a reduction in molecular weight of Slit on N-glycosidase treatment.

Functional significance of Slit glycosylation

Although we have sufficient data to suggest that Slit is glycosylated, it was important to analyze the functional significance of glycosylated Slit.

GLYCOSYLATION OF SLIT IS NOT REQUIRED FOR BINDING TO ITS RECEPTOR ROUNDABOUT.

We have reliably demonstrated that Slit undergoes post translational glycosylation. The next step was to determine the biological significance of this glycosylation. Since Slit has to bind to its receptor Robo to activate axonal repulsion (Kidd et al., 1998a; Kidd et al., 1999; Dickson and Gilestro, 2006), we wanted to check if

Slit glycosylation was required for binding to Robo. To analyze this, we immunoprecipitated the glycosylated Slit (from wildtype sample), and non-glycosylated Slit (from *mmy*^{slm} sample), and immunoblotted it with Robo. The western blot results showed that there was a significant down regulation of Slit-bound Robo in *mmy*^{slm} when compared to wildtype (Figure 1.13 A). However, *mmy*^{slm} has significantly reduced levels of Robo protein when compared to wildtype (refer to chapter 2, Fig 2.1). The reduction in non-glycosylated Slit-bound Robo in *mmy*^{slm} could either be because of reduction in binding efficiency of Slit-Robo binding due to lack of glycosylation or due to the reduction in Robo protein available for Slit to bind in *mmy*^{slm}. In order to determine the reason for reduced binding observed in *mmy*^{slm} (Figure 1.13 A), we decided to scale up the protein level in *mmy*^{slm}, such that both samples would have equal/ similar amounts of Robo protein to bind to glycosylated or non-glycosylated Slit. In this case, we standardize the Robo protein levels in both samples and can determine if there is a decrease in binding efficiency due to non-glycosylation of Slit.

To scale up the protein levels in *mmy*^{slm} , we optimized the immune-precipitation column by increasing the protein sample (wildtype) to determine the amount of embryo protein required to achieve saturation of Slit-Robo binding (Figure 1.13 B). Then, we decided to increase the Robo protein levels in *mmy*^{slm} by approximating the reduction in Robo protein levels compared to wildtype. This was determined by quantifying the Robo protein reduction in *mmy*^{slm} using several western blots. We estimated that there is almost 2.5 times reduction in Robo protein in *mmy*^{slm} when compared to that of the wildtype.

The total protein extract in *mmy*^{slm} was increased while keeping the wildtype protein levels constant (by a ratio of 1:4). This allowed us to make sure that there was enough Robo protein in both samples to bind to either glycosylated (wildtype) or non-glycosylated (*mmy*^{slm}) Slit. On analyzing the binding, it was found that both glycosylated and non-glycosylated Slit bound to Robo with the almost the same efficiency (Figure

1.13 C). This result suggests that glycosylation of Slit is not required for binding with its specific receptor, Robo.

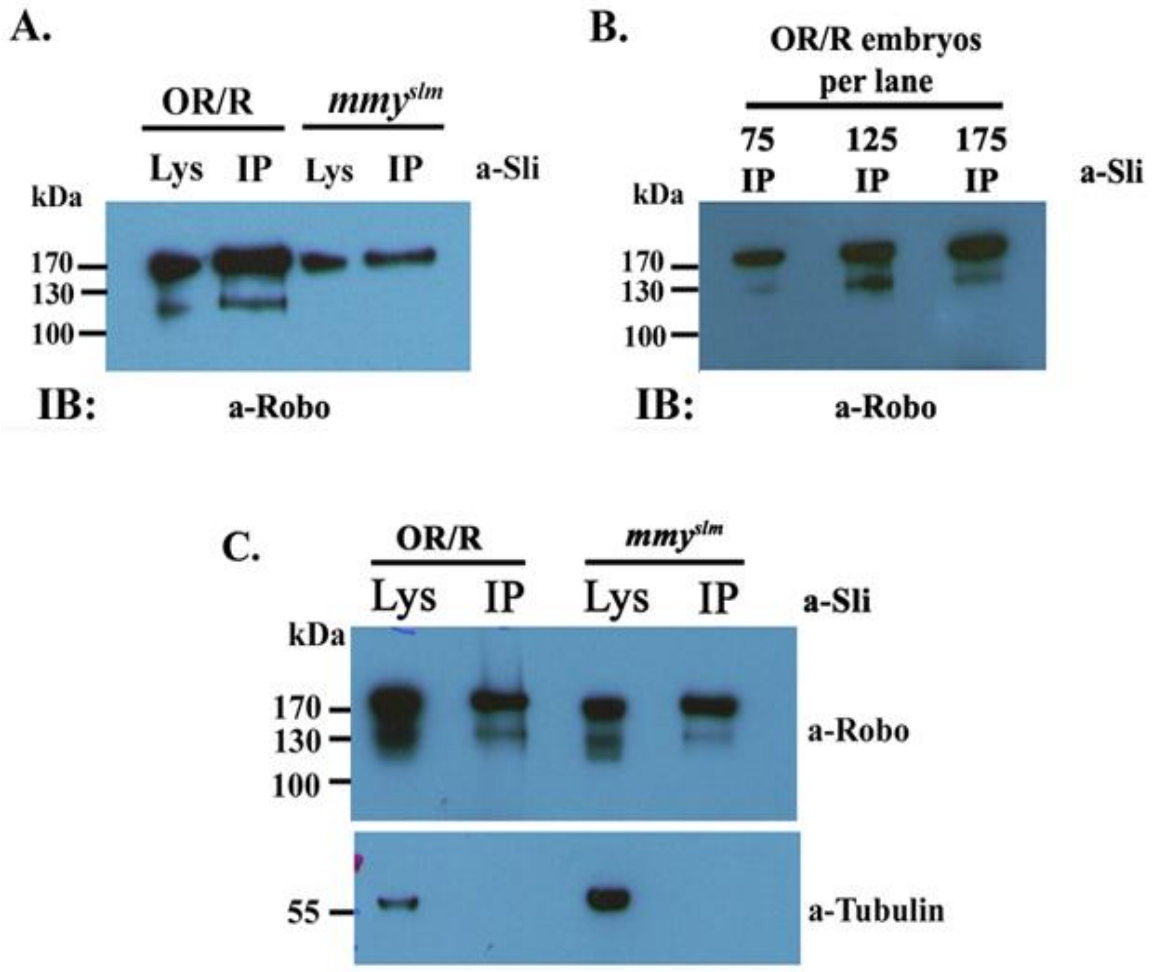

Figure 1.13: Slit glycosylation is not required for binding to Roundabout: Panel A Western Blotting analysis of wildtype and *mmy*^{slm} protein extracts immune-precipitated with anti-Sli-C and probed with anti-Robo. Panel B, Western Blotting analysis of wild-type sample with different protein concentrations to determine the saturation of the column used for immune-precipitation. Panel C, Western Blotting analysis of wild-type and protein adjusted *mmy*^{slm} sample immune-precipitated with anti-Sli-C and probed with anti-Robo.

GLYCOSYLATION IS ESSENTIAL FOR EFFECTIVE CLEAVAGE OF SLIT PROTEIN

Data in Figure 1.13 show that glycosylation of Slit is not required for its binding to Robo receptor. Next, we performed western blotting of protein extracts from both wild type and mmy^{slm}. They were blotted with both Slit antibody against N-terminal and C-terminal fragments to determine their cleavage patterns (Figure 1.14 A and B). We see that, in mmy^{slm}, there is an increase in levels of full length Slit and a decrease in the cleaved fragments when compared to that of the wild type. This suggests that cleavage of non-glycosylated Slit in mmy^{slm} is significantly reduced due to lack of functional Mummy. Due to the polyclonal nature of the anti-Sli-C, we see various non-specific bands at ~70kDa and ~100kDa (Figure 1.14 A) especially when the concentration of the loading protein was increased. The non-specific nature of these bands was reconfirmed by analyzing the cleavage pattern in *sli*Df where the *sli* gene is deleted (data not shown). Anti-Sli-N is more specific since it is a monoclonal antibody and shows the full length (~220kDa) and cleaved N-terminal fragment (~130kDa) (Figure 1.14 B).

The above results suggest that Mummy regulates axon guidance in the developing nerve via the Slit-Robo signaling pathway. It mediates glycosylation of Slit protein as well as Slit transport into the longitudinal tracts. Glycosylation of Slit is not essential for binding to its receptor Roundabout, but is required for effective cleavage of Slit. This suggests that Mummy plays an important role in fine-tuning the Slit-Robo signaling pathway.

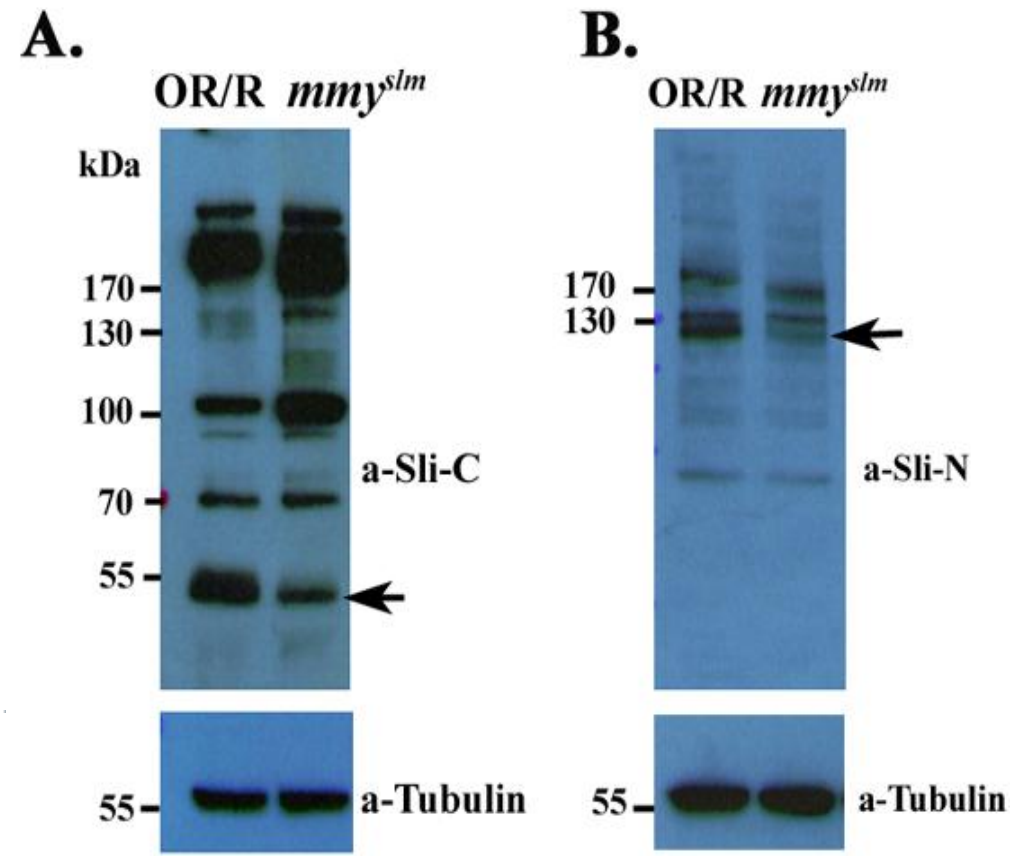

Figure 1.14: Mummy regulates Slit processing: ***Panel A:*** Western Blot analysis of wild type and *mmy*^{slm} with anti-Sli-C to determine processing of the C-terminal fragment (denoted by the arrow), ***Panel B:*** Western Blot analysis of wild type and *mmy*^{slm} with anti-Sli-N to examine processing of the N-terminal fragment (denoted by arrow).

RESULTS 1B: MUMMY REGULATES ROBO RECEPTOR LEVELS IN THE DEVELOPING DROSOPHILA NERVE CORD.

In the previous chapter, I have conclusively proved that Mummy (Mmy) has a direct effect on Slit glycosylation and Slit transport. Since Robo, Robo2 and Robo3 are specific receptors for Slit, I wanted to further examine the effect of Mummy on the different Robo family proteins.

Robo protein levels are reduced in *mmy^{slm}*.

mmy^{slm} exhibit significant axon guidance defects and lack of Slit at the commissural axon tracts. We have already established that there is non-glycosylation of Slit in *mmy^{slm}*. We decided to analyze if lack of functional Mummy protein in *mmy^{slm}* affects other players in the Sli-Robo signaling pathway. Since Roundabout is a specific receptor for Slit ligand, we decided to analyze Robo protein in *mmy^{slm}*. *mmy^{slm}* exhibits a reduction of Robo protein on the longitudinal tracts of the ventral nerve cord (Figure 2.1). This is an important finding, since it establishes a direct regulatory role for Mmy in the Slit-Robo signaling pathway. We analyzed the Robo protein levels in the developing nerve cord by Immuno-staining and Western Blotting experiments. On staining the nerve cord with anti-Robo, we observed a marked decrease in protein levels when compared to the wildtype (Figure 2.1 A,B). Also, Western blotting of wildtype and *mmy^{slm}* embryo extracts against Robo antibody reaffirms the reduction of Robo protein levels in the mutant (Figure 2.1C).

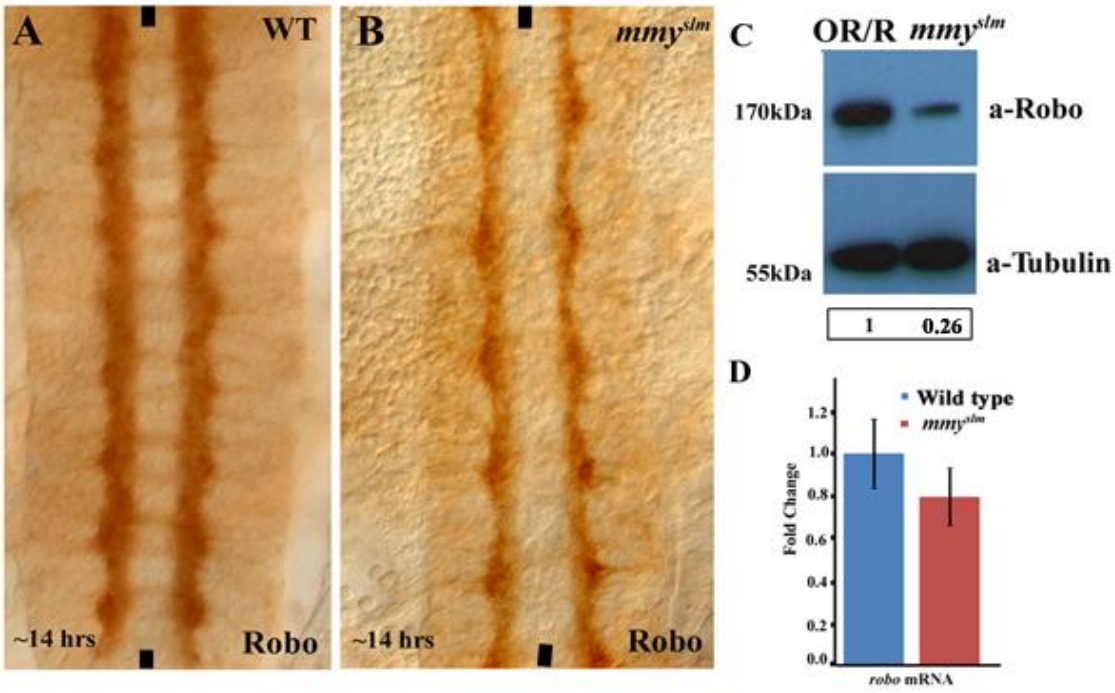

Figure 2.1: *mmy*^{slm} shows a significant reduction in Robo protein levels when compared to the wildtype. A) Wildtype embryos and B) *mmy*^{slm} stained with anti-Robo. Robo is present in the longitudinal axon tracts and there is a significant reduction of Robo in *mmy*^{slm} (Anterior is up and midline is marked by the black line). C) Western blot comparing Robo levels in Wildtype (OR/R) and *mmy*^{slm}. The intensities of the bands were quantified and show a marked reduction in Robo protein levels. D) mRNA levels of robo in wildtype and *mmy*^{slm} determined by qRT-PCR.

A reduction of protein levels may manifest due to a reduction in the production of transcript in the mutant. To eliminate transcriptional regulation as a cause for reduction in Robo protein levels, we performed a RT-PCR on embryo extracts from wild type and *mmy*^{slm} (Figure 2.1 D). The above results suggest that there is no reduction in the *robo* transcript levels which propose that the Robo protein levels are being regulated by *mmy*, either directly or indirectly, after translation.

Robo 2 and Robo 3 protein levels are regulated by *mmy^{slm}*.

Robo 2 and Robo 3 belong to the Robo receptor family and are additional specific receptors for the Slit ligand. These receptors have been reported to respond to long range Slit signaling and are required for the proper positioning of the longitudinal tracts with respect to the midline (Rajagopalan et al., 2000b).

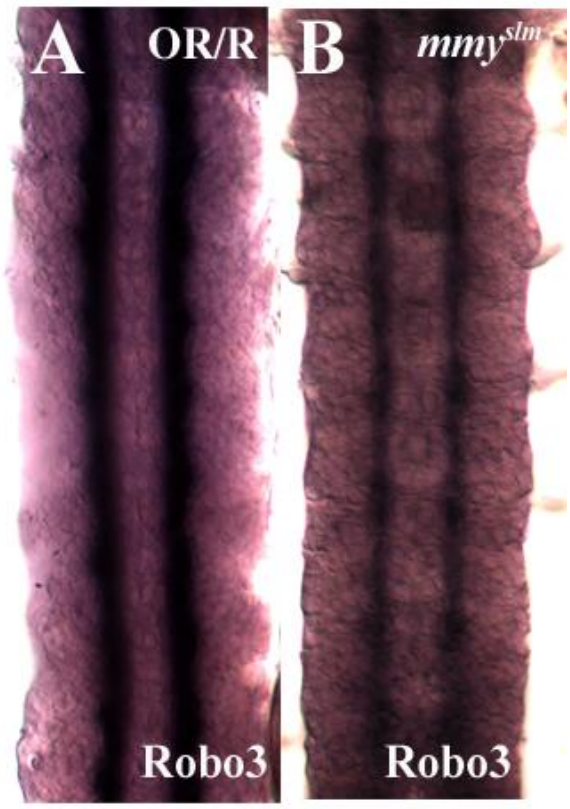

Figure 2.2: Mummy downregulates Robo3 protein level: *Panel A*, Wildtype and *Panel B*, *mmy^{slm}*embryos(age matched) stained with anti-Robo3 (developed by AP reaction). Robo3 is expressed in the longitudinal tracts and is significantly down-regulated in *mmy^{slm}*.

In *mmy^{slm}*, the nerve cord was observed to be "slim" or located much closer to the midline when compared to that of the wild type. This suggests that loss of repulsive

activity which might be due to lack of Slit function or reduction in Robo protein levels. We also observe that the lateral positioning of the longitudinal tracts is affected in *mmy*^{slm}. Rajagopalan et al have reported the importance of the localization of the three Robo receptors in the lateral positioning of the axonal tracts (Rajagopalan et al., 2000b). I decided to analyze Robo2 and Robo3 proteins in *mmy*^{slm}. This was done by immunostaining the *mmy*^{slm} embryos with anti-Robo2 (results not shown) and anti-Robo3 (Figure 2.2) to analyze the protein levels and compare it to the wild type embryos. We found that the Robo2 and Robo3 protein levels were downregulated in *mmy*^{slm}.

Maternal Mmy in *mmy*^{slm} maintains Robo protein levels comparable to Wildtype till ~9 hrs of development

One of our startling observations was that the development of the axon tracts continued normally until about ~10 hours of development. The axon guidance defects were more prominent after ~12 hours. This was an interesting question we wanted to explore.

Mummy has been reported to have maternal contribution (Tonning et al., 2006). As described earlier, we have shown the effect of maternal *mmy* on Slit glycosylation during early development. Similarly, we wanted to examine if the maternal *mmy* can influence the Robo protein levels early during development. We analyzed the Robo levels in embryos ~7-9 hours into development and ~12-14 hours into development. We observed that Robo levels in younger embryos are comparable to that of wildtype, but older embryos show a marked decrease in protein levels (Figure 2.3).

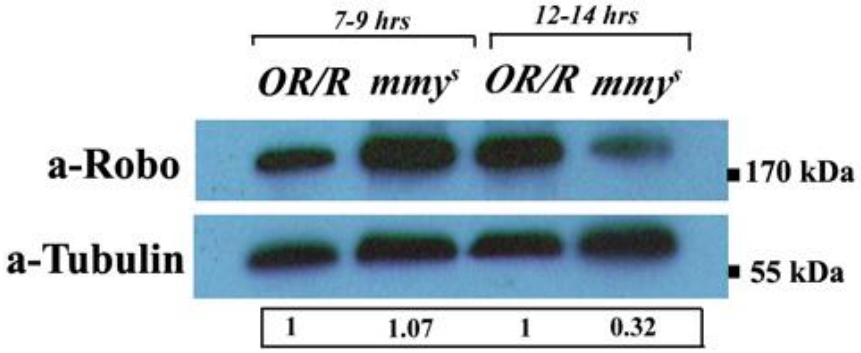

Figure 2.3: Analysis of effect of maternal Mummy on Robo protein levels: Western blot comparing protein levels in wildtype and *mmy*^{slm} aged for different time periods probed with anti-Robo. Tubulin is used as a loading control. Intensity of the bands were analyzed and denoted at the bottom of the blot.

Transport of Slit into the longitudinal tracts is not Robo-dependent.

We have previously reported that movement of Slit in to the longitudinal axon tracts is a Robo-independant process (Bhat et al., 2007). We came to this conclusion by examining the nerve cord of embryos deficient for *robo.* However, it was observed that the commissures and longitudinal axon tracts of these embryos also indicated presence of Slit. It is possible that in *robo* deficient fly lines, *robo2* and *robo3* may compensate for lack of Robo and may facilitate the transport of Slit into the longitudinal tracts. In order to answer that question, we generated fly lines which are deficient for *robo, robo2 and robo3.* However, lack of all three Robo proteins led to loss of repulsion and we observed the collapse of longitudinal tracts into the midline. Slit is secreted by a subset of glial cells in a specific pattern (as seen in Figure 2.4 A,C). Careful examination of the nerve cord of the triple mutant embryos (Figure 2.4 B,D) showed that the longitudinal tracts still showed presence of Slit outside of the Slit-secreting glial cells, which suggests that movement of Slit from the midline to longitudinal axon tracts. The above results indicate that Slit transport is truly a Robo receptor independent process.

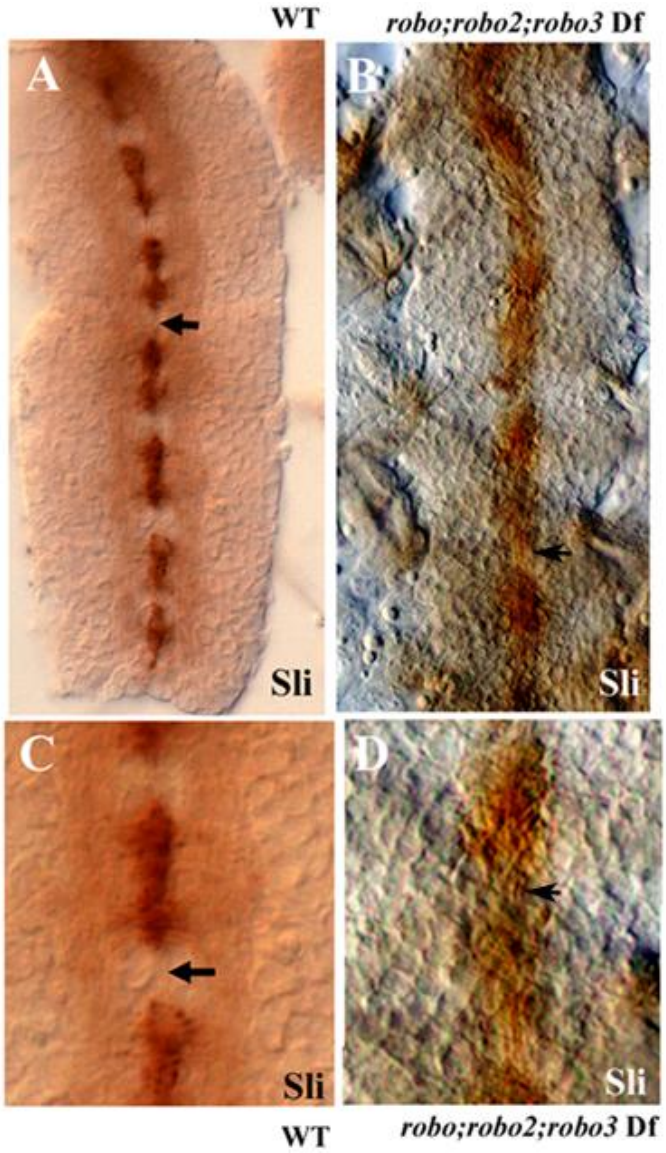

Figure 2.4: Movement of Slit from midline to the longitudinal tracts is a Robo receptor independent process: (A) Wild type embryos stained with Slit antibody. Slit is secreted in a midline by a particular subset of gial cells generating a pattern (Arrow indicates areas that do not secrete Slit). Slit staining is also observed in the longitudinal tracts. (B) *robo,robo2,robo3Df* embryos stained with Slit antibody. Slit staining is observed in the midline. The collapsed longitudinal tracts also show presence of Slit.(C) and (D) Magnified images of wild type and robo,robo2,robo3Df embryos stained with Slit antibody.. Arrow (in B, D) shows extra staining of Slit in midline, from the longitudinal axon tracts.

Ectopic pan-neural Robo expression does not rescue Slit transport or axon guidance defects.

As reported above, we see a decrease in Robo protein level in *mmy*^{slm}. To determine if the reduction in Robo levels is directly responsible for the axon guidance defects and aberrant Slit transport, we decided to ectopically express Robo pan-neurally and attempt to rescue the phenotype. Towards this, we first characterized the driver elav-Gal4.

Figure 2.5: *elav* Gal4 is a pan neural driver :*elav*Gal4 drives the expression of UAS-*lacZ* in a pan-neural manner.

To characterize the *elav*Gal4 driver, we crossed the driver flyline to UAS-*lacZ*. The *elav* driver will drive the production of *lacZ* (β−galactosidase protein) in the driver specific region of the embryo. This region was analyzed by staining with X-Gal. Blue color stained tissue indicate the driver specific tissues. It was observed that *elav*Gal4 is a pan-neural driver and drives the expression of the UAS-gene in most of the neurons (Figure 2.5).

The previous experiment showed that movement of Slit from the midline to the longitudinal tracts is a Robo-receptor independent process. To reconfirm this result, we ectopically expressed Robo protein in mmy^{slm} pan-neurally. Over-expression of the robo protein could reverse the phenotype, if the reduction of Robo receptors were the cause of defective axon guidance and lack of Slit movement from the midline to the longitudinal axon tracts.

This was done by exploiting the various genetic tools available in *Drosophila.* We introduced UAS-*robo* to the mmy^{slm} background and drove the over-expression of Robo using *elav-Gal4*. This experiment helped us determine the effect of Robo overexpression when *mmy* is mutated, thereby negated the effect of Robo down-regulation mediated by Mmy. If the phenotype was caused by the Robo down-regulation, the mmy^{slm} embryos would not exhibit the axon guidance phenotype in the presence of ectopic Robo. However, if mutant *mmy* induces this phenotype indirectly through another molecule, ectopic expression of Robo would not mediate the rescue of the phenotype.

We first examined the embryonic nerve cord by double staining with anti-FasII and anti-Robo. Staining with Robo antibody helped us pick the embryos which had ectopic Robo expression, while Fas II staining assisted in examining the rescue of the longitudinal tracts. Although, there is variability in the severity of the axon tract

phenotype, we did not see any significant rescue of the defective axon guidance phenotype (Figure 2.6 A,B).

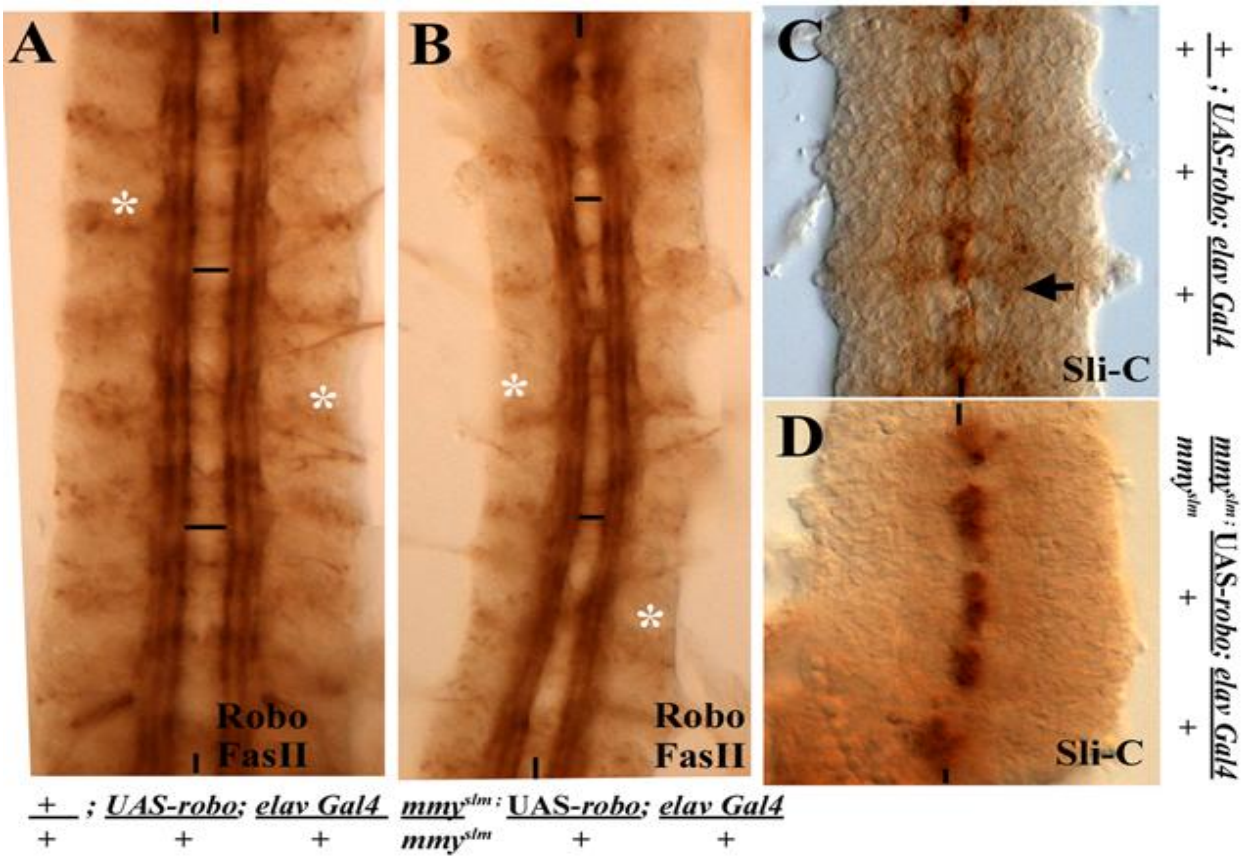

Figure 2.6: Ectopic expression of Robo does not rescue axon guidance phenotype: Nerve cord of A) Ectopic Robo expression in wildtype background, and B) mmyslm background, doublestained with Robo and FasII antibody. Arrow shows ectopic expression of Robo protein in the nerve cord. Black bars indicate the distance between the tracts.

These embryos were also examined to see if the movement of Slit was restored. As in case with the axon guidance phenotype, we did not observe significant movement of Slit into the longitudinal tracts with ectopic expression of Robo (Figure 2.6 C,D).

These results suggest that *mmy* does not regulate the axon guidance mechanisms directly by down-regulating Robo. It is possible that there is an indirect regulatory mechanism in play.

DISCUSSION

Neurons extend axons over large distances to form neuronal connections with their targets. This is mediated by chemotaxic molecules and their specific receptors present on the growth cones of axons. This process is called axon guidance and is one of the most critical steps during development, to pattern and maintain a functional nervous system. In this work, I used the *Drosophila* ventral nerve cord as a model to study axon guidance mechanisms and their regulatory molecules. I focused on the Slit-Robo signaling pathway, which is required to maintain the functional integrity of the ventral nerve cord. This signaling pathway has been well-studied. However, there are still different regulatory molecules that are still unknown and required for fine-tuning this pathway. In this work, I have uncovered a novel role for the molecule Mummy in mediating axon guidance through the Slit-Robo signaling pathway during ventral nerve cord development in *Drosophila* embryos.

Slit is transported from the midline to the longitudinal axon tracts:

The Bhat lab has previously reported the movement of Slit from the midline to the longitudinal axon tracts (Bhat et al., 2007). We characterized Slit movement in wild type

embryos. Whole mount RNA *in situ* hybridization studies show that *sli* mRNA is transcribed only in the midline and not in the longitudinal axon tracts. However, immunohistochemistry clearly suggests that Slit moves from the midline to the longitudinal axon tracts (Bhat et al., 2007). This led us to conclude that the hypothesis conceived by Rajagopalan (Rajagopalan et al., 2000b) about the Slit gradient model for specifying the lateral positioning of the axon tracts may not be viable *in vitro*. We have clear evidence suggesting that Slit is moved to the longitudinal axon tracts via commissural tracts.

Study of Slit movement became possible after mmy^{slm} was discovered in a genetic screen for aberrant Slit transport conducted in the lab (Figure 1.6). At that point, we had an allele which show lack of Slit movement which could be mapped to find the gene responsible for this phenomenon. The allele was mapped to a mutation in the gene *mmy* (Figure 1.7). This was an interesting turn of events since Mummy had no previously reported role in Slit-Robo signaling pathway.

Analysis of mmy^{slm} axon guidance phenotype:

Along with reduction of Slit levels at the longitudinal tracts, mmy^{slm} also exhibits significant axon guidance defects. The longitudinal tracts are located closer to the midline and there are significant midline crossings. The axon guidance defects vary in severity, from mutants where longitudinal tracts are completely collapsed to mutants with less severe phenotype.

On developmental analysis of the phenotype, it was found that the patterning of the nerve cord in mmy^{slm} was similar to the wild type until ~10 hours of development. The axon guidance defects only arise later, at ~12-14 hours of development (Figure 1.8).

We have demonstrated that Slit protein is glycosylated in wild type embryos (Figure 1.9). On examining mmy^{slm}, Slit was found to be non-glycosylated (Figure 1.9)

which establishes an important and novel role for Mummy in the Slit-Robo signaling pathway.

The occurrence of axon guidance defects later in development could be explained by maternal deposited *mmy* transcript. Maternal Mummy is available until ~10 hours of development and mediates the guidance of axons during early development (Araújo et al., 2005; Tonning et al., 2006). However, zygotic transcription begins after ~10 hours, and loss of functional Mummy manifests as axon guidance defects. Also, the variation in the axon guidance phenotype could be explained by the maternal deposition of *mmy*. Since we had observed individual variations in the maternal *mmy,* the severity of the phenotype may depend on how long the maternal *mmy* lasts. It was also observed that in early mmy^{slm} embryos, Slit was found to be glycosylated (Figure1.11). This is presumably due to functional Mummy protein due to maternal *mmy* transcription. However at later stages, Slit is found to be non-glycosylated (Figure 1.11) which suggests that continuous supply of functional Mummy is required to maintain Slit glycosylation.

Glycosylation of Slit by Mummy mediates Slit translocation and Slit-mediated axon repulsion

We have demonstrated that Mummy is required for Slit glycosylation in wild type embryos. From our results, we conclude that it serves two important purposes;

FINE REGULATION OF AXON GUIDANCE: In mmy^{slm}, the early axon guidance in the ventral nerve cord is similar to that of the wild type until ~10 hours of development due to maternally deposited Mummy *mmy* and Slit is glycosylated (Figure 1.8 and 1.9). After 12 hours of development, we see significant axon guidance defects (Figure 1.8) which is roughly around the time when zygotic *mmy* transcription begins (mutant nonfunctional Mummy protein which prevents glycosylation of Slit)(Araújo et al., 2005; Tonning et al.,

2006). It also shows that the continuous supply of functional Mummy is required for the proper patterning of the ventral nerve cord.

Another interesting result was the efficiency of Slit binding to its receptor, Roundabout. Initially, we thought that lack of Slit glycosylation would prevent it from binding to Robo. However, we found that the efficiency of Slit binding is not reduced by lack of Slit glycosylation (Figure 1.13 C). This poses a very interesting question since Slit can bind to its receptor Robo, but cannot not mediate axonal repulsion.

To address the question of axonal repulsion, I then analyzed the proteolytic cleavage of Slit protein. I found that proteolytic cleavage of Slit is significantly reduced in mmy^{slm} (Figure 1.14 A,B). I propose that full length as well as N-terminal cleaved product is required for activation of the Robo receptor and promotion axonal repulsion.

It could be argued that Mummy affects the function of another downstream molecule in the Slit-Robo signaling pathway or that there is another important player in the Slit-Robo binding complex which is required for activating the downstream repulsive pathway. Another hypothesis is that non-glycosylation of Slit could change its spatial structure, which might be reason for non- activation the Robo receptor.

This model system shows the importance of Mummy and Slit glycosylation for axon guidance.

SLIT TRANSLOCATION: We have previously reported that Slit gets translocated from the midline in to the longitudinal axon tracts. Immunostaining of mmy^{slm} suggests that translocation of Slit is significantly reduced due to loss of functional Mummy protein.

We know that proteolytic cleavage of Slit does not have a role in Slit translocation. Both antibodies, raised against the N- and the C-terminal epitope of Slit protein detect the presence of Slit in the longitudinal tracts which suggest that it is the full length Slit protein that moves into the longitudinal tracts. However, the mechanistic

importance of glycosylation for Slit translocation is not clear at this point. It is likely that glycosylation may alter the binding of Slit protein to a transporter molecule, which prevents its movement to the longitudinal tracts.

Future work would include an extensive genetic screen for other Slit distribution mutants to identify other molecules involved in this pathway.

Mummy mediates Robo down-regulation and affects Slit-Robo signaling pathway.

Apart from its effect on Slit, Mummy also regulates the Slit specific receptors Robo, Robo2 and Robo3. Loss of functional Mummy leads to reduced levels of all the three Robo receptors. The reduction in Robo proteins would also add to the axon guidance defects. As previously discussed, non-glycosylated Slit binds to Robo efficiently. However, reduction of Robo together with non-glycosylated Slit worsens the guidance defects in mmy^{slm}. This highlights the importance of Mummy in the developing embryo.

I have shown that the reduction of Robo in mmy^{slm} is a post-transcriptional process. However, the mechanism by which this occurs is not known. Commissureless (*comm)* has a reported function for downregulation of Robo in the commissural axons (Keleman et al., 2002)) . It is possible that there is another protein which regulates Robo protein level in the longitudinal axon tracts.

Although, it has been previously reported that the movement of Slit into the longitudinal tracts is a *robo* independent process (Bhat et al., 2007), it is possible that *robo2* and *robo3*gene products could bind to Slit and transport it in the absence of *robo.* To determine this, we generated triple *robo* mutants which are null for all the three *robo*

genes. I wanted to examine the tracts for the presence of Slit, which would determine if the Robo protein is involved in the movement of Slit into the tracts. Lack of all three *robo* proteins resulted in the longitudinal tracts collapsing in to the midline, as expected. However, I compared the pattern of wild type Slit expression in the midline and triple *robo* mutants stained with anti-Slit. Careful analysis showed that the tracts still showed presence of Slit even though all three *robo* gene products were absent. This suggests that Robo, Robo2 and Robo3 have no functional role in the movement of Slit from the midline to the longitudinal tracts.

I also tried to ectopically over-express Robo in mmy^{slm} to analyze if axon guidance defects could be reversed. I used the UAS-Gal4 system to pan-neurally express Robo in the mmy^{slm} background and analyzed the axon guidance phenotype. There was no significant change in the axon guidance phenotype. This could be due to the fact that both Slit glycosylation and reduction in Robo levels is the cause of axon guidance phenotype. Only increasing the Robo levels would not rescue the axon guidance phenotype of the mutants. To address this, we could also generate Slit protein which does not have glycosylation sites and thus do not get glycosylated. However, this in itself would be a mutant Slit protein and may not be regulated by Mummy as in wild type. This is one of the inherent drawbacks of the model.

To summarize, Mummy regulates both Slit and Robo proteins to mediate axon guidance in the developing ventral nerve cord. Glycosylation of Slit affects its movement in to the longitudinal axon tract as well as effective proteolytic cleavage. Both of these have an effect on the guidance of the tracts. Robo proteins are reduced in-directly by Mummy, which also contributes to the axon guidance phenotype observed in the mutant.

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