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Genetic Mechanisms Regulating Neural Stem Cell Self-renewal and Differentiation in the Central Nervous System of Drosophila

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**Genetic Mechanisms Regulating
Neural Stem Cell Self-renewal and
Differentiation in the
Central Nervous System
of *Drosophila*.**

A thesis submitted to King's College London in fulfilment of the degree of

Doctor of Philosophy

(Neuroscience)

By

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- March 2013 -

Declaration

I hereby declare that all of the work presented in this thesis is my own.

Dongwook Kim

April 2013

Aknowledgements

I would like to thank all the people who have supported me through this process. First of all, I would like to thank Dr Frank Hirth, whose supervision has facilitated my development as a researcher over the past four years. I would also like to thank members of my group for their help and advice, both scientific and personal. I would like to express my gratitude to Dr Brenda Williams, who was always available to provide support and encouragement when I needed it the most. I would also like to thank my family and friends for supporting me in the final stages. Finally, I would like to express my heartfelt gratitude to my family and friends outside the IoP.

Abstract

Asymmetric cell division plays a fundamental role in maintaining a balance between stem cell self-renewal and differentiation. A failure of this balance results in over-proliferation of stem cells, which could eventually lead to neoplastic over-growth and metastasis, i.e. tumourigenesis.

Key components of this genetic machinery in *Drosophila* CNS involves unequal segregation of differentiation factors such as brain tumour (Brat) and prospero (Pros), with their adaptor protein miranda (Mira). Using post-embryonic neuroblasts (NBs) as a model, I demonstrate basal co-localisation of Mira/Brat/Pros during late metaphase. RNAi-mediated knockdown of Brat or Pros result in excess stem cell self-renewal at the expense of neuronal differentiation, leading to over-proliferation of NBs. These data suggest Mira/Brat/Pros are likely to form a complex during post-embryonic NB division. However, how these cell fate determinants complexes are basally targeted remain unknown. Previous studies in the embryonic CNS implied a role of actin-myosin based transport in basal targeting. To investigate whether this is true for post-embryonic NBs, I conducted pharmacological interference experiments. Application of 2, 3-Butanedione monoximine (BDM), a non-muscle myosin inhibitor, or Latrunculin B, an actin polymerisation inhibitor to larval CNS demonstrated a failure in asymmetric segregation of Mira, indicating that both actin and myosin are required for basal targeting of cell fate determinants during NB division. To identify which *Drosophila* myosin motor(s) are involved, I studied the function of non-muscle myosin II, myosin V and myosin VI, that were previously implicated in basal targeting of the cell fate determinants by RNAi targeted knockdown. Mitotic spindle defects were observed in myosin V and myosin VI knockdown, suggesting a common functional pathway for the two myosin motors. Double knockdown of both myosin V and VI appeared to

exacerbate the mitotic spindle defect and affected neural stem cell self-renewal, causing a mild over-proliferation phenotype in the larval central brain, but did not result in tumourigenesis. My data suggest that synergistic activity of myosin V and myosin VI regulate neural stem cell self-renewal and differentiation decision in the post-embryonic central nervous system of *Drosophila* by regulating mitotic spindle orientation.

Table of Contents

<u>Declaration</u>	2
<u>Aknowledgements</u>	3
<u>Abstract</u>	4
<u>Table of Contents</u>	6
<u>List of figures</u>	12
<u>List of tables</u>	15
<u>Chapter 1: Introduction</u>	17
<u>1.1 Introduction</u>	17
<i>1.1.1 The importance of stem cells research</i>	<i>17</i>
<i>1.1.2 Stem cells and tumourigenesis</i>	<i>18</i>
<i>1.1.3 Using Drosophila NBs as a model system for stem cell research</i>	<i>18</i>
<u>1.2 Modes of stem cell division</u>	20
<u>1.3 Neural stem cells in Drosophila</u>	22
<i>1.3.1 Embryonic NBs</i>	<i>23</i>
<i>1.3.2 Post-embryonic NBs</i>	<i>24</i>
<i>1.3.3 Types of post-embryonic NBs</i>	<i>25</i>

<u>1.4 Establishment of NB polarity during asymmetric division of neural stem cells in <i>Drosophila</i> CNS</u>	30
1.4.1 <i>Polarity formation of NBs</i>	30
1.4.2 <i>Coupling cortical polarity to mitotic spindle positioning</i>	34
1.4.3 <i>Telophase rescue</i>	35
<u>1.5 The mechanism of asymmetric neural stem cell division in <i>Drosophila</i></u>	36
1.5.1 <i>The role of apical protein complex in asymmetric division of NBs</i>	36
1.5.2 <i>Basal cell fate determinants</i>	39
1.5.3 <i>Mechanisms of basal protein targeting</i>	44
1.5.4 <i>Asymmetric cell division in the mammalian brain</i>	49
1.5.5 <i>Role of Brat and Pros in vertebrates</i>	51
<u>1.6 <i>Drosophila</i> myosins</u>	52
<u>1.7 Candidate <i>Drosophila</i> myosins implicated in basal targeting of cell fate determinants</u>	55
1.7.1 <i>Myosin II</i>	55
1.7.2 <i>Myosin V</i>	57
1.7.3 <i>Myosin VI</i>	61
<u>1.8 Principle objectives of research</u>	65
<u>Chapter 2: Materials and Methods</u>	66
<u>2.1 <i>Drosophila</i> culture</u>	66
2.1.1 <i>Fly food</i>	66
2.1.2 <i>Fly husbandry</i>	67

<u>2.2 Genetics</u>	67
2.2.1 <i>Drosophila</i> lines.....	67
2.2.2 The <i>Gal4/UAS</i> system for tissue-specific gene activation.....	70
2.2.3 RNA interference.....	72
2.2.4 Target sequences of the RNAi constructs.....	74
<u>2.3 Pharmacological interference</u>	77
2.3.1 Drug treatments	77
2.3.2 Quantification of neuroblasts	77
2.3.3 Statistical analysis of drug treatments.....	78
<u>2.4 Immunohistochemistry</u>	79
2.4.1 Embryo staging and collections.....	79
2.4.2 Embryo fixations	80
2.4.3 Larval dissections and immunostaining	82
2.4.4 Antibodies used.....	85
2.4.5 Microscopy and image analysis.....	86
2.4.6 Quantification of Neuroblasts.....	86
2.4.7 Statistical analysis of RNAi experiments	87
2.4.8 Quantification of spindle orientation.....	87
<u>Chapter 3: Characterisation of the role of cell fate determinants in asymmetric division of post-embryonic NBs</u>	88
<u>3.1 Introduction</u>	88
<u>3.2 Identification of post-embryonic NB lineages in the larval CNS of <i>Drosophila</i></u>	89

3.2.1 Overview of post-embryonic NB lineages in the third instar larval CNS.....	89
3.2.2 1407 Gal4 targets mCD8-GFP expression to NB lineages in the third instar larval CNS.	90
<u>3.3 Mira is asymmetrically segregated in dividing post-embryonic NBs in a cell cycle-dependent manner.....</u>	92
<u>3.4 1407 Gal4 driven Mira-GFP expression partially overlaps with endogenous Mira in a basal crescent during asymmetric division of post-embryonic NBs</u>	94
<u>3.5 Brat and Pros co-localise with Mira in a basal crescent during asymmetric division of post-embryonic NBs</u>	95
<u>3.6 Genetic knock-down of Brat or Pros leads to over-proliferation of central brain post-embryonic NBs in 3rd instar larval CNS</u>	98
3.6.1 Mutation in Brat or Pros leads to excess self-renewal at the expense differentiating neurons.....	98
3.6.2 Genetic knock-down of Brat or Pros leads to increased number of Mira positive central brain NBs in late larval stages.	99
3.6.3 Type II NB lineages are susceptible to over-proliferation.....	106
3.6.4 Co-expression of dicer with pros RNAi, which significantly reduced protein expression, hence enhancing the tumour phenotype.....	108
<u>Chapter 4: Role of actin in basal targeting of cell fate determinants during asymmetric division of post-embryonic NBs</u>	110
<u>4.1 Introduction.....</u>	110

<u>4.2 F-actin co-localises with Mira during asymmetric division of post-embryonic NBs</u>	111
<u>4.3 Pharmacological interference of actin functions disrupts asymmetric segregation of Mira in dividing post-embryonic NBs</u>	113
<u>4.4 Pharmacological interference of actin function does not lead to an over-proliferation phenotype</u>	117
<u>Chapter 5: Identifying the candidate myosin motor responsible for basal targeting of cell fate determinants during asymmetric division of post-embryonic NBs</u>	123
<u>5.1 Introduction</u>	123
<u>5.2 Pharmacological interference of myosin functions disrupts asymmetric segregation of Mira in dividing post-embryonic NBs</u>	126
<u>5.3 Validating the specificity of RNAi mediated knockdown experiments using BLAST search</u>	130
<u>5.4 Genetic knockdown of Myosin II leads to partial cellular over-growth of post-embryonic NBs due to endomitosis</u>	132
<u>5.5 Establishing the role of the candidate myosin motors in asymmetric division of post-embryonic NBs in <i>Drosophila</i></u>	137
5.5.1 <i>Identifying the candidate myosin motor responsible for basal targeting of Mira</i>	137
5.5.2 <i>RNAi mediated knockdown of Myosin V or Myosin VI does not lead to over-proliferation of central brain post-embryonic NBS</i>	139

5.5.3 Genetic knockdown of Myosin V or Myosin VI leads to mitotic spindle mis-orientation	145
5.5.4 Double Knockdown of Myosin V and Myosin VI leads to increased mitotic spindle defect.	147
5.5.5 Double knockdown of Myosin V and Myosin VI leads to increased number of NBs at late third instar larval central brain.	152
Chapter 6: General discussion.....	158
<u>6.1 Summary of findings</u>	158
<u>6.2 Cell fate determinants Brat and Pros basally segregate into GMC as cargo proteins of their adaptor protein Mira during asymmetric division of post-embryonic NBs.</u>	160
<u>6.3 Defective NB division: a model for tumour formation</u>.....	161
<u>6.4 Pharmacological inhibition of actin or myosin results in cortical de-localisation of Mira.</u>	163
<u>6.5 Myosin II is required for cytokinesis in post-embryonic NBs.</u>.....	164
<u>6.6 Myosin V or Myosin VI do not directly contribute to basal targeting of the cell fate determinants.</u>	165
<u>6.7 Myosin V and Myosin VI may act in a common pathway to regulate mitotic spindle alignment during asymmetric division of NBs.</u>.....	168
<u>6.8 Evaluation of the RNAi mediated knockdown experiments.</u>.....	172
<u>6.9 Conclusion</u>	173
<u>References</u>	177

List of figures

Chapter 1

Figure 1.1 Modes of stem cell division	Error! Bookmark not defined.
Figure 1.2 Type I NB lineage	21
Figure 1.3 Overview of the <i>Drosophila</i> CNS.	25
Figure 1.4 Molecular mechanisms involved in asymmetric division of NBs.	31
Figure 1.5 Structure of the basal cell determinants.....	39
Figure 1.6 Mechanism involved in basal targeting of cell fate determinants.....	46

Chapter 2

Figure 2.1 The Gal4/UAS system for tissue-specific gene expression in <i>Drosophila</i>	70
Figure 2.2 RNAi mediated knockdown of the gene of interest in <i>Drosophila</i>	72

Chapter 3

Figure 3.1 1407 Gal4 driven expression of mCD8::GFP reveals post-embryonic NB lineages in 3rd instar larval CNS	90
Figure 3.2 Mira localisation is cell cycle dependent during asymmetric division in post-embryonic NBs.....	91
Figure 3.3 Cell fate determinants Brat and Pros co-localise with their adaptor protein Mira during post-embryonic NB division in a 3rd instar larval CNS.....	93
Figure 3.4 Expression of 1407 Gal4 driven Mira-GFP partially overlaps with endogenous Mira expression during post-embryonic NB division in 3rd instar larval CNS.....	95

Figure 3.5 Mutation in cell fate determinants Brat or Pros, as well as their adaptor protein Mira leads to excess self-renewal at the expense of differentiating neurons.....	97
Figure 3.6 RNAi mediated knockdown of Brat or Pros leads to over-proliferation of central brain NBs.....	99
Figure 3.7 RNAi mediated knockdown of Brat or Pros leads significantly increase in the number of post-embryonic NBs in the central brain.....	101
Figure 3.8 knockdown of Pros or Brat leads to over-proliferation of INPs in type II post-embryonic NBs at the expense of differentiation.	106
Figure 3.9 Co-expression of 1407 Gal4 driver with Dcr2 increases efficiency of Pros RNAi.....	108

Chapter 4

Figure 4.1 F-actin co-localises with mira during asymmetric division of post-embryonic NBs	110
Figure 4.2 The effect of Latrunculin B treatment on asymmetric Mira localisation in telophase NBs, in vivo.....	113
Figure 4.3 Pharmacological interference of actin does not lead to over-proliferation of central brain post-embryonic NBs.	118
Figure 4.4 Pharmacological interference of actin does not result in a significant increase in the number of central brain post-embryonic NBs.....	120

Chapter 5

Figure 5.1 BDM treatment disrupts asymmetric division of NBs at third instar larval central brain	127
Figure 5.2 validation of target specificity of Myosin II, Myosin VI and Myosin V RNAi constructs using BLAST search.....	130

Figure 5.3 Genetic knockdown of Myosin II affects asymmetric Mira segregation during NB division in larval central brain	133
Figure 5.4 RNAi mediated knockdown of Myosin V or Myosin VI does not lead to over-proliferation of central brain NBs	139
Figure 5.5 RNAi mediated knockdown of Myosin V or Myosin VI do not significantly increase the number of central brain NBs.....	142
Figure 5.6 RNAi mediated knockdown of Myosin V or Myosin VI leads to mitotic spindle defect	145
Figure 5.7 RNAi mediated double knockdown of Myosin V and Myosin VI leads to increased mitotic spindle defect	147
Figure 5.8 Double knockdown of Myosin V and Myosin VI leads to increased spindle defect during asymmetric division of post-embryonic NBs at third instar larval stage.....	148
Figure 5.9 Double knockdown of Myosin V and Myosin VI leads to increased number of NBs at late third instar larval central brain.....	152
Figure 5.10 Double knockdown of Myosin V and Myosin VI leads to significantl increase in number of post-embryonic central brain NBs at 96h ALH.....	154

List of tables

Chapter 1

Table 1.1 Physiological functions myosin genes expressed in <i>Drosophila</i>	52
--	-----------

Chapter 3

Table 3.1 Number of NBs undergoing asymmetric division in control vs Latrunculin B treated group.....	103
--	------------

Chapter 4

Table 4.1 Number of post-embryonic NBs undergoing asymmetric division in control vs Latrunculin B treated group.....	115
---	------------

Table 4.2 Mean numbers of Mira positive central brain post-embryonic NBs in Control vs. Latrunculin B treated group.....	121
---	------------

Chapter 5

Table 5.1 Number of post-embryonic NBs undergoing asymmetric division in control vs BDM treated group.....	128
---	------------

Table 5.2 Mean number of Mira positive post-embryonic NBs in wild type vs. Myosin V IR brain or Myosin VI IR brain	143
---	------------

Table 5.3 Quantification of mitotic spindle angles mis-positioned away from apical-basal axis.	149
--	------------

Table 5.4 Mean number of post-embryonic NBs in RNAi mediated Myosin V and Myosin VI double knockdown brains vs. wild type	156
--	------------

Chapter 1: Introduction

1.1 Introduction

1.1.1 The importance of stem cells research

Extensive studies have been conducted to investigate the two fundamental properties of stem cells; their ability to undergo self-renewal and differentiation. It is these unique properties of stem cells that make them an attractive tool for researching aetiology and treatment of various diseases. As for potential therapeutic uses of stem/progenitor cells, among the most promising applications include cell replacement treatment for neurodegenerative disorders. With high prevalence in the aging population, neurodegenerative disease is notable for progressive loss of neurons in the central nervous system (CNS). However, most of the conventional therapeutic approaches are aimed at alleviating motor and psychiatric symptoms, rather than to prevent or halt the progression of the disease (Bjorklund and Lindvall, 2000; Lindvall and Kokaia, 2006). This is mainly due to lack of knowledge on the underlying pathophysiology of the diseases, coupled with the nature of the mammalian CNS that does not allow for extensive regeneration. The self-renewing capacity and multi-potency may allow for stem cells to provide an unlimited source of neurons and glia for functional restoration.

1.1.2 Stem cells and tumourigenesis

Stem cells have recently become implicated as the causal factor for solid tumours found in various types of cancers. This is particularly evident in the case of human malignant gliomas. Histological features of the disease indicate that tumours may arise from neural progenitor cells (Galli et al., 2004) and/or de-differentiated astrocytes (Dufour et al., 2009). Several treatment options include surgical interventions, radiotherapy or chemotherapy. However, further course of the disease is often overshadowed by the recurrence of cell over-proliferation, and subsequent infiltration of neighbouring tissues (reviewed in Merlo, 2003). This observed therapeutic resistance have led to the ‘cancer stem cell’ hypothesis, in which aberrant activation of the self-renewal pathways of stem/progenitor cells may account for tumourigenesis in some cancers (reviewed in Visvader and Lindeman, 2008). However, insights into the underlying mechanisms is yet to be elucidated, and will require better understanding of the genetic control of stem cell self-renewal and differentiation.

1.1.3 Using *Drosophila* NBs as a model system for stem cell research

The *Drosophila* central nervous system (CNS) is an excellent simple model system for studying the cellular and molecular mechanisms that control stem cell divisions. Specifically, the *Drosophila* neural stem cells, called neuroblasts (NBs) that undergo repeated rounds of asymmetric cell division (Reviewed in Egger et al., 2008). NBs are one of the most extensively used model system for understanding stem cell biology during normal development, as well as

for stem cell derived brain tumours that arise due to loss of control of the asymmetric division of stem cells (reviewed in Caussinus and Hirth, 2007; Wu et al., 2008).

Here, I aim to summarise recent key discoveries in this field mostly from *Drosophila* neural stem cells, known as neuroblasts, with focus on different modes of stem cell proliferation, establishment of cell polarity and the regulation of mitotic spindle orientation and the asymmetric segregation of cell fate determinants. In addition, I will explore how these events contribute to the key decision on the switch between neural stem cell self-renewal and differentiation.

1.2 Modes of stem cell division

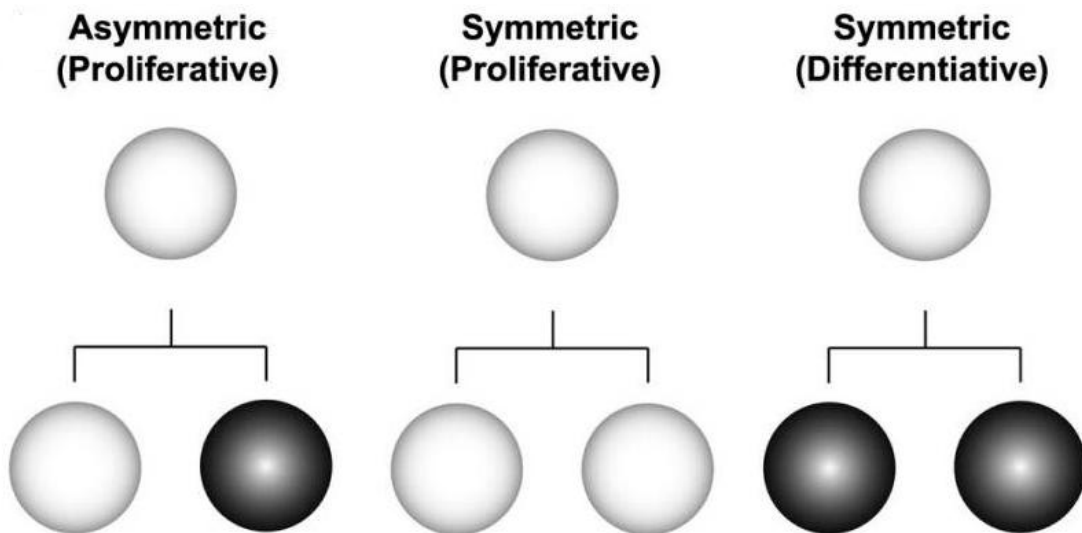


Figure 1.1 Modes of stem cell division. Stem cells employ either symmetric or asymmetric modes of division to regulate a balance between maintaining the number of stem cell pool available and the supply of differentiated cells. Asymmetric division results in generation of a self-renewing daughter (white circles), and a differentiating daughter (black circles). The symmetric division generates two identical daughters, which can be either self-renewing daughters or differentiating daughters (adapted from Kim and Hirth, 2009).

Stem cells in both invertebrates such as *Drosophila*, or mammals share the defining hallmark of self-renewal, which either expands or maintains the available stem cell pool, but can also differentiate to generate more specialised progeny that become increasingly restricted in their lineage potential during development (Eckfeldt et al., 2005). Employing different modes of division allows the stem cells to extensively increase their numbers by undergoing ‘proliferative’ symmetric division that generate two daughter cells with the same fate, or to increase the supply of differentiating cells via asymmetric division (Figure 1.1). Regulating the balance between asymmetric and symmetric modes of division is crucial during development and in adulthood. Depletion of the critical stem cell pool can lead to under-development of tissues and organs, whereas over-proliferation of stem cells may lead to tumourigenesis (reviewed in Caussinus and Hirth, 2007).

1.3 Neural stem cells in *Drosophila*

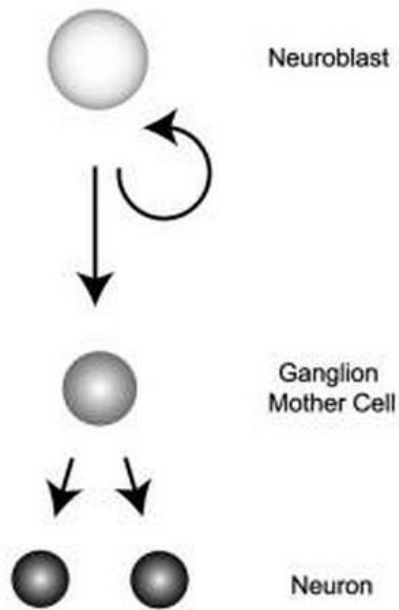


Figure 1.2 Type I NB lineage. During *Drosophila* neurogenesis, NB divides in a stem cell like fashion to simultaneously give rise to a self-renewing daughter, as well as one ganglion mother cell (GMC, shown in gray). GMCs are intermediate precursor cells that undergo terminal division to generate two post-mitotic neurons (black) (adapted from Kim and Hirth, 2009).

1.3.1 Embryonic NBs

During the embryonic period of neurogenesis, about 30 NBs in each hemi-segment delaminate from the ventral neuroectoderm and undergo asymmetric division to generate two daughter cells with different fates. The larger daughter retains stem-cell like properties and self renews, while the smaller daughter, called ganglion mother cell (GMCs). Each GMCs divides once more to generate two post-mitotic daughter cells that differentiate into neurons and/or glial cells (Figure 1.2). Each embryonic NBs produce multiple cell lineages during development that form motor neurons, interneurons and glial cells (Schmidt et al., 1997). Gliogenesis in *Drosophila* embryonic CNS requires concomitant activation of glial and repression of neuronal differentiation genes. The glial cell fate is specified by the master regulatory gene, *glial cell missing/glial cell deficient* (*gcm/glide*, henceforth *gcm*). *Drosophila gcm* is transiently expressed in cells destined to differentiate into glia and are required for gliogenesis of most glia in the CNS and all glia in the peripheral nervous system (PNS) (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996). *Gcm* promotes differentiation of glial cells by activating downstream target genes such as *reversed polarity* (*repo*) and *pointed* (*pnt*) (Campbell et al., 1994; Klaes et al., 1994), and represses the neuronal fate through the activation of *tramtrack* (*ttk*) (Giesen et al., 1997; Yuasa et al., 2003), each of which appear to be expressed in all *gcm* positive glia.

Embryonic NBs divide up to 20 times, decreasing in size at each division until the end of embryogenesis, when they stop dividing. A subset of NBs become quiescent until the larval stage, when they enlarge and divide to generate the adult nervous system.

1.3.2 Post-embryonic NBs

During the post-embryonic phase of neurogenesis, larval NBs undergo repeated asymmetric division, but do not shrink with each division, unlike their embryonic counterpart (Bossing et al., 1996). Instead, self-renewing larval NBs have the capacity to re-grow back to the size of their parental NBs, thus, can proliferate for extended periods of time during larval stages. Consequently, post-embryonic NBs generate larger lineages of post-mitotic progeny that constitute majority of the adult *Drosophila* CNS through repeated rounds of ‘classic’ asymmetric divisions (Ito and Hotta, 1992), making them an attractive model system for studying stem cell self-renewal and differentiation.

In contrast to embryonic gliogenesis (Giesen et al., 1997), the genesis of glia in the *Drosophila* post-embryonic nervous system is poorly understood, particularly in the brain. Gliogenesis is thought to restart during the second instar and increases exponentially throughout the third instar (Pereanu et al., 2005; Awasaki et al., 2008). During this period, glia are generated both from neuroglioblast precursors and by glial cell division. Clonal analysis reveals that distinct glial types derive from different precursors, and that most adult perineurial, ensheathing and astrocyte-like glia are produced after embryogenesis. With the exception of perineurial glial cells that are made locally on the brain surface without the involvement of *gcm*, the widespread ensheathing and astrocyte-like glia derive from specific brain regions in a *gcm*-dependent manner (Awasaki et al., 2008).

1.3.3 Types of post-embryonic NBs

The *Drosophila* larval brain can be divided into the paired optic lobes and the central brain (Figure 1.3). The neurons that constitute these structures derive from NBs that undergo multiple rounds of stem cell-like divisions. Several types of larval brain NBs can be distinguished on the basis of their lineage and position within the brain (Figure 1.3). Most prevalent are the type I NBs that undergo classical asymmetric division to generate a self-renewing daughter and a GMC, which subsequently divides into two differentiating neurons/glial cells.

Central Brain NBs

Central brain NBs are responsible for generating neurons that differentiate into specific functional domains of the adult brain such as the central complex involved in locomotor control and the antennal glomeruli involved in olfactory information processing (Ito and Awasaki, 2008). The proliferative activity of type I central brain NBs during embryonic and post-embryonic stages is comparable and relies on asymmetric division.

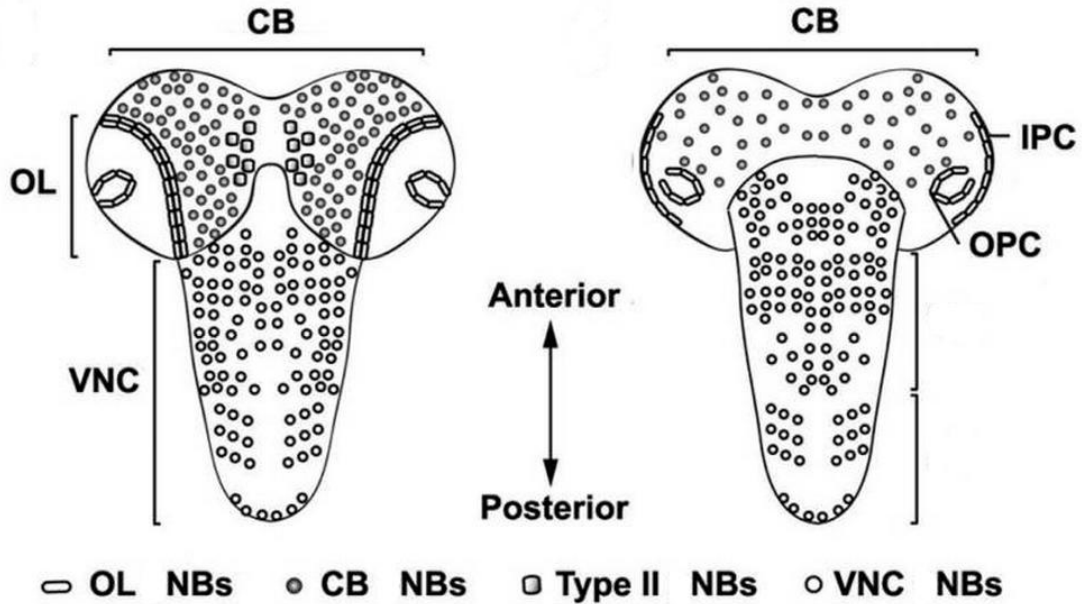


Figure 1.3 Overview of the *Drosophila* CNS. The *Drosophila* larval CNS consists of the central brain (CB), ventral nerve cord (VNC), and optic lobes (OL) that consists of inner- (IPC) and outer- (OPC) proliferation centres. Post-embryonic NBs can be found in the optic lobe, central brain and ventral nerve cord. Recently identified type II central brain NBs are positioned within the dorso-medial region of the central brain (Adapted from Kim and Hirth, 2009).

The type II central brain NBs have recently been discovered in dorso-medial region of the two central brain hemispheres (Bello et al., 2008; Bowman et al., 2008; Boone and Doe, 2008). The type II central brain NBs give rise to a different cell lineage to that of type I NBs. The smaller daughter cell initiates expression of the proneural gene *asense* and becomes intermediate neural progenitor cell (INP) that undergoes a limited number of self-renewing asymmetric divisions with each division resulting in one INP and one GMC (Bello et al., 2008). Recent discovery of INPs in mammalian brain development suggests that evolutionarily conserved mechanism underlies neurogenesis in *Drosophila* and vertebrates (Gotz and Huttner, 2005; Merkle and Alvarez-Buylla, 2006).

Clonal analysis of the neuronal progeny generated by 8 pairs of type II NBs during post-embryonic development indicates that the adult specific secondary neurons in these lineages form complex and wide-spread longitudinal and commissural projections in the brain. Interestingly, a subset of these secondary neurons form major arborizations in the central complex neuropile (Izergina et al., 2009; Bayraktar et al., 2010; Boyan and Reichert, 2011), which is involved in information processing, memory, as well as co-ordination of motor control in *Drosophila* (Strauss, 2002; Liu et al., 2006a).

The neurons generated from type II NB lineage was proposed to have dual roles in the development of complex brain neuropile (Riebli et al., 2013). During larval stages, type II NB lineages contribute to the formation of a specific central complex primordium (Victorin et al., 2011). During subsequent pupal development, type II NB lineages undergo extensive growth and differentiation and integrate into the modular circuitry of the central complex of the adult brain.

Thus, in addition to generating a large number of structurally diverse neurons, which comprise the intrinsic neurons of the central complex, type II NBs also generate specific glial cells that ensheath the neuropile components of the central complex (Izergina et al., 2009; Bayraktar et al., 2010; Viktorin et al., 2011). Therefore, the type II NBs appear to generate the neural primordium, the mature neuronal cells, and the glial cells for the central brain complex.

Optic lobe NBs

Type I NBs of the optic lobes derive from the neuroepithelial cells known as the inner- and outer-proliferation centres adjacent to the central brain during larval stages (Figure 1.3) (White and Kankel, 1978). The lobe neuroepithelial cells initially undergo proliferative symmetric division to expand the pool of precursor cells that transform into optic lobe NBs in an ordered and highly localised manner in response to a wave of pro-neural gene expression that traverses the neuroepithelium (Ceron et al., 2001). Subsequent to their formation, the optic lobe NBs switch to a neurogenic mode and proliferate by undergoing a limited number of asymmetric cell divisions to generate neuronal progeny in a manner similar, but not identical to that of the asymmetrically dividing NBs in the central brain (Egger et al., 2007)

Other type I NBs

Other specialised kinds of post-embryonic type I NBs include the mushroom body NBs that give rise to Kenyon cells involved in learning and memory formation (Ito and Hotta, 1992; Ito et al., 1997; Egger et al., 2007). Moreover, in the ventral nerve chord (VNC), around 60 NBs repeatedly divide in an asymmetric manner to form the neurons of the thoracic ganglia, some of which later innervate the wings and legs of the adult animal (Truman et al., 2004).

1.4 Establishment of NB polarity during asymmetric division of neural stem cells in *Drosophila* CNS

The basic mechanism of asymmetric division is common to all types of NBs in *Drosophila* CNS. Among the identified key intrinsic and extrinsic factors that contribute to asymmetric division of NBs, the most important trait appears to be the polarised distribution of two evolutionarily conserved protein complexes that defines axis of polarity. The next step entails coupling cell polarity to the segregation of cell fate determinants into the smaller daughter cell, the GMC (Figure 1.4). Tight co-ordination of spindle orientation and asymmetric localisation ensures that cell fate determinants are inherited by only one of the two daughter cells.

1.4.1 Polarity formation of NBs

At metaphase, NBs localise factors that specify differentiation to one end of the cortical domain, whereas the opposite cortical domain is occupied by a complex of proteins that are ultimately partitioned into the self-renewing daughter (Figure 1.4 A). During embryogenesis, NBs delaminate from the ventral neural ectoderm, which are polarised epithelial cells that divide symmetrically in the plane of the epithelium. The delaminating NBs re-align their mitotic spindle perpendicular to the epithelial plane and inherit the evolutionarily conserved Par protein complex consisting of Par-3 (Bazooka) (Schober et al., 1999), Par-6 (DmPar6) (Petronczki and Knoblich, 2001), and the *Drosophila* atypical protein kinase C (DaPKC) (Betschinger et al., 2003; Rolls et al., 2003; Izumi et al., 2004) from the over-lying neuroectoderm. The Par protein complex localises in a crescent at the apical cortex of embryonic NBs, where they are required for

establishing and maintaining apical-basal polarity (Wodarz et al., 1999; Petronczki and Knoblich, 2001). During NB delamination, an adaptor protein called Inscuteable (Insc) becomes expressed and binds the Par protein complex through Bazooka. Upon binding the Par protein complex, Insc recruits another adaptor protein called Partner of Inscuteable (Pins) (Nipper et al., 2007), which in turn binds the heterotrimeric G-protein subunit Gai through its GoLoco domain (Yu et al., 2005). Subsequently, Pins recruits an additional protein called Mushroom body defect (Mud) (Bowman et al., 2006; Izumi et al., 2006; Siller et al., 2006), the *Drosophila* homolog of the microtubule and dynein binding protein NuMA (Zheng, 2000; Sun and Schatten, 2006). Mud is thought to provide a ‘docking site’ for astral molecules, enabling one end of the spindle pole to attach to the apical cortex of the NBs, thus contributing to the apical-basal orientation of the mitotic spindle (Bowman et al., 2006). The final sequence of events leading to polarity formation involves recruitment of a membrane associated guanylyl kinase protein, Discs large (Dlg) and Kinesin heavy chain 73 (Khc-73) through Pins into the apical protein complex (Siegrist and Doe, 2005). These interactions polarise the complex of proteins localised at the apical cortex of a NB and define the orientation of the mitotic spindle in an apical-basal manner, aligned perpendicular to the overlying epithelial plane (Siegrist and Doe, 2005; Yu et al., 2005). Apical protein complex therefore direct apical-basal spindle orientation in dividing NBs, and establish an axis of polarity along which cytokinesis takes place. This sets a platform for cell fate determinants to exclusively segregate along the polarity axis into the basal cortical domain in subsequent mitotic divisions (Figure 1.4). Although asymmetric division in larval NBs are not uniformly oriented relative to the organismal axis, I will refer to the cortical domain that becomes GMC as the basal, and the other as the apical cortex.

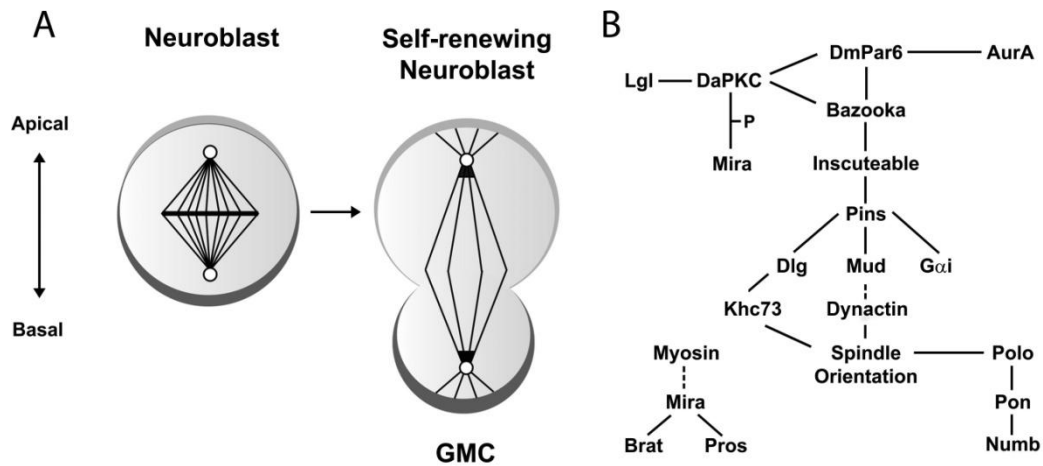


Figure 1.4 Molecular mechanisms involved in asymmetric division of NBs. (A) Cell fate determinants (Dark crescent) localise basally at metaphase, and segregate into GMC by telophase. The evolutionarily conserved apical protein complex (light gray crescent) ensures stem cell self-renewal and maintains spindle orientation. (B) Summary of key proteins involved in asymmetric NB division (adapted and updated from Kim and Hirth, 2009).

Improper establishment of polarisation of NBs lead to failure in cell specification; It can result in over-proliferation of NBs, eventually leading to tumourigenesis (Caussinus and Gonzalez, 2005; Bello et al., 2006; Lee et al., 2006a). However, as discussed in the following section, apical protein complex do not directly contribute to basal cell fate determination, but instead are responsible for regulating basal polarity and maintaining mitotic spindle alignment along the polarity axis.

1.4.2 Coupling cortical polarity to mitotic spindle positioning

Proper asymmetric division of NB requires alignment of the mitotic spindle along the apical-basal polarity axis (Figure 1.4 A) (Siller and Doe, 2008). The importance of spindle alignment can be highlighted with the formation of a cleavage furrow between the apical and basal cortical domains, enabling exclusive segregation of factors into two daughter cells. Cortical polarity and spindle orientation is connected by an intermediary protein called Inscuteable that interacts with both bazooka of the Par protein complex, and Pins (Kraut et al., 1996) that in turn recruits Mud to establish a cortical attachment site for astral microtubules to orient the mitotic spindle in metaphase (Bowman et al., 2006; Nipper et al., 2007). Accordingly, mutation in Mud protein “un-links” cortical polarity and spindle positioning, as the spindle orientation becomes less correlated with the polarity axis, leading to over-proliferation of larval central brain and mushroom body NBs, presumably due to mis-segregation of cell fate determinants (Bowman et al., 2006; Izumi et al., 2006; Siller et al., 2006). However, the over-proliferation phenotype observed in mud mutants is very mild compared to the over-proliferation phenotypes in basal cell fate determinants. In fact, mutation in any component of the apical complex similarly results in mis-localisation of cell fate determinants in metaphase, but undergoes ‘telophase rescue’ to correctly segregate proteins into only one daughter cell (Wang et al., 2006b).

1.4.3 Telophase rescue

Although the exact mechanisms of this rather enigmatic phenomenon is still unknown, the currently proposed model suggests that two distinct pathways exist for polarisation of embryonic NBs: a well established microtubule-independent cortical pathway involving Par protein complex interacting with mitotic spindle via Inscuteable, and the microtubule-dependent pathway (Siegrist and Doe, 2005). In *Drosophila* NBs, interaction of G-protein subunit Gai, Pins and Mud establish a cortical attachment site for astral microtubules to orient the mitotic spindle in metaphase. In telophase, however, it is the mitotic spindle that influences cortical polarity of NBs through a microtubule-dependent pathway. In this case, Khc73 which is transported on astral microtubules binds Dlg, this in turn recruits G-protein subunit Gai and Pins, which then interacts with Mud. This mutual microtubule-cortex interaction is believed to stabilise spindle orientation (Figure 1.4B). Interestingly, the secondary microtubule-dependent pathway is not required for asymmetric protein localisation in wild type NBs, as pharmacological interference of microtubules did not result in disruption of asymmetric division in metaphase (Siegrist and Doe, 2005).

1.5 The mechanism of asymmetric neural stem cell division in *Drosophila*

Establishment of polarity and alignment of mitotic spindle orientation along the apical-basal NB polarity axis sets a platform for asymmetric protein localisation, and subsequent exclusive segregation of fate determinants into two daughter cells with different fate. According to the embryonic NB axis of polarity, apical protein complex are retained in the self-renewing daughter, whereas basal cell fate determinants are segregated into the differentiating GMC, which is destined to exit the cell cycle to generate two post-mitotic neurons or glial cells that constitute the adult CNS in *Drosophila*.

1.5.1 The role of apical protein complex in asymmetric division of NBs

The key substrates required for the asymmetric segregation of cell fate determinants include tumour suppressor protein Dlg, and Lethal (2) giant larvae (Ohshiro et al., 2000; Peng et al., 2000; Albertson and Doe, 2003; Betschinger et al., 2003). Lgl is a cytoskeletal protein known to specify the basolateral domain and to restrict DaPKC, Bazooka, and DmPar6 to the apical cortex (Wirtz-Peitz and Knoblich, 2006). Although Lgl does not directly influence spindle orientation and apical localisation of the Par complex, phosphorylation of Lgl by DaPKC leads to Lgl inactivation, or exclusion of Lgl from the apical cortex (Betschinger et al., 2003), thereby restricting cortical recruitment of basal cell fate determinants. This is in line with Lgl mutant studies, in which the cell after determinant adaptor protein Miranda (Mira) mis-localises to the cytoplasm. As a result, Lgl mutant neural lineages results in multiple neuroblasts due to occasional ectopic self-renewal (Rolls et al., 2003; Lee et al., 2006a), suggesting that Lgl inhibits

uncontrolled neural stem cell self-renewal. Furthermore, an over-expression of a membrane targeted DaPKC, but not a kinase-dead mutant isoform results in increased numbers of larval brain NBs, whereas a decrease in DaPKC expression reduces NB numbers. Genetic interaction experiments showed that Lgl; DaPKC double mutants have normal numbers of NBs and that DaPKC is fully epistatic to Lgl suggesting that DaPKC directly promotes NB self-renewal (Lee et al., 2006a).

Together, these data suggest that DaPKC and Lgl are key players in the establishment and maintenance of apical polarity, thereby, providing NBs with the capacity to self-renew. A main question arising from these studies is which mechanisms and molecules are directing DaPKC and Lgl to the apical cortex of a dividing neural stem cell? A partial answer to that comes from recent data suggesting that the mitotic kinase Aurora-A (AurA) is required for the asymmetric localisation of DaPKC (Lee et al., 2006c; Wang et al., 2006b; Wirtz-Peitz et al., 2008). These data suggest that AurA does so via phosphorylation of Par-6, a member of the apical complex, which in turn prevents the interaction between Par-6 and DaPKC. Subsequently, phosphorylated DaPKC can act independently of Par-6 and is able to phosphorylate Lgl, leading to Lgl inactivation/exclusion of Lgl from the apical cortex, a crucial step in restricting cortical recruitment of basal cell fate determinants (Betschinger et al., 2003; Lee et al., 2006a). Within the Par complex, this sequence of events lead to the exchange of Lgl for Bazooka, which in turn enables phosphorylation of the cell fate determinant Numb and its subsequent segregation into the differentiating GMC (Wirtz-Peitz et al., 2008), where it prevents self-renewal by an unknown mechanism.

The importance of these new data is that they provide a direct link between asymmetric protein localisation and mitotic spindle orientation. A linkage between mitotic spindle and apical cortex had already been established with the identification of the Mud/NuMa protein and its role in regulating NB self-renewal via proper spindle-orientation. However, mutant Mud does not alter cortical polarity (Bowman et al., 2006; Izumi et al., 2006; Siller et al., 2006), whereas mutant AurA does (Lee et al., 2006c; Wang et al., 2006b; Wirtz-Peitz et al., 2008). This difference is far from being obvious, as both proteins localise to the centrosomes and mutants of AurA and Mud exhibit similar defects in spindle orientation (Berdnik and Knoblich 2002; Giet et al. 2002; Izumi et al., 2006; Siller et al., 2006; Bowman et al., 2006; Lee et al., 2006c; Wang et al., 2006b; Wirtz-Peitz et al., 2008). A possible explanation to this apparent discrepancy comes from genetic interaction data indicating that AurA controls mitotic spindle orientation in dividing neuroblasts by regulating the asymmetric localisation of Mud (Wang et al., 2006b). Moreover, AurA seems not only to act on Mud and Par-6, but also on Notch signalling. Mutational inactivation of AurA leads to ectopic activation of Notch (Wang et al., 2006b). Upon activation of the Notch receptor, the intracellular domain of Notch (N^{IC}) translocates to the nucleus and activates the transcription of Hes family proteins, such as Hairy and Enhancer of split are known to mediate Notch signalling (Fischer and Gessler, 2006). Increasing Notch activity by expressing the intracellular domain of Notch significantly increases the numbers of type II post-embryonic NBs (Lee et al., 2006c; Wang et al., 2006b; Zhu et al., 2012).

Based on these data, it is conceivable that AurA acts via Mud to orient mitotic spindles required for the establishment of a proper division plane, which is a prerequisite for unequal segregation of cell fate determinants during NB cytokinesis. Simultaneously, asymmetric protein localisation

is achieved, at least in part by AurA acting on Par-6 and in turn via phosphorylation of DaPKC followed by that of Lgl. Such a dual role of AurA linking asymmetric protein localisation and mitotic spindle orientation could explain to some extent why in AurA and Mud, but also in DaPKC and Lgl mutants, results in similar over-proliferation phenotypes with increased number of NB-like cells at the expense of differentiating neurons.

1.5.2 Basal cell fate determinants

The fate of GMCs are determined by the exclusive inheritance of key differentiation factors such as the Notch repressor Numb (Uemura et al., 1989), the NHL-domain protein Brain tumour (Brat) (Arama et al., 2000) and the homeodomain transcription factor Prospero (Doe et al., 1991; Vaessin et al., 1991; Matsuzaki et al., 1992) which are collectively known as the cell fate determinants. Basal targeting of these cell fate determinants in dividing NBs is achieved via their adaptor proteins, Partner of Numb (PON) (Lu et al., 1998) and Miranda (Shen et al., 1997; Ikeshima-Kataoka et al., 1997), respectively.

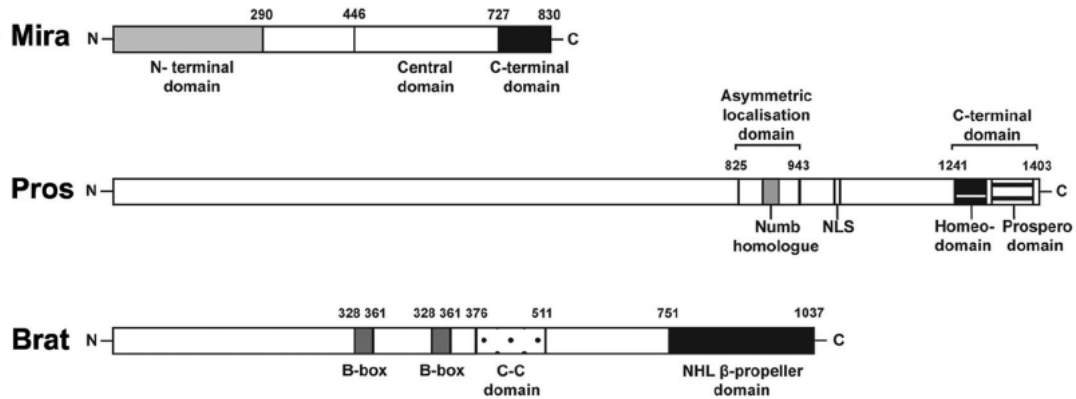


Figure 1.5 Structure of the basal cell determinants. Mira protein binds to its Brat and Pros via its central domain. The N-terminal domain of Mira is required for its association to the membrane, and the C-terminal domain is crucial for cortical localisation of Mira, and release of its cargo proteins upon cytokinesis. Pros protein is characterised by the asymmetric localisation domain required for its binding to Mira, as well as the nuclear localising signal (NLS), the homeodomain required for DNA binding. Brat protein consists of two N-terminal Zinc binding B-boxes, a coiled-coil region, and the C-terminal NHL β-propeller domain, which bind the central domain of Miranda (adapted from Kim and Hirth, 2009).

Numb

Numb is also one of the key factors that regulate self-renewal and differentiation of NBs (Spana et al., 1995). Partner of Numb (Pon) acts as an adaptor protein to assist in segregation of Numb into the GMC, although Pon is not strictly required for this to occur (Lu et al., 1998; Wang et al., 2007). Interestingly, direct phosphorylation of Numb by DaPKC has been found to control asymmetric localisation of Numb (Smith et al., 2007).

Recently, Polo kinase was shown to phosphorylate Pon and indirectly regulate the asymmetric localisation of Numb (Wang et al., 2007). In Polo mutants, the number of type I NBs significantly increases; however, over-expression of Polo phosphorylates Numb, impairing its tumour suppressor activity, resulting in over-proliferation of type II NBs (Ouyang et al., 2011).

Ectopic expression of phosphor-mimetic form of Numb or genetic manipulation that boosts phosphorylated Numb levels, attenuates endogenous Numb activity and results in over-proliferation of type II NBs (Ouyang et al., 2011), which is consistent with Numb primarily acting in type II lineage to restrict the proliferation of INPs (Bowman et al., 2008).

It is conceivable that Numb is also phosphorylated by Polo kinase in type I NB lineage. However, certain unidentified factors might block the effect of phosphorylated-Numb on type I NBs. It is also possible that type I and type II lineages might employ different molecular mechanisms to control their stem cell self-renewal and differentiation, considering their different

origin and modes of neurogenesis. Consistent with this notion, the Numb/Notch pathway has been suggested to be dispensable in the type I NB lineage (Bowman et al., 2008).

Prospero

Upon cytokinesis, Pros exclusively segregates into the GMC by its adaptor protein Mira. Subsequent degradation of Mira releases Pros from the cortex, which then translocates into the nucleus (Hirata et al., 1995). In the nucleus, Pros controls the cell cycle progression of the GMC by repressing cell cycle regulators such as *cyclin A*, *cyclin E* and the *Drosophila cdc25* homologue, *string* (Li and Vaessin, 2000). Simultaneous activation of the expression of a cyclin-dependent kinase inhibitor dacapo leads to terminal differentiation of the GMC into two post-mitotic neurons/ or glial cells (Liu et al., 2002).

Expression profiling of Pros using loss and gain-of function indicate that Pros represses NB-specific apical polarity genes such as *inscuteable*, *bazooka* and *DaPKC*, and activates expression of neural differentiation genes such as *fushi tarazu* and *even skipped* (Choksi et al., 2006). Moreover, loss of Pros function results in over-proliferation of NBs in the central brain, with the majority of cells within these mutant clones showing sustained expression of stem cell markers and increased mitotic activity, eventually leading to tumourigenesis (Bello et al., 2006; Betschinger et al., 2006; Choksi et al., 2006; Lee et al., 2006b). Whereas, gain of Pros function can prematurely terminate the proliferation of post-embryonic NBs (Maurange et al., 2008; Cabernard and Doe, 2009; Colonques et al., 2011). Pros, therefore acts as a binary switch between regulating self-renewal and differentiation in neural stem cells.

Brat

Brat is a member of the conserved NHL family of proteins (Arama et al., 2000; Sardiello et al., 2008) that has a C-terminal NHL domain, a coiled-coil region and two N-terminal Zinc binding B-boxes (Figure 1.5). Mutation of Brat also results in over-proliferating NB lineages at the expense of differentiating neurons (Betschinger et al., 2006; Lee et al., 2006b; Bello et al., 2006; Bowman et al., 2008). Interestingly, in Brat mutant NB clones, Mira is mis-localised from the cortex and loss of nuclear Pros occurs (Bello et al., 2006), suggesting that these proteins may play a role in the same molecular pathway. To bolster this view, ectopic expression of Pros can rescue the tumourigenic phenotype in Brat mutants in the larval central brain (Bello et al., 2006). Moreover, mutation of Mira lead to mis-localisation of Brat and Pros (Bello et al., 2006; Betschinger et al., 2006).

These results indicate that Mira is essential for the asymmetric localisation of Brat and Pros. In line with this, Pros binds to the central Pros-binding domain of Miranda (Fuerstenberg et al., 1998), and Brat binds to the coiled-coil cargo binding domain of Miranda (Lee et al., 2006b) (Figure 1.5). Moreover, the interaction between the NHL domain of Brat and the C-terminal domain of Mira appears to be essential for promoting asymmetric localisation of Pros to the GMC, where it is required for cell cycle exit and neuronal fate determination (Lee et al., 2006b). Thus, it is conceivable that the cell fate determinants Brat and Pros maybe transported across a dividing NB as cargo proteins of their adaptor protein Mira. However, the exact mechanism involved in the basal targeting of the cell fate determinants remains elusive.

In addition, Brat has also been identified as a potent differentiation factor in *Drosophila* ovarian germline stem cells (GSCs). When the GSC divides one daughter cell remains with the niche, continuing to receive Decapentaplegic (Dpp) self-renewal signal (Xie and Spradling, 1998), while the differentiating daughter, cystoblast moves posteriorly away from the signal (Ohlstein and Mckearin, 1997). Translational repressors such as Pumilio and Nanos also contribute to stem cell identity (Forbes and Lehmann, 1998; Wang and Lin, 2004; Chen and McKearin, 2005; Szakmary et al., 2005).

Following GSC division, a reduction in Dpp signalling allows expression of the key differentiation factor, Bag of marbles (Bam), which in turn down-regulates Nanos in the cystoblast, subsequently allowing translation of Brat. In succession, Brat interacts with Pumilio to translationally repress Mad and *Drosophila Myc* mRNAs, which lowers cellular responsiveness to Dpp signalling allowing the cystoblast to differentiate (Harris et al., 2011). Together with these findings, it maybe feasible to speculate that Brat has a global function in promoting cell fate changes in stem cell systems.

1.5.3 Mechanisms of basal protein targeting

Previous studies suggested that basal localisation/anchoring of Mira depend on intact actin cytoskeletons (Broadus and Doe, 1997) and myosin motors (Ohshiro et al., 2000; Petritsch et al., 2003).

Disrupting actin cytoskeleton with Cytochalasin D does not completely abolish the asymmetric localisation Numb and Pros (Knoblich et al., 1995). However treatment with a more potent inhibitor of actin polymerisation Latrunculin A, in embryos, results in a complete depolymerisation of F-actin as Numb and Pros are no longer asymmetrically localised, suggesting that asymmetric localisation of numb and Pros require an intact actin filaments for basal cortical anchoring, in vivo (Knoblich et al., 1995). When cytoskeleton inhibitors were applied to NBs cultured in vitro, Pros and Numb were de-localised from the cortex and were localised to the cytoplasm instead (Broadus and Doe, 1997). Moreover, treatment with the weaker inhibitor Cytochalasin D resulted in partial disruption of microfilaments and aberrant localisation of Numb and Pros in some NBs. Latrunculin B treatment, however, resulted in complete disruption of microfilaments with virtually all of the NBs showing uniform cytoplasmic localisation of Numb and Pros. These data were consistent with previous results showing Latrunculins to be more effective at inhibiting microfilaments than Cytochalasins (Knoblich et al., 1995; Knoblich et al., 1997). Based on in vivo and in vitro experiments, the integrity of the microfilament cytoskeleton has to be intact, in order for Pros to anchor to the basal cortex (Broadus and doe, 1997).

Subsequent studies implicated Myosin II as one of the myosin motors involved in basal localisation of the cell fate determinants (Barros et al., 2003). Firstly, Mira physically interacts with the non-muscle Myosin, Zipper (Ohshiro et al., 2000). Secondly, in Myosin II mutant studies, cell fate determinants failed to form a basal crescent in embryonic NBs (Ohshiro et al., 2000), notably Mira is mis-localised uniformly around the cortex (Erben et al., 2008). Similarly,

reduced Myosin VI (Jaguar) activity in embryos, leads to a failure in basal crescent formation as well, with Mira mis-localising to the cytoplasm in patches (Petritsch et al., 2003).

The exact role of Myosin II in basal targeting remains unclear, however, Barros et al. (2003) proposed a model involving phosphorylation of Lgl by DaPKC that in turn activates Myosin II at the apical cortex leading to cortical displacement of Mira and its cargo proteins into the cytoplasm. The model also suggests that Myosin II moves along the cortex towards the cleavage furrow to exclude Mira protein from the apical cortex into the cytoplasm. Hence, Myosin II appears to be responsible for cortical exclusion of Mira rather than direct basal transport.

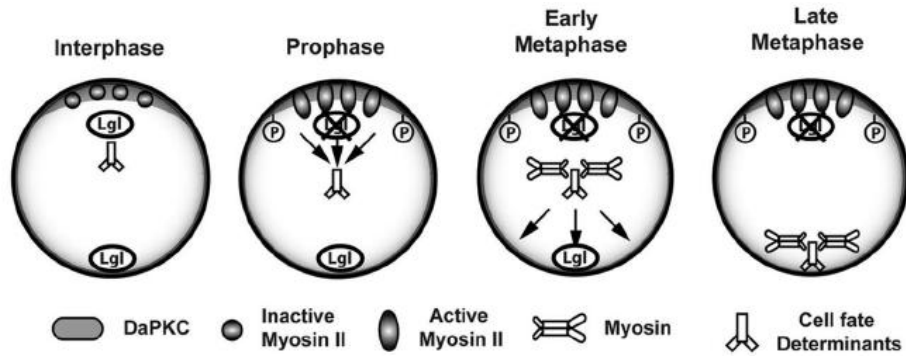


Figure 1.6 Mechanism involved in basal targeting of cell fate determinants. Phosphorylation of Lgl by DaPKC activates Myosin II in the apical cortex, leading to cortical displacement of Mira from the apical cortex. Once Mira and the cell fate determinants are in the cytoplasm, Myosin VI directly binds Mira, and transport Mira and its cargo proteins to the basal cortex (adapted from Kim and Hirth, 2009).

Myosin VI transiently accumulates in the basal cortex and partially co-localises with Mira during metaphase (Figure 1.6) and in vitro studies using *Drosophila* embryonic extracts also showed physical interaction with Mira. It is therefore feasible that Myosin VI may be the myosin motor protein responsible for transporting Mira to the basal cortex of NBs (Erben et al., 2008).

The distinct phenotype, mode of action, and sub-cellular localisation of Myosin II and Myosin VI suggests that they may act at consecutive steps in a single pathway to localise Mira and its cargo proteins to the basal side of dividing NBs. In addition, Erben et al (2008) provide some evidence that Myosin II acts upstream of Myosin VI in this common pathway. Thus, the proposed model for the basal protein targeting (Fig. 5B) involves activation of Myosin II around early prophase by DaPKC phosphorylating Lgl, leading to cortical exclusion of Mira and its cargo proteins into the cytoplasm. Subsequent binding of Myosin VI to Mira is thought to transport Mira to the basal cortex along the actin filaments (Erben et al., 2008).

However, the current proposed models for basal targeting of cell fate determinants are not without its controversies. Previously, Mayer et al. (2005) suggested that actin-myosin based cortical transport is incompatible with photo-bleaching experiments that have determined the dynamics of asymmetric Pon localisation. Whilst the experiment failed to detect any directional lateral mobility of some of the segregating determinants, such as Numb and Pon, it cannot rule out that acto-myosin based segregation is required for basal targeting of Mira, Pros and Brat. Moreover, Erben et al. (2008) proposed that while PON requires Myosin II, its localisation does not depend on Myosin VI, thus maybe utilising a distinct mode of localisation. Therefore, further

experiments employing Brat-Mira-Pros transport are required to address this question, and the validity of the current model proposed by Erben et al. (2008) remains to be seen.

1.5.4 Asymmetric cell division in the mammalian brain

Asymmetric cell division is a key feature of mammalian stem cells as well. Most of the information regarding asymmetric cell division in mammals is derived from more rapidly dividing embryonic progenitor cells in the developing mouse cortex (reviewed in Knoblich, 2010).

Early during mouse brain development, at embryonic day 9 (E 9.0), the cortex consists of neuroepithelial progenitors, which extend from the apical ventricular surface to the basal surface of the neural tube. Before these neuroepithelial cells divide, their nuclei undergo interkinetic nuclear migration and move apically to undergo mitosis at the apical-most position. Early divisions are symmetric and results in expansion of the progenitor pool. When neurogenesis starts, at around E11.0, neuroepithelial progenitors start expressing characteristics features of glial cells (Mori et al., 2005) and turn into the so called radial glial (RG) cells. RG cells also extend apical and basal processes, and are restricted to the most apical area of the cortex: the ventricular zone (VZ). They continue interkinetic nuclear migration and divide asymmetrically into one self-renewing daughter cell and one cell that migrates into the more basally located cortical plane to differentiate into a neuron, during a process known as the direct neurogenesis (Reviewed in Rakic, 2009)

Interestingly, the pre-dominant mode of neurogenesis that occurs in the mouse cortex during late stages utilises a process known as the indirect neurogenesis. During this mode of division, RG cell generates a self-renewing RG cell and one intermediate neural progenitor (INP) cell. INPs reside in the cortical area between the ventricular zone and intermediate zone, where they form the sub-ventricular zone. INPs undergo at least one more symmetric division, generating two terminally differentiating neurons. The indirect neurogenesis in the mouse cortex resembles the mode of division utilised by *Drosophila* type I NBs (Reviewed in Knoblich, 2010).

Moreover, the polarity and spindle machinery are conserved in the mammalian brain. Mammalian Par-3, Par-6 and aPKC are important for both apical-basal polarity and for spindle orientation (Suzuki and Ohno, 2006). Pins has two mammalian homologs, Ags3 and Lgn (Yu et al., 2003; Sanada and Tsai, 2005). Although *Ags3*-null mice show no defects in brain morphology or function (Blumer et al., 2008), knocking out *Lgn* randomises the orientation of normally planar neuroepithelial divisions, bolstering the view that Pins has a conserved role in mitotic spindle orientation in the developing brain (Morin et al., 2007; Konno et al., 2008). The mammalian Mud homolog nuclear protein that associates with the mitotic apparatus, NuMa has a role in the establishment and maintenance of spindle poles (Sun and Schatten, 2006; Silk et al., 2009). Finally, the single vertebrate homolog of *Drosophila* Inscuteable is required and sufficient for inducing non-planar spindle orientation (Zigman et al., 2005; Konno et al., 2008; Postiglione et al., 2011). Thus, the molecular machinery regulating the orientation of progenitor divisions appears to be conserved in vertebrates. However, how this conserved molecular machinery influences cell fate in vertebrates remains to be elucidated.

1.5.5 Role of Brat and Pros in vertebrates

During asymmetric cell division in mouse neural progenitor cells, the mammalian TRIM-NHL protein TRIM32 is enriched in one of the two daughter cells. It is thought that TRIM32 suppresses cell proliferation and induces neuronal differentiation of neural progenitor cells by two mechanisms: Firstly, by ubiquitinating the transcription factor *c-Myc*, targeting it for degradation and secondly, by enhancing miRNA protein particle (miRNP) activity by interacting with AGO1 via the NHL domain (Schwamborn et al., 2009).

In *Drosophila*, the TRIM-NHL proteins Brat and Mei-P26 control growth and differentiation of NBs and ovarian stem cells, respectively, similar to the role of TRIM32 in mouse neural progenitor cells. Like TRIM32, both Brat and Mei-P26 interact with AGO1 and function post-transcriptionally to inhibit *Drosophila Myc* in differentiating daughter cells (Neumuller et al., 2008).

Similarly, vertebrate ortholog of Pros, prospero homeobox 1 (Prox1) is expressed in newly differentiating neurons in developing mammalian CNS (Torri et al., 1999). Prox1 is thought to inhibit proliferation of neural progenitor cells (Dyer, 2003) and has been proposed as a candidate tumour suppressor gene in mammals (Shimoda et al., 2006; Takahashi et al., 2006; Laerm et al., 2007).

1.6 *Drosophila* myosins

Myosins are a large super-family of motor proteins that move along actin filaments, while hydrolysing ATP. About 24 main classes of myosins have been distinguished on the basis of the sequence of amino acids in their ATP hydrolysing motor domains (Foth et al., 2006). All known myosins comprise an N-terminal head motor domain, a neck regulatory domain, and a carboxy-terminal tail domain. The head/motor domain contains ATP- and actin-binding sites, thus converting the energy stored in ATP into mechanical force to move myosin molecules along the actin filament or to translocate other molecules (Hasson and Mooseker, 1994). The neck domain contains regulatory sites, composed of isoleucine-glutamine (IQ) motifs (Rhoads and Friedberg, 1997), which provides a binding site for a calmodulin. The highly divergent tail domain differ among myosin classes, which gives them variety of cell functions, including membrane trafficking, signal transduction, and maintenance of the cell architecture (Tzolovsky et al., 2002).

Myosin Class	Gene/Protein	CNS expression	Cargo binding domain	Functions	References
IA	<i>Myo61F</i>	N	N	Function in microvilli of brush border	Hegan et al., 2007
IB	<i>Myo31DF</i>	Y	N	Left-right asymmetry establishment	Speder et al., 2006
IC	<i>Myo95E</i>	Y	N	in embryo gut	Petzoldt et al., 2012
II muscle	<i>Myosin heavy chain</i> <i>Zipper</i>	N	N	smooth or skeletal muscle contraction	Hasson and Mooseker, 1995
II non-muscle		Y	N	Maintenance of cell architecture, cell motility and phagocytosis asymmetric division of NBs	Escudero et al., 2007 Tang et al., 2011a Barros et al., 2003
III	<i>Nina C</i>	Y	N	phototransduction	Hardie et al., 2012
V	<i>Didum</i>	Y	Y	polarised cell growth component of yeast cell polarisation	Toth et al., 2005 Yin et al., 2000
VI	<i>Jaguar</i>	Y	Y	Epithelial morphogenesis spermatid individualisation asymmetric division of NBs	Millo et al., 2004 Lenartowska et al., 2012 Erben et al., 2008
VIIA	<i>Crinkled</i>	Y	N	Shaping of denticles in embryonic epidermis	Bejsovec and Chai, 2012
VIIIB	<i>Myo28B1</i>	N	N	N/A	
XV	<i>Myo10A</i>	Y	N	Hair cell function	Mustafa et al., 2007
XVIII	<i>Myosin heavy chain-like (Mhcl)</i>	N	N	Possible function in mature muscle cells	Bonn et al., 2013
Unclassified	<i>Myo29D</i>	N	N	N/A	Tzolovsky et al., 2002

Table 1.1 Physiological functions myosin genes expressed in *Drosophila*. All of the myosin motors present in the *Drosophila* and their physiological functions.

Multi-cellular organisms appear to express 10-40 myosin genes, encoding at least 6 different classes of myosins. In humans, around 40 myosin genes grouped into 12 classes are expressed, whereas in *Drosophila*, 13 different myosin genes across 8 classes have been identified (Table 1) (Tzolovsky et al., 2002).

Only the *Drosophila* myosin motors that are expressed in the CNS and with appropriate functional domains were considered as the possible candidate myosin motor for basal targeting of cell fate determinants during asymmetric division of NBs. Myosin V (Thirumurugan et al., 2006) and Myosin VI (Isaji et al., 2011) were chosen based on their cargo binding domain. Interestingly, Myosin VI was proposed to be the candidate myosin motor for basal targeting of Mira (Erben et al., 2008). Moreover, Myosin V (Wu et al., 2005) and Myosin VI (Lantz and Miller, 1998) interacts with microtubule plus end tracking proteins. This interaction may provide a direct link between microtubules and the cortical actin cytoskeletons, which could function to anchor astral microtubules to cortical actins and/or could function as a transition point for transport of vesicles or other cytoplasmic structures from microtubules to actin filaments in the NB cortex or vice versa (Koonce, 1996). Thus, maintaining this link could be important for the stable localisation of the basal cell fate determinants during asymmetric division of NBs.

In addition, Myosin II was also analysed based on recent studies implicating its role in asymmetric segregation of cell fate determinants (Barros et al., 2003), which is further discussed in the following chapters.

1.7 Candidate *Drosophila* myosins implicated in basal targeting of cell fate determinants

1.7.1 Myosin II

Most myosins belong to class II, and together with actin, make up the major contractile proteins of cardiac, skeletal, and smooth muscle. Importantly, myosin molecules that resemble their muscle counterparts, referred to as the non-muscle myosin II, with respect to both structure and function are present in all non-muscle eukaryotic cells (Conti and Adelstein, 2008). Similar to their muscle counterparts, non-muscle myosin II molecules are comprised of three pairs of peptides; two heavy chains of 230 kDa, two 20 kDa regulatory light chains that regulate its activity, as well as two essential light chains that stabilise the heavy chain structure (reviewed in Krendel and Mooseker, 2005). Interestingly, these non-muscle myosin IIs are also present in muscle cells, where they contribute to skeletal muscle development, as well as in the maintenance of tension in smooth muscles (Morano et al., 2000). Here, I will be focusing mainly on their activities in the central nervous system.

Domain structure of non-muscle myosin II

Non-muscle myosin II has two globular head domains which contain binding sites for both ATP and actin, followed by neck regions, which binds the two functionally different light chains. The neck domain acts as a lever arm to amplify head rotation while the chemical energy of ATP is converted into the mechanical movement of the myosins head. This neck domain is then

followed by a long α -helical coiled coil domain, which forms an extended rod-shaped domain that effects dimerisation between the heavy chains and terminates in a relatively short non-helical tail, which is believed to contain cargo binding domain (Svitkina et al., 1995).

There are three genes in mammalian cells that encode different isoforms of non-muscle myosin II heavy chains. Myosin heavy chain 9 (MYH9), MYH10, MYH14 encode non-muscle myosin heavy chains (NMHC) IIA, IIB, IIC, respectively, although, there is only one NMHC gene in *Drosophila*, known as zipper (Mansfield et al., 1996). The NMHC isoforms in turn determine the whole non-muscle myosin II (NM II) molecules that include both heavy and light chains. Thus the three isoforms in mammalian cells are named NM IIA, NM IIB, NM IIC, accordingly.

Physiological functions of non-muscle myosin II

Non-muscle myosin II is an important regulator of adhesion (Pollard et al., 2003) and polarity (Yam, 2007) in cell migration. These processes involve the dynamic remodelling of the actin cytoskeleton and the interaction of the cell with its environment, as well as in stabilising actomyosin ring involved in cytokinesis (Goldbach et al., 2010). Each of the non-muscle myosin II isoforms affects these processes differently; however, I will not go into too much detail regarding this, as there is only one known form of non-muscle myosin II present in *Drosophila*.

An interesting function of a non-muscle myosin II is its involvement in formation and constriction of a contractile composed of filamentous (F) actin and non-muscle myosin II, known as the actomyosin contractile ring. Bipolar filaments of myosin II draw F-actin together in a

purse string like manner to constrict the contractile ring at cleavage furrow. It is thought that a tight link is established between the acto-myosin ring and the plasma membrane at the equator, and this attachment is maintained during late stages of cytokinesis (Goldbach et al., 2010).

Implication of myosin II involvement in asymmetric segregation of cell fate determinants (Barros et al., 2003) rendered it to be shortlisted as a possible candidate myosin motor responsible for basal targeting of cell fate determinants during asymmetric NB division. Firstly, the regulatory light chain of Myosin II, Spaghetti Squash is required in embryonic NBs to organise the actin cytoskeleton (Barros et al., 2003). Secondly, Mira physically interacts with the heavy chain of Myosin II, Zipper (Ohshiro et al., 2000). Moreover, disruption of Myosin II function by Rho kinase inhibitor, Y-27632 caused Mira to mis-localise uniformly around the cortex (Barros et al., 2003). But because Myosin II and Mira localise almost exclusively during asymmetric division in embryonic NBs, it has been suggested that Myosin II is responsible for exclusion of Mira from the apical cortex rather than for direct basal targeting of Mira (Barros et al., 2003). Interestingly, basal localisation of Pon requires Myosin II as well, but not Myosin VI (Erben et al., 2008).

1.7.2 Myosin V

Myosin V is present in most eukaryotes excluding plants (Reck-Peterson, et al, 2000). In vertebrates it exists in three distinct subclasses (myosin Va, Vb, Vc) which are differentially expressed but it is not known whether they have distinct or overlapping functions (Wu et al, 2000). However, only one isoform of myosin V has been discovered in *Drosophila* so far

(Bonafe and Sellers, 1998). Phylogenetic analysis of the head domain reveals that *Drosophila* myosin V is more closely related to mammalian myosin Va and Vb than to other invertebrate class V myosins; nevertheless, it is not significantly more related to myosin Va than to Vb. While vertebrates would require two different isoforms to accomplish specific functions, it is speculated that *Drosophila* myosin V may provide the equivalent functions by itself (Bonafe and Sellers, 1998).

Myosin V is a dimeric molecule consisting of conserved motor domains followed by 6 IQ motifs which bind specific light chains and calmodulin (Reck-Peterson et al., 2000). The tail domain is important for cellular localisation and cargo binding and can be divided into a α -helical coiled coil region which in vertebrates contains a PEST site (a calpain protease sensitive site) (Espreafico, et al, 1992) and a C-terminal globular region containing an AF-6 homology domain (Ponting, 1995).

Role of myosin V in intracellular signalling

Myosin Va is a processive motor protein with a high duty ratio (high proportion of its ATPase cycle spent attached to its actin filament track) and a large step size (Walker et al, 2000). These properties make it ideally suited for its role in membrane trafficking, polarised cell growth and specific transport pathways.

Most of our information on the functions of myosin V has been gained by analysing mutants in mouse and yeast. In the dilute mouse, functional myosin V is absent (Mercer et al, 1991) and the

resulting loss of coat colour and neurological disorders are due to defects in melanosome transport in melanocytes and smooth ER trafficking in neurons (Wu et al, 1997). Available data support a model where melanosomes are transported from the cell body to the melanocyte dendrites by microtubule based motors and their subsequent movement and tethering at the cell periphery is dependent on myosin V and actin (Wu et al, 2000). Further support for such a model has been provided by co-localisation and binding studies which have shown direct interaction of the myosin V tail with kinesin tail (Huang et al, 1999).

The cytoskeletal transport mechanisms in mammalian cells involve interactions of molecular motors on microtubules, for long range transports, as well as the actin tracks, for shorter transport that are confined more to the cell periphery. This process was clearly demonstrated in *Xenopus* melanocytes, where pigment granules are transported between the cytoskeletal tracks in both directions (Gross et al., 2002a). At the single molecule level, myosin V is thought to navigate through the microtubule-actin intersection by executing a turn onto a crossing actin-filament, by stepping over the crossing actin filament (Ali et al., 2007). At the intersection, unbound motor head searches for a new binding site by undergoing diffusional search to become the new leading head. However, as demonstrated in *Xenopus* study in vivo, cargo will likely contain multiple motors (over 60 myosin V motors are thought to be present on one melanosome), although only a few of these are expected to be engaged at one time (Gross et al., 2002a).

So, how does myosin V contribute to transfer of cargo from microtubule to actin tracks? One possibility has been suggested based on in vitro observation that myosin V can undergo a one

dimensional diffusional search along microtubules to facilitate cargo transfer onto actin (Ali et al., 2007). Otherwise, the microtubule plus-end tracking protein EB1 has been thought to play a crucial role in this process instead. A C-terminal region of melanophilin, an adaptor protein that binds myosin Va contains a binding site for EB1. This binding could allow melanophilin to be transported by EB1 to the end of the microtubule, at which point the protein complex consisting of myosin Va, melanophilin, and melanosome-bound Rab27a could be assembled for short-range transport along actin filaments (Wu et al., 2005).

Other physiological functions of myosin V

Recent study in *Drosophila* oocytes have clearly demonstrated the utilisation of long range mitochondrial track based transport of oskar mRNA across the oocyte towards the posterior cytoplasm, where myosin V was thought to engage in short range actin based transport (Krauss et al., 2009). What makes this phenomenon interesting is that, firstly, mechanisms displayed here are in line with the processes described above. Secondly, oskar is responsible for assembling the germ plasm, a specialised cytoplasm required for germ cell formation. During the syncytial phase of the early embryo the germ plasm induces germ cell fate on a number of zygotic nuclei adjacent to the posterior pole, a mechanism that may be comparable to the asymmetric division in NBs. These features indicate myosin V as a likely candidate of the myosin motor involved in basal targeting of cell fate determinants in neuroblasts.

1.7.3 Myosin VI

Myosin VI is the only class of myosin that moves towards the minus end of actin filaments (Wells et al., 1999), and therefore appears to have unique functions in the cell. Given the polarity of actin filaments in the cell with their plus (barbed) ends inserted or into or at membrane and their minus (pointed) ends projecting inwards, it may be feasible to speculate that Myosin VI would move cargo from the plasma membrane into the cell and away from the surface of organelles (Buss et al., 2004).

Myosin VI is composed of an N-terminal canonical motor domain with an ATP binding pocket and actin binding interface, a short neck region with a single IQ motif that binds calmodulin, a tail with helical regions and a C-terminal cargo binding domain (Hasson and Mooseker, 1994). Between the motor domain and the IQ motif lies the 53 amino acid insert known as the reverse gear. This insert is an integral part of the converter region that directs the lever arm of myosin VI by 120° towards the minus end of the actin filament, thus allowing myosin VI to move towards the pointed or minus end of actin filaments, (in the opposite direction of all other myosins) (Menetrey et al., 2005).

How is Myosin VI regulated?

Since Myosin VI is involved in such a wide variety of cellular functions, it must be tightly regulated so that it is only active where and when it is required in the cell. For the cell to maintain its organisation and the correct distribution of components, the activities of all the motor proteins involved must be tightly regulated and coordinated. For instance, establishing how myosin VI regulates the numerous proteins that bind C-terminal domain may explain the mechanisms involved in basal targeting of cell fate determinants in neuroblasts.

Firstly, major conformational changes in the tail could be used to regulate/block these protein-protein interactions. Non-muscle Myosin IIs and Myosin V can exist in vitro in a folded inactive state, with the tails interacting with the heads (Liu et al., 2006b; Thirumurugan et al., 2006). However, whether these myosins exist in folded states in cells is yet to be established. So far, there is no conclusive proof that Myosin VI can exist as a folded molecule, but its tail region contains helical coiled-coil regions that might allow the tail to fold.

Cellular localisation studies on Myosin VI (Buss et al., 1998) support the idea that Myosin VI may exist in an 'inactive' state in the cell, since they show that a considerable proportion of the endogenous Myosin VI is present as a diffuse cytoplasmic pool, not associated with any obvious cellular compartments or structures. One might speculate that in this cytoplasmic pool, Myosin VI is in a folded inactive state with the binding sites on the cargo binding domain. Thus, binding to cargo or membrane may activate this molecule.

Physiological functions of Myosin VI.

The functional diversity of Myosin VI in cells varies to a great extent. Few of the main functions that have been established include, clathrin-mediated endocytosis, exocytosis in golgi complex, and cell migration (Reviewed in Buss and Kendrick-Jones, 2004). However, I will only focus on the functions of Myosin VI that maybe of interest to my project.

Myosin VI is suggested to play a vital role in asymmetric division of NBs (Petritsch et al., 2003; Erben et al., 2008). Reduced Myosin VI activity in embryos results in Mira failing to form a basal crescent in metaphase, with Mira mis-localising to the cytoplasm in patches (Petritsch et al., 2003). Moreover, in vitro studies using *Drosophila* embryonic extracts showed that Myosin VI physically interacts with Mira. Accordingly, Myosin VI transiently accumulates in the basal cortex and partially co-localises with Mira at metaphase (Erben et al., 2008). Taken together, these data implicate Myosin VI as a strong candidate myosin motor directly responsible for basal targeting of Mira during asymmetric division of post-embryonic NBs.

Interestingly, down-regulation of Myosin VI by RNAi resulted in mis-orientation of the mitotic spindle at metaphase (Erben et al., 2008). Myosin VI may regulate the orientation of the mitotic spindle via its interaction with -CLIP-190, an orthologue of human CLIP-170. In *Drosophila*, Myosin VI was shown to co-immunoprecipitate with D-CLIP-190 (Lantz & Miller, 1998). The human CLIP-170 is a microtubule-actin linker protein that binds preferentially to the growing (plus ends) of the microtubules (Perez et al., 1999). Thus, CLIP-170 may capture and transport cargo via the unconventional myosin motor, Myosin VI from/to microtubules/actins (Lantz and

Miller, 1998). In addition, CLIP-170 directly binds to (Busch and Brunner, 2004), and functions downstream of EB1 (Komarova et al., 2005), which is another microtubule plus end tracking protein. Interference of EB1 function produces effects on spindle structure, most common being an overall decrease in the length of the spindle, as well as the mis-orientation of the spindle poles (Rogers et al., 2002). This is in line with phenotypes observed in my own myosin VI RNAi experiments, where the mitotic spindle orientation was effected. My hypothesis is that functional interference of EB1 disrupts vesicular transport between growing ends of microtubule and actin filaments via CLIP-170 and myosin VI, which in turn de-focuses mitotic spindle.

1.8 Principle objectives of research

Drosophila post-embryonic NBs are a great model system for studying asymmetric division of neural stem cells. Although extensive research has been undertaken in order to validate the mechanisms involved in asymmetric division of NBs, the crucial mechanisms involved in basal targeting of the cell fate determinants that may be the key to the binary switch between proliferation and differentiation of post-embryonic NBs have remain unclear. Accordingly, the major aim of my thesis is to identify and characterise the candidate myosin motors that are directly responsible for basal targeting of the adaptor protein Mira and its cargo proteins Brat and Pros into the GMC during asymmetric division of post-embryonic NBs.

Chapter 2: Materials and Methods

2.1 *Drosophila* culture

2.1.1 Fly food

Cornmeal medium

1L	dH ₂ O
17.5g	Yeast
10g	Soy flour
73.1g	Cornmeal
6g	Agar (Sigma)
46.2g	Light malt extract
32g	Sugar
2.5g	Nipagen (Methyl 4-hydroxybenzoate - Sigma)
5ml	Propionic acid (Sigma)
25ml	100% Ethanol (VWR)

Dry ingredients were brought to the boiling temperature for approximately 10 mins, followed by addition of propionic acid, and nipagen dissolved in ethanol. The food is then transferred into the plastic vials and left to cool overnight at room temperature. The cooled food is then stored at 4°C up to two weeks.

2.1.2 Fly husbandry

Fly stocks were kept at 25°C in an incubator (LMS) during 12 hour light/dark cycle, and the generation time for the stock to reach adulthood was approximately 10 days using the standard cornmeal food.

2.2 Genetics

2.2.1 *Drosophila* lines

All RNAi lines were obtained from Vienna *Drosophila* RNAi centre (VDRC) and raised at 25°C, unless otherwise stated.

Control lines used were wild type *w*; 1407 Gal4; + (Broadie et al., 1995), a Gal4 driver that has P-element insert in *inscuteable* locus, which in turn, drives Gal4 activity in neuroblast lineages from embryogenesis onwards.

Post-embryonic neuroblast lineage was visualised by crossing neuroblast specific 1407 Gal4 driver with *yw*; UAS-mCD8::GFP/ CyO; + to generate *yw*; UAS-mCD8::GFP/ 1407 Gal4; +.

For co-localisation study of *miranda*/*brat*/*prospero*, expression of *miranda* in neuroblast was visualised by using *w*; 1407 Gal4; + in combination with *w*1118; UAS-*miranda*-GFP; + (a gift from J. Knoblich) to generate *w*1118; UAS-*miranda*-GFP/1407 Gal4; +.

Characterising the functions of miranda/brat/prospéro in post-embryonic neuroblasts were achieved by analysing RNAi mediated knockdown of miranda, brat, prospéro, individually. Thus, w; UAS-miranda-RNAi / CyO; + (VDRC #51485) and w; UAS-brat-RNAi; + (Bello et al., 2006, VDRC #105054) and w; UAS-prospéro-RNAi; + (VDRC #101477) were crossed with w, UAS-Dcr2; 1407 Gal4; MKRS/TM6B to generate w, UAS-Dcr2; UAS-miranda-RNAi /1407 Gal4 ; MKRS/+ and w, UAS-Dcr2; UAS-brat-RNAi /1407 Gal4 ; MKRS/+ and w, UAS-Dcr2; UAS-prospéro-RNAi /1407 Gal4 ; MKRS/+, respectively. The component lines UAS-Dcr2 (VDRC #6007) (Dietzl et al., 2007) and 1407 Gal4 were combined by multiple genetic crosses with double balancer line w; Sco/CyO; MKRS/TM6B Tb, Hu (a gift from J. Bateman).

Studies to characterise myosin II function in neuroblasts were conducted by using w; 1407 Gal4 or w, UAS-Dcr2; 1407 Gal4; MKRS/TM6B, in combination with w; UAS-zipper-RNAi; + (VDRC #7819) to generate w; UAS-zipper-RNAi/1407 Gal4i; + and w, UAS-Dcr2; UAS-zipper-RNAi/1407 Gal4i; MKRS/+, respectively.

Analysis of myosin VI function also involved w; 1407 Gal4 or w, UAS-Dcr2; 1407 Gal4; MKRS/TM6B, subsequently crossed with w; UAS-jaguar-RNAi; + (VDRC #37534) to generate w; UAS- jaguar-RNAi/1407 Gal4; + and w, UAS-Dcr2; UAS- jaguar-RNAi/1407 Gal4i; MKRS/+, respectively.

Functional analysis of myosin V was carried out using w, UAS-Dcr2; 1407 Gal4; MKRS/TM6B crossed with w; +; UAS-myosin V-RNAi/TM6B (Li et al., 2007) (a gift from D. Ready) to generate w, UAS-Dcr2; UAS-1407 Gal4; myosin V-RNAi/MKRS.

Double RNAi-mediated knock-down of myosin V and myosin VI was accomplished by generating *w*, UAS-Dcr2; UAS- jaguar-RNAi; UAS-myosin V-RNAi/TM6B. This was subsequently crossed with *w*, UAS-Dcr2; 1407 Gal4; MKRS/TM6B to generate *w*, UAS-Dcr2; UAS- jaguar-RNAi/1407 Gal4; UAS-myosin V-RNAi/MKRS. The component lines UAS-Dcr2, UAS-jaguar-RNAi, UAS-myosin V-RNAi were combined through series of genetic crosses using the double balancer line *w*; Sco/CyO; MKRS/TM6B Tb, Hu (a gift from J. Bateman).

2.2.2 The Gal4/UAS system for tissue-specific gene activation

A Gal4-dependent target gene can be constructed by sub-cloning any sequence behind Gal4 binding sites. The target gene is silent in the absence of Gal4. To activate the target gene in cell- or tissue-specific manner, flies carrying the target UAS gene are mated to male flies expressing Gal4 driver line. The resulting progeny then express the responder gene in a transcription pattern that reflects the Gal4 pattern of the respective driver (Fig 2.1).

The Gal4/UAS system is often associated with the analysis of gain of function phenotypes. However, its recent combination with RNA interference-mediated gene knockdown is emerging as a powerful tool for analysis of loss of function phenotypes as well. In addition, dominant-negative or dominant-interfering versions of the protein of interest have also been used in flies using the Gal4/UAS system to disrupt gene activity (Reviewed in Duffy, 2002).

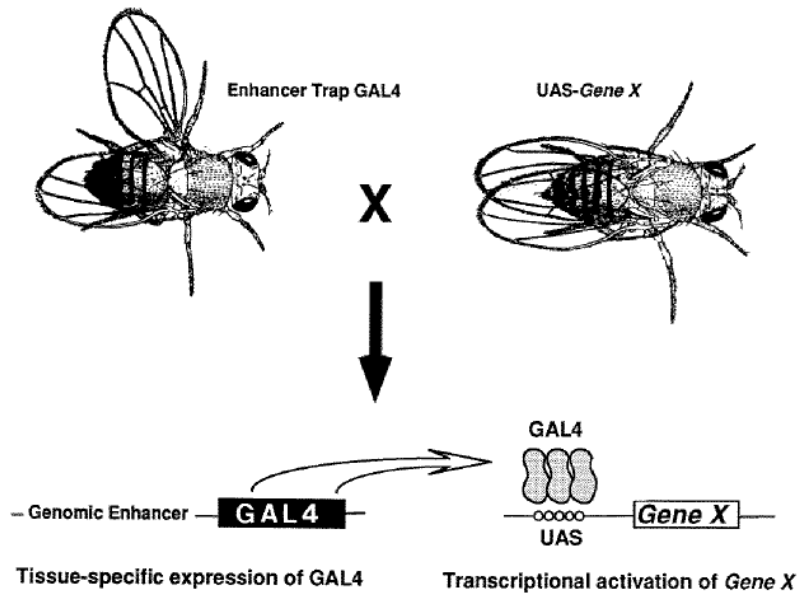


Figure 2.1 The Gal4/UAS system for tissue-specific gene expression in *Drosophila*.

(Adapted from Brand and Perrimon, 1993)

The Gal4/UAS system requires the ‘driver’ line that contains the yeast transcriptional activator Gal4 and the Gal4 responsive Upstream Activating Sequence (UAS) target gene to be separated in two distinct transgenic lines. To activate the transcription of the target gene, a fly carrying the Gal4 driver strain is mated with a fly carrying the UAS responder strain. In the progeny of this cross, it is possible to activate UAS-genes in cells where Gal4 is expressed and to observe the effects of this directed targeted mis-expression.

2.2.3 RNA interference

Targeted expression of RNAi constructs using the Gal4/UAS system can be used for cell or tissue specific knockdown of gene function. Following a cross between a target-specific Gal4 driver and a UAS-transpondent line containing hairpin RNA (hpRNAs) construct allows to drive expression of double stranded RNA (dsRNA) (Kennerdell and Carthew, 2000) (Figure 2.2). The transcribed dsRNAs are processed by dicer into small interfering RNAs (siRNAs) which subsequently suppresses the expression of a target protein by stimulating the specific degradation of the target mRNA, thus silencing gene expression. Such knockdowns are often incomplete, however, this approach provides a powerful way to analyse a gene with no available mutants. Interestingly, when the RNAi lines of interest were co-expressed with Dicer-2, reduction in protein expression was greater, thus UAS-Dcr2 was used in following RNAi experiments.

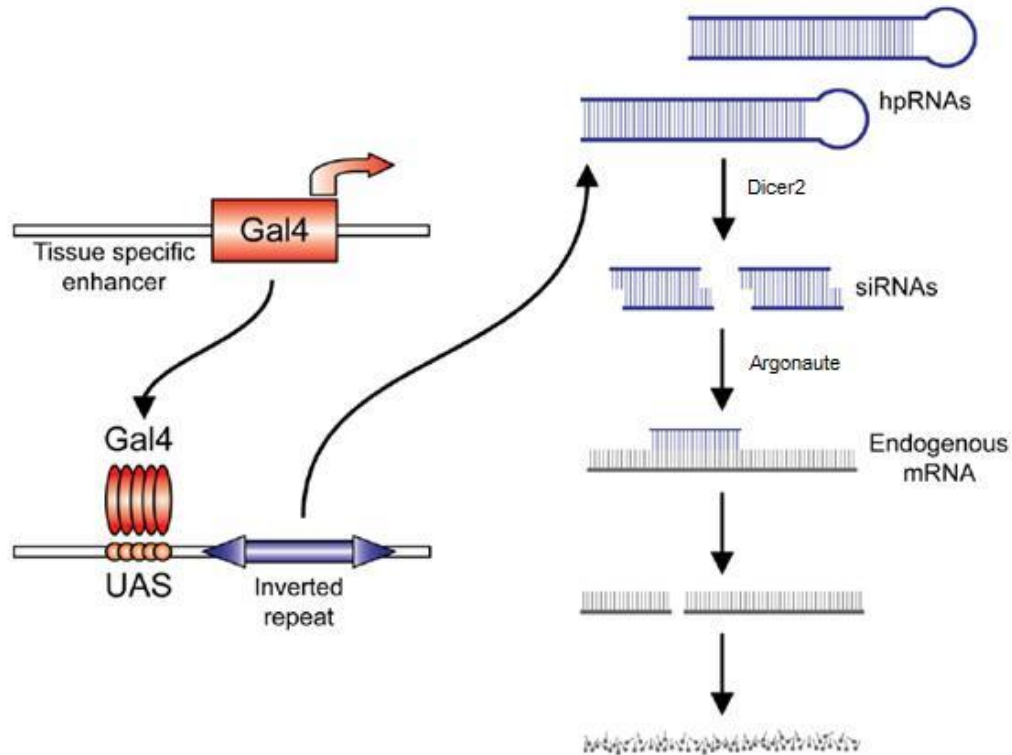


Figure 2.2 RNAi mediated knockdown of the gene of interest in *Drosophila*.

(Adapted from images at <http://www.vdrc.at/rnai-library>)

Tissue specific knockdown of gene function can be achieved by using RNA interference-mediated genetic knockdown in conjunction with Gal4/UAS system. Crossing flies carrying the Gal4 driver and UAS-transpondent line drives the expression of the double stranded RNA (dsRNA). Subsequently, Dicer-2 processes dsRNAs into small interfering RNA (siRNAs). In turn, Argonaute binds the siRNAs that guides Argonaute proteins to their target complementary mRNA, which leads to cleavage of the mRNA molecule and its consequent degradation, thereby preventing translation of the target protein.

2.2.4 Target sequences of the RNAi constructs

These are the target sequences of the RNAi constructs used in the knockdown experiments of

Myosin motors:

Myosin II (Zipper)

Landmark of the targeted gene: 2R:20880779..20885037

CG number: CG15792

Inserted chromosome: 2

Viability: Viable

ON targets: 1

OFF targets: 0

```
GCCTTCTGTCGTGGCTTCCTTGCGCGTCGCAACTACCAAAAGCGCTTGCAGCAACTA
AACGCTATTCGAATCATCCAGCGGAATTGTGCCGCCTACCTTAAGCTTCGGAAGCTGG
CAGTGGTGGCGCCTCTATACTAAGGTCAAGCCCCTGTTGGAAGTTACAAAGCAGGA
AGAGAAGCTTGTCCAGAAGGAGGATGAGCTGAAGCAGGTACGCGAGAAGCTTGAC
ACTCTGGCCAAGAATACGCAGGAGT ACGAGCGCA
```

Myosin V (Didum)

Landmark of the targeted gene: 2R:3392354..3396684

Inserted chromosome: 3

Viability: Viable

ON targets: 1

OFF targets: 0

CGTGTTATTTGGCTCGTCAATTCCATTACGCTGCTAAATCTTATGAAGCAATACGGC
GACGTGGATGAGTACGTCAAGTTCAATACTGAGAAGCAGAATCAGCAGCAGCTGAA
GAACTTCAATCTCTTTGAATACCGTCGCGTAATTCTTGATTTAATTGTGAACCTGTAC
CAGGCGCTGATCATGCAGATCCAGGGTCTGTTGGACCCAAAAATAGTGCCAGCGAT
TCTCAACAATGATGAGATTCAGCGTGGGCGGCAGGCGCACGGAATGCGTAGTCGGG
CCACGTCGATTGGAGCATCCTCGTCACCGGAGCACGGTGGCGGTCCGGCCTGGAAG
CAACTGATCGGGCAGCTGGAG

Myosin VI (Jaguar)

Landmark of the targeted gene: 3R:20080225..20084551

CG number: CG5695

Inserted chromosome: 2

Viability: Viable

ON targets: 1

OFF targets: 0

GCCTCAATGTGCCCGAGATCACTTTCACGGATAACCAGGACATCATCGAGCTGATCG
AGGCCAAGTCGAATGGCATCTTTACGCTGCTCGACGAGGAGTCCAAGCTTCCGAAG
CCCTCGTACTCGCATTTACCGCCGAGGTGCACAAGTCCTGGGCGAATCACTATCGC
CTGGGTCTGCCGAGATCATCGCGACTAAAGGCCACCGCACTCTGCGCGATGAGGA
GGGCTTCCTGGTGCGTCACTTTGCCGGAGCTGTGTGCTACAACACGGAGCAGTTCAT
TGAGAAGAACAACGACGCCCTGCATGCTTCGCTGGAGGGTTTGG

2.3 Pharmacological interference

2.3.1 Drug treatments

Sixty wild-type 3rd instar larvae (96h ALH) with same starting age were chosen and allocated into three groups of 20. While the control group was only treated with 1ml of PBS, Latrunculin B (Broadus and Doe, 1997, Merck LOT D00028575) was applied to the second treatment group and 3-Butanedione monoximine (BDM) (Ohshiro et al., 2000, Merck, LOT D00003178) was injected into the third treatment group. Drug delivery methods were adapted to optimise the efficacy.

1 mg of Latrunculin B powder was dissolved in 12,642 ml of PBS (MW=395.5, 200 μ M) and aliquoted 1ml of drug solution in 1 ml eppendorfs to be stored at -20°. 1ml of the Latrunculin B-PBS drug solution was directly applied to 3rd instar larval CNS for 10 minutes at room temperature. Whereas, BDM solution was always prepared fresh. BDM powder was dissolved in PBS (MW=101.1, 200 mM) and the wild-type third instar larvae were incubated in BDM-PBS solution for 90 min at room temperature prior to dissection. The same procedure was repeated five times in order to increase sample size, thereby increasing statistical power.

2.3.2 Quantification of neuroblasts

Control group, as well as BDM and Latrunculin-B treated CNS were then fixed and immunolabelled (as described below) using anti-Mira antibodies to examine cell fate determinant localisation during the cell cycle with anti-pH3 antibodies as a cell cycle marker. Late metaphase post-embryonic NBs were identified based on the typical anti-pH3 pattern. Total of 100 NBs in

late metaphase were randomly chosen and analysed for each treatment groups. Late metaphase Mira localisation in wild-type post-embryonic NBs were compared to that of drug treated groups.

2.3.3 Statistical analysis of drug treatments

The statistical analysis was performed using SigmaStat software (v3.01; SPSS Inc, Chicago, USA). Thus, a student t-test was used to compare the control group against the drug treated group, with overall significance level of $P < 0.05$.

2.4 Immunohistochemistry

2.4.1 Embryo staging and collections

Approximately 150 flies were caged in a modified food bottle with ventilation holes, with Petri dishes containing apple juice agar with yeast paste at the bottom, on which flies laid their eggs. Subsequently, Petri dishes were changed every 2 hours and incubated at 25°C, until embryos reached appropriate stage for collection.

Agar plates

1.5l	Filtered water
25g	Sugar
42.5g	Agar
0.5l	Apple juice
4g	Nipagen

Mixture of water, sugar and agar are autoclaved in a 2 litre conical flask, followed by addition of pre-warmed apple juice and Nipagen which is stirred at ~50°C until Nipagen is fully dissolved. The warm mixture is transferred onto 55mm Petri dishes, with just enough portions to cover the bottom of the dishes. The plates are then left to cool overnight at room temperature, before being stored at 4°C in a sealed container for up to one month.

Yeast paste

5g	Dried active yeast
----	--------------------

0.2g Sugar

Add appropriate amount of distilled H₂O until yeast become moist.

2.4.2 Embryo fixations

Once embryos develop into an appropriate stage, dechorination of embryos are achieved by immersing in a solution of diluted household bleach (50%) for approximately 5 minutes. Dechorinated embryos are collected on a plastic mesh, remaining bleach are washed off with distilled water. Subsequent fixation of embryos involve placing the plastic mesh with retained embryos in a 2ml eppendorf tube containing 1ml PEM/FA solution and 1ml heptane (Fisher) to be rotated at 100rpm for 10-30 minutes. Lower and upper aqueous phase is then carefully removed, followed by addition of 1ml heptanes and 1ml 100% methanol (VWR). Vigorous shake of the tube for 1 minute allows devitellinised embryos to sink to the bottom. The embryos undergo three 5 minute washes, and a final 30 minute wash in 100% methanol in room temperature. Embryos are then immersed in fresh methanol to be stored at -20°C.

Solutions for embryo fixation

PEM:

100mM PIPES (Sigma)

2mM EGTA (Sigma)

1mM MgSO₄ (Fluka)

Make up to 1L with dH₂O

Adjust pH to 7 with HCl

PEM/FA:

9ml PEM

1ml 37% formaldehyde solution (Calbiochem)

2.4.3 Larval dissections and immunostaining

Larval immunohistochemistry

Larval CNS at 3rd instar stage (96h ALH or 120h ALH) were dissected in PBS, and were fixed for 1 hr in PLP at room temperature, followed by 3x5 min washes in PBT. They were then incubated in PBT-NGS to block non-specific binding for 30 min on a rotator at room temperature. Subsequently, PBT-NGS was removed, followed by addition of primary antisera, diluted at their required concentrations to a final volume of 300 µl to be left overnight at 4°C.

On the following day, larvae were washed again for 3x5 min in PBT, followed by incubation in secondary antisera, diluted at their required concentrations to a final volume of 300 µl for 3 hrs at room temperature. After 3 hours, larvae were washed for 2x15 min in PBT, and with PBS for 2x15 min. Finally two drops of vectashield mounting medium (Vector Labs) were added, and left to be incubated overnight at 4°C.

On the third day, larvae were fine dissected on a slide, and arranged in an easy location for fluorescent microscope. Coverslips were placed, and sealed with clear nail varnish.

Solutions for larval dissection

Sodium phosphate buffer (PBS):

500ml 0.1M Na₂HPO₄ (Fluka)

Adjust pH to 7.4 with NaH₂PO₄ (Fisher)

Phosphate buffer with Lysine (PBL):

1.8g Lysine HCL

50ml of distilled H₂O

Adjust pH to 7.4 with Na₂HPO₄

Store at 4°C for 3 months

8% Paraformaldehyde (Sigma)

1.6g PFA in 20ml dH₂O +140µl 1M NaOH

Incubate in water bath (37°C) until completely dissolved

Aliquot into 2ml micro-centrifuge tubes to be stored at -20°C

PLP (Make fresh):

1200 µl PBL

400 µl 8% Paraformaldehyde

PBT:

500ml PBS

0.5ml Triton X-100 (Fluka)

0.5g BSA (Sigma)

stored at 4°C for 3 months

PBT-NGS:

9.5ml PBT

0.5ml Normal Goat Serum (Invitrogen)

2.4.4 Antibodies used

Primary antibodies

Mouse anti-Pros (mAbMr1A, Developmental Studies Hybridoma Bank, DSHB, 1:10), Polyclonal Rabbit anti-Phospho-Ser28 histone H3 (Upstate Biotechnology, 1:200), Monoclonal Mouse anti-Mira mAb81 (a gift from P. Overton; Matsuzaki et al., 1992, 1:50), Monoclonal Mouse 3C7 Anti-Myosin VI/Jaguar (a gift from K. Miller, 1:20), Polyclonal Rabbit anti-Mira (a gift from J. Knoblich, 1:200), Polyclonal Rabbit anti-Brat (a gift from F. Hirth, 1:200), Phalloidin-Alexa Fluor 568 (Invitrogen, 1:1000)

Secondary antibodies

Goat anti-mouse, Goat anti-rabbit or goat anti-guinea conjugated to Alexa fluorophores 488, 568 or 647 (Invitrogen) were used. They were pre-absorbed at 1:15 with w1118; +; + embryos to decrease background staining, followed by addition of 0.02% sodium azide to be stored at 4°C. The pre-absorbed antibodies were used for secondary staining at concentration of 1:10.

2.4.5 Microscopy and image analysis

Confocal microscopy

Images were taken on a Leica TCS SP5 confocal microscope, using Leica Application Suite Advanced Fluorescence (LAS AF) version 2.0.2 software. Images were acquired at resolution of 512x512 on 4x line average.

Image processing

Confocal data sets were analysed using Image J software. Figures were arranged and labelled using Microsoft PowerPoint and Adobe Photoshop CS3. Schematic diagrams were generated using Adobe Illustrator CS3, graphs with Microsoft Excel.

2.4.6 Quantification of Neuroblasts

Using Miranda antibody as a NB marker, central brain NBs (the focus of this study) were distinguished from optic lobe NBs due to their medial superficial location, larger size, and dispersed pattern (optic lobe NBs are laterally positioned in the brain and spaced very closely to each other, forming a ribbon that flanks and encircles the highly stereotypical epithelial-shaped optic lobe cells). Quantification of central brain NBs were conducted in 3rd instar larval CNS (Lee et al., 2006a) (96h ALH and 120h ALH) of wild type and 1407>Pros IR, 1407>Brat IR, 1407> Jag IR, 1407>MyoV IR, as well as 1407>Jag IR; myoV IR. I quantified central brain NBs from each gene defective groups were compared against the wild-type.

2.4.7 Statistical analysis of RNAi experiments

The statistical analysis was performed using SPSS software (v18; SPSS Inc, Chicago, USA). A one-way ANOVA was used to compare the wild type group against the gene defective groups. Turkey HSD post-hoc test was applied for multiple comparisons against the wild type group, with overall significance level of $p < 0.05$.

2.4.8 Quantification of spindle orientation

To quantify spindle orientation defect, I analysed the angle between a line bisecting the crescent of Mira and phosphorylated histone H3 expression during metaphase and telophase. Quantification of spindle orientation was conducted in central brain NBs of 3rd instar larval CNS (96h ALH) in wild-type NBs, as well as in 1407>Jag IR, 1407>myoV IR, 1407>Jag IR; myoV IR. Numbers of NBs from each gene defective groups were compared against the wild-type using Student's t-test.

Chapter 3: Characterisation of the role of cell fate determinants in asymmetric division of post-embryonic NBs

3.1 Introduction

Asymmetric cell divisions play a key role in giving rise to cellular diversity and specificity of numerous cell types in the brain during development. Recent studies have shown that the post-embryonic central nervous system (CNS) of *Drosophila* is an excellent model to study the genetic mechanisms involved in these processes, as they undergo classic asymmetric divisions, with the daughter cells exhibiting differential gene expression and cell size, including tumorigenesis, if these processes are perturbed (Reviewed in Gonzalez, 2007; Egger et al., 2008).

Embryonic and post-embryonic NBs divide into two daughters with different fates. Establishment of apical-basal polarity and alignment of the mitotic spindle along the apical-basal axis enables exclusive basal segregation of cell fate determinants into the smaller daughter known as ganglion mother cell (GMC) that divides once more into two differentiating neurons, whilst giving rise to a larger daughter cell that retains NB characteristics and continues to divide in a stem cell like manner (Reviewed in Jan and Jan, 1998; Knoblich, 2010).

During each NB division, Pros and Brat (Betschinger et al., 2006; Lee et al., 2006b), collectively known as the cell fate determinants localise to the basal cell cortex and segregate into the GMC (Bello et al., 2006) (Figure 3.2 A). Binding of these basal cell fate determinants to their adaptor

protein Mira is essential for their basal localisation to the cortex and asymmetric segregation into the smaller daughter cell (Betschinger et al., 2006; Lee et al., 2006b). In line with this, disruption in asymmetric segregation of the cell fate determinants leads to over-proliferation of NBs at the expense of differentiating neurons (Bello et al., 2006; Betschinger et al., 2006), indicating that exclusive partitioning of cell fate determinants are the general mechanism in which stem cells regulate self-renewal and control the balance between proliferating and differentiating daughter cells. In this chapter, I aim to establish the relationship between the cell fate determinants and their adaptor protein Mira in the post-embryonic NBs in third instar larval CNS.

3.2 Identification of post-embryonic NB lineages in the larval CNS of *Drosophila*

3.2.1 Overview of post-embryonic NB lineages in the third instar larval CNS

The CNS of *Drosophila* is mainly composed of two brain hemispheres and the ventral ganglia. The central brain develops in the medial regions of each hemisphere, while the olfactory lobes are located laterally (Figure 3.1 A). Most of the cells of the adult central brain originate from NBs located in the medial region of the hemispheres, which in turn proliferates from the first instar stage until reaching pupal development (Urbach et al., 2003; Maurange et al., 2008).

Within the central brain, two different types of NBs have been identified. The type I NBs constitute majority of the central brain NBs, which follows similar patterns of proliferation to those of embryonic counterpart. A smaller group of type II NBs also exist within the central brain that undergoes a different proliferative mode to generate intermediate progenitors with transit amplifying divisions (Bello et al., 2008; Bowman et al., 2008) (Figure 3.1A).

3.2.2 1407 Gal4 targets mCD8-GFP expression to NB lineages in the third instar larval CNS.

In *Drosophila* larval CNS, NBs can be identified by staining for Mira and by the expression of membrane bound CD8-GFP in NBs and all their progeny by using the 1407 Gal4 driver (Figure 3.1 B). The expression patterns of 1407 Gal4 driven membrane bound CD8-GFP co-labelled with anti-Mira antibody reveal that endogenous Mira expression is mainly limited within the NBs and only transiently expressed in the GMC before degrading and subsequently releasing Pros (Figure 3.1 B, C). This is in line with the published findings that Mira expression concomitantly decreases with Pros translocation into the GMC nucleus (Ikeshima-Kataoka et al., 1997). Moreover, strong Pros labelling was observed in the nuclei of the ganglion cells progeny located below the anti-Mira positive parental NBs (Figure 3.1 C, D). Expression of Pros was also detected in dividing NBs in a basal crescent from Pro-metaphase to telophase (Figure 3.4).

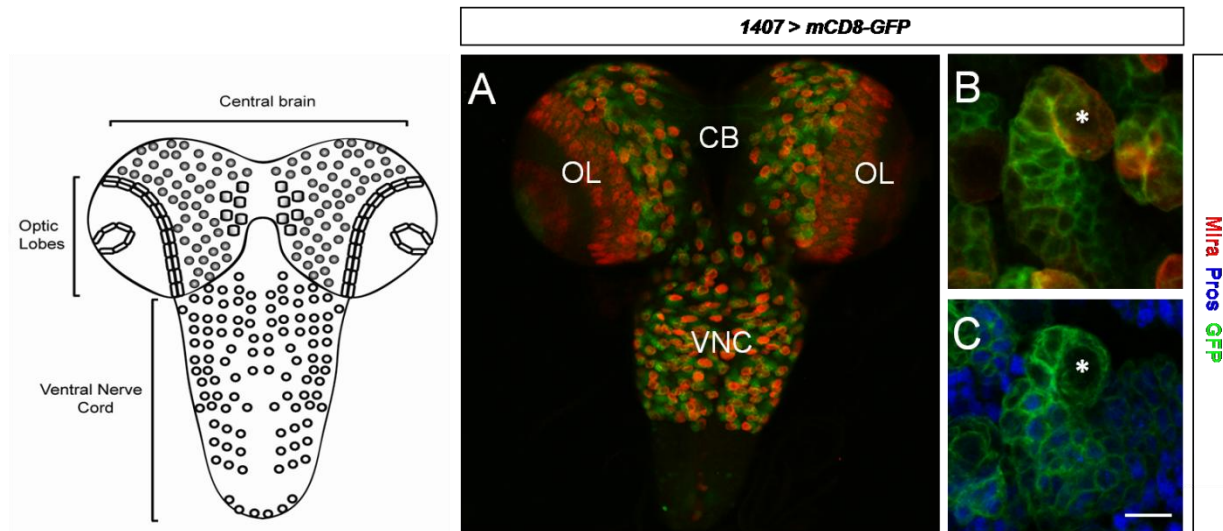


Figure 3.1 1407 Gal4 driven expression of mCD8::GFP reveals post-embryonic NB lineages in 3rd instar larval CNS. A schematic diagram of the third instar larval CNS of *Drosophila* (A), displaying the location of post-embryonic NBs (circles) in optic lobes (OL), central brain (CB) and ventral nerve cord (VNC) (adapted from Kim and Hirth, 2009). Type I NBs are shown in grey circles, whereas, type II NBs are represented by squares in the dorso-medial region of the central brain. Confocal Z-projections of a whole mount 3rd instar larval CNS (B) labelled with anti-Mira antibody and 1407 Gal4 Driven expression of mCD8-GFP. A close up view of the Type I post-embryonic NB lineage (C-D) in the central brain reveal that Mira expression is mostly limited within the NBs, whereas, Pros is predominantly expressed in the ganglion cells. The scale bar is 10 μ m.

3.3 Mira is asymmetrically segregated in dividing post-embryonic NBs in a cell cycle-dependent manner

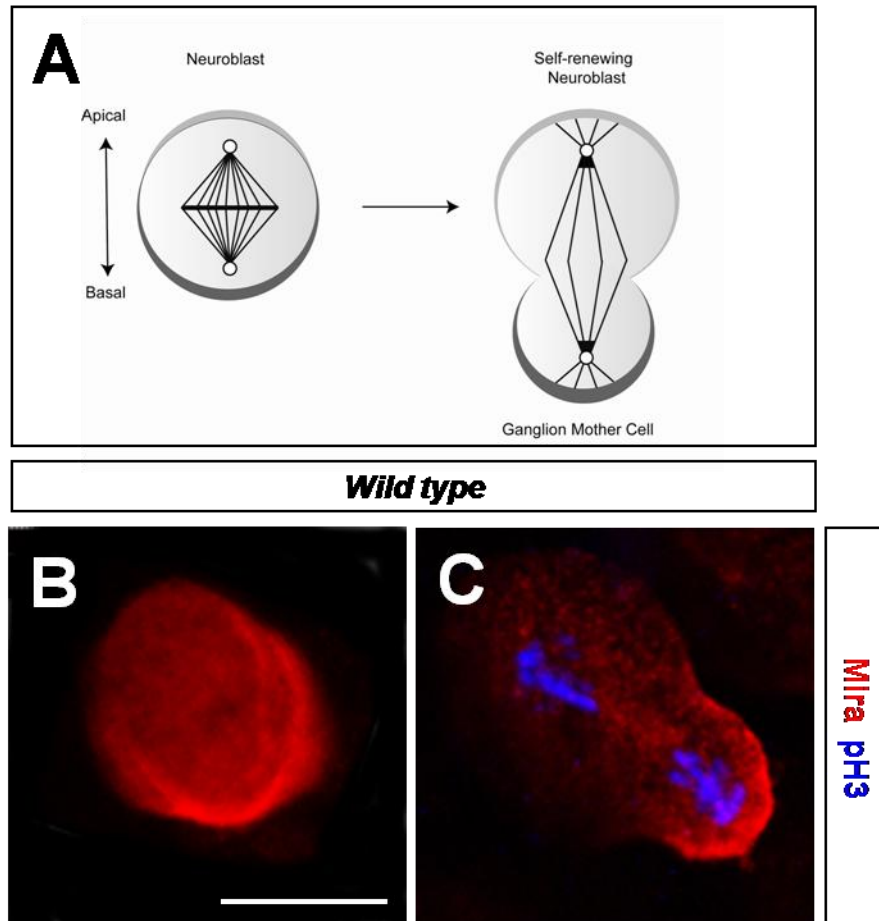


Figure 3.2 Mira localisation is cell cycle dependent during asymmetric division in post-embryonic NBs.

A schematic representation of cell fate determinant localisation (dark gray crescent) during asymmetric division of neuroblasts (A). Basal crescent formation of Mira at metaphase (B). Basal segregation of Mira into the smaller daughter, GMC at telophase (C), as indicated by pH3 labelling; All images shown are in a single confocal plane. Scale bar is 10µm.

Sub-cellular localisation of the wild-type Mira protein in the 3rd instar larval CNS was analysed by co-labelling with anti-Mira antibody and the cell cycle marker phosphohistone H3 (pH3). By metaphase, Mira protein pre-dominantly localises to the basal cortex as a crescent (Figure 3.2 B). As the mitotic stage proceeds, increased amounts of Mira appear to be incorporated into the basal crescent. While some Mira protein is still observed in the apical daughter, most Mira protein segregates to the budding GMC by telophase (Figure 3.2 C).

3.4 1407 Gal4 driven Mira-GFP expression partially overlaps with endogenous Mira in a basal crescent during asymmetric division of post-embryonic NBs

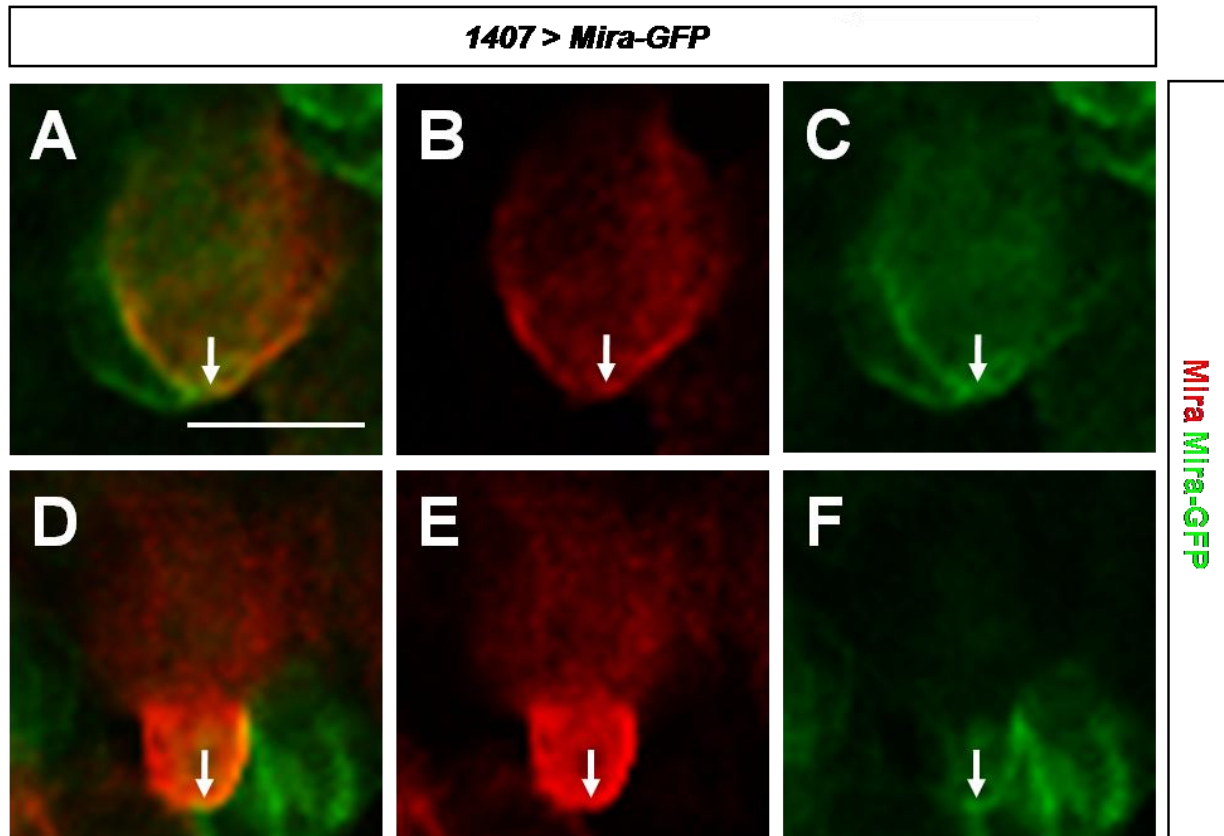


Figure 3.3 Expression of 1407 Gal4 driven Mira-GFP partially overlaps with endogenous Mira expression during post-embryonic NB division in 3rd instar larval CNS. 1407 Gal4 driven expression of Mira-GFP (green) is shown at metaphase (A) and telophase (D). Immunolabelling with anti-Mira (red) shows endogenous Mira expression at metaphase (B) and telophase (E). Expression of 1407 Gal4 driven Mira-GFP partially overlaps with endogenous Mira-expression in a basal crescent (in their merged image) during asymmetric division of post-embryonic NB at metaphase (C) and telophase (F); arrows indicate basal crescent; All images shown are in a single confocal plane. The scale bar is 10 μ m.

Sub-cellular expression of 1407 Gal4 driven Mira-GFP were analysed against endogenous Mira protein expression. At metaphase, basal enrichment of Mira-GFP is observed (Figure 3.3 C), which is comparable to that of endogenous Mira expression (Figure 3.3 B), and partially overlaps, in a basal crescent (Figure 3.3 A). Similarly, partial overlap of Mira-GFP expression with endogenous Mira expression is seen in the cortex of recently born GMC (Figure 3.3 A, D). Interestingly, GFP expression appeared to be slightly delayed and persistent, as Mira-GFP expression can be detected in the some post-mitotic ganglion cells (Figure 3.3 C, F).

3.5 Brat and Pros co-localise with Mira in a basal crescent during asymmetric division of post-embryonic NBs

It has been well established that Brat and Pros are exclusively segregated into the GMC as cargo protein of Mira during asymmetric division of embryonic NBs (Lee et al., 2006b). To analyse the sub-cellular localisation of the cell fate determinants in dividing post-embryonic NBs, whole mounts of 3rd instar larval CNS were immunolabelled with an antibody raised against the N-terminal of the Brat Protein, anti-Pros antibody, in combination with 1407 Gal4 driven expression Mira-GFP.

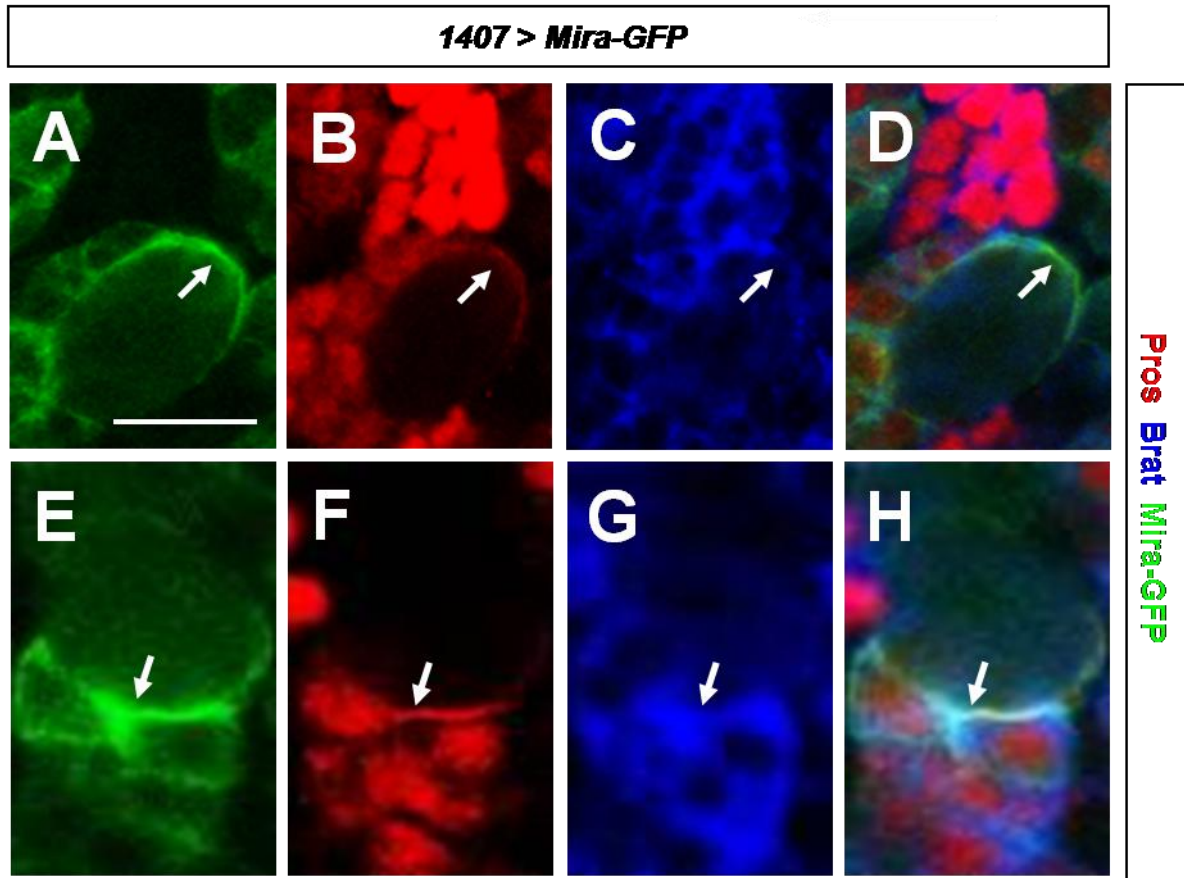


Figure 3.4 Cell fate determinants Brat and Pros co-localise with their adaptor protein Mira during post-embryonic NB division in a 3rd instar larval CNS. 1407 Gal4 driven expression of Mira-GFP (green, A, E) was co-labelled with anti-Mira (red, B, F) and anti-Brat (blue, C, G). Merged image (D, H) reveal that cell fate determinants Brat and Pros co-localise in a basal crescent with their adaptor protein Mira during asymmetric division of two different individual post-embryonic NB in 3rd instar larvae, irrespective of their orientation towards the larval body wall; arrows indicate basal crescent in post-embryonic NB; All images shown are in a single confocal plane. The scale bar is 10 μ m.

During mitosis, Mira localises basally and forms a crescent at metaphase (Figure 3.4 A, E; indicated by arrows). Brat is a uniformly expressed cytoplasmic protein that can be observed in both NBs and the ganglion cells (Figure 3.4 C, G), whereas Pros expression is limited to the ganglion cells, but can be seen in a basal crescent in a few NBs (Figure 3.4 B, F; indicated by arrows). Moreover, I was able to demonstrate that Brat and Pros co-localise in a basal crescent with their adaptor protein Mira at metaphase during post-embryonic NB division (Figure 3.4 D, H). This is consistent with the notion that Mira is essential for basal anchoring of Pros (Ikeshima-Kataoka et al., 1997; Shen et al., 1998) and bolsters the view that Brat and Pros are segregated into the GMC as cargo proteins of Mira.

Rarity in observation of Pros expression in the basal crescent in dividing post-embryonic NBs suggests that Pros expression in NBs are very transient. Immediately following cytokinesis, Mira expression is observed in the cortex of GMCs. This event is fleeting, as Mira quickly becomes undetectable while Pros translocates to GMC nuclei (Figure 3.4 D, H). Thus, in asymmetrically dividing post-embryonic NBs, Mira, Brat, Pros co-localise at the basal cortex just prior to being exclusively segregated into the GMC cortex, then concomitant with a decrease in Mira expression in the GMC, Pros translocates into the GMC nucleus.

3.6 Genetic knock-down of Brat or Pros leads to over-proliferation of central brain post-embryonic NBs in 3rd instar larval CNS

3.6.1 Mutation in Brat or Pros leads to excess self-renewal at the expense differentiating neurons.

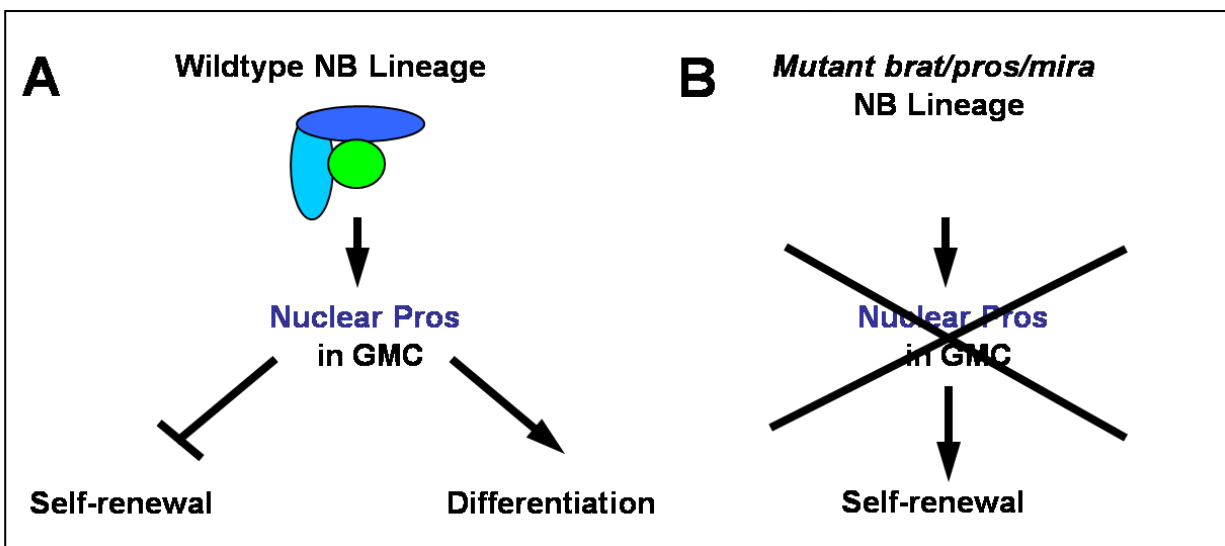


Figure 3.5 Mutation in cell fate determinants Brat or Pros, as well as their adaptor protein Mira leads to excess self-renewal at the expense of differentiating neurons. A Schematic diagram demonstrating mechanisms involved in GMC differentiation. Upon asymmetric segregation of the cell fate determinants, (A) Pros translocates to the nucleus to initiate GMC differentiation. (B) Failure in nuclear localisation of Pros leads to excess self-renewal at the expense of differentiating ganglion cells.

Exclusive segregation of the cell fate determinants, more specifically, translocation of Pros into the newly born GMC nucleus is believed to be the binary switch that regulates self-renewal and differentiation of GMC (Figure 3.5 A). However, if a mutation occurs in Brat or Pros, NBs over-proliferate leading to tumorigenesis (Bello et al., 2006; Betschinger; et al., 2006). The observed common tumorigenic phenotype in Pros and Brat mutants suggests that disruption in asymmetric segregation of the cell fate determinants maybe the cause of this phenomenon. The currently proposed hypothesis indicate that nuclear translocation of Pros is crucial for initiating differentiation of the newly born GMC

3.6.2 Genetic knock-down of Brat or Pros leads to increased number of Mira positive central brain NBs in late larval stages.

Approximately, 106 NBs are formed in the central brain during embryogenesis (Urbach and Technau, 2008), where they proliferate briefly before entering quiescence. The central brain NBs re-enter the cell cycle by mid larval stages (72h ALH). Thus, from mid to late larval stages, central brain NBs undergo asymmetric division to generate majority of the neurons that constitute adult brain.

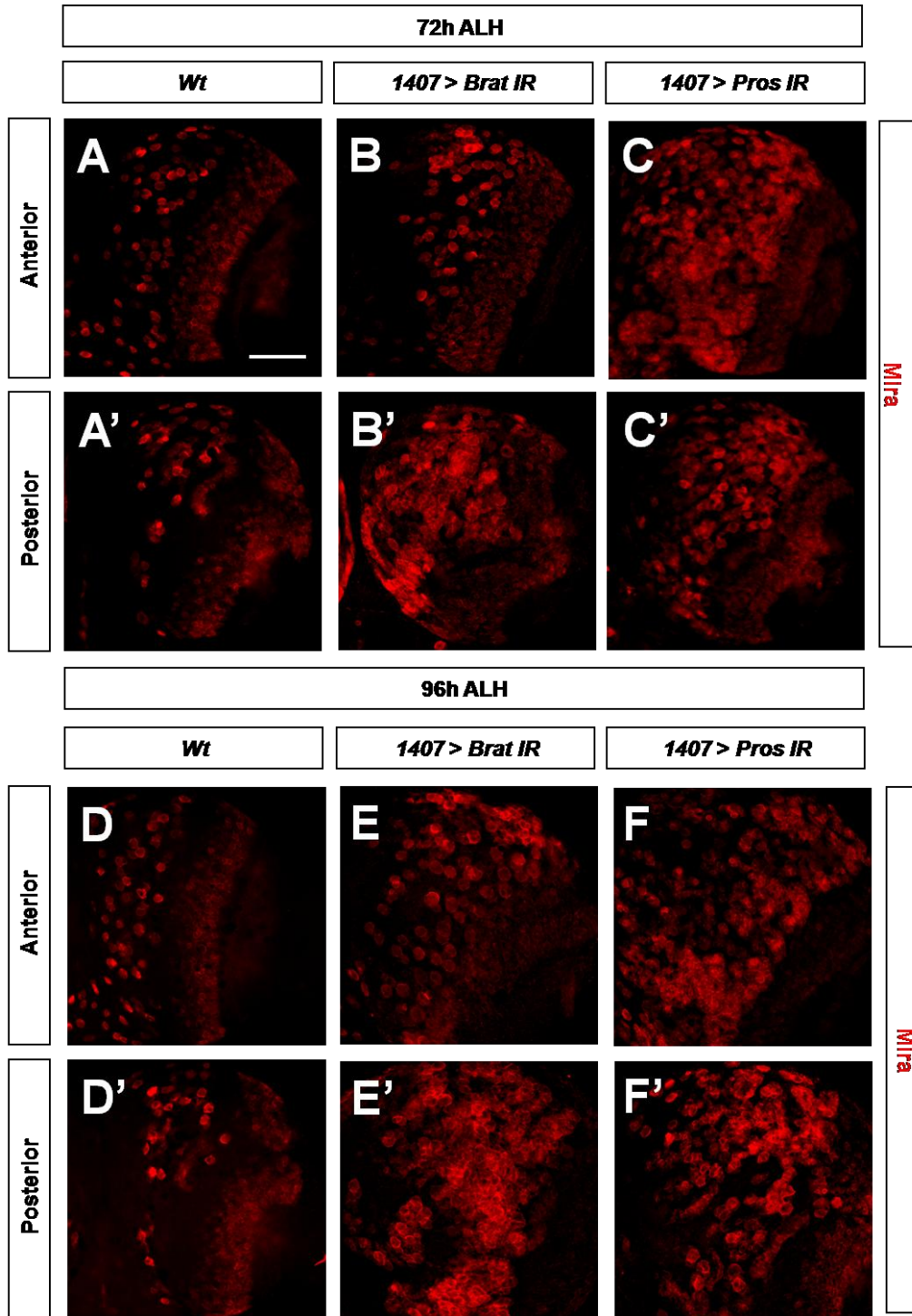


Figure 3.6 RNAi mediated knockdown of Brat or Pros leads to over-proliferation of central brain NBs. Post-embryonic NBs in the central brain were analysed for over-proliferation phenotypes at mid to late larval stages (at 72h or 96 h after larval hatching, ALH) in in confocal

Z-stacks of whole mount wild type (A and D), 1407 > Brat IR (B and E), 1407 > Pros IR (C and F) larval brains. Post-embryonic NBs were identified by the expression of Mira. Posterior view of the larval brains were also analysed to assess for tumour phenotypes in type II NBs (A'–F'). RNAi mediated knockdown of Brat resulted in over-proliferation of post-embryonic central brain NBs at 72h ALH (B) and 96h ALH (E). Similarly, reduced function of Pros by RNAi knockdown also lead to a over-proliferation phenotype of central brain NBs at 72h ALH (C) and 96h ALH (F); The images shown are in a single confocal plane. The scale bar is 50µm.

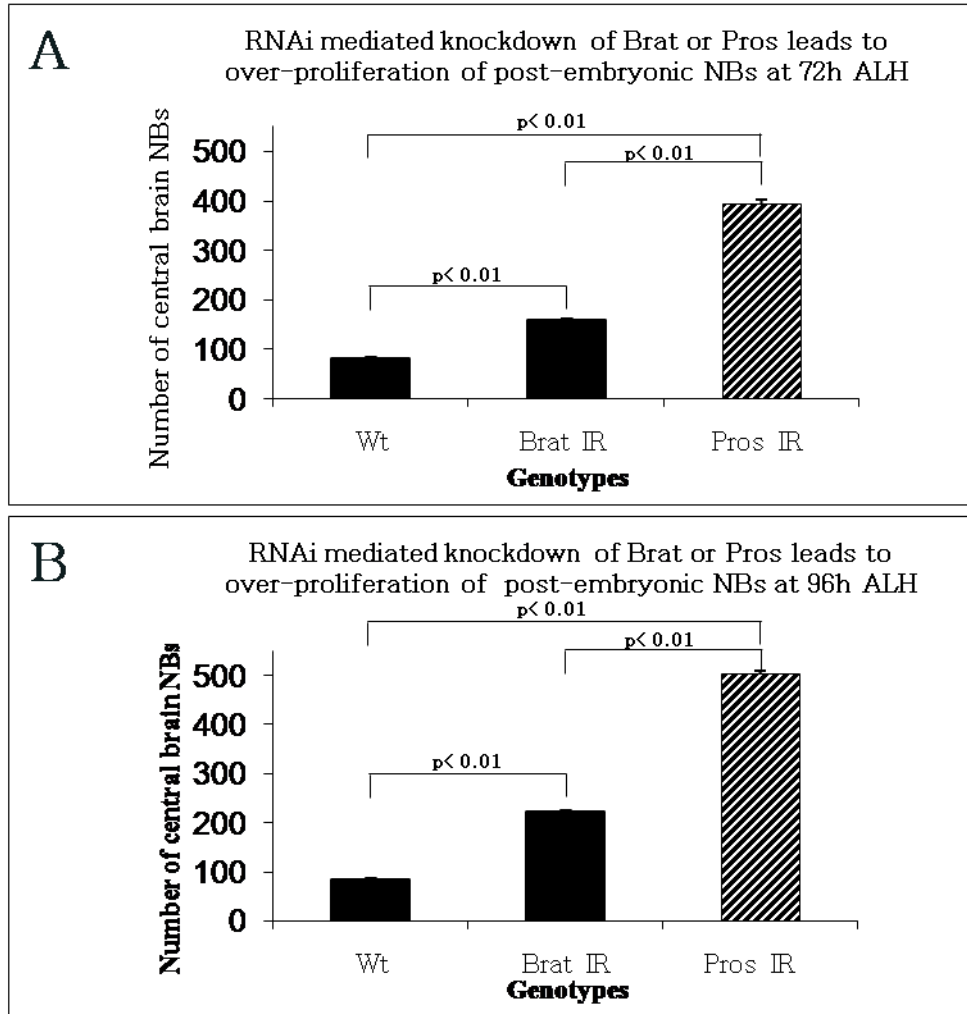


Figure 3.7 RNAi mediated knockdown of Brat or Pros leads significantly increase in the number of post-embryonic NBs in the central brain. Quantification of central brain NBs numbers were conducted at mid to late larval stages (at 72h or 96 h ALH) in wild type , 1407 > Brat IR , 1407 > Pros IR larval brains. RNAi mediated knockdown of Brat or Pros resulted in a significant increase in the mean number of Mira positive central brain NBs at 72h ALH (A) and 96h ALH (B) compared to the wild type. Moreover, the number of Mira positive central brain NBs in 1407 > Pros IR larval brains were significantly higher than that of 1407 > Brat IR brains at 72h ALH (A) and 96h ALH (B). Error bars are standard errors of the mean.

I therefore characterised RNAi mediated knockdown of Brat and Pros in 3rd instar larval stage by using Mira as post-embryonic NB marker. I quantified mira positive post-embryonic central brain NBs in mid to late larval stages (72 h and 96 h ALH). In wild type central brains (Figure 3.6 A), there were 81.4 ± 1.9 at 72h ALH and by 96h ALH, NB numbers slightly increased to 84.1 ± 1.4 (Figure 3.6 D). RNAi mediated knockdown of Brat significantly increased the number of post-embryonic NBs in central brain to 160 ± 7.6 at 72h ALH (Figure 3.6 B), and by 96h ALH reached 222.8 ± 6.7 (Figure 3.6 E). Similarly, RNAi mediated knockdown of Pros resulted in a significant increase in the number of Mira positive post-embryonic NBs in the central brain at 72h ALH 393.5 ± 13.2 (Figure 3.6 C), which increases dramatically by 96h ALH to 502.65 ± 17.1 (Figure 3.6 F). Therefore, RNAi mediated knockdown of Brat and Pros lead to a significant increase in the number of proliferating post-embryonic NBs from 72h ALH to 96h ALH, compared to that of the wild type counterpart (Figure 3.7) (see table 3.1). The increased number of post-embryonic central brain NBs is mostly likely due to transformation of the GMC into a continuously proliferative state. Interestingly, in Brat or Pros knockdown brains, the post-embryonic NBs continues to proliferate and the mean number of central brain NBs significantly increase from mid to late larval stages (Table 3.1 C). These data suggest that both Brat and Pros play an important role in regulating proliferation control of post-embryonic NBs. Disrupting the function of either cell fate determinants, Brat or Pros results in over-proliferation of post-embryonic NBs at the expense of differentiating neurons that may eventually lead to tumourigenesis in the larval brain.

A				
72h ALH				
Genotype	N	Mean	SEM	p Value
Wt	30	81.4	1.9	1
1407>Brat IR	20	160.0	7.6	<0.01
1407>Pros IR	20	393.5	13.2	<0.01

B				
96h ALH				
Genotype	N	Mean	SEM	p Value
Wt	30	84.1	1.4	1
1407>Brat IR	20	222.8	6.7	<0.01
1407>Pros IR	20	503.7	17.1	<0.01

C				
72h ALH				
Genotype	N	Mean	SEM	p Value
1407>Brat IR	20	160	7.6	1
1407>Pros IR	20	393.5	13.2	<0.01

D				
96h ALH				
Genotype	N	Mean	SEM	p Value
1407>Brat IR	20	222.8	6.7	1
1407>Pros IR	20	503.7	17.1	<0.01

E		
Genotype	72h ALH	96h ALH
Wt	1	>0.05
1407>Brat IR	1	<0.01
1407>Pros IR	1	<0.01

Table 3.1 RNAi mediated knockdown of Brat or Pros leads to a significant increase in the number of central brain post-embryonic NBs. Genetic knockdown of Brat or Pros results in a significant increase the mean number of post-embryonic NBs in the larval central brain at 72h ALH (A) and 96h ALH (B). Over-proliferation phenotype appears to be significantly exacerbated in 1407> Pros IR brains compared to the 1407> Brat IR brains at 72h ALH (C) and 96h ALH (D). Moreover, in Pros or Brat knockdown brains, the number of post-embryonic NBs continues to proliferate and the number significantly increases from mid (72h ALH) to late (96h ALH) larval stages (C). A one-way ANOVA was used to compare the wild type group against

Brat or Pros knockdown groups. Turkey HSD post-hoc test was applied for multiple comparisons against the control group, with overall significance level of $p < 0.05$.

3.6.3 Type II NB lineages are susceptible to over-proliferation

Analysis of Type II NB lineages in 3rd instar larval CNS was conducted using anti-Mira Immunolabelling of 96h ALH larval CNS. Type II NBs are located in dorso-medial region of the brain, which generates an INP and a GMC, resulting in a much larger lineage than type I NBs. Mira is expressed in their progeny located adjacent to their parental NBs (Figure 3.8 A). Loss of Brat (Figure 3.8 B) or Pros (Figure 3.8 C) by RNAi mediated knockdown resulted in marked increase in Mira positive intermediate neural progenitor cells, which is indicative of an over-proliferation phenotype.

Moreover, previous findings suggest that only type II NBs are susceptible to loss of Brat (Bowman et al., 2008; Bayraktar et al., 2010). This may be a plausible explanation for exacerbated over-proliferation phenotype observed in 1407> Pros IR brains compared to the 1407> Brat IR brains (Figure 3.7).

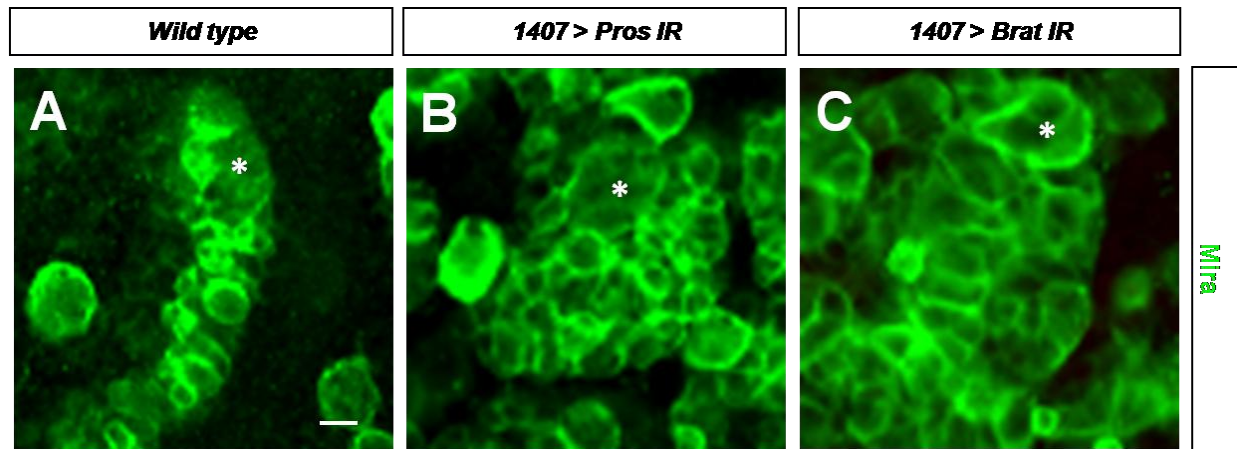


Figure 3.8 knockdown of Pros or Brat leads to over-proliferation of INPs in type II post-embryonic NBs at the expense of differentiation. Close inspection of type II post-embryonic NBs reveal that compared to a wild type (A), RNAi mediated knockdown of Pros (B) and Brat (C) leads to increased number of mira positive intermediate neural progenitor cells (Parental NBs shown in asterisks); All images shown are in a single confocal plane. The scale bar is 10 μ m.

3.6.4 Co-expression of dicer with pros RNAi, which significantly reduced protein expression, hence enhancing the tumour phenotype.

The efficiency of RNAi can be improved by concomitant over-expression of Dcr2, which cuts double-stranded RNAs into siRNAs (Dietzl et al. 2007). The observed over-proliferation phenotype in Pros knockdown brains were obtained in the absence of Dcr2 over-expression, indicating that this is not necessary for effective RNAi mediated knockdown of Pros. However, when 1407 gal4 was used to drive the expression of both Pros RNAi and Dcr2, it resulted in greatly exacerbated over-proliferation phenotype in the larval central brain at 72h ALH (Figure 3.9 C). It is also interesting to note that the severity of the over-proliferation phenotype directly correlates with the level of Pros knockdown. Thus, in the subsequent chapters Dcr2 was co-expressed with all RNAi lines to increase RNAi efficiency, in order to ensure optimal level of protein is being knocked down in the following RNAi experiments.

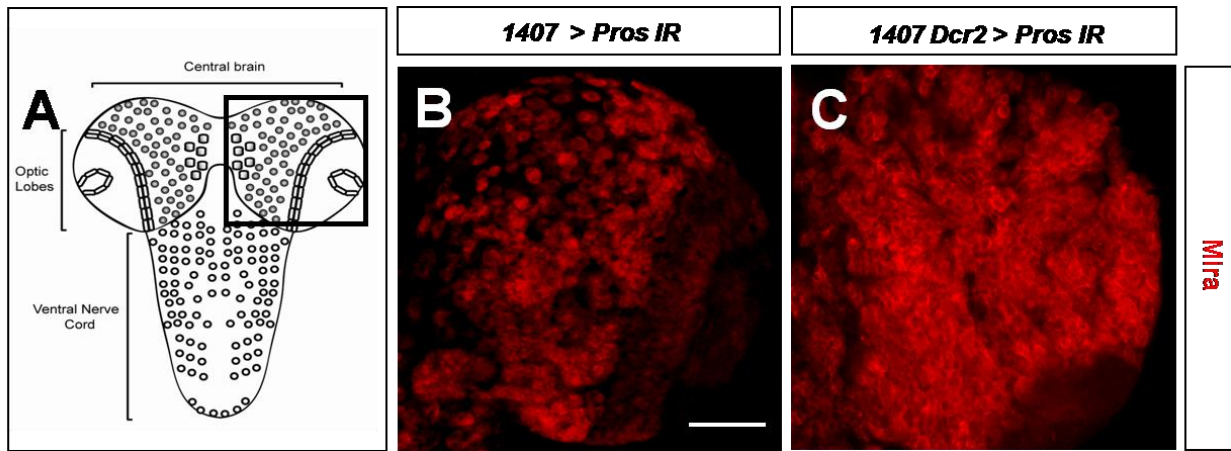


Figure 3.9 Co-expression of 1407 Gal4 driver with Dcr2 increases efficiency of Pros RNAi. RNAi mediated knockdown of Pros results in increased number of Mira positive NBs (A) in the central brain at 72h ALH compared to the wild type. Interestingly, co-expression of with Dcr2 greatly enhanced the Pros RNAi efficiency (B), as increased Mira expression is visibly noticeable, to the extent that it is no longer possible to distinguish between outer proliferating centre and the central brain; All images shown are in a single confocal plane. The scale bar is 50 μ m.

Chapter 4: Role of actin in basal targeting of cell fate determinants during asymmetric division of post-embryonic NBs

4.1 Introduction

How the Brat/Mira/Pros complex are targeted and anchored to the basal cortex is not well understood. However, an intact filamentous actin (F-actin) cytoskeleton is thought to be required for asymmetric segregation of Pros in embryonic NBs, in vitro (Broadus and Doe, 1997). In this chapter, I aim to establish the role of F-actin in basal targeting/anchoring of Mira during asymmetric division of post-embryonic NBs, in vivo.

4.2 F-actin co-localises with Mira during asymmetric division of post-embryonic NBs

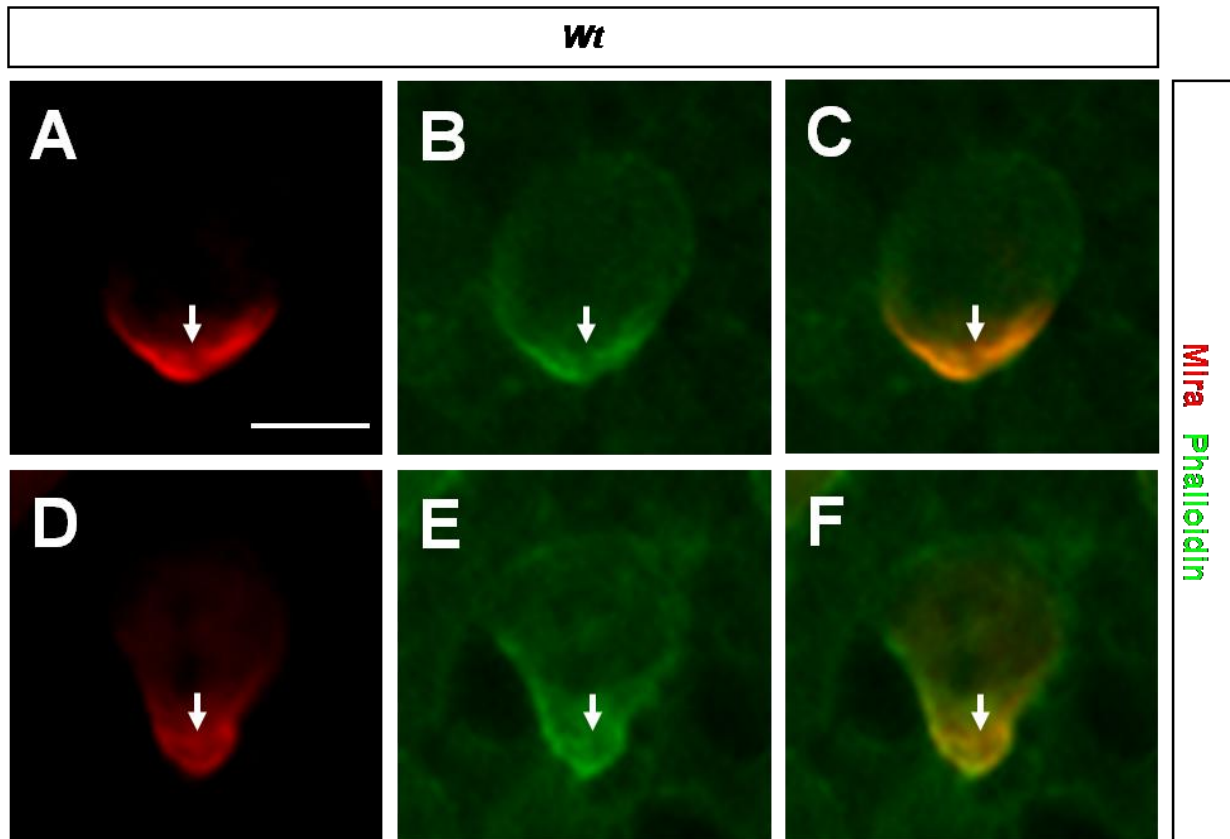


Figure 4.1 F-actin co-localises with mira during asymmetric division of post-embryonic NBs. Mira forms a basal crescent during metaphase (A) and is exclusively segregated into GMC at telophase (D). Although F-actin is uniformly expressed in the cytoplasm, F-actin expression enriched at the basal cortex at metaphase (B) and at telophase (E). Merged image reveals that Mira and F-actin co-localise during asymmetric division; arrows indicate basal enrichment of Mira and F-Actin; All images shown are in a single confocal plane. The scale bar is 10 μ m.

Sub-cellular localisation of F-actin in the 3rd instar larval CNS was analysed by co-labelling with F-actin marker Phalloidin, along with anti-Mira antibody. As shown in the previous chapter, Mira forms a basal crescent at metaphase (Figure 4.1 A), before segregating into the smaller daughter, GMC (figure 4.1 D). Careful analysis of Phalloidin staining reveals that F-actin appears to be enriched around the cortex of post-embryonic NBs (Figure 4.1 B, E). Interestingly, basal enrichment of F-actin is observed at metaphase (Figure 4.1 B, indicated by arrows). Moreover, strong phalloidin expression is observed in the cortex of budding GMC (Figure 4.1 E).

In line with the previous findings that intact actin cytoskeleton is required for asymmetric division of embryonic NBs (Broadus and Doe, 1997), the merged image demonstrate that Mira and F-actin co-localise in a basal crescent during metaphase (Figure 4.1 C) and telophase (Figure 4.1 F).

4.3 Pharmacological interference of actin functions disrupts asymmetric segregation of Mira in dividing post-embryonic NBs

To assess the requirement for an intact microfilament cytoskeleton for the basal anchoring of the cell fate determinants Brat and Pros, localisation of their adaptor protein Mira was examined in wild-type third instar larval CNS treated with a potent actin depolymerising drug, Latrunculin B (Broadus and Doe, 1997). Ten third instar larval CNS were incubated in 1ml of Latrunculin B solution (200 μ M) for 10 minutes at room temperature. A sample of randomly chosen central brain post-embryonic NBs at late anaphase/ early telophase were analysed and the number of NBs that had undergone asymmetric division was recorded. The same experimental procedure was repeated five times (See table 4.1).

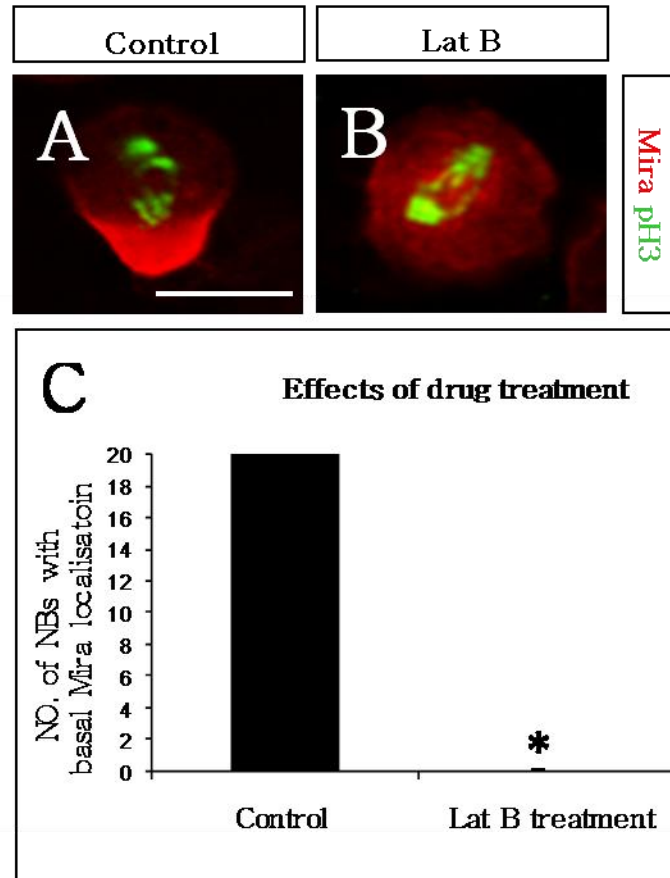


Figure 4.2 The effect of Latrunculin B treatment on asymmetric Mira localisation in telophase NBs, in vivo. Asymmetric localisation of Mira was analysed at a sub-cellular level.

Wild type post-embryonic NBs undergo asymmetric division to segregate Mira into the GMC at telophase (A), whereas in Latrunculin B treated NBs, Mira was de-localised from the cortex (B); Cell cycle stage indicated by pH3 labelling. (C) Quantification of NBs in control vs. Latrunculin treated groups undergoing asymmetric division in telophase; Images shown are in a single confocal plane. The scale bar is 10 μ m.

Analysis of Mira localisation at a sub-cellular level reveals that by late anaphase/ early telophase, as indicated by pH3 labelling, Mira segregates into the budding GMC (Figure 4.2A). Treatment with Latrunculin B, for 10 mins resulted in complete disruption of F-actin cytoskeleton, in that, no phalloidin staining was detected (no data shown). In absence of intact F-actin cytoskeleton, Mira no longer appeared to be cortically detached and was mis-localised to the cytoplasm, in telophase (Figure 4.2B). Interestingly, quantification of NBs undergoing asymmetric division in telophase demonstrates that in the control group, virtually all of the NBs had undergone asymmetric division, whereas, in the Latrunculin B treated group, none of the NBs divide asymmetrically (Figure 4.2 C and table 4.1). Nonetheless, in some NBs, cortical enrichment of Mira was observed, however, by telophase, no cytokinesis was recorded. Thereby, I conclude that the ability to anchor Mira at the basal cortex is correlated with the integrity of the F-actin cytoskeleton.

Mira localisation											
1		2		3		4		5		Σ	
Asymmetric	De-localised	Asymmetric	De-localised	Asymmetric	De-localised	Asymmetric	De-localised	Asymmetric	De-localised		
Control	20	0	20	0	20	0	20	0	20	0	100
Latrunculin	0	20	0	20	0	20	0	20	0	20	100

Table 4.1 Number of post-embryonic NBs undergoing asymmetric division in control vs. Latrunculin B treated group. Total of 100 post-embryonic NBs were analysed for Mira localisation at late anaphase/ early telophase for each treatment groups, over five sets of experiments. In the control group, all of the NBs undergo asymmetric division, whereas, depolymerisation of F-actin resulted in cortical de-localisation of Mira, leading to failure in segregation of Mira into the GMC. Since the difference between the control group against the Latrunculin B treated group was complete, no statistical test was conducted.

4.4 Pharmacological interference of actin function does not lead to an over-proliferation phenotype.

As demonstrated in the previous chapter, disruption in asymmetric division of post-embryonic NBs in the third instar larval CNS leads to over-proliferation, thereby, increasing the number of central brain NBs, as well as displaying a notable over-proliferation phenotype in the type II NBs. As the drug induced disruption in F-actin cytoskeleton leads to failure in asymmetric segregation of Mira into the GMC, it may be feasible to speculate that a similar phenotype may occur.

To assess whether the disruption in F-actin cytoskeleton in dividing post-embryonic NBs leads to an over-proliferation phenotype in the central brain, quantification of the number of Mira positive post-embryonic central brain NBs were performed in Latrunculin treated larval CNS (Figure 4.3 B, D), and was compared against the control (Figure 4.3 A, C), in 20 randomly chosen brains for each treatment groups that was used in the previous experiments (Figure 4.2). In wild type central brains, there were 82.1 ± 1.9 NBs at 72h ALH (Figure 4.3 A), and slightly increased to ALH 84.3 ± 2.1 by 96h (Figure 4.3 C). Interestingly, there was no significant increase in the number of central brain NBs in Latrunculin B treated brains (Figure 4.4) which amounted to 85.5 ± 1.5 by 72h ALH (Figure 4.3 C), and 86.1 ± 1.6 at 96h ALH (Figure 4.3 D) (see table 4.2). Moreover, analysing the posterior brain revealed that there was no notable increase in intermediate neural progenitor cells in type II NB lineages in the Latrunculin treated larval CNS (Figure 4.3 B', D'), compared to that of the control (Figure 4.3 A', C').

Taken together, my data on in vivo localisation of F-actin during post-embryonic NBs cytokinesis and the drug inhibition study suggest that intact actin cytoskeleton is required for proper asymmetric division of post-embryonic NBs. It is interesting to note that the 10 minute incubation of third instar larval CNS in Latrunculin B was sufficient to affect asymmetric segregation of Mira in all of the post-embryonic NBs that was analysed. However, the drug induced de-polymerisation of F-actin did not lead to a significant increase in the number of Mira positive post-embryonic central brain NBs in third instar larval CNS.

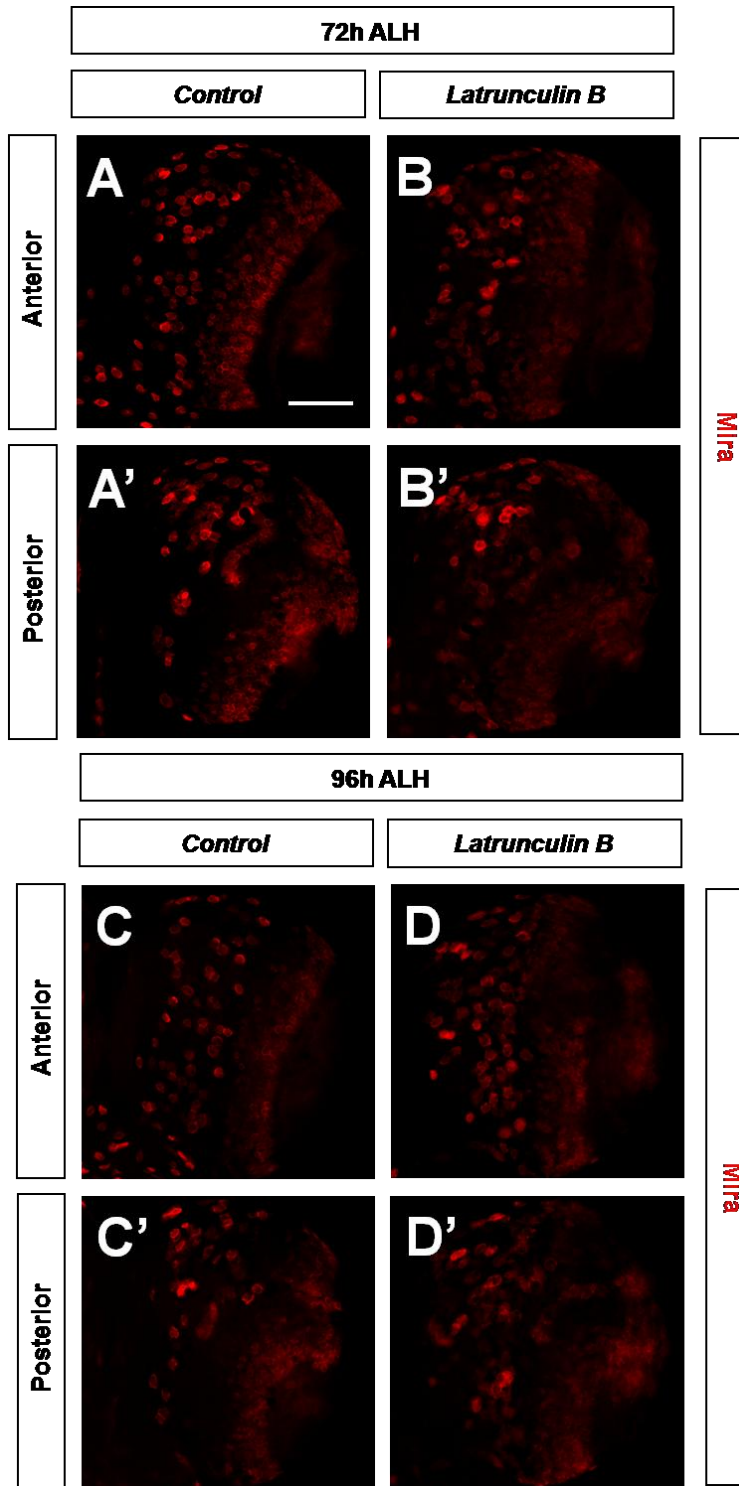


Figure 4.3 Pharmacological interference of actin does not lead to over-proliferation of central brain post-embryonic NBs. Central brain post-embryonic NBs were analysed in confocal Z-stacks of whole mount wild-type (A and C) and Latrunculin B treated (B and D)

larval CNS; Post-embryonic NBs were immunolabelled with Mira. Moreover, posterior view of the larval brains were analysed to assess for tumour phenotypes in type II NBs (A'-D'). All images shown are in a single confocal plane. The scale bar is 50µm.

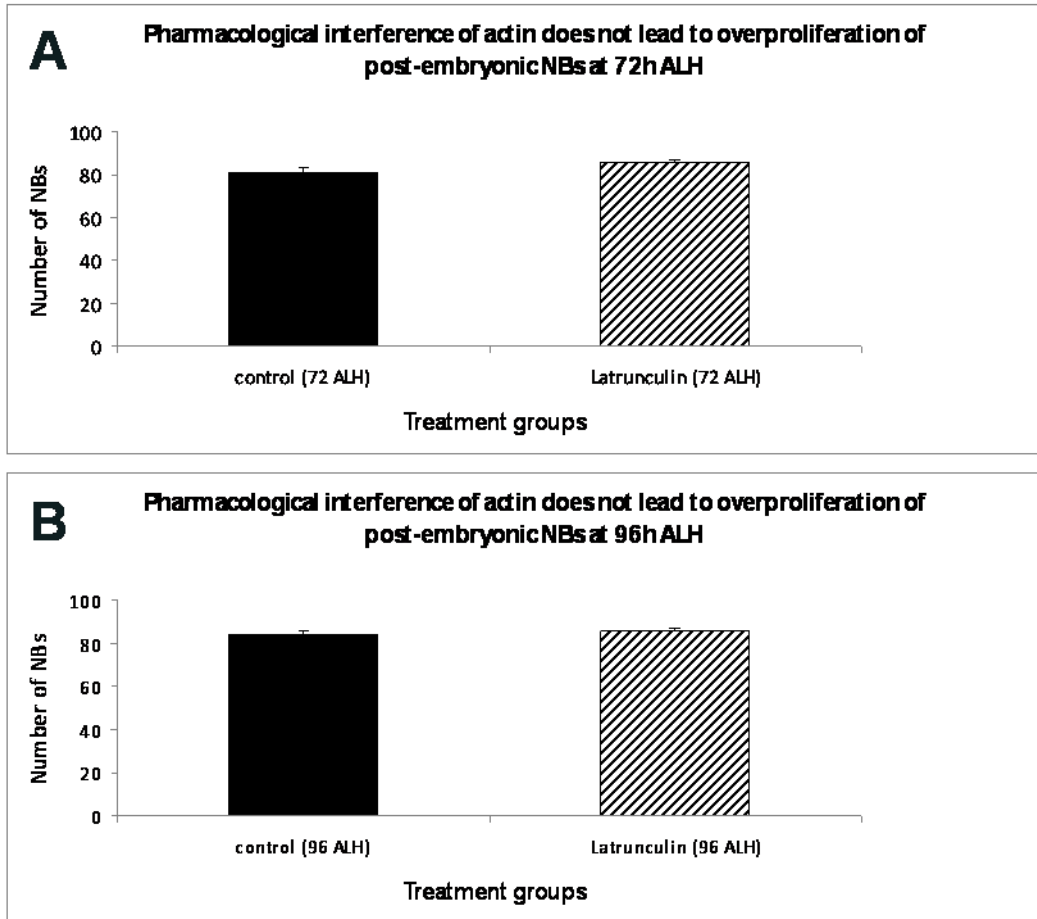


Figure 4.4 Pharmacological interference of actin does not result in a significant increase in the number of central brain post-embryonic NBs. Quantification of central brain NBs reveal that pharmacological interference of actin does not significantly increase the number of central brain NBs at 72h ALH (A) or at 96h ALH (B).

72h ALH				
Treatment Groups	N	Mean	SEM	p Value
Control	20	82.1	1.9	1
Latrunculin B	20	85.5	1.5	0.15

96h ALH				
Genotype	N	Mean	SEM	p Value
Control	20	84.3	2.1	1
Latrunculin B	20	86.1	1.6	0.66

Table 4.2 Mean numbers of Mira positive central brain post-embryonic NBs in Control vs. Latrunculin B treated group. The number of post-embryonic NBs were quantified in 20 larval central brain for each treatment groups at 72h ALH (A) and 96h ALH (B). A one-way ANOVA was used to compare the control group against Latrunculin treated groups. Turkey HSD post-hoc test was applied for multiple comparisons against the control group, with overall significance level of $p < 0.05$.

Chapter 5: Identifying the candidate myosin motor responsible for basal targeting of cell fate determinants during asymmetric division of post-embryonic NBs

5.1 Introduction

Establishment of apical-basal polarity and alignment of the mitotic spindle along the apical-basal axis enables exclusive segregation of cell fate determinants such as Brat and Pros to the basal cortex. Pros and Brat are not able to directly associate with the cortex, but require the adaptor protein Mira (Ikeshima-Kataoka et al., 1997; Shen et al., 1997). Mira sequesters these factors to the basal cortex until cytokinesis is complete, whereupon it is degraded, subsequently releasing Pros and Brat (Fuerstenberg et al., 1998). These observations led to a model in which key differentiation factors such as Brat and Pros are recruited to the basal cortex in an inactive form as cargo proteins of Mira (Lee et al., 2006b). The fundamental question that remains to be determined is the mechanisms involved in basal cortical targeting of Mira.

What are the proposed basal targeting mechanisms that lead to recruitment of Pros and Brat to the basal cortex? The current proposed model implicates Myosin VI to be responsible for transporting Mira to the basal cortex (Petritsch et al., 2003), as Myosin VI accumulates in the basal region of the cell and interacts directly with Mira. Moreover, Erben et al. (2008) proposed a rather complex model involving the tumour suppressor Lethal giant larvae (Lgl) and non-muscle myosin II (Zipper) for aPKC-mediated displacement of Mira and its cargo proteins from

the apical cortex into the cytoplasm. Subsequent binding of Myosin VI to Mira is thought to transport Mira to the basal cortex along the actin filaments. Although, Myosin VI is likely to be involved in transporting Mira to the cortex, the physical association mechanisms that anchors Mira to the basal cortex is currently unknown.

Another Myosin motor candidate that is capable of cargo transport is Myosin V. It has been proposed that Myosin V mediates a short-range actin-myosin V dependent transport or entrapment of oskar mRNA at the posterior cortex in *Drosophila* oocytes (Krauss et al., 2009), a vital process for embryonic development (Ephrussi and Lehmann., 1992).

Interestingly, posterior localisation of oskar mRNA and Staufen is impaired with reduced Myosin V activity (Krauss et al., 2009). Staufen is an RNA binding protein that co-localises with oskar mRNA in oocytes and is also a cargo protein of Mira during asymmetric division of NBs (Slack et al., 2007).

Although the function of Myosin V in NBs remain unclear, its involvement with posterior localisation of oskar mRNA in oocytes displaying its ability to transport cargo, coupled with its association with Staufen, promotes it as a potential candidate myosin motor responsible for basal targeting of Mira in dividing post-embryonic NBs.

As previous studies implicate Myosin II, Myosin V and Myosin VI to have a role in basal localisation of the cell fate determinants during asymmetric division of NBs, in the embryo (Barros et al., 2003; Petritsch et al., 2003; Erben et al., 2003), I hypothesise that actin-based

myosin motor is required for asymmetric division of NBs, and that Mira is basally transported as cargo protein of the proposed myosin motor along actin filaments during asymmetric division of post-embryonic NBs. Thus, in this Chapter, I aim to determine the requirement of myosins in basal localisation of the cell fate determinants and to characterise the functions of the candidate myosin motors in asymmetric division of post-embryonic central brain NBs.

5.2 Pharmacological interference of myosin functions disrupts asymmetric segregation of Mira in dividing post-embryonic NBs

As a way of directly testing for the involvement of myosin motors in asymmetric division of NBs, I examined drug induced disruption of myosin function, by applying 2, 3-Butanedione monoxime (BDM), a well established ATPase inhibitor of myosin super-family in late third instar larval brains (Ohshiro et al., 2000).

To assess the role of myosin motors in basal targeting/ anchoring of the cell fate determinants Brat and Pros, localisation of their adaptor protein Mira in BDM treated brains were analysed and evaluated against the control group. Ten wild type 3rd instar larvae were incubated in 1ml of BDM solution (200mM) at room temperature prior to dissection. Subsequently the BDM treated brains were co-labelled with anti-Mira antibody and anti-phosphohistone H3 (pH3, used as a DNA marker). 20 randomly chosen NBs were analysed for Mira localisation at telophase, and the number of NBs undergoing undisrupted asymmetric division was recorded in control as well as BDM treated groups (see table 5.1), the same experimental procedure was repeated 5 times.

Consistent with my previous observations, undisrupted asymmetric division of Mira occurs in the control group. Mira is exclusively inherited by the GMC in telophase in 100% of the NBs (Figure 5.1 A and C). Whereas, perturbation of Myosin motor activity resulted in disruption of asymmetric division of Mira (Figure 5.1 B), where Mira is no longer localised to the basal cortex, and is mis-localised to the cytoplasm instead.

Quantification of the NBs undergoing asymmetric division reveal that in the control group, 100% of the NBs undergo asymmetric division, whereas, disruption of NBs occur in majority of the NBs in BDM treated group, only a small amount of NBs (26%) undergo asymmetric division, which is a significant decrease compared to that of the control group (Figure 5.1 C) ($P < 0.05$).

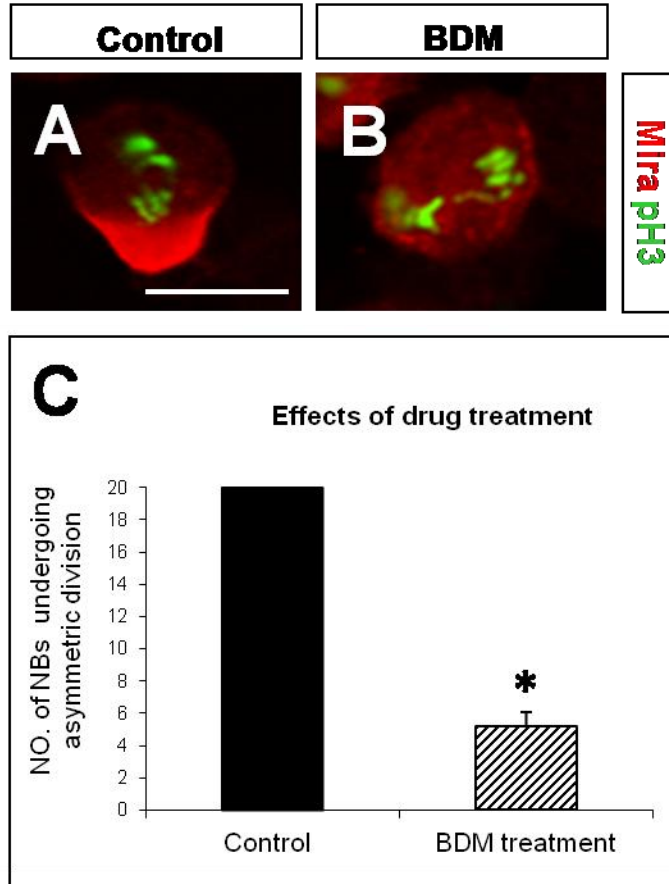


Figure 5.1 BDM treatment disrupts asymmetric division of NBs at third instar larval central brain. Wild type post-embryonic NBs undergo asymmetric division to segregate Mira into the GMC at telophase (A), whereas in BDM treated NBs, Mira was de-localised from the cortex (B); Cell cycle stage indicated by pH3 labelling. (C) Quantification of NBs in control vs BDM treated groups undergoing asymmetric division in telophase. Error bars are standard errors of the mean; Images shown are in a single confocal plane. The scale bar is 10 μ m.

Mira localisation											
1		2		3		4		5			
	Asymmetric	De-localised	Asymmetric	De-localised	Asymmetric	De-localised	Asymmetric	De-localised	Asymmetric	De-localised	Σ
Control	20	0	20	0	20	0	20	0	20	0	100
BDM	5	15	6	14	2	18	7	13	6	14	100

Table 5.1 Number of post-embryonic NBs undergoing asymmetric division in control vs. BDM treated group. In total, 100 randomly chosen central brain NBs were analysed in post-embryonic larval brain, over five sets of experiments. In the control group, all of the NBs undergo asymmetric division, with Mira forming a basal crescent at metaphase, whereas, in the BDM treated group, cortical de-localisation of Mira was observed in majority of the NBs, indicating perturbed asymmetric division of NBs. A student t-test was used to compare the control group against the drug treated group, with overall significance level of $P < 0.05$.

5.3 Validating the specificity of RNAi mediated knockdown experiments using BLAST search

In the following experiments I used transgenic *Drosophila* strains, each containing an inducible UAS-RNAi construct against a single protein coding gene. Off target effects are of great concern in targeting a single gene to demonstrate that a particular effect is due to knockdown of the targeted protein. Thus, I conducted BLAST searches available on Flybase (www.Flybase.net) to ensure the target sequences of Myosin II, Myosin VI and Myosin V transgenic RNAi strains did not have matches to other genes of the same species of 16 or more nucleotides.

Analysis of BLAST search results display that target sequence of Myosin II/Zipper RNAi includes all 9 of its isoforms (Figure 5.2A). Similarly, the target sequence of Myosin VI/Jaguar RNAi covers all 5 of its isoforms (Figure 5.2B), as does the sequence of Myosin V/Didum RNAi (Figure 5.2C). Moreover, no off target genes were identified in any of the target sequences. Thus, in the set of knock-down experiments shown below, any phenotypic change that occurs is unlikely to be due to off target effects.

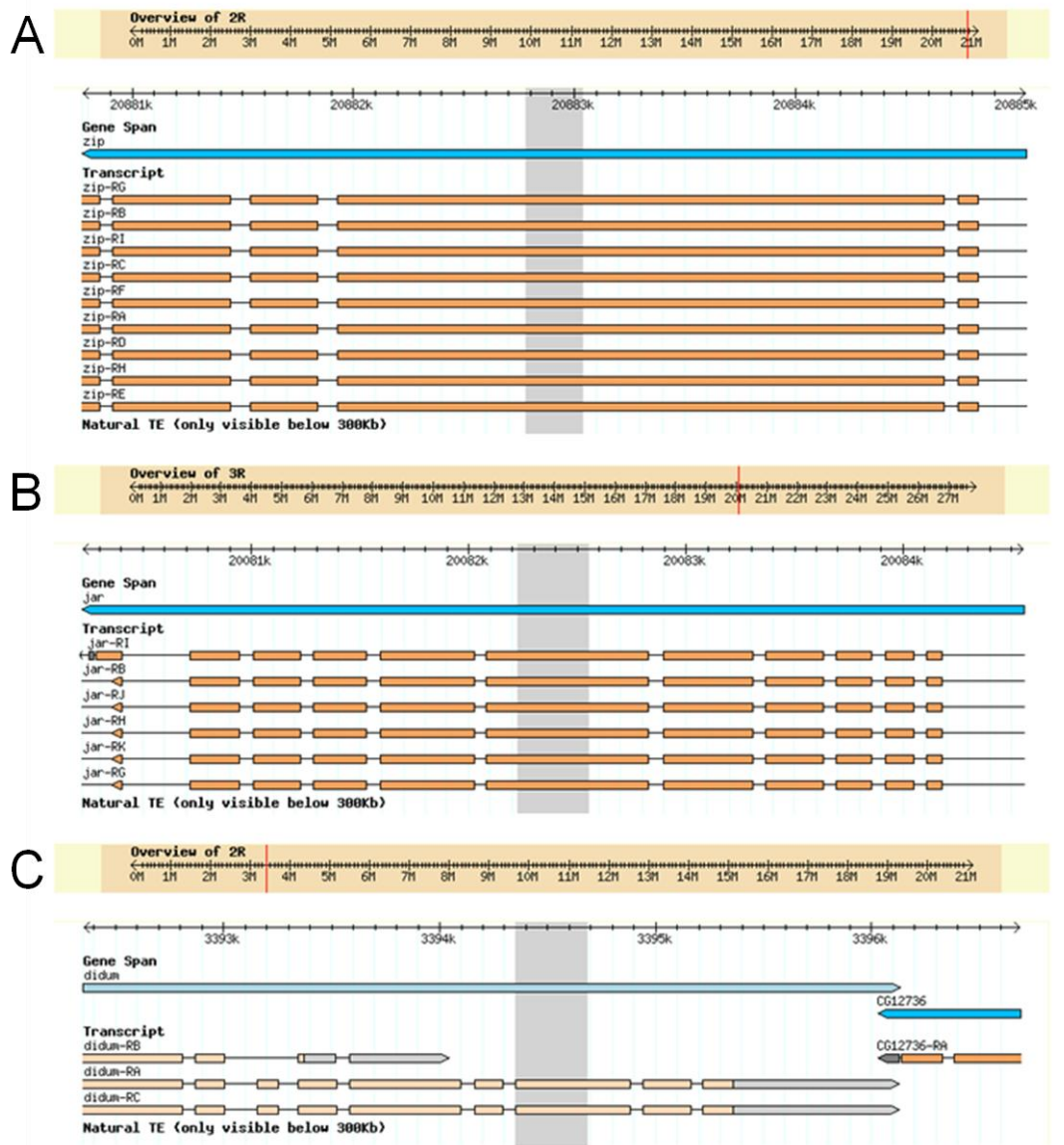


Figure 5.2 Validations of target specificity of Myosin II, Myosin VI and Myosin V RNAi constructs using BLAST search. Gene span shows the total extent of the transcribed region of the annotated genes, non-muscle Myosin II (Zipper) (A), Myosin VI (Jaguar) (B), Myosin V (Didum) (C). The gene structures shown here correspond to respective gene isoforms contained within the target sequence: exon is displayed by the wider bar and intron in black line.

5.4 Genetic knockdown of Myosin II leads to partial cellular over-growth of post-embryonic NBs due to endomitosis

Class II myosins are barbed end-directed motors that form bipolar filaments. The filaments bind actin and initiate contraction when the two ends of the bipolar filament pull in opposite directions. The mode of action of Myosin II suggests that it is unlikely to transport cargo from one side of the cell to other, although passive cargo transport along the cortex by progressive contraction maybe a possible (Barros et al., 2003).

It is proposed that Non-muscle Myosin II (Zipper) regulates the basal targeting of cell fate determinants in dividing NBs, not by direct transport but by cortical exclusion. During prophase and metaphase, Myosin II forms an apical crescent, where Myosin II negatively interacts with Lethal giant larvae (Lgl). Inactivation of Lgl leads to phosphorylation of aPKC, which excludes Mira from the apical cortex into the cytoplasm (Barros et al., 2003).

Cytoplasmic localisation of Mira at early metaphase enables direct interaction with Myosin VI and subsequent basal targeting to the cortex, completing the asymmetric division; allowing exclusive segregation of cell fate determinants. Thus, Myosin II may act upstream of Myosin VI in the same pathway to localise Mira (Erben et al., 2008).

If Myosin II plays a role in asymmetric division of cell fate determinants by excluding Mira from the apical cortex, which occurs upstream of Myosin VI led basal targeting of Mira, reduced activity of Myosin II by RNA mediated knockdown would hinder proper exclusion of Mira from

the apical cortex. Mira would fail to interact with Brat and Pros in the cytoplasm, leading to improper basal segregation of the cell fate determinants into the GMC, which in turn results in over-proliferation of the post-embryonic NBs at the expense of neurons.

Thus, in order to characterise the function of Myosin II in asymmetric division of the central brain NBs of late instar larvae, I used RNAi in combination with Gal4/UAS system (Brand and Perrimon, 1993) using pre-existing *Drosophila* strain to genetically knock down Zipper (the heavy chain of non-muscle myosin II) with 1407 Gal4 driver to reduce Myosin II activity specifically within the embryonic and post-embryonic NB lineages.

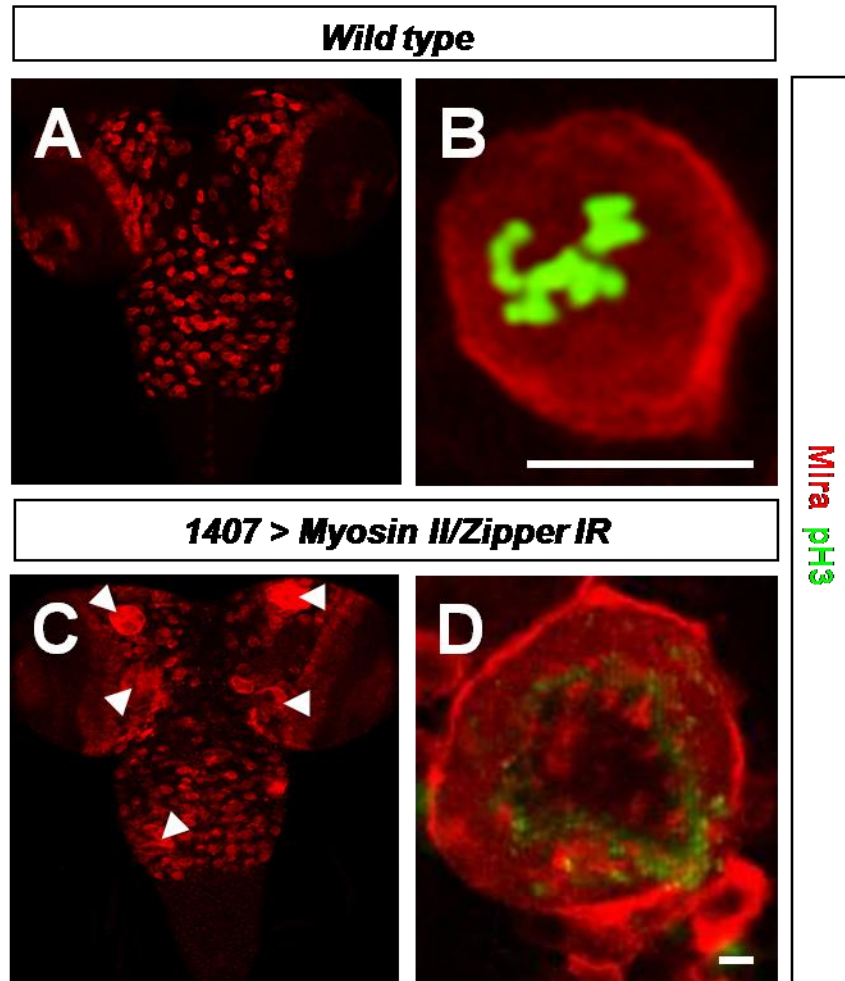


Figure 5.3 Genetic knockdown of Myosin II affects asymmetric Mira segregation during NB division in larval central brain. A whole mount 3rd instar larval CNS (A) was immunolabelled with anti-Mira and anti-phosphohistone H3. (A) Wild-type larval CNS displaying NB localisation in central brain and ventral nerve chord. 1407 Gal4 driven RNAi mediated knockdown of Myosin II leads to partial cellular over-growth in the central brain and ventral nerve chord (C, Arrow heads). A close up view of the central brain NB in wild type (B) and the enlarged NB in 1407 > Zipper IR brain (D) reveal that besides the vast difference in cell size, multiple proliferative activity is shown, indicative of multiple proliferative activity, possibly due to endomitosis; The images shown are in a single confocal plane. The scale bar is 10µm.

All of the Mira positive post-embryonic NBs that can be visualised in the wild type CNS (1407 Gal4 > +) are approximately the same size (Figure 5.3 A). However, knockdown of Zipper (1407 Gal4> UAS Zipper IR) leads to partial cellular over-growth in some NBs in the central brain, as well as in the VNC (Figure 5.3 C, Arrowheads), indicating that Myosin II function is not limited to type I and type II post-embryonic NBs, but also plays a role in NB cell division in the VNC.

The partial cellular over-growth of post-embryonic NBs was observed in all of the Myosin II knockdown brains. However, there was no significant increase in the number of central brain NBs in any of the Myosin II knockdown brains (no data shown) , indicating that reduced Myosin II function in the larval central brain did not result in over-proliferation of post-embryonic NBs.

Interestingly, a pupal lethal phenotype was observed in 1407 Gal4> UAS Myosin II/Zipper IR flies, with arrested pupae dying as pharate adults. This, coupled with the expression of early mitotic marker pH3 suggests that the most plausible cause for ectopic chromosomal materials observed in the enlarged cell may be due to endomitosis, a phenomenon in which the cell undergoes aspects of mitosis, i.e. DNA replication, without cytokinesis. Duplicated chromosomes generated by endomitosis exist as discrete units in a single polyploid nucleus or maybe packaged into separate nuclei.

Other than the obvious vast difference in the cell size, a closer inspection of the enlarged cell in the Zipper RNAi knockdown central brain reveals large clusters of ectopic pH3 labelled punctae (Figure 5.3 D), which may be indicative of multiple proliferative activity within the NB cell, as opposed to that of the control (Figure 5.3 B). Moreover, increasing DNA content by endomitosis

often correlates with increased cell size, which may explain the enlarged NBs observed in Myosin II knockdown brains.

Furthermore, Myosin II constitute an integral part of the acto-myosin contractile ring, required for the generation of force for cytokinesis in eukaryotic cells (Barr and Gruneberg, 2007; Pollard, 2010). Thus, it may be possible to speculate that genetic knockdown of Myosin II disrupted the structure of the acto-myosin ring, leading to cytokinesis failure in some of the NBs in the Myosin II knockdown brains.

5.5 Establishing the role of the candidate myosin motors in asymmetric division of post-embryonic NBs in *Drosophila*

5.5.1 Identifying the candidate myosin motor responsible for basal targeting of Mira

There are two candidate myosin motors that I have identified to be associated with basal targeting/anchoring of Mira. The first candidate is Myosin VI, the only class of myosin that moves towards the minus end of actin filaments that can also function as an actin based anchor (Sweeney and Houdusse, 2007). Myosin VI protein is abundantly expressed in NBs, where it transiently accumulates in the basal half of the metaphase NBs and partially co-localises with Mira (Petritsch et al., 2003). Myosin VI forms a complex with Mira and Pros in *Drosophila* embryonic extracts and shows direct physical interaction with Mira, in vitro (Petritsch et al., 2003). Moreover, reduction of Myosin VI activity in embryos led to mis-localisation of Mira to the cytoplasm, while failing to form a basal crescent (Petritsch et al., 2003). These observations suggested that Mira might be transported from the apical to basal cortex as Myosin VI cargo (Erben et al., 2008).

Moreover, previous findings implicating a role for Myosin V in basal targeting/anchoring of *oskar* mRNA in *Drosophila* oocytes (Krauss et al., 2009), along with the presence of cargo binding domain has rendered Myosin V as another possible myosin motor capable of conducting direct basal transport of Mira during asymmetric division of post-embryonic NBs.

Thus, in this chapter, I aim to characterise the function of the candidate myosin motors Myosin V and Myosin VI during asymmetric division of post-embryonic NBs, in order to determine the candidate myosin motor responsible for basal targeting of Mira. Initially, Myosin V and Myosin VI knockdown brains were analysed for NB over-proliferation phenotype in the central brain. However, RNAi-mediated genetic knockdown of Myosin V or Myosin VI did not lead to a significant increase in the number of post-embryonic central brain NBs, thus, basal targeting of Mira did not appear to be affected by RNAi mediated single knockdown of the candidate Myosin motors.

Interestingly, absence of either Myosin V or Myosin VI led to defects in mitotic spindle alignments along the apical-basal polarity axis at anaphase (Figure 5.6), suggesting that both Myosin V and Myosin VI may have a role in regulating mitotic spindle positioning during asymmetric division of NBs. Furthermore, double knockdown of Myosin V and Myosin VI seemed to exacerbate the spindle defects (Figure 5.7), implying that both Myosin motors may act sequentially in the common pathway or in parallel pathways at distinct steps to basally localise Mira in an indirect way, by aligning the mitotic spindle along the apical-basal polarity axis during asymmetric division of post-embryonic central brain NBs.

5.5.2 RNAi mediated knockdown of Myosin V or Myosin VI does not lead to over-proliferation of central brain post-embryonic NBS

As demonstrated in the previous chapter, disruption in basal targeting of the cell fate determinants Brat and Pros as well as their adaptor protein Mira leads to over-proliferation of post-embryonic NBs in central brain. Therefore, it is feasible to speculate that reduced activity of the candidate myosin motor responsible for basal targeting/anchoring of Mira would cause over-proliferation of NBs.

In order to determine the candidate myosin motor required for basal targeting of Mira in asymmetric division, I conducted RNAi mediated knockdown of Myosin VI and Myosin V using 1407 Gal4 driver in 3rd instar larvae to screen for changes in the number of central brain NBs compared to the wild type. I co-expressed 1407 Gal4 driver with Dicer 2 (Dcr2) to improve the knockdown efficiency (Dietzl et al., 2007).

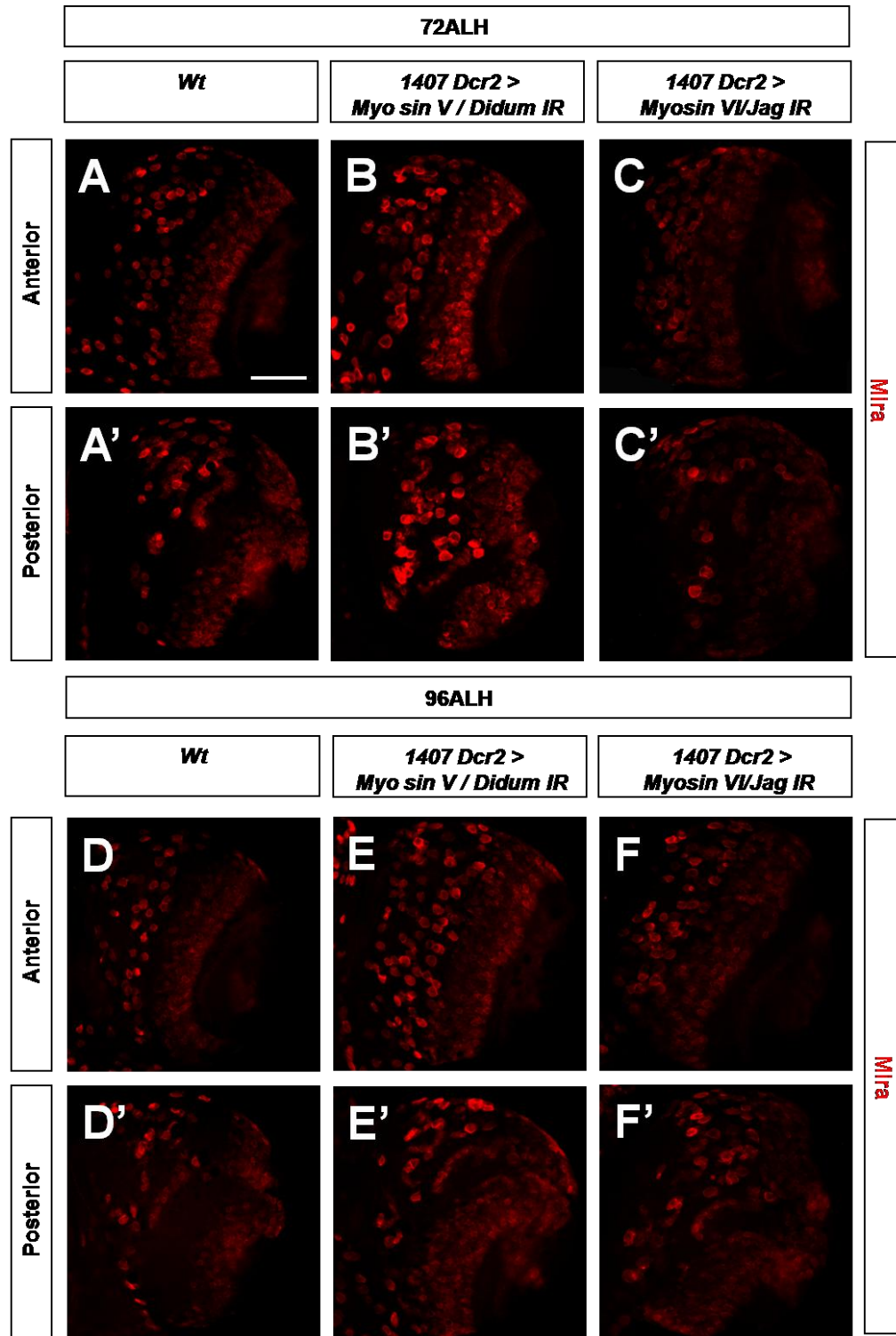


Figure 5.4 RNAi mediated knockdown of Myosin V or Myosin VI does not lead to overproliferation of central brain NBs. The number of NBs were manually counted through confocal Z-stacks of whole mount larval central brains from wild type, 1407 Dcr2 > Myosin

V/Didum IR, 1407 Dcr2 > Myosin VI/Jaguar IR flies dissected at mid (at 72h ALH) to late larval stages (96h ALH). At mid larval stages, 1407 Dcr2 > Myosin V/Didum IR (B, B') and 1407 Dcr2 > Myosin VI/Jaguar IR (C, C') larval brains were evaluated against the wild type (A, A'), but over-proliferation phenotype was not observed. Similarly, at late larval stages, there were no obvious over-proliferation phenotypes displayed in 1407 Dcr2 > Myosin V/Didum IR (E, E') and 1407 Dcr2 > Myosin VI/Jaguar IR (F, F') brains, compared to the wild type (C, C'); The images shown are in a single confocal plane. The scale bar is 50µm.

From mid (72h ALH) to late (96h ALH) larval stages, the number of Mira positive NBs were counted in 20 randomly chosen brains for each group. At 72h ALH, the mean number of type I central brain NBs in the Myosin V knockdown group (87.1 ± 1.6 ; Figure 5.4 B) and Myosin VI knockdown group (88.1 ± 2.4 ; Figure 5.4C) were evaluated against the wild-type group (85.9 ± 1.7 ; Figure 5.4 A), but did not show over-proliferation defect (Figure 5.5 A). Similarly, there was no significant difference in the number of type I central brain NBs at 96h ALH (Figure 5.5 B), between the wild type group (83.5 ± 1.4 ; Figure 5.4 D) and the Myosin V knockdown group (85.8 ± 1.7 ; Figure 5.4 E) or the Myosin VI knockdown group (88.5 ± 2.4 ; Figure 5.4F). Moreover, examination of type II NBs did not reveal any over-proliferation phenotype either (Figure 5.4 A'-F'). Thus, the proposed candidate myosin motors Myosin V or Myosin VI do not appear to directly regulate basal targeting of Mira during asymmetric division of post-embryonic central brain.

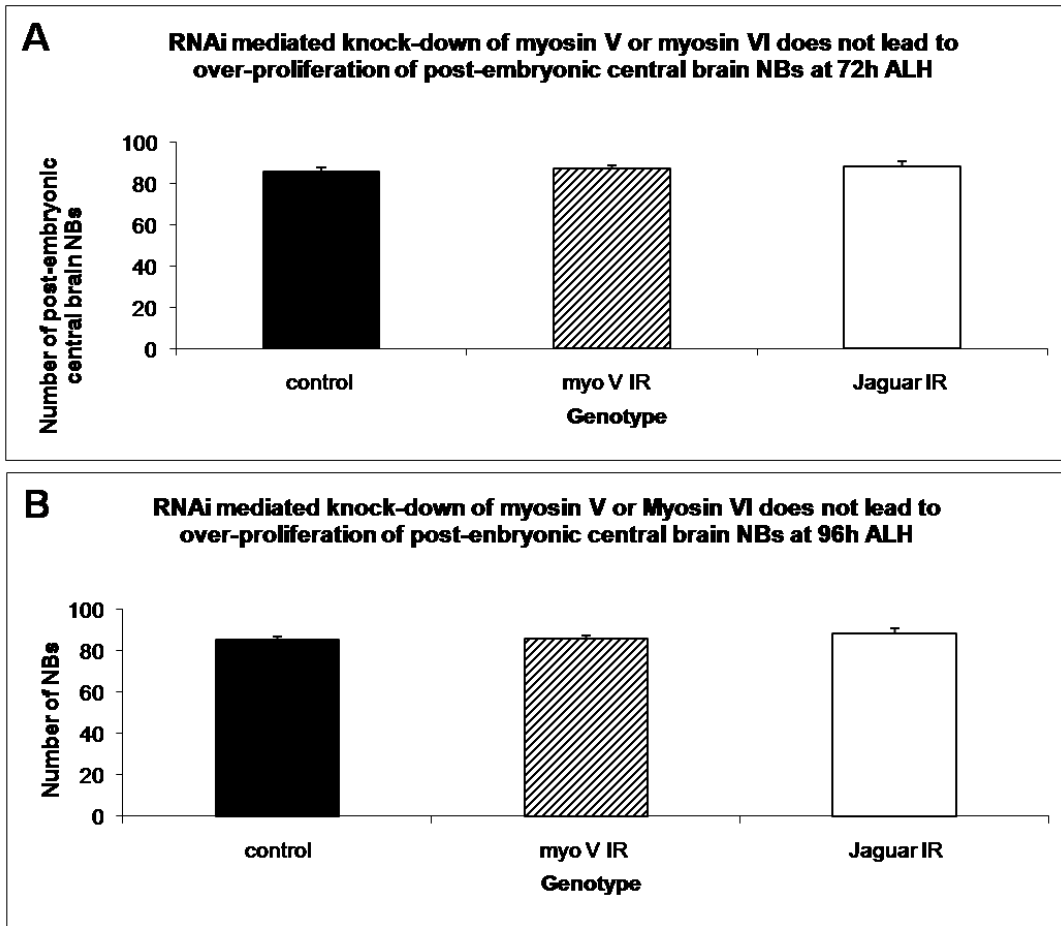


Figure 5.5 RNAi mediated knockdown of Myosin V or Myosin VI do not significantly increase the number of post-embryonic central brain NBs. Quantification of the central brain NBs was conducted at mid (72h ALH) and late (96h ALH) larval stages. There was no significant increase in the mean number of Mira positive central brain NBs at (n=20) 72h ALH (A) nor at 96h ALH (B). Error bars are standard errors of the mean.

A	72h ALH				
	Genotype	N	Mean	SEM	p Value
	Wt	20	85.9	1.7	1
	1407 Dcr2>Myosin V/ Didum IR	20	87.1	1.6	0.21
	1407 Dcr2>Myosin VI/Jaguar IR	20	88.1	2.4	0.09

B	96h ALH				
	Genotype	N	Mean	SEM	p Value
	Wt	20	83.5	1.4	1
	1407 Dcr2>Myosin V/Didum IR	20	85.8	1.7	0.89
	1407 Dcr2>Myosin VI/Jaguar IR	20	88.5	2.4	0.62

Table 5.2 Mean number of Mira positive post-embryonic NBs in wild type vs. Myosin V IR brain or Myosin VI IR brain. The number of post-embryonic NBs were quantified in 20 larval central brain for each treatment groups at 72h ALH (A) and 96h ALH (B). A one-way ANOVA was used to compare the Myosin V or Myosin VI knockdown group against the wild type group. Turkey HSD post-hoc test was applied for multiple comparisons against the wild type group, with overall significance level of $p < 0.05$.

5.5.3 Genetic knockdown of Myosin V or Myosin VI leads to mitotic spindle mis-orientation

Orientation of the mitotic spindle along a pre-defined apical-basal polarity axis is essential for the reliable partitioning of cell fate determinants during asymmetric cell division (Bowman et al., 2006). Interestingly, previous study showed that the mitotic spindle is mis-oriented in embryos lacking fully functional Myosin VI (Petritsch et al., 2003). Moreover, Myosin V is also shown to regulate mitotic spindle orientation in yeast (Yin et al., 2000). Thus, it may be possible that Myosin V and Myosin VI contribute to basal localisation of cell fate determinants during asymmetric division by regulating mitotic spindle orientation.

To test for a potential indirect function of Myosin V and Myosin VI in Mira basal localisation during asymmetric division of post-embryonic NBs, I conducted RNAi mediated knockdown of Myosin V and Myosin VI in late larval stages. The brains were co-immunolabelled with anti-Mira to identify anaphase NBs and anti-pH3 to mark the chromosomes as a mitotic reference point, as well as to indicate mitotic spindle orientation. Only NBs with clear mitotic chromosome segregation were counted.

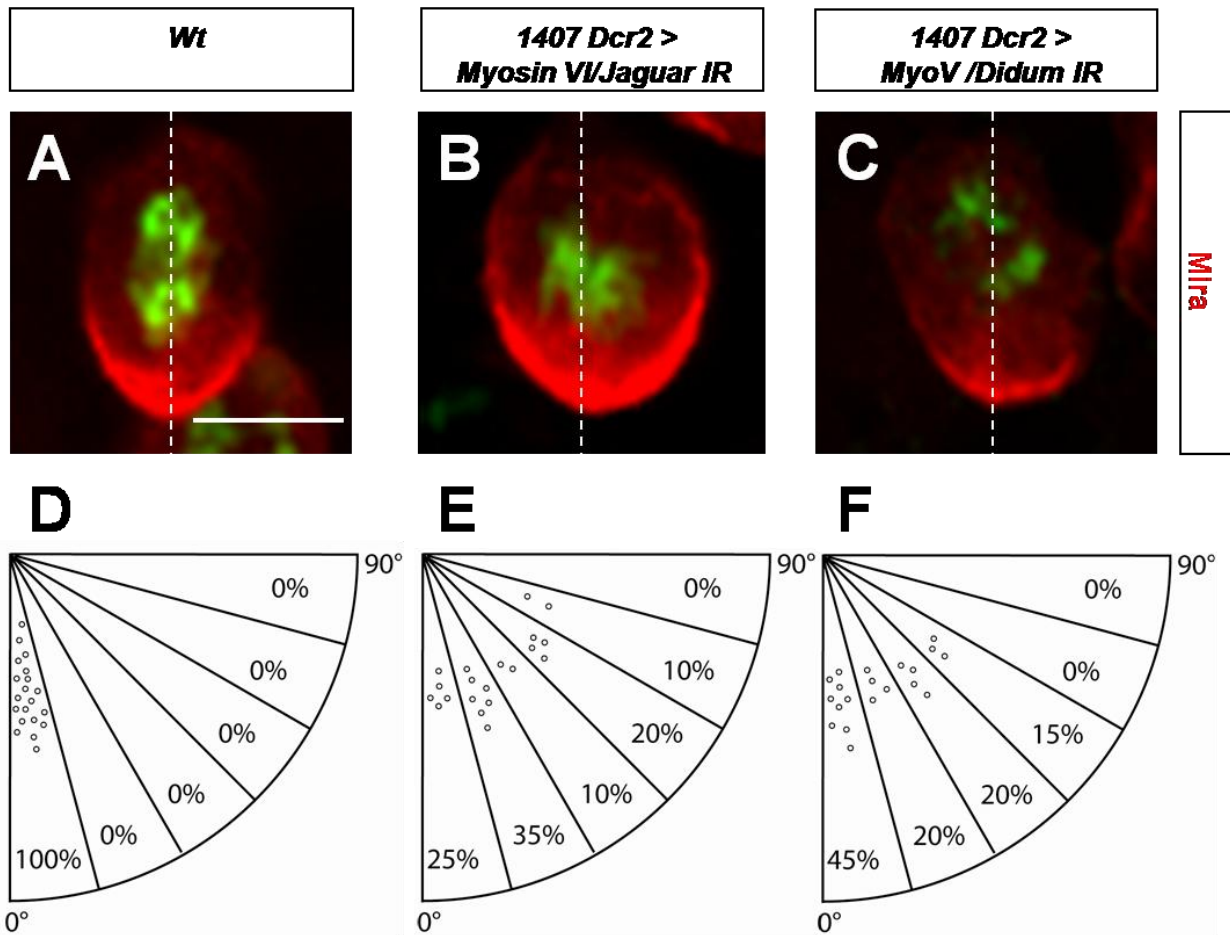


Figure 5.6 RNAi mediated knockdown of Myosin V or Myosin VI leads to mitotic spindle defect. Sub-cellular analysis of Mira localisation at anaphase reveal that in wild type central brain NBs (A) chromosomal segregation occurs along the mitotic spindle aligned with the apical basal polarity at anaphase. In both 1407 Dcr2 > Jaguar IR 1407 (B) and 1407 Dcr2 > Myosin V IR (C) central brain NBs, spindle is mis-aligned relative to the basal Mira crescent. Quantification of mitotic spindle orientation (D-F; n=20). Spindle angles were measured relative to the Mira basal crescent, i.e. angles away from the dotted line. All images are single Confocal sections, with anterior on top and lateral to the right. The scale bar is 10 μ m.

In wild-type anaphase NBs, the mitotic spindle is aligned within 15° of the centre of the apical/basal cortical polarity axis (100% of NBs, n = 20; Figure 5.6 A, D). However, in NBs of 1407 Dcr2>Myosin VI IR, mitotic spindles fail to align with the apical-basal polarity axis, with the majority of spindles showing more oblique orientations (75%, n = 20; Figure 5.6 B, E). Only a minority of spindles have measured in angles less than 15° . Similarly, in NBs of 1407 Dcr2>Myosin V IR, mitotic spindles also show oblique angles over 15° (55% of NBs, n=20; Figure 5.6 C, F). It appears that NBs polarise correctly, but in absence of either Myosin V or Myosin VI the polarised cortical domains cannot direct the orientation of the mitotic spindle. In line with these observations, both Myosin V and Myosin VI may have a role in regulating mitotic spindle positioning during asymmetric division of post-embryonic NBs

5.5.4 Double Knockdown of Myosin V and Myosin VI leads to increased mitotic spindle defect.

Observed defects in mitotic spindle alignment along the apical-basal polarity axis in absence of either Myosin V or Myosin VI suggests that both Myosin motors may act either sequentially in the common pathway or in parallel pathways at distinct steps to basally localise Mira in an indirect way, by aligning the spindle formation.

Thus, in order to characterise the function of the two myosin motors in a common pathway, RNAi-mediated double knockdown of Myosin V and Myosin VI was conducted, driven by 1407 Gal4 driver, co-expressing Dcr2.

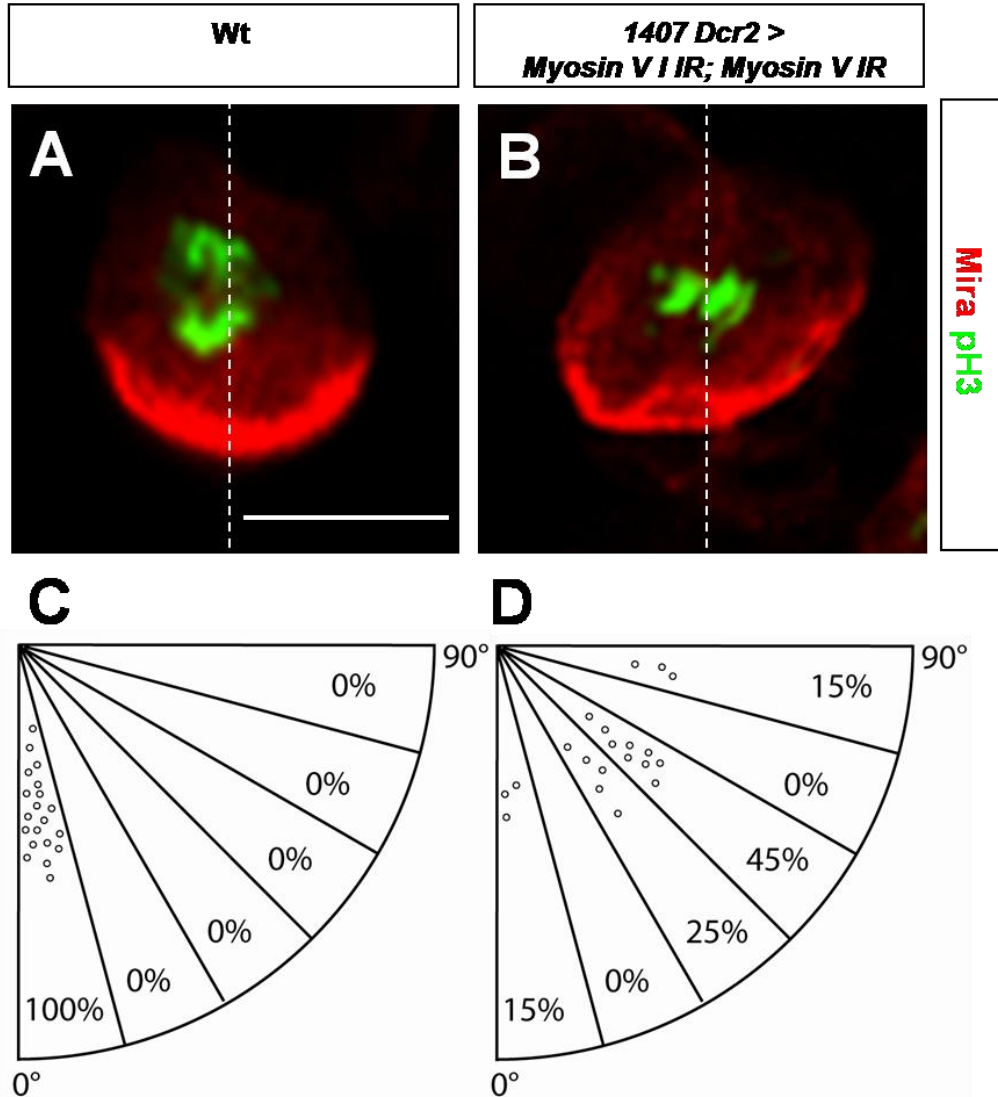


Figure 5.7 RNAi mediated double knockdown of Myosin V and Myosin VI leads to increased mitotic spindle defect. Analysis of anaphase NBs in late larval central brains reveal that mitotic spindle orientation aligns with the apical-basal polarity in wild type (A). In the 1407 Dcr2 > Jaguar IR; Myosin V IR brains (B), severe mitotic spindle defect is observed in NBs. Quantification of spindle orientation (C, D; n=20). Spindle angles were measured relative to apical-basal axis, based on Mira basal crescent. All images are single Confocal sections, with anterior on top and lateral to the right. The scale bar is 10 μ m.

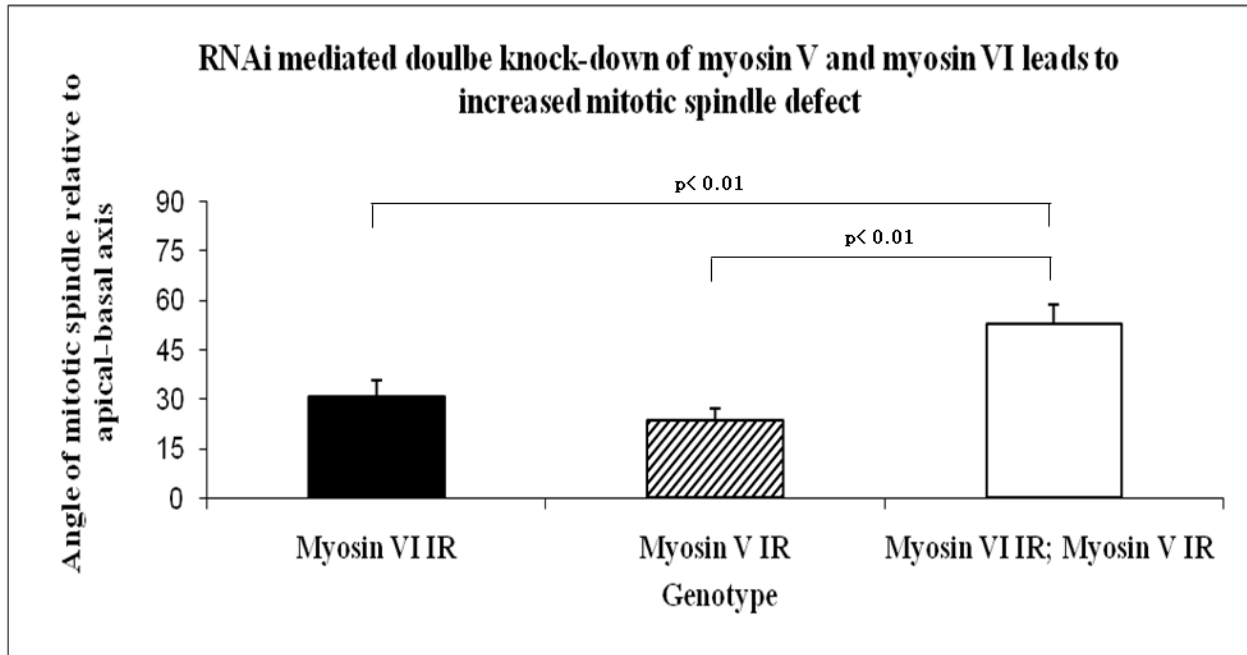


Figure 5.8 Double knockdown of Myosin V and Myosin VI leads to increased spindle defect during asymmetric division of post-embryonic NBs at third instar larval stage. Quantification of spindle orientation. The plot is generated from random sample of mitotic spindle angles mis-positioned relative to the apical-basal axis at anaphase in 1407 Dcr2> Myosin VI IR, 1407 Dcr2> Myosin V IR and 1407 Dcr2> Myosin VI IR; Myosin V IR. The double knockdown of Myosin VI and Myosin VI leads to increased spindle defect compared to that of single knockdown of Myosin VI or Myosin V.

Genotype	N	Mean	SEM	p Value
1407 Dcr2>Myosin VI IR; Myosin V IR	20	53	5.5	1
1407 Dcr2>Myosin VI IR	20	30.9	3.8	0.005
1407 Dcr2>Myosin V IR	20	23.4	4.8	0.0001

Table 5.3 Quantification of mitotic spindle angles mis-positioned away from apical-basal axis. Mitotic spindle angles at anaphase in 20 randomly chosen NBs were quantified from 1407 Dcr2> Myosin VI IR, 1407 Dcr2> Myosin V IR and 1407 Dcr2> Myosin VI IR; Myosin V IR brains. A one way ANOVA was used to compare the Myosin V or Myosin VI single knockdown groups against the double knockdown group. Tukey HSD post-hoc test was applied for multiple comparisons against the double knockdown group, with overall significance level of $p < 0.05$.

As previously shown, in wild type I central brain NBs, virtually all mitotic spindle lay within 15° away from the apical-basal polarity axis (Figure 5.7 A, C). However, RNAi mediated double knockdown of Myosin VI and Myosin V induce mitotic spindle defect (Figure 5.7 B, D). Quantification of spindle defect shows in double knockdown NBs, majority of mitotic spindles were mis-aligned (85%, Figure 5.7 D). Moreover, in 60% of the double knockdown NBs, angle of mitotic spindle relative to the apical-basal axis was greater than 45°. Whereas, RNAi mediated single knockdown of Myosin VI resulted in only 30% of the NBs with mis-aligned spindles over 45° (Figure 5.6 E). Likewise, only 15% of NBs in the RNAi mediated Myosin V knockdown group had spindle mis-orientation greater than 45° (Figure 5.6 F). Moreover, quantification of angles mis-positioned away from the apical-basal axis in NBs of 1407 Dcr2> Myosin VI IR; Myosin V IR reveal that mean angle ($53.0^{\circ} \pm 5.5$; Figure 5.7 B and D) significantly increased (Figure 5.8) compared to that of single knockdown of Myosin VI ($30.9^{\circ} \pm 3.8$; Figure 5.6 B and E) or Myosin V ($23.4^{\circ} \pm 4.8$; Figure 5.6 C and F) (See Table 5.3). However, there was no significant difference between the angles in the single knockdown groups. Thus, my results suggests there is a more severe spindle defect phenotype in post-embryonic NBs of the Myosin V and Myosin VI double knockdown brains, in comparison to that of spindle mis-orientation phenotype observed in single knockdown brains of respective myosin motors (Figure 5.8).

5.5.5 Double knockdown of Myosin V and Myosin VI leads to increased number of NBs at late third instar larval central brain.

In individual knockdowns of Myosin V and Myosin VI, as well the double knockdown of Myosin V and Myosin VI, Mira forms a basal crescent. But spindle mis-orientation can lead to occasional mis-segregation of Mira in some NBs, causing a mild over-proliferation of NBs (Lee et al., 2006c). The ‘mild’ over-proliferation phenotype can be explained by a phenomenon known as the telophase rescue, with spindle orientation ‘correcting’ itself at telophase, thereby, allowing asymmetric segregation of Mira and cell fate proteins to GMC (Peng et al., 2000).

In line with the previous findings, if the exacerbated mitotic spindle defect observed in RNAi mediated double knockdown of Myosin V and Myosin VI lead to over-proliferation of post-embryonic NBs in the central brain, it would bolster the view that Myosin V and Myosin VI act in a common pathway. Myosin V and Myosin VI are double headed motor proteins that transport cargo in opposite directions along polarised actin filament tracks. It may be feasible to speculate that Myosin V and Myosin VI co-operate to transport a cargo in a “tug of war” manner, similar to the mechanisms observed in axonal transport by the microtubule motors kinesin and dynein (Malik and Gross, 2004; Welte, 2004).

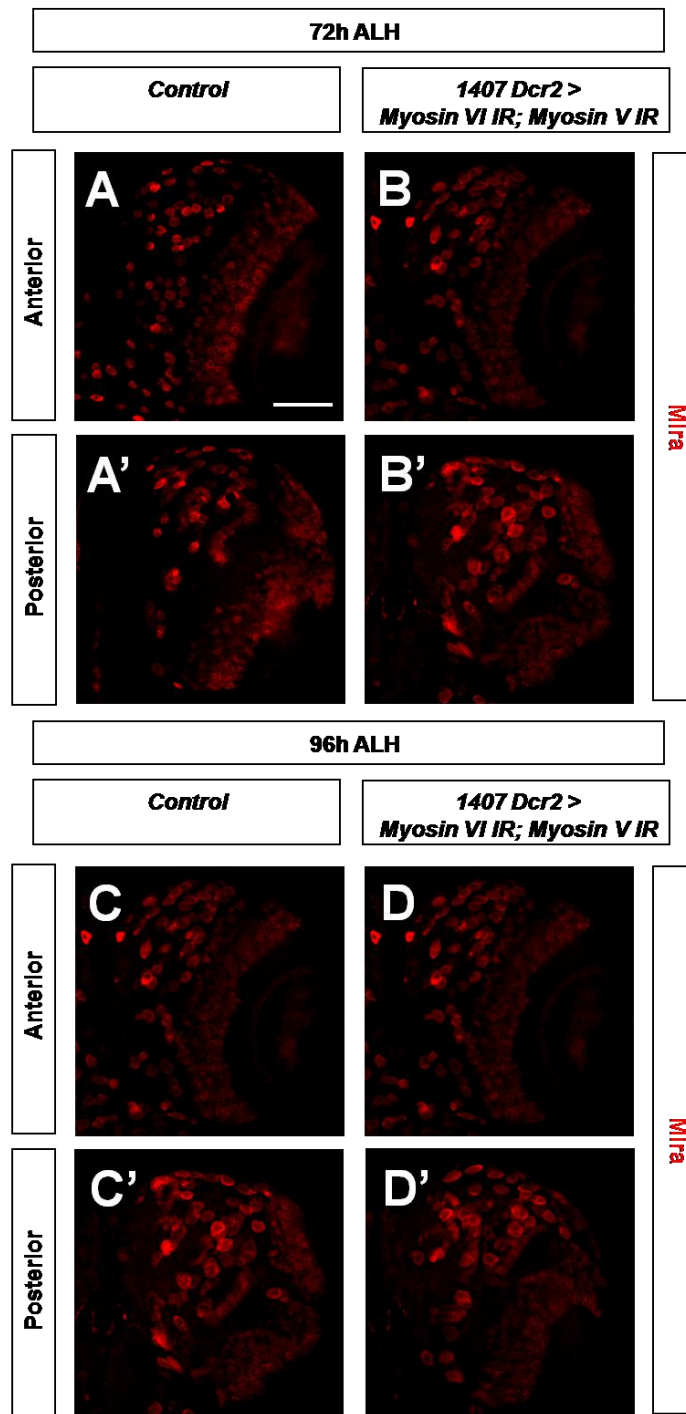


Figure 5.9 Double knockdown of Myosin V and Myosin VI leads to increased number of NBs at late third instar larval central brain. Central brain NBs were analysed and quantified through confocal Z-stacks of whole mount larval central brains from wild type (A) and 1407

Dcr2 > Myosin VI IR; Myosin V IR brains (B) at mid larval stages (72h ALH). Similarly, quantification was conducted at late larval stages (96h ALH) from wild type (C) and Dcr2 > Myosin VI IR; Myosin V IR brains (D). Mira immunolabelling was used as a marker for post-embryonic NBs. Posterior view of the larval brains were also analysed (A'-D') but did not display substantial tumour phenotypes in type II NBs. All images shown are in a single confocal plane. The scale bar is 50µm.

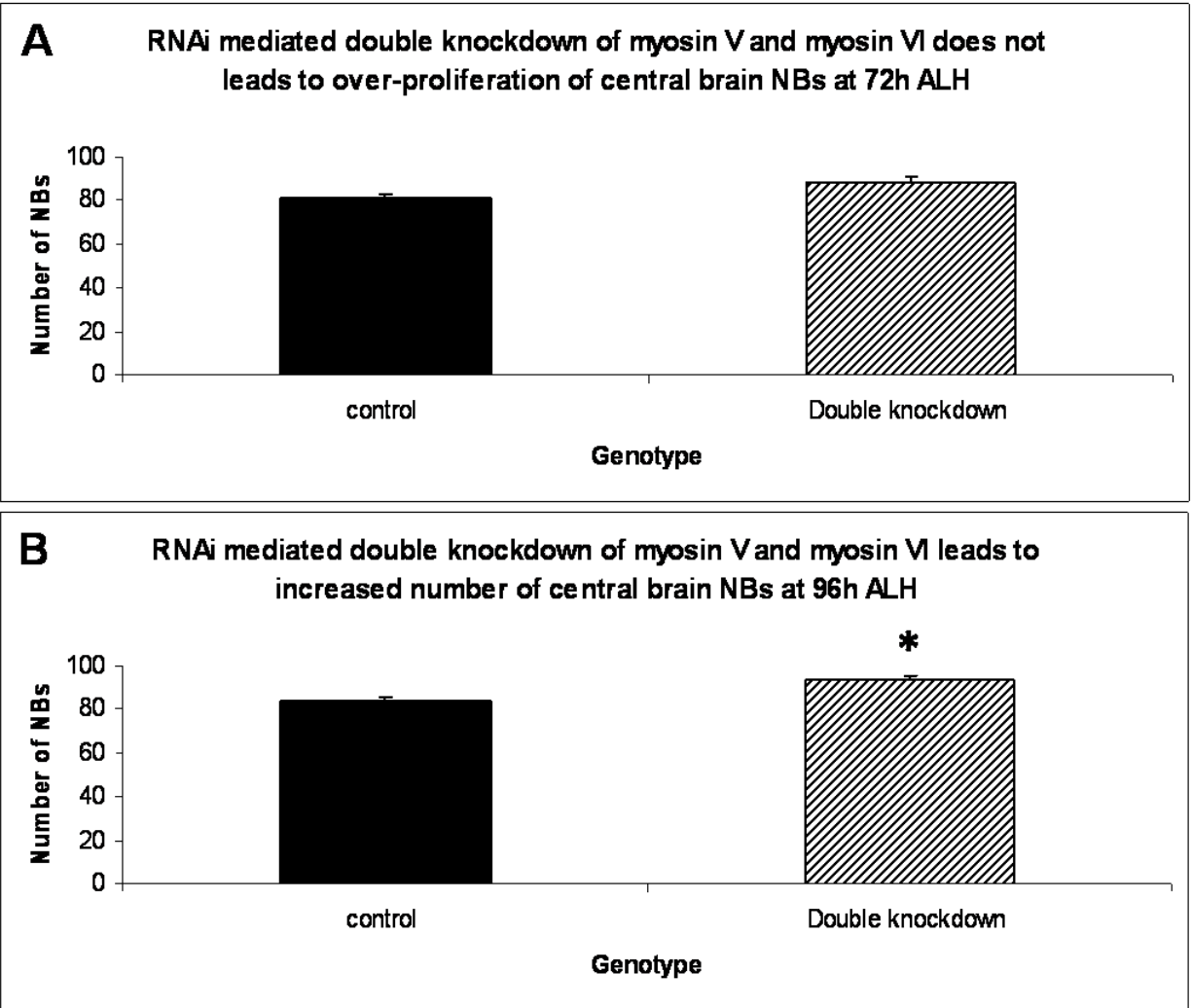


Figure 5.10 Double knockdown of Myosin V and Myosin VI leads to significantly increase in number of post-embryonic central brain NBs at 96h ALH. Quantification of central brain NBs reveal that the double knock down of Myosin V and Myosin VI significantly increased the number of Mira positive central brain NBs at 96h ALH (B) ($P < 0.01$), but not at 72h ALH (A). Error bars are standard errors of the mean.

Quantification of Mira positive central brain NBs was conducted in 20 randomly chosen brains for each group at mid (72h ALH) to late (96h ALH) larval stages. At 72h ALH, RNAi mediated double knockdown of Myosin V and Myosin VI did not significantly affect the number of post-embryonic central brain NBs (88.7 ± 2.8 ; Figure 5.9 B), compared to the wild type (83.1 ± 1.9 ; Figure 5.10 A). However, at 96h ALH, the double knockdown leads to significant over-proliferation of NBs (93.6 ± 1.8 ; Figure 5.9 D; Figure 5.10 B) when evaluated against the control (85.8 ± 1.7 ; Figure 5.9 C) (See table 5.4). Taken together, these findings augment my hypothesis that Myosin V and Myosin VI act in a common pathway to indirectly contribute to basal targeting of cell fate determinants by regulating mitotic spindle orientation during asymmetric division of post-embryonic NBs.

		72h ALH			
A	Genotype	N	Mean	SEM	p Value
	Wt	20	83,1	1.9	1
	1407 Dcr2>Myosin VI IR; Myosin V IR	20	88.7	2.8	0.06

		96h ALH			
B	Genotype	N	Mean	SEM	p Value
	Wt	20	85.8	1.7	1
	1407 Dcr2>Myosin VI IR; Myosin V IR	20	93.6	1.8	<0.01

Table 5.4 Mean number of post-embryonic NBs in RNAi mediated Myosin V and Myosin VI double knockdown brains vs. wild type. Central brain NBs were quantified in 20 wild-type (A) and 1407 Dcr2 > Jaguar IR; Myosin V IR brains at 72h ALH (A) and 96h ALH (B). A one-way ANOVA was used to compare the Myosin V and Myosin VI double knockdown group against the wild type group. Turkey HSD post-hoc test was applied for multiple comparisons against the wild type group, with overall significance level of $p < 0.05$.

Chapter 6: General discussion

6.1 Summary of findings

In order to gain insight into the genetic mechanisms regulating neural stem cell self-renewal and differentiation in the CNS of *Drosophila*, characterisation of the molecular control of asymmetric division in post-embryonic NBs were carried out;

I focused my study on the post-embryonic NBs of the central brain of third instar larvae. Immunolabelling assay was conducted to study in detail the asymmetric division of these NBs in terms of the expression and sub-cellular localisation of Mira, Brat and Pros. In line with previous findings that cell fate determinants Brat and Pros are basally segregated as cargo proteins of Mira in embryonic NBs, I detected basal co-localisation of the cell fate determinants Brat and Pros with their adaptor protein Mira at metaphase during asymmetric division of post-embryonic NB, *in vivo*.

Baseline measures of the number of post-embryonic NB in the central brain were established in controlled conditions, so that these parameters could be reliably applied in comparing observations using pharmacological treatments and genetic knockdown models. Experiments based on existing genetic knockdown model of Pros and Brat were carried out in order to provide a comparison for any tumourigenic phenotypes observed in the following genetic knockdown models. Targeted knockdown of Pros or Brat by RNAi in third instar larvae resulted in

significantly increased number of NBs in the central brain, leading to tumourigenesis, validating previous results that exclusive segregation of Brat or Pros is crucial in differentiating GMC.

Pharmacological interference of actin polymerisation or myosin phosphorylation led to mislocalisation of Mira, suggesting that asymmetric division of cell fate determinants may require an intact actin cytoskeleton and myosin motors. However, drug-induced interference of actin and myosin motor function did not result in an over-proliferation of central brain NBs. Moreover, RNAi mediated knockdown of Myosin V or Myosin VI did not increase the number of central brain NBs either, which conflicts with published findings that these Myosin motors are directly responsible for basal targeting/anchoring of Mira. Interestingly, in single RNAi-mediated knockdown of Myosin V or Myosin VI, mitotic spindle fail to align with the apical-basal polarity axis. The observed defects in mitotic spindle alignment in absence of either Myosin V or Myosin VI suggest that both Myosin motors act in a common pathway. Double genetic knockdown of Myosin V and Myosin VI was conducted, resulting in more significant spindle orientation defects than the single knockdown. These data suggests that Myosin V and Myosin VI indirectly contribute to basal targeting of cell fate determinants by regulating mitotic spindle orientation during asymmetric division of post-embryonic NBs.

6.2 Cell fate determinants Brat and Pros basally segregate into GMC as cargo proteins of their adaptor protein Mira during asymmetric division of post-embryonic NBs.

I initially set out to understand the mechanisms involved in self-renewal and differentiation of the post-embryonic *Drosophila* NBs. The different fate of two NB daughter cells is thought to be induced by the unequal segregation of Brat and Pros into the GMC following mitosis (Matsuzaki et al., 1998; Lee et al., 2006b). Due to their combined activity in specifying GMC fate, these proteins are referred to as cell fate determinants. Asymmetric segregation of the cell fate determinants is mediated by their adaptor protein Mira. Mira prevents Pros from regulating transcription in the NB by tethering it to the basal cortex during mitosis (Shen et al., 1997). Like Pros, Brat also binds to Mira and hence is co-segregated into the GMC during NB division (Lee et al., 2006b).

In my study, I have established that cell fate determinants Brat and Pros co-localise with their adaptor protein Mira in a basal crescent from pro-metaphase through telophase in post-embryonic NBs, in vivo. This is in line with previous findings that both Brat and Pros are specifically partitioned into the new born GMC as cargo proteins of Mira in embryonic NBs (Matsuzaki et al., 1998; Lee et al., 2006b). Moreover, in Pros or Brat mutants, Mira localisation is unaffected (Bello et al., 2006). However, in Mira mutants, both Pros and Brat are uniformly cytoplasmic and segregate equally into both daughter cells (Lee et al., 2006b). Thus, Mira acts as an obligatory molecular adaptor that connects Brat and Pros to the machinery for asymmetric protein localisation in *Drosophila* neural stem cells. In summary, these extensive studies have

shown that asymmetric cell division is controlled intrinsically in NBs and that a combination of cortical determinants is segregated into one daughter cell during mitosis to restrict self-renewal capacity following asymmetric division in NBs.

6.3 Defective NB division: a model for tumour formation

The cell fate determinants Brat and Pros segregate exclusively into the GMC at telophase and act to specify GMC fate. In the GMC, Pros and Brat are all thought to inhibit self-renewal and promote cell cycle exit and differentiation. Pros enters the GMC nucleus after degradation of its cortical anchor protein Mira and represses expression of cell cycle genes, and activates pro-neural genes required for terminal differentiation (Choksi et al., 2006). Brat acts as a post-transcriptional regulator during embryogenesis (Arama et al., 2000; Lee et al., 2006b), but how it acts in NBs is not yet understood.

Consistent with the functions of these genes in repressing growth and self-renewal, my studies have shown that asymmetric cell division is controlled intrinsically in NBs and that a combination of basal cell fate determinants are segregated into one daughter cell during mitosis to restrict self-renewal capacity following asymmetric division in post-embryonic NBs. It is thought that the inability of GMCs to undergo terminal differentiation results in their de-differentiation into additional NBs. Accordingly, genetic knockdown of Brat or Pros in the larva results in NB lineages that divide to generate a large number of daughter cells capable of self-renewal. This excessive self-renewal occurs at the expense of neuronal differentiation, leading to tumourigenesis (Bello et al., 2006; Betschinger et al., 2006; Lee et al., 2006b).

Although genetic knockdown of Pros or Brat result in similar terminal phenotypes, the precise cellular events leading to over-proliferation of post-embryonic NBs in the central brain vastly differs. Close analysis of the Brat mutant phenotype indicates that the over-growing cells arise in a specific location in the central brain (Bello et al., 2006; Betschinger et al., 2006), suggesting that type II NBs are particularly sensitive to loss of Brat (Bowman et al., 2008).

In type I NB lineage, Pros is imported into the nucleus of the basal NB daughter cell. One cell fate determinant in addition to Pros is enough to establish GMC fate, because loss of Brat alone does not prevent neural differentiation in type I NBs (Bowman et al., 2008). Whereas, type II NBs divide to self-renew and generate an immature intermediate neural progenitor (INP) which lacks Asense and nuclear Pros. After maturation, INPs start expressing the transcription factor Asense and divide to generate GMCs. In the absence of Brat, immature INP reverts back to a fate similar to its parental NB. Instead of becoming a mature INP, the asense negative daughter commits to unlimited self-renewal, causing a strong over-proliferation defect. (Bayraktar et al., 2010). In type II NB lineages, immature INP lacks nuclear Pros, so it uses only Brat or Numb to differentiate itself from its parental cell. The weak commitment of immature INP to its fate may be the reason why type II NB lineages are more susceptible to loss of Brat. Moreover, this could explain why Brat mutant NBs do not appear to express Pros or segregate it asymmetrically (Bello et al., 2006; Betschinger et al., 2006), this could explain why expressing Pros rescues tumour phenotype induced by loss of Brat (Bello et al., 2006), as it enables immature INP to fully commit to its fate.

6.4 Pharmacological inhibition of actin or myosin results in cortical de-localisation of Mira.

The proposed model for direct basal targeting of the cell fate determinants implicated myosin based cargo transport along the actin filaments (Erben et al., 2008). The basis of this model stems from initial experiments using pharmacological inhibitors indicating that asymmetric division of Mira require an intact actin (Broadus and Doe., 1997; Shen et al., 1998) and myosin (Ohshiro et al., 2000). In line with this hypothesis, in my study, pharmacologically induced functional inhibition of F-actin resulted in cortical de-localisation of Mira, failing to segregate into the GMC. In addition, BDM induced inhibition of myosins resulted in a similar phenotype where Mira failed to form a basal crescent at metaphase. With presumption that asymmetric segregation of the cell fate determinants rely upon direct basal targeting via actin-myosin motor based transport, Mira cortical de-localisation phenotype observed in my study would indicate that basal cell fate determinants failed to be segregated into the GMC, as a result of drug induced inhibition of actin or myosin motor function.

However, these conclusions must be treated with caution. Although BDM is a supposed general myosin inhibitor, especially a well studied inhibitor of the ATPase of skeletal muscle Myosin II, the efficacy of BDM towards non-muscle Myosins such as Myosin V or Myosin VI remains controversial (Ostap, 2002). Moreover, BDM has been shown to block actin polymerisation at the leading edge of mammalian cells (Yarrow et al., 2003). Thus, I cannot rule out that the cortical de-localisation of Mira observed in BDM treated NBs may have been due to inhibition of actin polymerisation by BDM.

6.5 Myosin II is required for cytokinesis in post-embryonic NBs.

RNAi mediated genetic knockdown of Myosin II resulted in partial cellular over-growth of post-embryonic NBs in the central brain as well as in the VNC. Closer look into the morphology of the enlarged cell revealed ectopic pH3 labelled punctae, indicative of increased chromosomal materials. These evidences suggest that cellular over-growth observed in post-embryonic NBs in Myosin II/Zipper RNAi knockdown CNS may have been due to endoreplication.

Endomitotic phenotype observed in Zipper RNAi knockdown central brain NBs may be explained by involvement of Myosin II in formation and constriction of the acto-myosin contractile ring (Reviewed in Eggert et al., 2006), a transient structure built with a network of actin filaments and myosin II motor proteins required for the generation of force for cytokinesis in animal cells and yeast (Reviewed in Field et al., 1999; Barr and Gruneberg, 2007; Pollard, 2010). Bipolar filaments of myosin II draw F-actin together in a purse string like manner to constrict the contractile ring at cleavage furrow. It is thought that a tight link is established between the acto-myosin ring and the plasma membrane at the equator, and this attachment is maintained during late stages of cytokinesis (Goldbach et al., 2010). As myosin II constitute an integral part of the acto-myosin ring, knocking down myosin II would have disrupted the structure of the contractile ring, which in turn would debilitate cytokinesis to occur. Accordingly, it is possible to speculate that Mira basal localisation defect observed in NBs treated with drugs to inhibit function of actin and myosin may have been due to cytokinesis failure.

It is interesting to note that the endomitotic phenotype was observed in all of the Myosin II/Zipper RNAi knockdown CNS in the absence of Dcr2 over-expression, indicating that it is not

necessary for effective knockdown of Myosin II. But why the cellular over-growth was observed in only a few post-embryonic NBs and how rest of the NBs had undergone cytokinesis, hence, RNAi mediated Myosin II knockdown did not cause embryonic lethality are still unknown. It maybe possible that only certain types of NBs are more susceptible to loss of Myosin II, however, an important future experiment would be to use 1407 Gal4 to drive expression of both Myosin II/Zipper RNAi and Dcr2, as increased RNAi efficiency may affect the outcome.

Nevertheless, the direct involvement of Myosin II in cytokinesis makes it an unlikely candidate myosin motor responsible for basal targeting of Mira. Moreover, given the lack of resources and the limited I had available, it was not feasible for me to continue investigating the role of Myosin II in asymmetric division of post-embryonic NBs.

6.6 Myosin V or Myosin VI do not directly contribute to basal targeting of the cell fate determinants.

The mechanisms that lead to basal targeting of the cell fate determinants Brat and Pros by their adaptor protein Mira has remained a mystery for many years. Actin-myosin dependent mechanism was proposed for basal targeting of cell fate determinants along the cell cortex (Jan and Jan, 2001). Support for this model came from the demonstrations that Myosin VI is important for asymmetric cell division and from the findings that Lgl binds and inhibits non-muscle Myosin II (Petritsch et al., 2003). Consistent with this hypothesis, when Myosin II is inhibited by mutation or chemical inhibitors of Rho-associated protein kinase (ROCK), Mira no longer localises basally (Barros et al., 2003).

Although the actin-myosin cortical transport model is attractive, it has been challenged by several recent observations, including my study. Firstly, the ROCK inhibitor that was used to demonstrate the requirement of myosin for Mira localisation can also inhibit aPKC (Atwood and Prehoda, 2009). Recent work has uncovered a direct mechanism for polarisation of basal domain proteins by aPKC in which phosphorylation of Numb or Mira leads to release from the cortex. In NBs, phosphorylation by aPKC was recently shown to promote cortical displacement of Mira (Atwood and Prehoda, 2009). Although a complex model involving a tumour suppressor Lgl and non-muscle Myosin II has been proposed for aPKC-mediated Mira polarisation (Erben et al., 2008), direct Mira phosphorylation appears to be necessary and sufficient for cortical displacement. Mira contains an N-terminal cortical localisation domain that specifies cortical recruitment in NBs and this domain is specifically phosphorylated by aPKC, *in vitro* (Atwood and Prehoda, 2009). Mutation of the phosphorylation sites to alanine causes Mira to fail to respond to the presence of aPKC, with Mira mis-localised to the apical cortex. When these sites are changed to phosphomimetic residues, Mira remains in the cytoplasm, even in the absence of aPKC (Atwood and Prehoda, 2009). This leads to a model in which Mira and the cell fate determinants Brat and Pros as its cargo are basally targeted via direct phosphorylation of Mira by aPKC.

Secondly, fluorescence recovery after photobleaching (FRAP) experiments did not reveal uni-directional cortical transport of the Numb adaptor PON (Lu et al., 1998; Mayer et al., 2005). Instead, FRAP recovery rates showed that PON and Numb rapidly exchange between cortex and cytoplasm and that local difference cortical 'on' and 'off' rates are responsible for basal targeting

of cell fate determinants rather than direct cortical transport. Therefore, the cortical transport model has been replaced by more dynamic models, in which the differential mobility or cortical attachment of protein determinants to the apical and basal plasma membrane regulates the asymmetric localisation of the cell fate determinants. This is in line with my study, where RNAi mediated single knockdown of the candidate myosin motors, Myosin V or Myosin VI did not result in mis-localisation of Mira or increased number of Mira positive NBs in the post-embryonic central brain of *Drosophila*. In addition, phenotypic alterations caused by RNAi mediated knockdown of Myosin V or Myosin VI in mitotic spindle orientation of NBs during asymmetric division indicate that the candidate Myosins were successfully knocked down via RNAi. Thus, it is highly unlikely that cell fate determinants Brat and Pros utilise myosin motor for direct basal targeting during asymmetric NB division. However, all I could deduce from my data is that the aforementioned candidate Myosin motors do not appear to be directly responsible for basal targeting of Mira. Therefore, in order to further validate the hypothesis that basal targeting of Mira is achieved through rapid exchange between cytoplasm and the basal cortex, quantifying the exchange of Mira between cell cortex and the cytoplasm should be conducted through FRAP analysis of Mira-GFP. I have already shown that Mira-GFP co-localises with endogenous Mira throughout mitosis (Figure 3.3), thus, rescue experiment using Mira-GFP should be conducted to show that it is functional. Subsequently, fluorescence intensity should be recorded in wild-type post-embryonic NBs expressing Mira-GFP at metaphase, after photobleaching the basal cortex to assess whether the movement of Mira-GFP is dynamic or static. If the Mira-GFP fluorescence recovers to pre-bleached level it would indicate a dynamic Mira-GFP movement is taking place. Then, photobleaching should be applied to a larger area covering basal half of the NBs at metaphase. An overall reduction in cortical Mira-GFP signal

would indicate that refilling of the cortical Mira GFP was due to movement of Mira-GFP from adjacent cytoplasm. Alternatively, lateral cortex could be photobleached to determine whether Mira-GFP movement occurs along the cortex.

6.7 Myosin V and Myosin VI may act in a common pathway to regulate mitotic spindle alignment during asymmetric division of NBs.

Observed defects in mitotic spindle alignment at anaphase in post-embryonic NBs of the single knockdown Myosin V IR brains or Myosin VI brains suggested that both Myosin V and Myosin VI may act in a common pathway or in parallel pathways at distinct steps to basally localise Mira in an indirect way, by aligning the mitotic spindle formation along a predefined cortical polarity axis for the reliable partitioning of the cell fate determinants Brat and Pros during asymmetric NB division. In line with this hypothesis, the double RNAi-mediated knockdown of Myosin V and Myosin VI resulted in exacerbated mitotic spindle defect, suggesting that Myosin V and Myosin VI may act in a common pathway.

Myosin V and Myosin VI transport cargo in opposite directions along polarised actin filament tracks (Reviewed in Walker et al., 2000), thus, I hypothesize that Myosin V and Myosin VI motors may engage in a virtual “tug of war” to transport a common cargo in a bi-directional manner, similar to the mechanisms observed in axonal transport by the microtubule based motors, kinesin and dynein (Malik and Gross, 2004; Welte, 2004). If so, how do these oppositely

directed Myosin motors mechanically interact so that cargo is efficiently delivered to its destination?

Previous studies using an *in vitro* model system that physically linked a single Myosin V and a single Myosin VI to a common cargo revealed that as these two classes of motors attempted to carry the same cargo in opposite directions, Myosin V won this “tug of war” most of the time (Ali et al., 2011). Myosin V dominating suggests that intra-cellular cargo transport would be unidirectional when equal numbers of both Myosin motors are present. The ability of one Myosin V motor to dominate over oppositely directed Myosin VI motor raises questions of how opposing motors attached to intracellular cargo are regulated to ensure proper cargo delivery. Regulation could involve varying the number of motors of a given type. Varying the number of a given motor type could be accomplished by regulating the active state of one motor type relative to the other (Schroeder et al., 2010). Such shift in local Myosin motor numbers on a given actin track on which the Myosin motors travel, could differentially impact different classes of Myosin motors.

As recently reported (Clayton et al., 2010), the differential localisation of tropomyosin in yeast can enhance the activity of two class V Myosins (Myo51p and Myo 52p) while inhibiting that of class I Myosin (Myo1p), providing a means for spatially regulating Myosin function and cargo delivery. Modifiers that alter the number of engaged motors in effect would eliminate or bias (Gross et al., 2002b; Ally et al., 2009; Laib et al., 2009) the inherent “tug of war” that may exist between opposing Myosin V and Myosin VI motors.

In addition, the double knockdown resulted in significant increase in the number of Mira positive NBs in late instar larval central brain. The over-proliferation phenotype observed in double knockdown brain is very ‘mild’ compared to that of over-proliferation phenotypes observed in brains with knockdown of basal cell fate determinants. The ‘mild’ over-proliferation of the central brain NBs observed in the double Myosin knockdown brain may be attributed to a compensatory mechanism known as ‘telophase rescue’ (Peng et al., 2000). Similar to my findings, mutation in any component of the apical protein complex leads to mitotic spindle defect resulting in mis-localisation of the cell fate determinants in metaphase, however, great majority of the mutant NBs redistribute the cell fate determinants Pros and Brat into the GMC as basal crescents, starting from anaphase (Schober et al., 1999; Wodarz et al., 1999; Peng et al., 2000).

There are two pathways for generating cortical polarity in NBs: a well established primary microtubule independent pathway that is initiated at late interphase by the apical Par complex (Siegrist and Doe, 2006), and the secondary microtubule-dependent pathway initiated later at pro-metaphase/metaphase by astral molecules, which only acts at late in mitosis (anaphase and telophase) and is required for telophase rescue. The exact mechanisms regulating telophase rescue is yet to be discovered, although Discs Large has been implicated to be involved (Siegrist and Doe, 2005). The microtubule-dependent pathway induces Pins/Gai crescent formation over one spindle pole, providing a docking site for attracting astral microtubules nucleated from the apical spindle pole (Reviewed in Knoblich, 2008; Kim and Hirth, 2009). Since Mud interacts with microtubules and the minus end-directed microtubule motor dynein, it is thought that cortical Mud either recruits dynein as the force generator or tethers the captured astral microtubules as a means to achieve alignment of the mitotic spindle along the apical–basal axis

(Siller et al., 2006). Mitotic spindle orientation has been speculated to involve a balancing of forces generated either by growing astral microtubules pushing against the cell cortex (Lu et al., 1998). In the absence of microtubule-dependent pathway, Insc can still recruit Pins/Gai to the apical cortex, yet the spindle may not be properly aligned with the cortical polarity (Siegrist and Doe, 2005). Mitotic spindle orientation would be ‘corrected’ and aligned along the apical-basal polarity axis in most but not all NBs in the absence of microtubule-dependent pathway during asymmetric division. Hence, this explains the mild over-proliferation of post-embryonic central brain NBs observed in RNAi mediated double knockdown of Myosin V and Myosin VI. Moreover, these results implicate Myosin V and Myosin VI to be involved with the apical protein complex to regulate orientation of the mitotic spindle during post-embryonic NBs asymmetric division.

How does Myosin V and Myosin VI regulate mitotic spindle alignment along the apical-basal polarity axis during asymmetric NB division? My hypothesis is that Myosin V and Myosin VI act in a common pathway involving +TIPs, EB1 and CLIP-170, respectively, to regulate mitotic spindle orientation of post-embryonic NBs in *Drosophila*.

Firstly, Myosin VI was shown to co-immunoprecipitate with D-CLIP-190, an orthologue of human CLIP-170 (Lantz and Miller, 1998). The human CLIP-170 is believed to be a microtubule-actin linker protein that binds preferentially to the growing (plus ends) of the microtubules (Perez et al., 1999). Thus, CLIP-170 may capture and transport cargo via the unconventional myosin motor, Myosin VI from/to microtubules and actins (Lantz and Miller, 1998). In addition, CLIP-170 directly binds to (Busch and Brunner, 2004), and functions

downstream of EB1 (Komarova et al., 2005), which is another microtubule plus end tracking protein (+TIPs). EB1 influences microtubule dynamics and plays a particularly critical role in the assembly, dynamics, and positioning of the mitotic spindle. Interference of EB1 function produces effects on spindle structure, most common being an overall decrease in the length of the spindle, as well as the mis-orientation of the spindle poles (Rogers et al., 2002).

Secondly, Myosin V binds to and transports EB1 and plus end of astral microtubules as cargo along polarised actin cables to the apical protein complex in yeast (Reviewed in Vaughan, 2005). Although the mechanisms involved in regulation of spindle orientation by +TIPs is yet to be unveiled, it may be feasible to speculate that localisation of +TIPs to the microtubule plus ends is essential to induce apical cortical polarity and establish mitotic spindle orientation in *Drosophila* NBs by linking astral microtubules to Pins/Gai via Mud.

6.8 Evaluation of the RNAi mediated knockdown experiments.

As my data relies on the specificity and efficiency of the RNAi mediated knockdowns, it may be crucial to discuss the potential caveats of this approach and how they could be solved. Firstly, I conducted BLAST searches available on Flybase (www.Flybase.net) to ensure the target sequences of Myosin II, Myosin VI and Myosin V transgenic RNAi strains did not have matches to other genes of the same species of 16 or more nucleotides. The BLAST search did not reveal any off targets (Figure 5.2), hence the RNAi transgenic lines that were used in my experiments are supposedly specific. However, some additional control experiments could be conducted in the future to prove that the observed phenotypes are indeed due to knockdown of RNAi target

genes. Although I could not include such experiments in my thesis due to lack of resources and time constraint, immunohistochemistry using appropriate antibodies for Myosin II, Myosin V, Myosin VI should be conducted. Moreover, RT-PCR should also be conducted in order to show whether and how much the RNAi transgenic lines do indeed knockdown/reduce the amount of transcript/protein. Furthermore, MARCM clonal analysis could be conducted using the available mutant lines for Myosin V and Myosin VI to characterise the role of respective Myosin motors during asymmetric division of post-embryonic NBs. Given that similar phenotype is observed in the Myosin V and Myosin VI mutation analysis compared to my data shown above, conducting rescue experiments using Myosin V and Myosin VI should reveal whether only the gene of interest is involved in the observed phenotype. Otherwise there is a small possibility that the observed phenotypes from my studies could be due to position effects of the RNAi transgene used.

In addition, in the experiments where Dicer2 was used to increase the RNAi efficiency, I used uncrossed 1407 Dcr2 Gal4 driver lines as control, labelled with anti-Mira antibody. I did not observe any increase in the number of NBs nor any spindle defects. However, conducting a MARCM clonal analysis on 1407 Dcr2> Mira-GFP may further validate my claims that there are no phenotypes by expression of Dicer2 alone.

6.9 Conclusion

The aim of my thesis was to gain insight into the mechanisms involved in self-renewal and differentiation of neural stem cells in *Drosophila* by identifying the myosin motor responsible for direct basal transport of the cell fate determinants during asymmetric division of post-

embryonic NBs. Although the actin-myosin cortical transport model was the basis of my hypothesis, the results from my studies, along with several recent publications indicate several fundamental flaws with the model.

Firstly, the Mira de-localisation phenotype observed in NBs due to drug-induced functional inhibition of actin (Broadus and Doe, 1997) and myosin (Barros et al., 2003) do not signify that basal segregation of cell fate determinants utilise actin-myosin based transport system for basal targeting. Instead, partial cellular over-growth was observed in Myosin II RNAi mediated knockdown brains. The enlarged NBs displayed increased phospho-histone H3 labelled DNA materials, indicating that cell division is ongoing without cytokinesis. Coupled with involvement of Myosin II in acto-myosin contractile ring suggests that partial cellular over-growth phenotype observed in my study may have been affected by failure in cytokinesis, due to disrupted structure of the acto-myosin contractile ring. Although endoreplication is a natural process, an active mechanism for polytenization such as in salivary glands (Reviewed in Lee et al., 2009), endoreplication does not occur in wild-type third instar larval NBs. Thus, it may be feasible to speculate that RNAi mediated knockdown of Myosin II contributed to endoreplication phenotype observed in my study. Accordingly, cortical de-localisation of Mira observed in drug-induced functional inhibition of actin may have been due to failure in cytokinesis as well. In line with this, an over-proliferation defect was not detected in any brains treated with functional inhibitors of actin or myosin.

Secondly, RNAi mediated genetic knockdown of the implicated myosin motors, Myosin V and Myosin VI did not result in over-proliferation of NBs in the post-embryonic central brain of

Drosophila. Thus, it is unlikely that the proposed myosin motors play a direct role in basal targeting of the cell fate determinants. Instead, I identified a possible role of Myosin V and Myosin VI in regulating mitotic spindle orientation during asymmetric division of NBs. Exacerbation in mitotic spindle defect phenotype observed in NBs of Myosin V and Myosin VI double knock down brains suggest that the two myosin motors may act in a common pathway to align mitotic spindle orientation along an apical-basal polarity axis in order for cell fate determinants to be basally segregated into the GMC. Correspondingly, double knock down of Myosin V and Myosin VI led to a mild over-proliferation NBs in the central brain, suggesting that the proposed myosin motors may indirectly contribute to asymmetric division of cell fate determinants in post-embryonic NBs by regulating mitotic spindle orientation.

Therefore, the exclusive segregation of the cell fate determinants into the GMC are not likely to utilise actin-myosin based cortical transport model as previously thought. The newly proposed model suggests a new role for aPKC as the driving force for asymmetric localisation of these determinants. A direct phosphorylation of Mira by aPKC leading to exclusion of Mira from the apical cortex, followed by subsequent cortical attachment of Mira to the basal cortex is the key mechanisms involved in basal targeting of the cell fate determinants Brat and Pros into GMC during asymmetric division of NBs in *Drosophila*.

Although we have learned the basic principles of asymmetric NB division, our understanding of the mechanisms involved in cell fate specification by Brat and Pros is limited. Moreover, it may be challenging to transfer our knowledge from flies to vertebrates. Although, RHAMM has recently been suggested to be a divergent mammalian ortholog of the anterior targeting domain

N-terminus of Mira (Chang et al., 2011), a functional ortholog of Mira in vertebrates remains to be identified. Further studies are required to enable a deeper understanding of the mechanisms involved in regulation of neural stem cell self-renewal and differentiation using *Drosophila* as a model system.

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