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HEM-PROTEIN REGULATES CELL MIGRATION AND ASYMMETRIC CELL DIVISION DURING DEVELOPMENT OF THE VENTRAL NERVE CORD IN *DROSOPHILA MELANOGASTER*

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by

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Dedication

I dedicated this dissertation to Feifan Zhang, my wife,

who has supported me throughout these years.

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I foremost would like to thank my mentor Dr. Krishna M. Bhat for his constant support and endless patience. It was his enthusiasm about science that helped to keep me focused even when things in lab were not working well. Special thanks are to my committee members, Dr. Kathleen M. Beckingham, Dr. Javier V. Navarro, Dr. Pomila Singh and Dr. Ping Wu for their insightful advice and constructive criticisms throughout this investigation. I want to express my gratitude to all members of Dr. Bhat's laboratory for helping with my experiments and being good friends. I would especially like to thank the postdocs, Ivana for her advice on genetics, Michal to whom I could always turn for advice on molecular biology problems. I also wish to thank Vatsala Jayasinghe for her help in editing the manuscripts and my dissertation. I thank Dr. Jennifer A. Zallen for providing WAVE antibody, Dr. Christian Klämbt for providing Hem antibody and *Hem* alleles, Dr. Eyal D Schejter for providing *WAVE* and *WASp* alleles and Dr. Manfred Frasch for providing Eve antibody.

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Cell migration and asymmetric cell division are two of the key events during development of the nervous system. I have focused on a typical neuronal lineage, NB4-2→GMC-1→RP2/sib, in the ventral nerve cord (VNC) of the *Drosophila* embryo to investigate the regulation of neuronal migration and asymmetric cell division during development of the nervous system. I have discovered a migration defect of RP2 neurons in *HEM-protein (Hem)* mutants: RP2 neurons cross the midline and migrate from the initial hemi-segment to the opposite hemi-segment. The same migration defect is observed in *WASP-family verprolin-homologous protein (WAVE/SCAR)* and *Abl tyrosine kinase (Abl)* mutants, suggesting that these three genes might act together to regulate neuronal migration in the VNC. I have found that Hem is required for maintaining the protein level of WAVE *in vivo* and is necessary for its proper localization in the cell. In

Hem mutants, WAVE is down regulated and mis-localized in RP2 neurons, resulting in the migration defect of RP2 neurons. Abl on the other hand negatively regulates the protein level of WAVE. When Abl is ectopically expressed, WAVE protein is down regulated. In *Abl* mutants, WAVE is up regulated and its hyperactivity may be responsible for the migration defect of RP2 neurons. Meanwhile, instead of asymmetric division in wild type embryos, a symmetric division of GMC-1 is observed in the "strong phenotype embryo" of $Hem^{J4\text{-}48}$ mutants. It was not observed in other *Hem* mutants and *Hem* deficiency alleles. The truncated Hem protein ($\Delta Hem^{J4\text{-}48}$) in $Hem^{J4\text{-}48}$ allele may behave as a neomorphic protein, resulting in the symmetric division of GMC-1. In $Hem^{J4\text{-}48}$ mutants, the apical localization of Inscuteable (Insc) is disrupted, suggesting that regulation of the asymmetric division of GMC-1 by Hem is mediated by Insc. The same symmetric division of GMC-1s was also observed in *Abl* mutants but not *WAVE* mutants, suggesting that Abl may act together with Hem to regulate the asymmetric division of GMC-1s. This study uses the *Drosophila* VNC as a model system and describes how neuronal migration and asymmetric cell division are regulated by Hem during development of the nervous system.

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Chapter I

HEM-protein regulates cell migration during development of the ventral nerve cord in *Drosophila melanogaster*

Abstract

Cell migration is one of the most fundamental events, present from simple unicellular organisms to complicated multi-cellular organisms including humans. It is involved in chemotaxis, embryonic development, tissue formation, immune responses, wound healing and many other biological processes. During the past decades, both *in vitro* and *in vivo* experiments have been vigorously carried out to understand cell migration as well as identifying players and signaling pathways involved in this process. However, it is still unclear as to how cell migration is dynamically regulated. In this study, I use *Drosophila melanogaster* as a model system to investigate the regulation of neuronal migration during development of the nervous system. NB4-2→GMC-1→RP2/sib, a typical neuronal lineage in the ventral nerve cord (VNC) of the *Drosophila* embryo, undergoes a 3-step migration during development of the VNC. By investigating the migration pattern of this typical neuronal cell lineage, I have found a migration defect of RP2 neurons in *HEM-protein (Hem)* mutants: RP2 neurons cross the midline and migrate from the initial hemi-segment to the opposite hemi-segment, resulting in two RP2 neurons in one hemi-segment at the expense of the opposite hemi-segment. The same migration defect was also observed in *WASP-family verprolin-homologous protein (WAVE/SCAR)* and *Abl tyrosine kinase (Abl)* mutants, suggesting that these three genes

might act together to regulate neuronal migration in the VNC. I have found that WAVE is required for the migration of RP2 neurons; RP2 neurons stop migration in the absence of WAVE. Hem is required for maintaining the protein level of WAVE *in vivo* and is necessary for its proper localization in the migrating RP2 neurons. In *Hem* mutants, WAVE is down regulated and mis-localized in RP2 neurons, resulting in the migration defect of RP2 neurons. Abl on the other hand regulates the migration of RP2 neuron in a different way. It negatively regulates the protein level of WAVE. When Abl is ectopically expressed, the level of WAVE protein is down regulated. In *Abl* mutants, the level of WAVE is up regulated. Therefore, its hyperactivity may cause the migration defect of RP2 neurons.

Introduction

Cell migration

Cell migration is one of the most fundamental processes, ranging from simple unicellular organisms such as *Yeast*, to more complex multi-cellular organisms including humans. In unicellular organisms, the function of cell migration lies in food searching and mating (Bagnat and Simons, 2002; Manahan et al., 2004). In multi-cellular organisms, it is required for embryonic development, tissue and organ formation, immune responses, wound healing and many other activities (Ridley et al., 2003; Vicente-Manzanares et al., 2005). During development of the nervous system, neurons and their precursor cells migrate from their initial locations to various positions, where they make

connections with other neurons or muscle cells to form functional neuronal circuits. For example, neurons generated in the ventricular zones of the developing cerebral cortex migrate in distinct radial and tangential routes to the top of the embryonic cortex, where they stop their migration and start to differentiate into different classes of cortical neurons (Ayala et al., 2007). Elucidating the mechanisms that govern the initiation, maintenance and termination of neuronal migration is crucial for our understanding of how a functional neuronal circuitry is established in the brain during development.

Cell migration is a highly complex, coordinated process in which extracellular signals and intracellular machineries act together to direct cells to move toward their targets. Since Abercrombie's series of studies of cell migration in 1953 (by investigating the locomotion of chick heart fibroblasts in culture) (Abercrombie and Heaysman, 1953, 1954; Abercrombie et al., 1970a, b, c, 1971), a large number of studies have been done in order to understand the complexities and regulations underlying cell migration. Most of these studies use a model of cells migrating on 2D substratum *in vitro*. More recently, cells migrating in 1D or 3D environments have also been used (Cukierman et al., 2001; Doyle et al., 2009). When investigated, different cell types exhibit very similar migration features despite their unique features. For example, leukocytes display an amoeba like movement and morphology, while keratocytes display a gliding motion. However, regardless of their unique features, almost all cells show similar characteristics when migrating and use similar molecules and mechanisms to regulate migration (Gunzer et al., 2000; Knight et al., 2000).

A typical cell migration in general, consists of five repeated steps: 1) front-back cellular polarization; 2) membrane protrusions such as lamella, lamellipodia and filopodia in the leading edge toward the direction of migration; 3) the assembly of cell adhesion in the newly formed membrane extensions; 4) adhesion disassembly in the rear end; 5) cell body retraction (Vicente-Manzanares et al., 2009). These five steps are repeated and are highly dynamically regulated by many signals and coordinate with each other to ensure proper cell migration. Any errors in these five steps may impair or abnormally enhance cell migration, both of which could easily result in developmental deficiencies, mental retardation, chronic inflammation, cancer, virus or bacterial infection, and dissemination (Vicente-Manzanares et al., 2005).

Actin polymerization

The protrusion of the cell membrane at the leading edge is one of the most significant features of migrating cells. It is also one of the key steps in cell migration (Abercrombie et al., 1970a, b; Chhabra and Higgs, 2007; Pollard and Borisy, 2003). Several different protrusive structures have been described previously in migrating cells: lamella, lamellipodia, filopodia and ruffles. Lamella are broad, flattened, and sheet-like membrane-enclosed cytoplasm, located at the anterior of migrating cells. Lamellipodia represent thinner (100 -160 nm) narrow regions at the edge of lamella, which are believed to play a role in leading cell migration. Different from lamella and lamellipodia, filopodia are long, thin and finger-like protrusions from the leading edge (Fig. 1.1). The functions of filopodia in cell migration remain unclear, due to the fact that they are only sometimes required for cell migration but not in all cases (Sepp and Auld, 2003). Ruffles are sheet

like membrane protrusions that do not attach to the substratum by any means, in a 2D environment. They instead assemble at the leading edge of motile cells, and move rearward (Abercrombie et al., 1970c).

Networks of actin filaments have been observed to be the dominant structures in all these protrusions, and growth of actin networks drive the formation of these protrusions. Consistent with their different morphology, the actin networks in the structures are significantly different. In general, dendritic actin networks with short, cross-linked actin filaments are present in lamella, lamellipodia and ruffles (Cano et al., 1991; Small et al., 1995; Svitkina, 2007) while long, parallel bundles of actin filaments are present in filopodia (Faix and Rottner, 2006) (Fig. 1.1).

Actin filaments (F-actin) are double helical polymers of globular actin monomers (G-actin). G-actin polymerizes into actin filaments that can then be assembled further into various structures. The polymerization starts with nucleation of G-actins into the dimeric and then the trimeric complexes. This nucleation process is somehow unfavorable. Without nucleation initiators, this process is extremely slow. Nucleation initiators are therefore necessary for actin polymerization. Currently three types of initiators have been identified: Arp2/3 complex (Machesky et al., 1994), formins (Watanabe and Higashida, 2004) and spire (Kerkhoff, 2006; Quinlan et al., 2005; Rosales-Nieves et al., 2006).

Once nucleated, more actin monomers are added and actin filaments start to grow rapidly. Since all actin subunits added are arranged in a head-to-tail manner, actin filaments are polar: with a barbed end and a pointed end. This polarity is of great importance to the mechanisms of actin assembly: actin filaments in cells are oriented in such a way that barbed ends always grow toward the cell membrane. Although G-actins could be added to or depolymerized from both ends, they are added much faster (~ 10 fold) at the barbed end. The action of profilin, which adds G-actin to the barbed end only, enhances this effect and effectively limits elongation only to the barbed end. Usually, there is a flux of G-actins, added to the barbed end and depolymerized from the pointed end.

Soon after the polymerization of actin filaments, other factors will further join in to finally form different types of actin structures based on actin filaments. Newly formed filaments are capped by abundant barbed-end capping proteins, preventing further elongation. Inhibition of capping proteins is therefore essential for structures requiring long filaments. Also, actin filaments can be cross-linked into networks. The initiators, Arp2/3 and formins, mentioned before, can perform these functions.

Arp2/3 complex

Arp2/3 complex (Machesky et al., 1994) is the primary candidate to nucleate new actin filaments (Ma et al., 1998; Mullins et al., 1998; Welch et al., 1998). It is composed of two actin related proteins (Arp2 and Arp3) with 5 other polypeptides (ARPC1-5). These two actin-related proteins are suggested to function similar to an actin dimer that

can elongate the barbed end. In addition to its function as a nucleation initiator, Arp2/3 complex can also bind to the side of a pre-existing actin filament and start to initiate a branched actin filament, with an angle of 70 degree between the newly formed and pre-existing filaments. By repeating this branching process, actin filaments could form a dendritic actin network, which is observed predominately in the lamellipodia at the leading edge (Fig. 1.1). By microinjecting antibodies that inhibit dendritic branching by Arp2/3 complex, lamellipodia formation is inhibited (Bailly et al., 2001). The Arp2/3 complex itself is intrinsically inactive because the two actin related proteins - Arp2 and Arp3 - are too far apart to form the dimer to which new G-actins can be added (Robinson et al., 2001). Therefore in order to activate Arp2/3, regulators are required.

Two of the endogenous regulators of Arp2/3 are WASp (Wiskott-Aldrich Syndrome protein) and WAVE/SCAR, both of which are members of the WASp family of proteins (Machesky and Insall, 1998). Studies have shown that WASp and WAVE cooperate with Arp2/3 complex to stimulate formation of new actin filaments or branching of a pre-existing actin filament. The activation of Arp2/3 by WASp and WAVE depends on their C terminal VCA/WCA domain (V: verprolin homology domain; C: cofilin homology sequence; A: acidic domain) (Marchand et al., 2001). VCA can bind to both actin monomers and Arp2/3 complex and then initiate actin polymerization. To avoid spontaneous activation of Arp2/3 complex without a signal input, WASp and WAVE are carefully regulated by the Rho family of small GTPases (Rho, Cdc42 and Rac). WAVE and WASp are believed to function in the formation of filopodia and lamellipodia respectively, which may not always be the case since people have also

observed WASp activity in lamellipodia and WAVE activity in filopodia (Takenawa and Miki, 2001) (Lorenz et al., 2004).

WASp

WASp proteins (ubiquitous WASp and hematopoietic specific N-WASp) are found predominantly in an auto-inhibition conformation. Their C-terminal VCA domain binds intra-molecularly to a basic sequence and to the GTPase binding domain (GBD) in the N-termini. WASp proteins activation of Arp2/3 complex is therefore, occluded. When small GTPase Cdc42 binds to the GBD domain, the VCA domain is released from inhibition and is able to activate Arp2/3 complex. Adapter proteins containing Src homology (SH) domain (SH2, SH3) such as NCK can also activate WASp by binding to its poly-proline region. By binding to the basic region of WASp, PIP2 can further activate WASp in cooperation with NCK or Cdc42. (Higgs and Pollard, 2000; Kim et al., 2000; Martinez-Quiles et al., 2001; Rohatgi et al., 2001).

WAVE complex

WAVE protein regulates actin polymerization by mediating the signal of Rac to Arp2/3 in lamellipodia. It is involved in forming branched and cross-linked actin networks. It was first identified in *Dictyostelium discoideum* as a suppressor of mutations in a cAMP receptor (SCAR) (Bear et al., 1998), and then its homologues were found in other organisms, including humans. There is only one isoform of WAVE protein in *C.elegans* and *Drosophila*, but there are three in mammals: WAVE1, WAVE2 and WAVE3. WAVE2 is reported to be expressed ubiquitously, whereas WAVE1 and

WAVE3 are mostly found in the brain (Suetsugu et al., 1999). All WAVEs contain a N-terminal WHD/SHD (WAVE/SCAR homologue domain), a central proline-rich region and a C-terminal VCA domain. Unlike WASp proteins, which are intrinsically inactive by auto-inhibition and activated by directly binding to Cdc42, PIP2 etc (Kim et al., 2000), WAVE proteins are intrinsically active *in vitro* (Eden et al., 2002; Machesky et al., 1999; Miki et al., 2000). In the cell however, the majority of WAVE proteins form a complex named "WAVE complex" with four other proteins: Hem/kette/Nap125/Nap1/NCKAP1, Sra-1/PIR121/CYFIP, Abelson interacting protein (Abi) and HSPC300 (Fig. 1.1). No direct binding of WAVE to Rac has been discovered. The molecular components of the WAVE complex are confirmed for both the ubiquitously expressed WAVE2 and the brain specific WAVE1, either *in vitro* or *in vivo* (Ismail et al., 2009; Padrick et al., 2008) by size exclusion chromatography, ultracentrifugation, immuno-precipitation and cell free reconstitution. In various cells and tissues, different paralogous subunits are recruited into the WAVE complex. In fact, even in a single cell the coexpression of several paralogous subunits might create different WAVE complexes (Derivery et al., 2009). Despite of this complication however, the WAVE complexes formed by paralogous subunits share the same molecular organization. Yet, different paralogous subunits might mediate different signals into the complex and mediate various responses.

In the WAVE complex, direct association between WAVE, Abi and HSPC300 represents the backbone of WAVE complex. Hem binds to Sra-1 forming a sub-complex, which is able to bind to Rac through Sra-1 (Bogdan et al., 2004; Kitamura et al., 1997). The interaction between Abi and Hem is what binds Hem and Sra-1 into the complex

(Derivery et al., 2009; Ismail et al., 2009). Nevertheless, controversy on how this complex is organized exists. For example, it is still unknown whether WAVE protein is active or inactive in this complex, how this complex is activated by Rac, whether this complex remains in contact or is dissociated into sub-complexes after activation and how this complex is site-directed to the leading edge of migrating cells.

When the WAVE complex (WAVE1) was first purified from bovine brain, it was found to be inactive and unable to promote actin polymerization *in vitro* (Eden et al., 2002). Reconstitution of the WAVE complex showed the same inhibition of WAVE protein, most likely due to the inhibition of VCA domain by Sra-1 or Hem (Derivery et al., 2009; Ismail et al., 2009). Genetic analysis in *Drosophila* supports this idea by showing that the removal of one copy of WAVE could rescue the loss of function of Hem phenotype (Bogdan and Klambt, 2003). Yet this inactive WAVE complex model is challenged by findings that the WAVE complex (WAVE1 and WAVE2) is active when purified from similar materials. Addition of subunits *in vitro* also failed to inhibit the constitutive activity of WAVE (Innocenti et al., 2004; Kim et al., 2006). Moreover, the depletion of one component either by RNAi treatment or mutation leads to degradation of the remaining subunits (Kunda et al., 2003; Rogers et al., 2003; Schenck et al., 2004). By fractioning cells and assaying WAVE protein from cytosolic and membrane fractions, more controversies are brought into this debate, which argues that the cytosolic pool of the WAVE complex is inactive when the WAVE complex in the membrane is active (Suetsugu et al., 2006).

How WAVE complex mediates the signaling from Rac to Arp2/3 complex is also controversial. Different from WASp, WAVE protein does not have a GBD domain. Therefore, there is no direct interaction between WAVE and Rac. IRSp53 has been shown to bind to WAVE directly and is an essential intermediate between Rac and WAVE, mediating the activation of WAVE by Rac (Miki et al., 2000). In addition to direct and positive regulation of WAVE, another indirect regulation model has been proposed. It suggests that the activation by small GTPase Rac and adapter protein NCK could disassemble the trans-inhibited WAVE complex. This will release WAVE associated with HSPC300 and a subcomplex of Hem, Sra-1. Released WAVE protein is then able to activate Arp2/3 complex (Eden et al., 2002). An engineered WAVE complex in which the WAVE PR region was replaced with a PreScission protease cleavage site further showed that it was the release of VCA domain from the WAVE complex that is responsible for the activation. VCA domain in the WAVE complex is initially masked by Sra-1 and/or Hem through weak interactions between WAVE and Sra-1-Hem sub-complex. Rac could bind to Sra-1 and remove the inhibition. However, no dissociation of the WAVE complex after Rac activation was observed (Ismail et al., 2009). Since the WAVE-Abi-HSPC300 sub-complex is active (Padrick et al., 2008), it is possible that dissociation of the WAVE complex either by releasing the WAVE-Abi-HSPC300 or by no dissociation but, the release of VCA domain of WAVE protein can both remove the inhibition on WAVE protein and activate Arp2/3 complex. Different to this but consistent with the active WAVE complex hypothesis, the activation of Rac has been shown to neither significantly increase the activity of WAVE complex, nor disassemble the complex. Instead, expression of a constitutive active Rac (Rac^{Q61L}) or stimulation with

EGF, induces re-localization of WAVE complex to the leading edge of ruffles. This is consistent with the finding that all components of the WAVE complex are found together at the lamellipodia (Steffen et al., 2004). Thus it was envisioned that rather than being activated by Rac, WAVE complex is inherently active. Through an interaction with Rac, WAVE was recruited to the lamellipodia where actin polymerization required for membrane protrusion is initiated and regulated. The integrity of the complex is critical for its proper localization since removal of either WAVE or Abi prevents its translocation to the leading edge of the lamellipodia (Innocenti et al., 2004). This is supported by RNAi treatment in S2 cells (Rogers et al., 2003). These controversies may be a consequence of the methods by which different groups have purified the WAVE complex or reconstituted the WAVE complex; the complex formed may not behave exactly as the native complex. To purify the WAVE complex, many steps of chromatography are usually applied, which may cause the dissociation of the complex or denaturation. For the reconstituted WAVE complex, subunits could bind to each other; however they might not assemble like the native complex. Moreover, the use of different cell lines and homologues proteins might also lead to these discrepancies. Furthermore, most of these experiments are based on *in vitro* assays, which may not accurately represent the true situation *in vivo*. Therefore, establishing a good *in vivo* model could provide better answers to these questions.

Phosphorylation provides additional regulation to the WAVE complex. The Abelson tyrosine kinase (Abl) has been shown to be recruited to WAVE2 by Abi after cell stimulation. This triggers the translocation of Abl together with the WAVE complex to the leading edge and the phosphorylation of WAVE2 by Abl. Mutation of tyrosine

residue Y150, the major site of phosphorylation by Abl in WAVE2, abrogated WAVE2 driven actin polymerization. This indicates that the phosphorylation of WAVE2 by Abl is required for the activation of WAVE complex (Leng et al., 2005; Stuart et al., 2006). The phosphorylation of WAVE3 by Abl has also been shown to be critical for the formation of lamellipodia and cell migration after growth factor stimulation (Sossey-Alaoui et al., 2007). In addition to Abl, WAVE1 has also been shown to be phosphorylated by Cdk5. The phosphorylation however inhibited its ability to activate Arp2/3 complex and initiate actin polymerization (Kim et al., 2006). Casein kinase 2 may phosphorylate the VCA domain of WAVE2 as well as that of WASp. Nevertheless, this phosphorylation of WAVE decreased Arp2/3 activity but increased Arp2/3 activity in the case of WASp (Cory et al., 2003; Pocha and Cory, 2009). The importance of the phosphorylation of WAVE, as well as other post-translation modifications however, need to be further elucidated.

HEM-protein

HEM-protein (Hem, also known as Kette/dhem2/Hem-2/NCKAP1/Nap1/ Nap125) belongs to a highly conserved Hem family from invertebrates to mammals ranging from 1118 to 1126 amino acid residues (Baumgartner et al., 1995). All Hem members, from *C. elegans* (gex-3) to humans (NCKAP1/NAP1) are preferentially expressed in the nervous system; only *Hem-1* gene in humans is expressed in hematopoietic cells (Weiner et al., 2006). In *Drosophila*, *Hem (kette/dhem2)* encodes a protein of 1126 amino acids with a calculated molecular mass of 129 kDa. Hem is maternally expressed during the early stages of embryogenesis but is then specifically

expressed in the nervous system: the brain and the VNC. Analysis of its sequence predicts it to have six transmembrane domains (Baumgartner et al., 1995) but has not been further confirmed. Most of the Hem is present in the cytosol and only very little is found to be localized to the membrane in *Drosophila* S2 cells (Bogdan and Klambt, 2003).

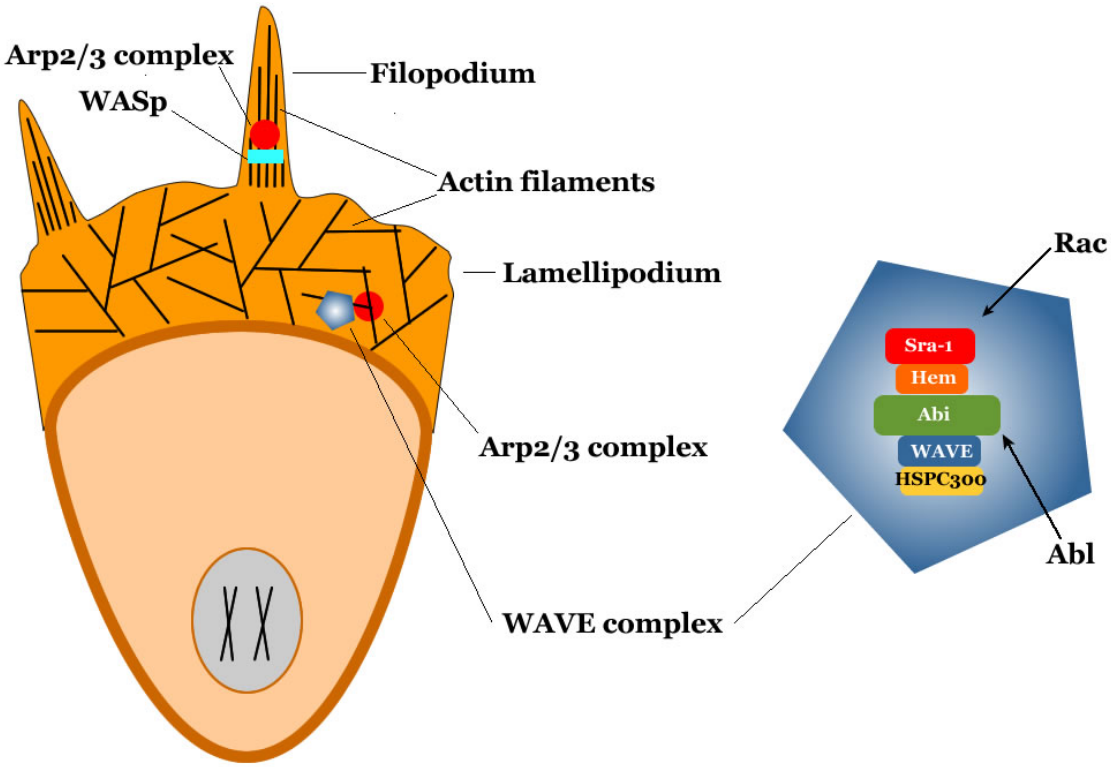


Fig. 1.1 WASp and WAVE complex are involved in the regulation of actin cytoskeleton networks in the leading edge of migrating cells. When activated by WASp, Arp2/3 is able to initiate actin polymerization, forming paralleled actin filaments in the filopodium. When activated by WAVE complex, Arp2/3 can initiate actin polymerization *de novo* or promote branching of existing actin filaments, forming a rigid, cross-linked actin cytoskeleton network in the lamellipodium. Different from auto-inhibition of WASp, WAVE and four other proteins (Sra-1, Hem, Abi and HSPC300) form the WAVE complex. Rac can activate the WAVE complex. Abl can be recruited to the WAVE complex by Abi and regulates the activity of the WAVE complex.

It has been shown that Hem and its homologues participate in many important biological processes. Analysis of human *NCKAP1*/*Nap1* gene shows that it is expressed preferentially in neurons and that the suppression of human *NCKAP1* transcripts could induce apoptosis in neuroblastoma cell lines (Suzuki et al., 2000). NCKAP1 has also been shown to be selectively expressed in the cortical plate region of the developing cortex and mediates the cytoskeleton rearrangements in the emerging cortical plate, which plays an essential role in cortical neuronal differentiation. In *Nap1* mutant cells, actin filaments accumulated at the edge of telencephalic neuroepithelial cells and a 90% reduction in lamellipodia was observed (Yokota et al., 2007). Observation of cell migration *in vitro* showed that Hem can also promote migration of U251 cells via OL-protocadherin-Nap1 interaction (Nakao et al., 2008). No knockout mice are available at this time (http://www.knockoutmouse.org/genedetails/MGI:1355333, 5/2010). Analysis of *Hem* alleles in mice exhibits growth arrest at midgestation, an open neural tube, cardia bifida, defective foregut development, defects in endoderm and mesoderm migration and sometimes duplication of the antero-posterior body axis (Rakeman and Anderson, 2006). In *Drosophila*, Hem is required for the formation and maturation of neuromuscular junction (NMJ). In certain hypomorphic *Hem* allele combinations, smaller neuromuscular junctions were observed (Bogdan et al., 2004). Hem has also been shown to be necessary for correct axon pathfinding during development of the nervous system. Axon pathfinding defects were observed in *Hem* mutants such that VUM neurons changed their growth directions (Hummel et al., 2000). Moreover, mutations in *Hem* led to enlarged foci that did not dissolve, similar to the observed block in myoblast fusion, which is

crucial for the formation and repair of skeletal muscle (Richardson et al., 2007; Schäfer et al., 2007; Schroter et al., 2004).

Hem is involved in these events mostly through dynamically modulating the actin cytoskeleton networks. As mentioned before, Hem forms the WAVE complex with four other subunits. The WAVE complex, by controlling the activity of Arp2/3 complex, regulates the initiation and/or branching of actin filaments. On this account, by regulating the activity of the WAVE complex and its localization in the cell, Hem is able to regulate the actin polymerization in the cell and thus the structure of the actin cytoskeleton networks. Although much is known, how Hem regulates the activity of the WAVE complex and its localization remains unclear. So far most of the evidence has been derived from in *vitro* cultured cell models and reconstitution analysis. Hem has been shown to inhibit the activity of WAVE by precluding its VCA domain from exposure in the WAVE complex. When cells are stimulated, this inhibition is removed by dissociation of Hem from the WAVE complex or, by conformational change that expose the VCA domain of WAVE. Consistent to this, people have shown that loss of function for *Hem* resulted in an excess of F-actin in the cytosol. This indicates the hyper-activity of WAVE protein. By reducing the *WAVE* gene dose, the *Hem* mutant phenotype can be suppressed (Bogdan and Klambt, 2003; Yokota et al., 2007). Depletion of Hem through RNAi or genetic mutations however, has been shown to lead to less WAVE (Innocenti et al., 2004; Schenck et al., 2004; Steffen et al., 2004). Since WAVE protein is required to promote polymerization of the actin filaments, less F-actin should be anticipated in *Hem* mutants. Different to inhibition, some researchers have also claimed that WAVE is not

inhibited but activated by Hem in the WAVE complex (Innocenti et al., 2004). Despite its role in the regulation of the activity of WAVE, Hem was also shown to be involved in the regulation of the translocation of WAVE2 in fibroblasts. In *Hem* knock-out cells, WAVE2 failed to be translocated to the cell periphery (Steffen et al., 2004). Furthermore, Hem has also been shown to bind to the first Src homology 3 (SH3) domain of Nck/Dock, which is an adaptor molecule containing one SH2 domain and three SH3 domains and links several receptor tyrosine kinases (RTKs) to the cytoskeleton (Kitamura et al., 1996; Li et al., 2001). Therefore, Hem may mediate extracellular signals into intracellular cytoskeleton. Furthermore, Hem has also been shown to activate WASp in the membrane (Bogdan and Klambt, 2003). Since WASp is also able to promote actin polymerization, Hem may then regulate the actin dynamics through regulating WAVE and/or WASp. How Hem regulates the dynamics of the actin cytoskeleton and is involved in biological processes such as axon pathfinding, cell proliferation, cell migration, cell differentiation etc. *in vivo* is yet to be discovered.

The active migration of RP2/Sib cells in the Drosophila Ventral Nerve Cord (VNC)

We have undertaken to study migration using the model organism *Drosophila melanogaster*. It is a widely used genetic model system for over 100 years, and has only three major chromosomes. Nearly all the genes in *Drosophila* have counterparts in humans; thus, our studies should be generally applicable across many organisms, including humans. *Drosophila* also has a short lifespan, ease of maintenance, strong genetics, and amenability to cell biological and biochemical analysis. These properties of *Drosophila* make it one of the best model systems to study the above questions.

Extensive research on cell migration has been carried out using *Drosophila* as an animal model (McDonald and Montell, 2005). The germ cells for instance, migrate as single cells in a stereotyped manner and are guided by repellent and attractive cues toward the somatic gonad in the mesoderm (Ricardo and Lehmann, 2009). Analysis of mesoderm migration during gastrulation provides additional insights into collective cell migration during development (Kadam et al., 2009; McMahon et al., 2008). Despite of these work, few studies have been done to illustrate how cell migration is regulated during development of the nervous system in *Drosophila*. The only published work on this topic was from our laboratory (Bhat, 2007).

The embryonic CNS of *Drosophila* has become an important model system for investigation of neuronal development. During development of *Drosophila* embryo, the precursors of the CNS drive from the neurogenic regions of the ectoderm: the ventral neurogenic region gives rise to the neuroblasts of the VNC and the pro-cephalic neurogenic region generates the brain (Hartenstein, 1993). So far, most of the investigations on development of the embryonic CNS have been carried on the VNC. The VNC of the *Drosophila* embryo consists of segmental repeated units, each of which contains about 320 neurons and around 30 glial cells generated by about 30 Neuroblasts (NBs). In the developing VNC, NB stem cells are delaminated from the neuroectoderm, and then are divided to produce ganglion mother cells (GMCs), which then divide again to form neurons. The NB4-2→GMC-1→RP2/sib lineage is one of the most typical and well-studied neuronal cell lineages in the *Drosophila* VNC (Bhat, 1996, 1999, 2007; Bhat and Apsel, 2004; Bhat et al., 2007; Buescher et al., 1998; Wai et al., 1999). A wealth of

information is available in terms of genetic regulation. Cells in this lineage can be distinguished either by specific gene expression patterns, size differences or their positions within the para-segment in the VNC (Buescher et al., 1998; Gaziova and Bhat, 2007). NB4-2 is formed as one of the thirty or so NB stem cells during the second wave of neuroblast delamination in mid stage 9 (approximately 4.5 hour old) during embryogenesis. The NB4-2 undergoes its first asymmetric division (approximately 6 hour old) to produce another NB and to its first GMC, GMC-1 (also called GMC4-2a). GMC-1 then divides (approximately 7.5 hour old) asymmetrically into a motor neuron RP2 neuron and its sibling cell sib. The ultimate identity of sib is unknown. There is a size difference between a GMC-1 (~7.5 μm), an RP2 (~5 μm) and a sib (~3 μm). Besides that, there is also a level difference in marker gene expression. Even-skipped (Eve) is a transcription factor that is expressed in GMC-1, a newly formed RP2 and sib. Sib begins to lose its Eve expression soon after formation and thus, only RP2 is positive for Eve in embryos at late stages (~14 hr). In addition, several marker genes are expressed in RP2 neurons but not sib cells like Mab 22C10 and Zfh-1. By using these genetic markers, cell size differences, as well as their position in the para-segment, we can very easily identify GMC-1, RP2 neuron and sib cell in the VNC (Bhat et al., 2000; Buescher et al., 1998; Gaziova and Bhat, 2007).

An active migration of the NB4-2→GMC-1→RP2/sib cell lineage during development of the *Drosophila* VNC was first reported by Bhat (Bhat, 2007). He divided the sequence of migration of this lineage into three steps. In step 1, immediately following formation from GMC-1, RP2 and sib move toward the midline. Then they

move downward crossing the parasegmental boundary (step 2). Following step 2, they migrate upward, re-crossing the parasegmental boundary (step 3) and then finally occupying their ultimate positions. The RP2 neuron migrates to its specific position within the anterior commissure and projects its axon antero-ipsilaterally to the intersegmental nerve bundle (ISN) and innervates muscle #2 on the dorsal musculature. The sib cell migrates to a position more posterior and dorsal to RP2 neuron. This neuronal migration pattern has been shown to be determined by *wingless (wg)* activity in the precursor neuroectodermal and neuroblast levels. In embryos mutant for *wg*-signaling, RP2 neurons are mislocated. Moreover, at least two downstream genes, Cut and Zfh1 are involved in this process at the downstream neuronal level (Bhat, 2007). I wished to expand on these results and understand more about the underlying mechanisms responsible for this complex migration pattern. Therefore, I sought to identify and study additional genes involved in this migration process. I found that in *Hem* mutants, RP2 neurons cross the midline and migrate from the initial hemi-segment to the opposite hemi-segment. This migration defect of RP2 neurons results in two RP2 neurons in one hemi-segment at the expense of the opposite hemi-segment. The same migration defect was observed in *WAVE* and *Abl* mutants, indicating that they may act together with *Hem* to regulate the neuronal migration. WAVE has been shown to be required for the migration of RP2 neurons. RP2 neurons stop migration in the absence of WAVE. Hem is necessary to maintain certain protein level of WAVE. In *Hem* mutants, the protein level of WAVE is down regulated and it is mis-localized in RP2 neurons, which may result in the migration defect. Abl negatively regulates the protein level of WAVE. The ectopic expression of *Abl* leads to the down regulation of WAVE. In *Abl* mutants, the protein

level of WAVE is up regulated. Since the ectopic expression of WAVE did not display any migration defect, Abl appears to be in part involved in the inactivation of activated WAVE. In *Abl* mutants, the hyperactivity of WAVE may cause the migration defect of RP2 neurons. My study uses *Drosophila* VNC as the model system and describes how cell migration is dynamically regulated in *vivo* during development of the VNC. It will not only help us understand the development of the *Drosophila* VNC better but may also improve our general understanding of genetic regulation of cell migration in the CNS.

Materials and Methods

Mutant strains and genetics

All flies and crosses were performed at 22 °C using standard methods unless otherwise indicated. Various mutant combinations were generated through standard genetics. The following alleles were used: $Hem^{J4\text{-}48}$, $Hem^{C3\text{-}20}$, *Df(3L)ED230, ,* wg^{ts}, en^{E}, $insc^{22}$, nb^{796}, Abl^{2}, *UAS-Abl.F, Df(3L)st-j7*, $WAVE^{\Delta 37}$, $WASp^{EY0623}$ from Bloomington Drosophila Stock Center; *UAS-AblRNAi, UAS-HemRNAi* from VDRC stock center*; UAS-WAVE* from Dr. Jennifer Zallen; *w;P{SCARK*^{13811}*,W+} FRT40A/Cy, w;*Wsp^{1}*/TM6B,* Wsp^{3}*/TM6B* from Dr. Eyal D Schejter. To induce the ectopic expression of genes of interest, the following Gal4 drivers were used: the proneural driver *sca-Gal4* and the maternally expressed driver *P{GAL4::VP16-nos.UTR}MVD2*. w^{1118}*;Gla/Cyo twi-GFP* and w^{1118}*;*Dr^{Mio}*/TM3 twi-GFP* were used as GFP balancers and *TM3/TM6B ftz-lacZ* and *TM3/TM6B ase-lacZ* were used as lacZ balancers. *hs-FLP;* Adv^{1}*/CyO, hsFLP;* Dr^{Mio}*/TM3*

Sb^{1}*, P{neoFRT}82B, P{neoFRT}82B P{ovoD1-18}3R, P{FRT(whs)}2A, P{ovoD1-18}3L P{FRT(whs)}2A* were used to generate mosaic flies. Homozygous embryos are GFP negative or lacZ negative. To exclude the possible maternal modifier effects of balancers (see Bhat et al., 2007;(Gaziova and Bhat, 2009), homozygous mutant embryos were also tested by out-crossing the balancer-bearing mutants (mutant/balancer) to wild type and backcrossing the non-balancer bearing mutant adults (mutant/+). Staging of embryos was done as described by Wieschaus and Nusslein-Volhard (Wieschaus and Nusslein, 1986).

Whole mount immunohistochemistry

Whole mount immunohistochemistry was done using standard procedures, with antibody-specific modifications. In brief, embryos were collected, dechorionated with 50% bleach for at least 3 minutes and then rinsed with water. The embryos were then fixed in formaldehyde and n-heptane, the detail of which depends on specification of the primary antibody. 0.8 mL CFB (Common fixation buffer: 0.1 M PIPES, 2 mM EGTA, 1 mM MgSO4, pH= 6.9-7.0) and 1.0 mL n-heptane for 30 seconds followed by addition of 0.2 mL formaldehyde for another 25 minutes (we designate this as E-fix) worked for anti-single-minded (Sim). 0.8 mL n-heptane and 0.8 mL formaldehyde for 6 minutes (we designate this as K-fix) worked for 22C10 (stain MAP1B), BP102, anti-Even skipped (Eve), anti-Zn finger homeodomain 1(Zfh-1) and anti-Fasciclin 2 (Fas II). A chemical "popping-off" by rapid shaking on a methanol-heptane interface was used to peal the vitteline membrane.

To use HRP conjugated secondary antibodies, endogenous peroxidase enzyme is inactivated through incubation with 3% hydrogen peroxide for 15 minutes [not necessary for alkaline phosphatase (AP) reactions or fluorescence-conjugated secondary antibodies]. Embryos were then rehydrated and permeabilized with PBST (PBS, 0.05% Triton X100), and were blocked in blocking buffer (PBST, 1% BSA and 5% NGS) for 2 hrs at room temperature. Primary antibodies were added after blocking, kept at 4 °C overnight and followed by secondary at room temperature on a rotator for 2 hrs. For HRP conjugated secondary antibodies, embryos were incubated with 0.5 mg/mL DAB (3,3'-diaminobenzidine) in PBST for 10 minutes and for another 3 ~ 5 minutes with additional 3 mL of 3% H_2O_2. Color reaction was inactivated by rinsing embryos with PBST twice and then PBS twice. Embryos were stored in PBS containing 70% glycerol. For fluorescence conjugated secondary antibody, no color reactions are needed; embryos were mounted in mounting medium (VectorShield) and kept at – 20 °C.

The following primary antibodies were used in whole mount immunochemistry: Eve (from Dr. Manfred Frasch rabbit, 1:2000 dilution), Eve (from DSHB antibody bank, mouse, 1:5 dilution), Zfh1 (from DSHB antibody bank, mouse, 1:400), 22C10 (from DSHB antibody bank, mouse, 1:4 dilution), LacZ (from DSHB antibody bank, rabbit, 1:3000 or mouse, 1:400), BP102 (from DSHB antibody bank, mouse 1:10) Fas II (from DSHB antibody bank, mouse, 1:5), Sim (rat, 1:200), Wave (from Dr. Jennifer A. Zallen, Genius pig, 1:100). For confocal microscopy of embryos, Cy5 and FITC-conjugated secondary antibodies were used. For light microscopy, alkaline phosphatase (AP) or DAB-conjugated secondary antibodies were used. Transmitted-light images were

obtained using a ZEISS Axioplan2 microscope. Fluorescent images were obtained using ZEISS Axioplan2 microscope and a Bio-Rad Radiance 2100 confocal system. Images were prepared using Adobe Photoshop.

Generating germline mosaic animals

Many genes involved in cell migration show maternal effect. The maternal contributions of the wild type gene products could be deposited in the oocyte, thereby rescuing the loss of function for these genes in homozygous zygotic mutant embryos to some extent. We overcome this problem by generating germline mosaics, in which the maternal contribution of wild type gene products are completely depleted, thus providing a powerful tool to study recessive lethal mutations in early developmental stages. The heat shock driven FLP recombinase (hs-FLP) and Flippase Recognition Target (FRT) mediated mitotic recombination, combined with DFS mutation ovo^{D1} were used in this study to generate germline mosaic flies. To generate WAVE germline clones, $WAVE^{K13811}$ neoFRT40A/CyO was crossed to hs-FLP;Gla/CyO, generating hs-FLP; $WAVE^{K13811}$, neoFRT40A/CyO. hs-FLP; $WAVE^{K13811}$ neoFRT40A/CyO were crossed to ovo^{D1} FRT40A and raised on standard apple juice-agar-yeast medium plates at 26 ºC. To obtain germline clones, early stage larvae (48h ~ 72h) were heat-shocked for 60 minutes at 37 ºC and then again 24 hours later. Heterozygous ovo^{D1} females do not lay eggs. When FLP-FRT driven mitotic recombination occurs, female germ cells eliminated the ovo^{D1} mutation and homozygous for $WAVE^{k13811}$ were generated, producing eggs without the maternal wild type WAVE contribution. Adult hs-FLP; $WAVE^{K13811}$, *neoFRT40A/* ovo^{D1}, FRT40A females were crossed to WAVE deficiency *Df(2L)BSC32* males. Embryos homozygous

for loss of function for WAVE and without the maternal contribution of wild type WAVE were collected for analysis, named *WAVE*^{mat}.

Western-blotting experiments

Embryos were collected, decorionated in 50% bleach for at least 3 minutes and rinsed with water. Twenty embryos were then collected under the microscope, homogenized in 37.5 μL Lysis Buffer (0.15 M NaCl, 0.02 M Tris pH=7.5, 0.001M EDTA, 0.001 M $MgCl_2$, 1% Triton-X-100 and PIC) and kept on ice for 10 minutes. After centrifugation for 1 minute at 13,000 rpm, the supernatant was collected and diluted with 12.5 μl 4×Laemmli sample buffer. The mixture was boiled in water for 10 minutes and kept in 4 ºC for 10 minutes. Equal amount of 10 μL lysates (4 embryos) were separated in 8% SDS-PAGE. Proteins were then transferred to a nitrocellulose (NT) membrane (Whatman). Ponceau S stain was used to determine transfer efficiency. After blocking in 5% milk overnight at 4 ºC, primary antibodies were incubated for 2 hours at room temperature and washed with PBST (PBS+0.02% Tween 20). The membrane was then incubated with HRP-conjugated secondary antibody for 2 hours at room temperature and washed again. Proteins were detected by ECL reaction (Thermo Scientific), scanned and analyzed.

Primary antibodies used were: Wave (from Dr. Jennifer A. Zallen, Genius pig, 1:1,500), Hem (from Dr. Klämbt Christian, rabbit 1:1,000), α–tubulin (Abcam, rabbit, 1:4,000) used as the loading control.

Generating transgenic animals

To determine the antimorphic phenotype of truncated Hem Δ Hem$^{J4\text{-}48}$, we used Gal4-UAS system to drive ectopic expression of Δ Hem$^{J4\text{-}48}$. The *UAS-* Δ *Hem*$^{J4\text{-}48}$ construct was made by amplifying the coding fragment of the first 489 amino acids of Hem by PCR with primers 5'-ATAAGAATGCGGCCGCTAAACTATTGCACGCCTCCCAATACG-3' and 5'-GCTCTAGATTAGTCCAGGCGGAATGGTC-3', which incorporated a NotI restriction site in the 5' end and an XbaI restriction site and a stop codon in the 3' end. The PCR product was then digested with NotI/XbaI (NEB) and subcloned into NotI/XbaI cut pUAST plasmid. The UAS promoter is contained in the pUAST plasmid therefore UAS-Δ Hem$^{J4\text{-}48}$ construct was generated. To avoid a possible error that might be introduced into the sequence by PCR reaction, the PCR product was sequenced (UTMB Molecular genomics core) and confirmed to be error free. The pUAST- Δ *Hem*$^{J4\text{-}48}$ plasmid harvested using Maxi prep kit (QIAGEN) was injected into y w; $\Delta^{2\text{-}3}$ embryos. $\Delta^{2\text{-}3}$ mediated P element transgenesis resulted in integration of the *UAS-* Δ *Hem*$^{J4\text{-}48}$ into the genomic DNA (Microinjection by Genetivision Inc., Houston). The transgenic lines were selected for expression of the *white* gene which gives rise to orange or red eyes. The chromosome of insertion was determined by analyzing segregation of the *white* gene with dominant markers on each chromosome, several independent transgenic lines were analyzed to avoid possible effect of insertion sites (position effect).

Gal4/UAS system

Gal4/UAS technique (Brand and Perrimon, 1993b) is widely used in *Drosophila* for studying ectopic expression of various genes. It contains two parts: the *Gal4 driver*, which encodes a yeast transcription activator Gal4 and the UAS (Upstream Activation Sequence), a short piece of promoter region to which Gal4 specifically binds to and therefore drives the expression of various genes. Since Gal4 is a yeast protein that is not normally expressed in *Drosophila*, when Gal4 is placed under the control of a native driver gene, it is only expressed where the driver should be on (Brand and Perrimon, 1993a; Fischer et al., 1988). As a result, genes of interest under the control of UAS are only expressed in those specific cells.

Flies carrying Gal4 drivers and UAS responders were collected and used to set up crosses. Progeny from these crosses will carry both Gal4 drivers and UAS responders. Therefore, Gal4 drivers could specifically induce the ectopic expression of genes of interest under the control of UAS. All Gal4-UAS crosses were performed at 26 °C.

Lambda protein phosphatase (Lambda PP) treatment

Embryos were collected, dechorionated in 50% bleach and washed to remove bleach completely. 80 embryos were collected, homogenized in 150 μL Lysis Buffer (0.15 M NaCl, 0.02 M Tris pH=7.5, 0.001M EDTA, 0.001 M $MgCl_2$, 1% Triton-X-100, PIC) and kept in ice for 10 minutes. After centrifugation, supernatant was collected and incubated with Lambda PP, NEB buffer and $MnCl_2$ according to the product description (NEB, Lamda PP). In brief, 75 μL of the protein extracted was incubated with 2.5 μl

Lambda PP (100 units), 10 μL NEB buffer (10×), 10 μL MnCl2 in a total of 100 μL reaction. In control group, 2.5 μL diH_2O was added instead of 2.5 μL Lambda PP. Both Lambda PP treatment group and control group were incubated at 30°C for 30 minutes. Protein lysate was then diluted in 4×Laemmli sample buffer and subject to further analysis.

Ectopic expression of WAVE in $Hem^{J4\text{-}48}$ mutants

Gal4/UAS system was used to ectopically express WAVE in *$Hem^{J4\text{-}48}$* mutants. *UAS-WAVE,$Hem^{J4\text{-}48}$* mutants were obtained by recombinant. Obtained *UAS-WAVE, $Hem^{J4\text{-}48}$* mutants were first crossed to *sca*-Gal4/Cyo. *sca*-Gal4/+;*UAS-WAVE, $Hem^{J4\text{-}48}$*/+ flies were selected and crossed to *UAS-WAVE, $Hem^{J4\text{-}48}$*/+ at 26 °C. Embryos of several genotypes were obtained: +/+;+/+ (wild type), +/+; *UAS-WAVE, $Hem^{J4\text{-}48}$/ UAS-WAVE, $Hem^{J4\text{-}48}$* (*$Hem^{J4\text{-}48}$* mutants without ectopic expression of WAVE), *sca*-Gal4/+; *UAS-WAVE, $Hem^{J4\text{-}48}$*/+ (wild type embryos with ectopic expression one copy of WAVE) and *sca*-Gal4/+; *UAS-WAVE, $Hem^{J4\text{-}48}$/ UAS-WAVE, $Hem^{J4\text{-}48}$* (*$Hem^{J4\text{-}48}$* mutants with ectopic expression of two copies of WAVE). The expression level of WAVE could identify embryos of different genotypes. Overnight embryos were then collected, fixed and stained as described above.

Real-time Polymerase chain reaction (PCR)

Embryos were collected, dechorionated in 50% bleach and washed before RNA isolation. About 100 embryos selected under microscope were subject to RNA isolation using RNAqueous Kit (Ambion). To avoid degradation of RNA, the whole process was

performed in RNase-free environment and was completed as soon as possible. Harvested RNAs were further cleaned by phenol: chloroform and was precipitated with ethanol. Using this method, approximately 10 μg RNA was produced and was stored at -80 ºC. Primers for real-time PCR were designed and the real-time PCR experiments were done by Molecular Genomic Core facility in UTMB. Prior to the real-time PCR detection, RNA samples were treated with DNase to avoid the possible contamination of DNA. RNA samples for Real Time Analysis were quantified using Nanodrop Spectrophotometer (Nanodrop Technologies) and qualified by analysis on RNA Nano chip using the Agilent 2100 Bioanalyzer (Agilent Technologies). Synthesis of cDNA was performed with 1 μg of total RNA in a 20 μl reaction using the reagents in the Taqman Reverse Transcription Reagents Kit from ABI (#N8080234). Reaction conditions were as follows: 25 ºC, 10 minutes, 48 ºC, 30 minutes and 95 ºC, 5 minutes. Real-time PCR amplifications (performed in triplicate) were done using 2 μl of cDNA in a total volume of 25 μl with SYBR green using the SYBR Green PCR Master Mix (ABI #4364344). Relative real-time PCR assays were performed with RpL32 as endogenous control. All PCR assays were performed in the ABI Prism 7500 Sequence Detection System and the conditions are as follows: 50 ºC, 2 minutes, 95 ºC, 10 minutes**,** 40 cycles of 95 ºC, 15 seconds and 60 ºC, 1 minute.

Primers used: WAVE (Forward: 5' ACGAAGAAGCCGGATACGG 3', Reverse: 5' GAAGCTGCTCGTAGGTGCTACC 3'), Abl (Forward: GCAATTTCATCGACGACCTCA, Reverse: GACTCTGCTCCAGACTATCGCC).

Results

In the *Drosophila* VNC, there are 14 segments, each of which consists of two hemi-segments. In each hemi-segment, NB4-2 divides to produce another NB and a GMC-1. GMC-1 then divides to produce one RP2 neuron and one sib cell. Eve is a transcription factor, expressed in GMC-1, RP2 neuron and sib cell. However, it is gradually down regulated in sib cells but not in RP2 neurons. Therefore in late stage embryos, RP2 neurons can be easily recognized according to their expression of Eve, their location in the VNC and their cell size. An active migration of the NB4-2→GMC-1→RP2/sib cell lineage is observed during development of the *Drosophila* VNC (Bhat, 2007). In this three-step migration process, immediately following formation from GMC-1, RP2 and sib move toward the midline (Step 1). Then they move downward crossing the parasegmental boundary (step 2). Following step 2, they migrate upward, re-crossing the parasegmental boundary (step 3) and then finally occupying their ultimate positions. In *Hem* mutants, this migration pattern of RP2/sib is disrupted.

RP2 neurons exhibit a migration defect in embryos mutant for Hem

In the VNC of the wild type embryos, in each hemi-segment, there is only one RP2 neuron as indicated by the arrow (Fig. 1.2A). In $Hem^{J4\text{-}48}$ mutants (Fig. 1.2B), however two RP2 neurons are present in one hemi-segment. There is no RP2 neuron in the counterpart - the other hemi-segment. This suggests one of two possibilities: 1) one RP2 neuron disappears in one hemi-segment and the RP2 neuron in the opposite hemi-segment is duplicated; 2) one RP2 neuron has moved away from its initial hemi-segment,

crossed the midline and finally stayed in the opposite hemi-segment. Observation of embryonic mutants for *Hem* at early developmental stages shows that RP2 neurons doesn't disappear but crosses the midline (Fig. 1.3H). It shows therefore that RP2 neurons exhibit a migration defect in *Hem* mutants.

Two RP2 neurons are observed in the right hemi-segment in Fig. 1.2B, showing that the RP2 neuron of the left hemi-segment has moved from the left side to the right hemi-segment. Another *Hem* allele $Hem^{C3\text{-}20}$ and *Hem* deficiency *Df(3L)ED230* mutants also show the same migration defect (Fig. 1.6C-E). To avoid possible background modifications, $Hem^{J4\text{-}48}$ mutants were outcrossed to wild type and the non-balancer bearing mutant adults were back-crossed ($Hem^{J4\text{-}48}$/+ × $Hem^{J4\text{-}48}$/+). These embryos also show the same migration defect.

An interesting question is whether RP2 neuron has any preference in the direction of migration: is it from the left hemi-segment to the right hemi-segment or vice versa? To answer this question, I counted the number of hemi-segments with 2 RP2 neurons on the right side (left to right migration) and the left side (right to left migration) in the VNC of ~ 500 embryos, with anterior of the embryo being up. I then calculated the penetrance by dividing this number by the total number of hemi-segments. My data shows a 6.7% penetrance for left to right migration, and a 6.2% penetrance for right to left migration. This displays no significant preference for migration direction.

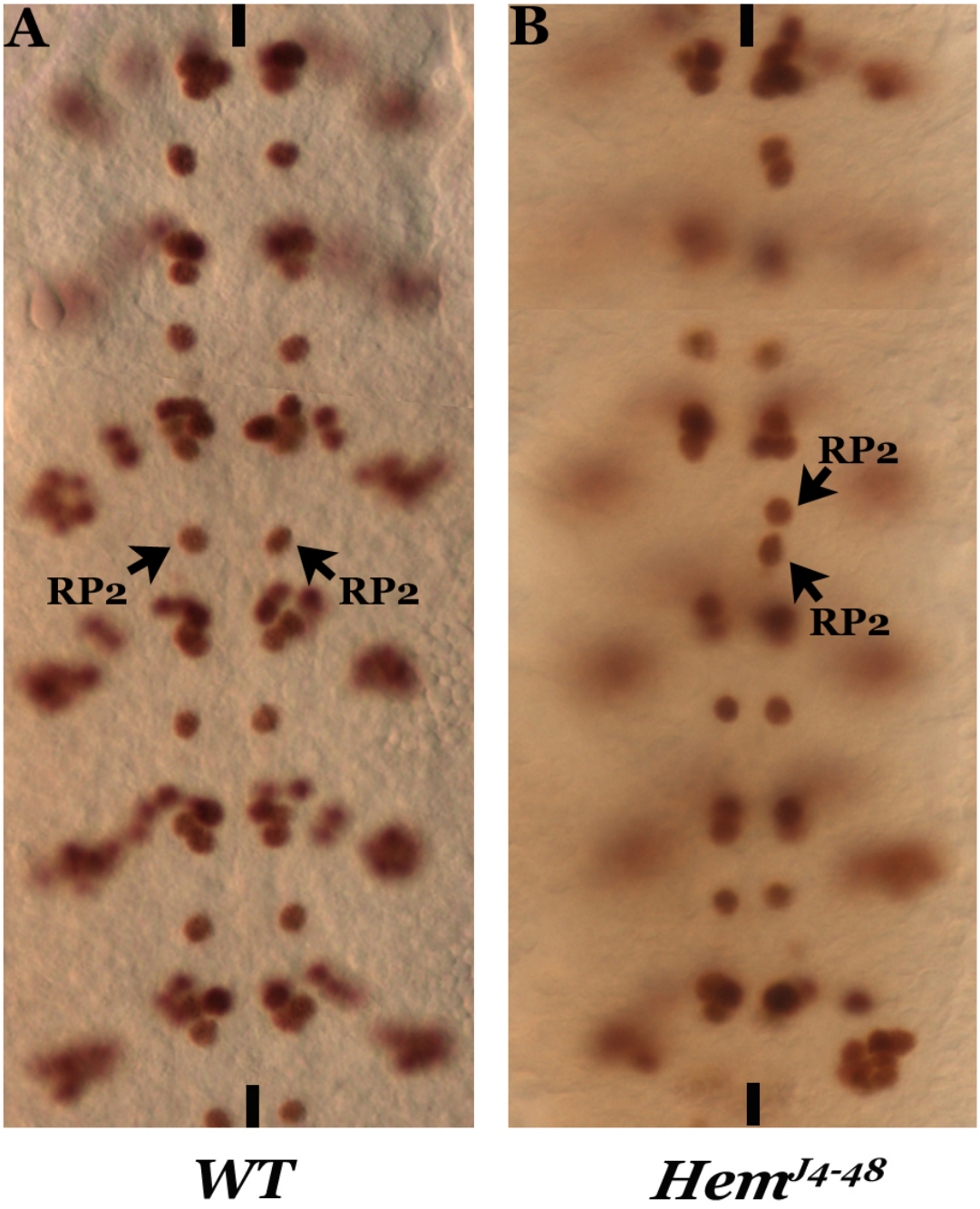


Fig. 1.2, RP2 cells display a migration defect in *Hem* mutants. Embryos are stained with antibody against Eve. A) In wild type embryos, only one RP2 neuron is present in each hemi-segment of the *Drosophila* VNC. B) In $Hem^{J4\text{-}48}$ mutants, RP2 neuron in one hemi-segment has aberrantly crossed the midline, migrated to the opposite hemi-segment, resulting in two RP2 neurons in one hemi-segment. (Anterior is up and midline is represented by the black line)

The temporal and spatial migration pattern of RP2 neurons in Hem$^{J4\text{-}48}$ mutants

Next, I wanted to follow the migration pattern of the RP2 neurons during lineage development. I carefully examined the position of RP2 neurons in *Hem* mutants at different developmental time points. As shown in Fig. 1.3A–E, in wild type embryos soon after its formation, GMC-1 begins to migrate toward the midline (Fig. 1.3A, Fig. 1.3B). During the migration, GMC-1 divides to produce an RP2 neuron and a sib cell. Both of them continue to migrate in the same direction toward the midline (Fig. 1.3B) (This is "step 1 migration", see Bhat, 2007). After that both RP2 neuron and sib cell start to migrate in the posterior direction along the midline (step 2 migration). By about 9 hours of development, both RP2 neuron and sib cell have crossed the parasegmental boundary and stopped their posterior migration (Fig. 1.3C). By about 11 hours of development, RP2 neuron begins its migration in the anterior direction, crossing the parasegmental boundary again (step 3), and residing in the location where the RP2 neuron and sib cell initiated their posterior migration (Fig. 1.3D, Fig. 1.3E).

This normal migration pattern is altered in *Hem$^{J4\text{-}48}$* mutant embryos (Fig. 1.3F – J). The step 1, where an RP2 neuron and its sib cell migrate towards the midline is not altered in *Hem$^{J4\text{-}48}$* embryos (Fig. 1.3F, Fig. 1.3G), nor the step 2 migration where the two cells migrate in the posterior direction and cross the parasegmental boundary (Fig. 1.3G, Fig. 1.3H). However, once the parasegmenatal boundary is crossed, the RP2 neuron migrates towards the midline, crosses it and resides in the opposite hemi-segment. By about 11 hour of development, RP2 neuron has completely crossed the midline and remains with the RP2 neuron of the contralateral hemi-segment (Fig. 1.3H, Fig. 1.3I).

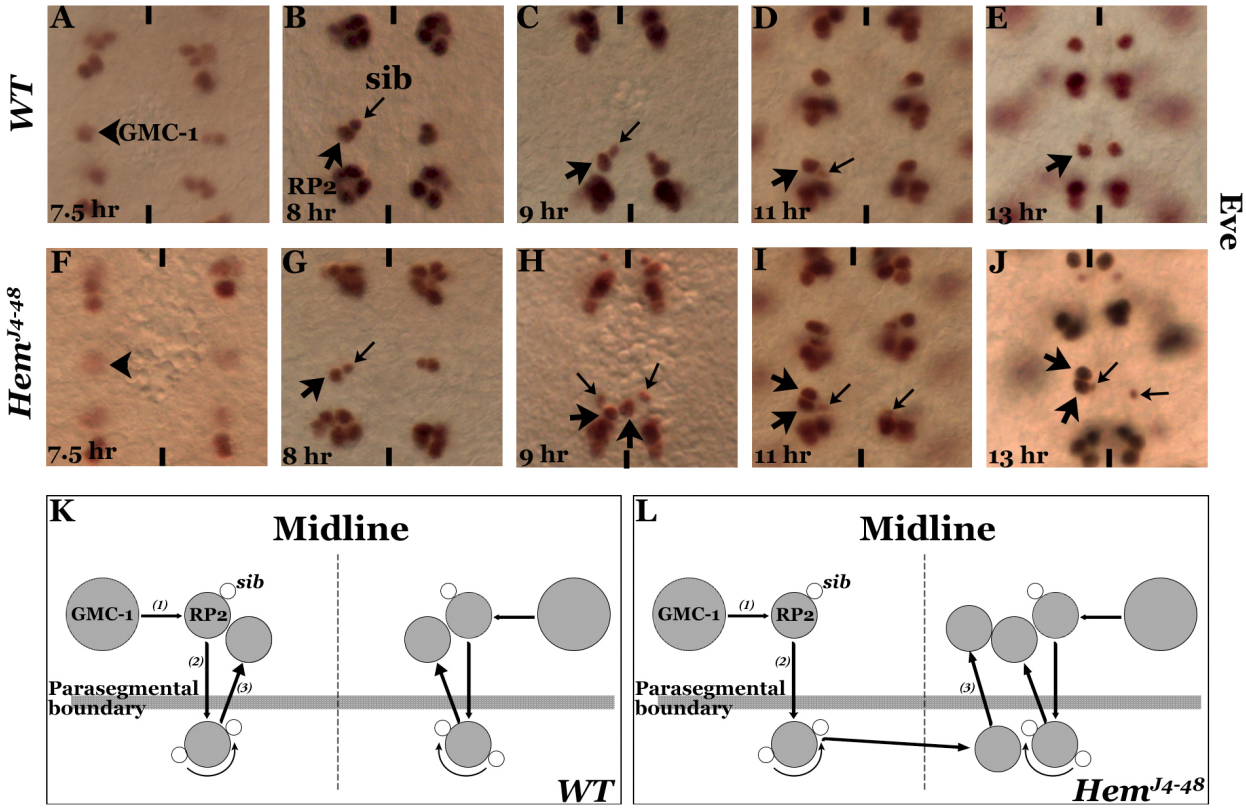


Fig. 1.3, The temporal and spatial migration pattern of RP2 neurons in wild type embryos and in *Hem*$^{J4\text{-}48}$ mutants. Embryos are stained with Eve antibody. GMC-1 is pointed by arrowhead with RP2 neuron by arrow and sib cell by small arrow. A-E) Wild type embryos in different developmental stages. GMC-1 divides to produce an RP2 neuron (arrow) and a sib cell (small arrow) around 7.5 hour (A). RP2 and sib migrate toward the midline (B), and then migrate in the posterior direction (C). After residing for about 2 hours in this location (D), they start to migrate in the anterior direction and reside in their final position (E). F-J) *Hem*$^{J4\text{-}48}$ mutant embryos in different developmental stages. In *Hem*$^{J4\text{-}48}$ mutants, after produced from GMC-1 (F), RP2 and sib cell migrate toward the midline (G), and then migrate posteriorly (H). In the mutant, RP2 neurons start to migrate towards the midline and then cross the midline to migrate to the contralateral hemi-segment (H, I). The two RP2 neurons then migrate in the anterior direction together and finally reside in the RP2 location (J). In contrast, sib cells in *Hem*$^{J4\text{-}48}$ mutant embryos keep their normal migration route. They do not cross the midline (H, I, J). K) The schematic migration pattern of RP2/sib cells in wild type embryos and L) in *Hem*$^{J4\text{-}48}$ mutants.

Both RP2 neurons then begin to migrate in the anterior direction, re-cross the parasegmental boundary and finally reside in their ultimate location in the VNC (Fig. 1.3I, Fig. 1.3J). The migration pattern of RP2 neuron in wild type embryos and in *Hem*$^{J4\text{-}48}$ mutant embryos are summarized in Fig. 1.3K and Fig. 1.3L.

The migration defect in Hem$^{J4\text{-}48}$ mutants is specific to RP2 neuron but not sib

Eve is expressed both in the RP2 neuron and the sib cell. However it is gradually down regulated in the sib cell therefore the sib cell cannot be detected in late stages of the embryos through Eve staining. By looking at early embryos, I have noticed that in *Hem*$^{J4\text{-}48}$ mutants, although the PR2 neuron migrates abnormally, the sib cell still keeps its normal migration route. As pointed out in Fig. 1.3H around 9 hour of development, while the RP2 neuron is crossing the midline toward the opposite hemi-segment, the sib cell remains in its usual position. By around 11 hour shown in Fig. 1.3I, when the RP2 neuron has completely crossed the midline, the sib cell is still in its original hemi-segment. By around 13 hours shown in Fig. 1.3J, when both the RP2 neurons have reached their ultimate position in the same hemi-segment, there is still only one sib cell in each hemi-segment. It therefore seems that the migration defect in *Hem*$^{J4\text{-}48}$ mutant embryos is specific to RP2 neurons.

To further test this conclusion, I generated double mutant embryos of *Hem*$^{J4\text{-}48}$*;insc*^{22} as well as *Hem*$^{J4\text{-}48}$*;numb*^{796}. In *insc* mutants, GMC-1 divides symmetrically to produce two RP2 neurons (Buescher et al., 1998; Mehta and Bhat, 2001) instead of one RP2 neuron and one sib cell in wild type embryos. As a result, there are two RP2 neurons

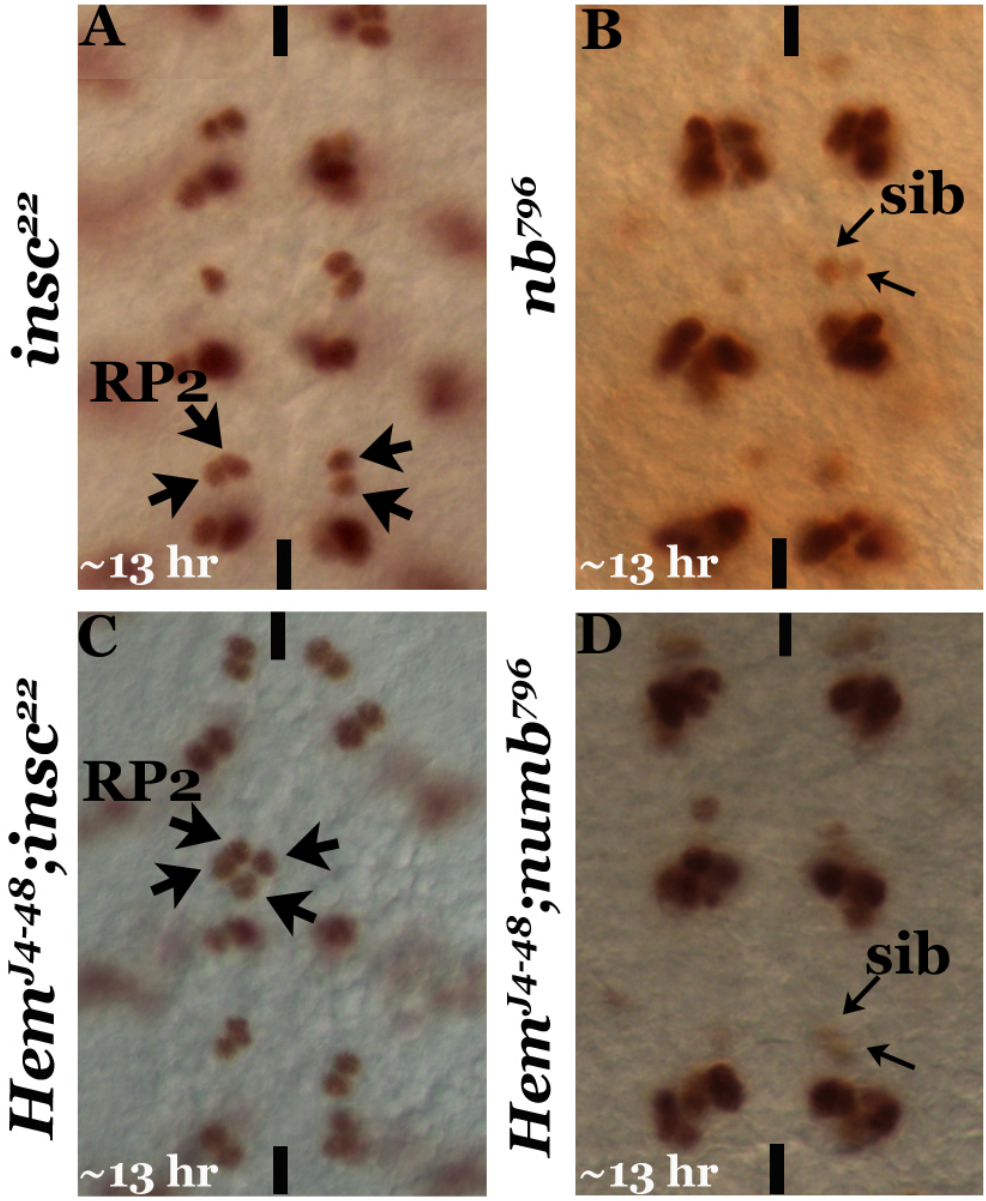


Fig. 1.4 The migration defect in *Hem*$^{J4-48}$ mutants is RP2 neuron specific. Embryos (~ 13 hr) are stained with Eve antibody. A) In *insc*^{22} mutant embryos, GMC-1 produces two RP2 neurons instead of an RP2 neuron and a sib cell due to the loss of function for *insc*. B) In *nb*^{796} mutant embryos, RP2 neuron is converted to be sib cell due to the loss of function for cell fate determinant *numb*. C) In double mutant embryos of *Hem*$^{J4-48}$;*insc*^{22}, both RP2 neurons produced by GMC-1 due to the loss of function for *insc* crossed the midline, resided in the opposite hemi-segment . D) In double mutant embryos of *Hem*$^{J4-48}$;*nb*^{796}, sib cell converted from RP2 neuron remain in their original hemi-segment and do not cross the midline.

in each hemi-segment (Fig. 1.4A). In *numb* mutants, GMC-1 divides symmetrically but to produce two sib cells in each hemi-segment (Wai et al., 1999). This is shown in Fig. 1.4B. In double mutants of $Hem^{J4\text{-}48};insc^{22}$ as shown in Fig. 1.4C, four RP2 neurons are present in one hemi-segment and none in the opposite hemi-segment, indicating that two RP2 neurons from one hemi-segment have migrated to the opposite hemi-segment. This result shows that when the sib cell is converted to an RP2 neuron, it is able to cross the midline and migrate from one hemi-segment to the opposite hemi-segment in $Hem^{J4\text{-}48}$ mutants. In comparison, in the double mutants of $Hem^{J4\text{-}48};numb^{796}$ (Fig. 1.4D), the "sib" cells show a normal migration pattern in $Hem^{J4\text{-}48}$ mutants. Therefore, the migration defect in $Hem^{J4\text{-}48}$ mutants is only specific to the RP2 neuron but not to the sib cell.

Migration defect in $Hem^{J4\text{-}48}$ mutants is mediated by an active cell migration process

Cell migration defects during development of the VNC might be caused passively by interruption of the midline glial cells, RP2 cell identity changes or disruption of the VNC structure. Is the migration defect of RP2 neurons in $Hem^{J4\text{-}48}$ mutants caused by any of the possibilities mentioned above? To answer this question, embryos were stained with antibodies that could help to determine whether any of these possibilities cause the migration defect in $Hem^{J4\text{-}48}$ mutants.

Embryos were first stained with Eve and Sim (single-minded) antibodies. Sim specifically stains the midline glial cells (Fig. 1.5A), thus can help determine if the midline gial cells are disrupted. In $Hem^{J4\text{-}48}$ mutants (Fig. 1.5B), the midline glial cells in the midline are not disrupted compared to the wild type (Fig. 1.5A), yet the RP2 cells

cross the midline. Therefore, the migration defect in *Hem*$^{J4\text{-}48}$ mutants is not caused by disruption of the midline glial cells. To examine if there is any changes in cell identity of the RP2 neuron that crosses the midline, embryos were stained with Eve and Zfh-1 antibody. Zfh-1 is a transcription factor that is expressed in RP2 neurons; therefore, it can be used to further confirm the cell identity of RP2 neurons. As shown in Fig. 1.5C in wild type embryos, both Eve (red) and Zfh-1 (green) are expressed in RP2 neurons (Yellow) and only one RP2 is observed in each hemi-segment. In *Hem*$^{J4\text{-}48}$ mutants (Fig. 1.5D), though one RP2 neuron from the right hemi-segment has crossed the midline, residing in the left hemi-segment with the other RP2 neuron, both of them express Eve and Zfh-1. Therefore, RP2 neurons do not appear to have changed their cell identity in *Hem*$^{J4\text{-}48}$ mutants. Embryos were also stained with Eve and 22C10. 22C10 is an antibody against MAP1B and it stains the axonal membrane, thus one can confirm the identity of an RP2 neuron by its axon projection. As shown in Fig. 1.5E, in wild type embryos, RP2 neurons (arrow) project their axons (small arrow) ipslaterally and fasciculate with axon of aCC neuron in the same hemi-segment. In *Hem*$^{J4\text{-}48}$ mutant embryos (Fig. 1.5F), even though RP2 neuron is mis-localized, it projects its axons contralaterally and fasciculate with axon of the aCC neuron in the opposite hemi-segment. This result not only confirms that the cell identity of RP2 neuron is unaffected in *Hem*$^{J4\text{-}48}$ mutants but also raises a very interesting question: How does an RP2 neuron remember its axon projection pattern during development even though its migration pattern has changed? We postulate that the axon projection pattern has already been established before RP2 neurons cross the midline. Further investigation is needed to provide more insight into this question.

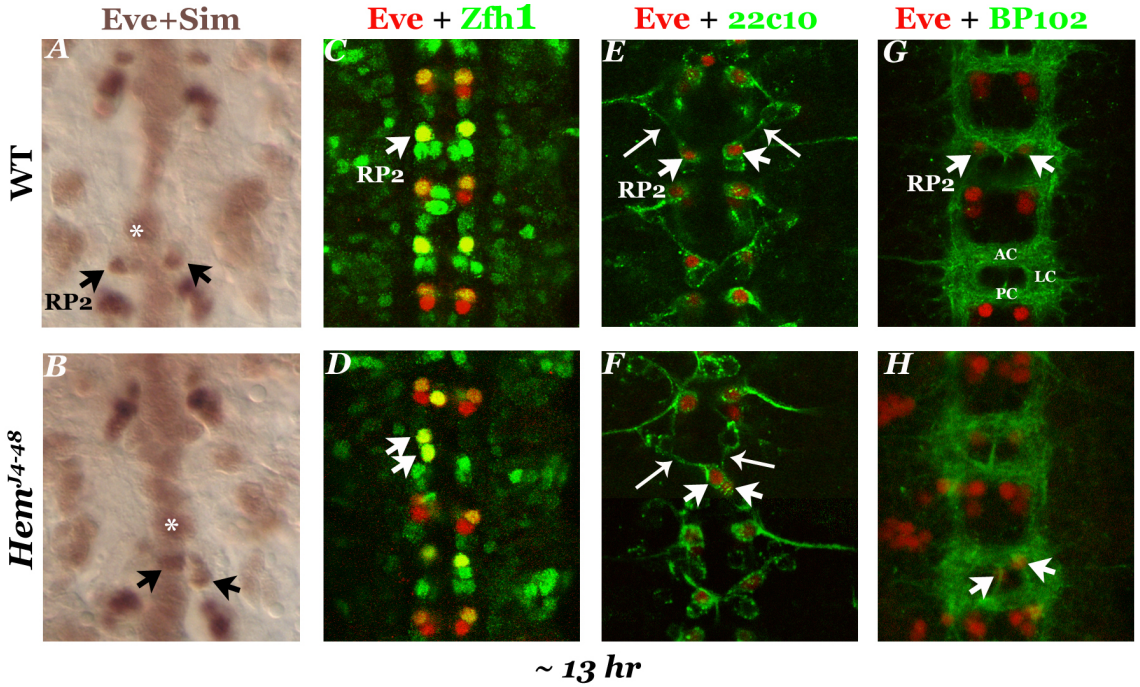


Fig. 1.5 The migration defect is not caused by any defect in midline glial cells, cell identity changes or VNC structure disruption but due to an active migration process. A). Embryos stained with Eve and Sim. RP2 neurons are pointed by arrow. Cells represented by star are midline glial cells and not RP2 neurons. B). Embryos stained with Eve and Zfh-1. C). Embryos stained with Eve and 22C10. An RP2 neuron is pointed by arrow and axon projections by small arrow. D). Embryos stained with Eve and BP102. AC: Anterior commissure; PC: Posterior commissure; LC: Longitudinal connective.

Finally the embryos were stained with Eve and BP102. BP102 specifically stains the CNS axonal scaffold: as shown in Fig. 1.5G in embryos of about 13 hour of development, axons in the VNC fasciculate to form longitudinal connectives (LC) and the anterior commissures (AC) and posterior commissures (PC). In wild type embryos, RP2 neurons are located at the armpit of anterior commissures. In *Hem*$^{J4\text{-}48}$ mutant embryos, as shown in Fig. 1.5H, RP2 neurons are mislocalized even though the VNC structure is not altered. Therefore, it is unlikely that the migration defect of RP2 neurons in the *Hem* mutant embryos is a result of midline glial cells, cell identity changes, or

disruption in the VNC structure, instead it is due to an active but aberrant migration of RP2 neurons.

The truncated Hem protein (ΔHem^{J4-48}) in Hem^{J4-48} allele behaves as an antimorphic protein

As mentioned in the previous sections, the migration defect of RP2 neurons is also observed in Hem^{C3-20} and *Hem* deficiency *Df(3l)ED230* embryos besides in Hem^{J4-48} (Fig. 1.6C-E). However, in none of them, the migration defect is fully penetrant; the penetrance is low. As shown in Table 1.1: 12.9% in Hem^{J4-48}, 8.3% in Hem^{C3-20} and 8.8% in *Df(3L)ED230*. In *Df(3L)ED230,* the entire *Hem* gene is deleted, therefore there is no zygotic Hem. However, the penetrance is still low. The maternal contribution of wild type Hem protein (Baumgartner et al., 1995) is likely responsible for this low penetrance. When examined using Western blotting analysis, as shown in Fig. 1.6G, wild type Hem protein is detected in *Df(3L)ED230* embryos at 6~10 hours and also detected at 12~16 hours old embryos. Because there is no zygotic expression of Hem in *Hem* deficiency *Df(3L)ED230*, this Hem is from the maternal contribution. Therefore, the maternally contributed Hem might function to partially rescue the migration defect of RP2 neurons in homozygous *Hem* mutants.

The *Hem* gene encodes a protein of 1126 amino acids in size. In *Df(3L)ED230,* the entire *Hem* gene is deleted. In Hem^{C3-20} mutants, Trp-256 is replaced by a stop codon; thus, a truncated Hem of 255 amino acids is produced. In Hem^{J4-48} mutants, Trp-490 is replaced by a stop codon therefore a larger truncated Hem (ΔHem^{J4-48}) of 489 amino acids

is produced (Fig. 1.6A). Interestingly the penetrance of 12.9% in *Hem*$^{J4\text{-}48}$ mutants is the highest compared to 8.9% in *Df(3L)ED230* and 8.3% in *Hem*$^{C3\text{-}20}$. In the transheterozygous *Hem*$^{J4\text{-}48}$*/Df(3L)ED230* mutants (Fig. 1.6B and Table 1.1) the penetrance of migration defect is about 9.29%, higher than homozygous *Df(3L)ED230* mutants but lower than homozygous *Hem*$^{J4\text{-}48}$ mutants. Based on this data, *Hem*$^{J4\text{-}48}$ might be an antimorphic mutation. Its product, the truncated Hem (ΔHem$^{J4\text{-}48}$) might function dominant negatively by interfering with the maternally contributed wild type Hem thus reducing the wild type activity of maternal Hem. This possibility is further supported by the result that the maternal protein level of Hem is less in *Hem*$^{J4\text{-}48}$ mutant embryos than in *Df(3L)ED230* embryos (Fig. 1.6G) (The Hem antibody recognizes the C-terminal of Hem so it will not detect the truncated Hem but only the full length Hem protein), indicating that ΔHem$^{J4\text{-}48}$ somehow reduces the levels of maternally deposited Hem.

To further test this possibility, transgenic flies carrying *UAS-ΔHem*$^{J4\text{-}48}$ were crossed to a maternally expressed Gal4 driver *P{GAL4::VP16-nos.UTR}MVD2* and the transgenic *ΔHem*$^{J4\text{-}48}$ was induced by this driver in the *Df(3L)ED230* mutant background. The expectation here is that the ectopically expressed truncated Hem (ΔHem$^{J4\text{-}48}$) might be able to interfere with the maternally contributed wild type Hem thereby increasing the penetrance of the migration defect of RP2 neurons. As shown in Fig. 1.7C, D and Table 1.1, with the ectopic expression of ΔHem$^{J4\text{-}48}$ in *Df(3L)ED230* mutant embryos, the penetrance of migration defect is increased from 8.8% in *Df(3L)ED230* to 17.2% in *UAS-ΔHem*$^{J4\text{-}48}$*/ P{GAL4::VP16-nos.UTR}MVD2; Df(3L)ED230* embryos. This doubling of the defect by the truncated Hem further supports the idea that *ΔHem*$^{J4\text{-}48}$ is an antimorphic

mutation and that its gene product could interfere with the maternally contributed wild type Hem.

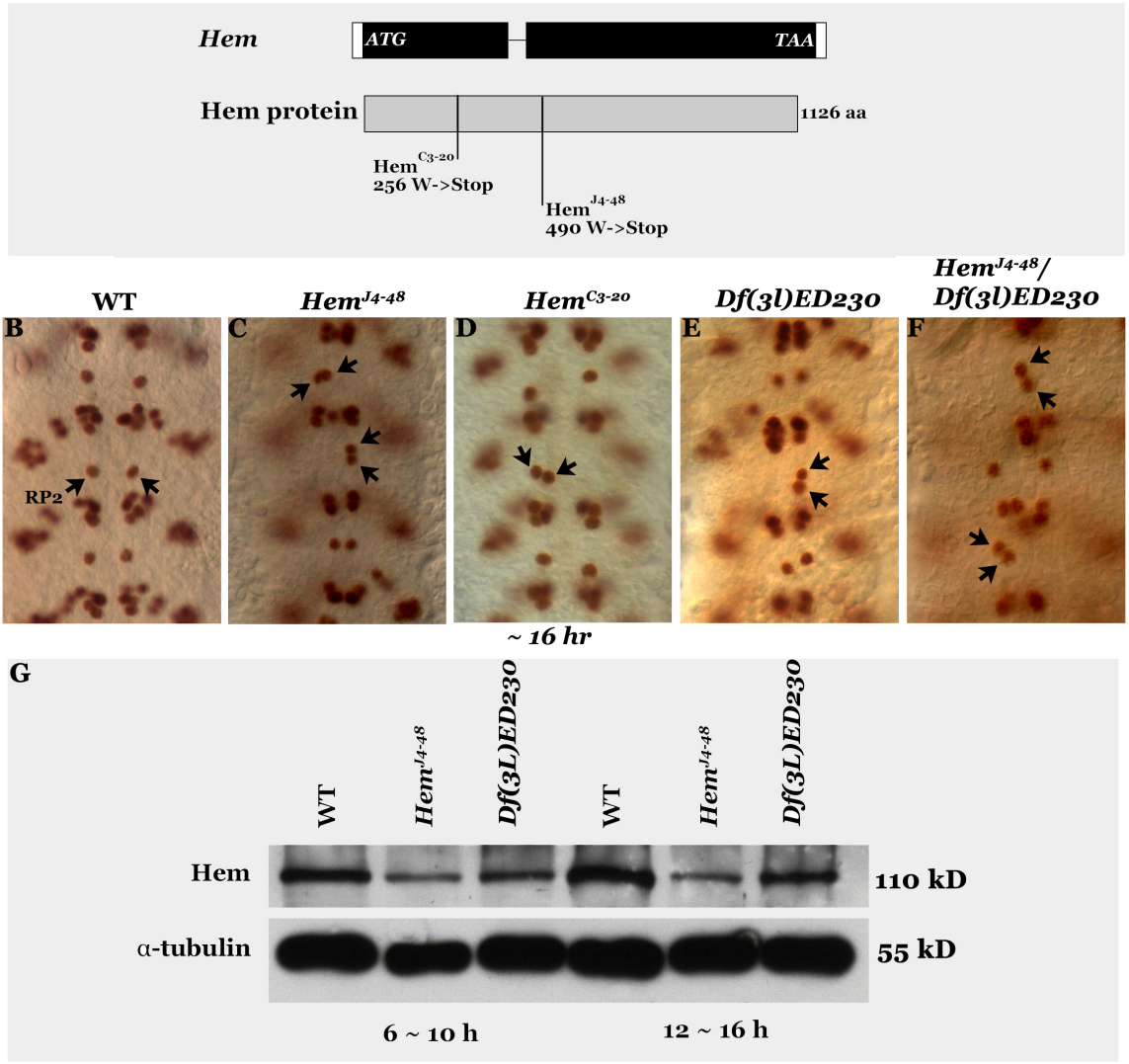


Fig. 1.6 The truncated Hem protein (ΔHem^{J4-48}) in *Hem^{J4-48}* allele behaves as an antimorphic protein. Embryos are stained with Eve antibody. A) Schematic view of *Hem* gene, Hem and two *Hem* alleles: *Hem^{J4-48} and Hem^{C3-20}*. B) In wild type embryos, there is one RP2 neuron (arrow) in each hemi-segment. C-F) Migration defect of RP2 neurons in *Hem^{J4-48}*, *Hem^{C3-20}*, *Hem* deficiency *Df(3L)ED230* in which the entire *Hem* gene is deleted and heterozygous *Hem^{J4-48}/Df(3L)ED230* mutants. G) The protein level of Hem in wild type, *Hem^{J4-48}* and *Df(3L)ED230* embryos. Embryos are collected at 6~10 hours and 12~16 hours of embryonic development.

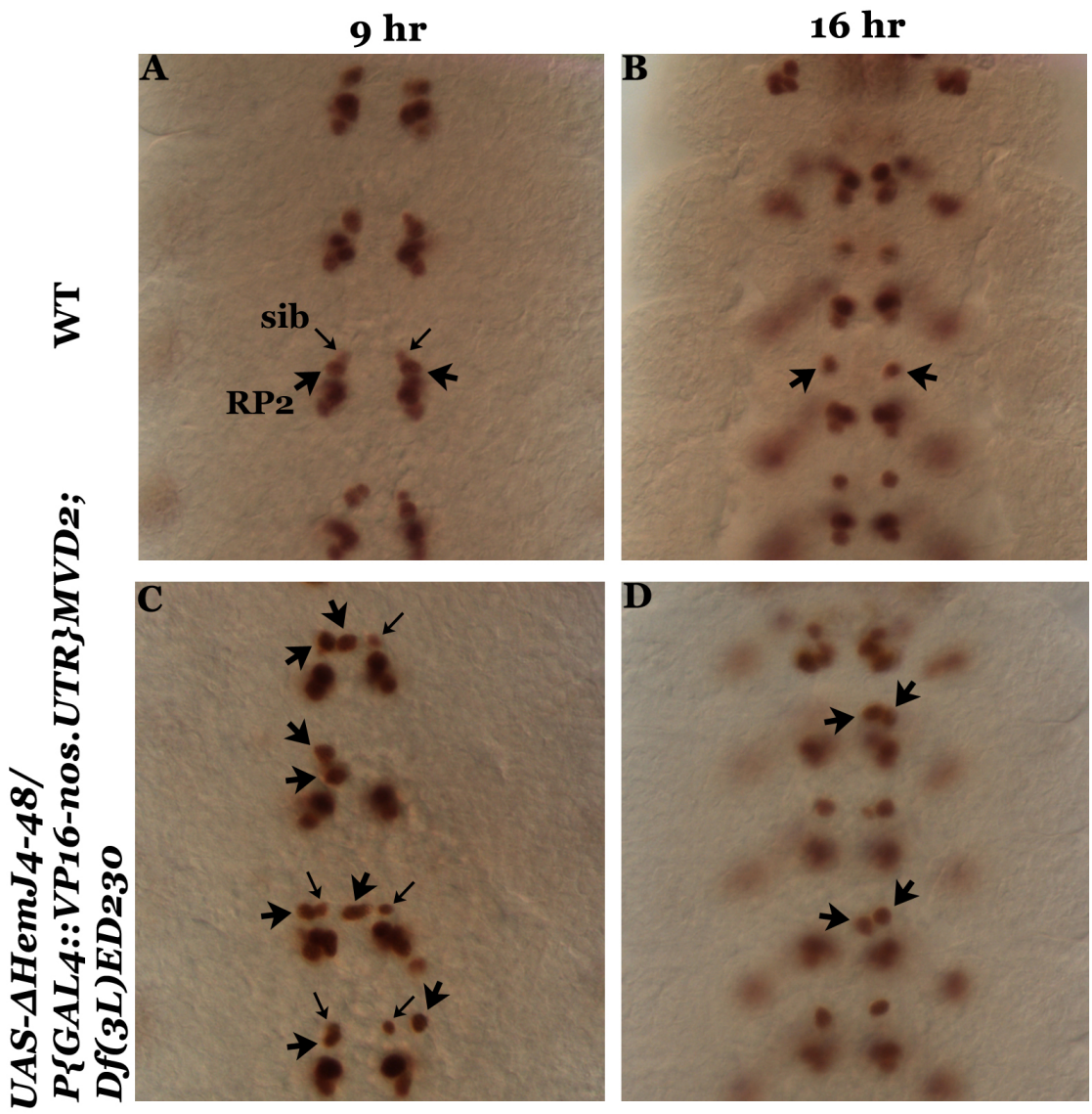


Fig. 1.7 Ectopic expression of ΔHem^{J4-48} in *Df(3L)ED230* mutants increases the penetrance of migration defect of RP2 neurons. Embryos are stained with Eve antibody. A) Wild type embryos around 9 hour. In each hemi-segment, there is one RP2 neuron (arrow) and a sib cell (small arrow). B) Wild type embryos around 16 hour. Only one RP2 neuron is present in each hemi-segment. C) *Df(3L)ED230* mutant embryos with ectopic expression of ΔHem^{J4-48} around 9 hour. RP2 neurons are crossing the midline but not sib cells. D) *Df(3L)ED230* mutant embryos with ectopic expression of ΔHem^{J4-48} around 16 hour. RP2 neurons migrate from the initial hemi-segments to the opposite hemi-segments.

Table 1.1 Penetrance of the migration defect of RP2 neurons in *Hem* alleles, *Hem* deficiency and in deficiency with ectopic expression of $\Delta Hem^{J4\text{-}48}$

Genotype	Penetrance
$Hem^{J4\text{-}48}$	12.9%
$Hem^{C3\text{-}20}$	8.3%
Df(3L)ED230	8.8%
$Hem^{J4\text{-}48}$/*Df(3L)ED230*	9.29%
UAS-DHem$^{J4\text{-}48}$*/ P{GAL4::VP16-nos.UTR}MVD2; Df(3L)ED230*	17.2%

Abl and WAVE mutants also show the same aberrant migration defect of RP2 neurons as Hem mutants

Hem forms the WAVE complex with four other proteins: WAVE/SCAR, Sra-1/PIR121/CYFIP, Abi and HSPC300. Abl might be recruited to this complex through interaction with Abi (Stuart et al., 2006). Hem has also been shown to activate WASp in the membrane (Bogdan and Klambt, 2003). Both WAVE and WASp are involved in actin cytoskeleton organization, which is very important for cell migration. Furthermore, Hem is able to bind to Dock (the homologue of Nck) that is linked to many receptor tyrosine kinases (RTKs) such as the epidermal growth factor (EGF) RTKs and platelet-derived growth factor (PDGF) RTks (McCarty, 1998). Therefore Hem may mediate the extracellular signaling. It seems possible that these proteins function together with Hem to regulate the migration of RP2 neurons during development. I examined all the alleles

and deficiencies of these *Hem* partner genes (obtained from the Bloomington stock center and private labs, listed in Table 1.2). Out of these mutants, the migration defect of RP2 neurons seen in $Hem^{J4\text{-}48}$ mutants is also observed in Abl^{2} mutants (9%) and $WAVE^{\Delta 37}$ *(7%)* mutants (Fig. 1.8C and D). Thus, Abl and WAVE might be involved in regulating the migration of RP2 neurons together with Hem. On the other hand, it is impossible to rule out a role for some of the other genes in migration because their mutants do not show any migration defects. This is mainly due to the fact that many of their encoded proteins are maternally deposited. The maternally contributed wild type gene products may then rescue the zygotic loss of function phenotype for these genes.

Table 1.2 All the alleles and deficiencies of *Hem* partner genes from the Bloomington stock center and private labs

Gene	Alleles and deficiencies
WAVE	$SCAR^{\Delta 37}$, $SCAR^{k03107}$, $P\{EP\}SCAR^{G12874}$ and *Df(2L)BSC32*
Sra-1	$P\{EPgy2\}Sra\text{-}1^{EY06562}$ and *Df(3R)Exel6174*
Abi	$P\{EPgy2\}Abi^{EY20423}$ and *Df(3R)Exel7359*
HSPC300	$P\{EP\}HSPC300^{EP506}$， $P\{EP\}HSPC300^{G19021}$ and *Df(2R)Exel6080*
Abl	Abl^{1}, *Abl2*, Abl^{4} and *Df(3L)st7*
WASp	$P\{EPgy2\}WASp^{EY06238}$, wsp^{1}, wsp^{3} and *Df(3R)3450*
dock	$P\{PZ\}dock^{04723}$, $P\{lacW\}dock^{k13421}$ and *Df(2L)ast1*

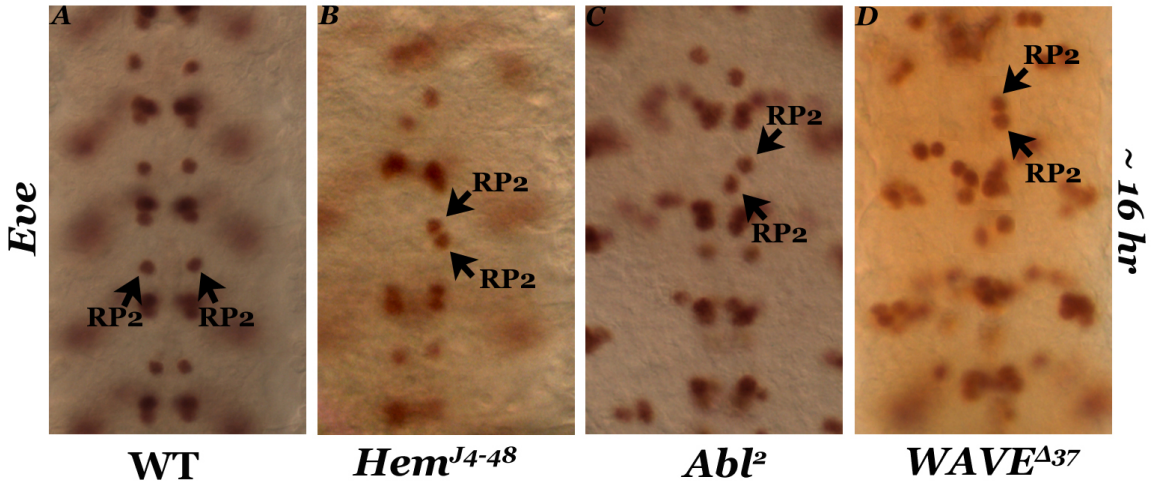


Fig. 1.8 The same migration defect was observed in *Hem*$^{J4-48}$, *Abl*^{2} and *WAVE*$^{\Delta 37}$ mutants. A) Wild type embryos. B) *Hem*$^{J4-48}$ mutant embryos. C) *Abl*^{2} mutant embryos. D) *WAVE*$^{\Delta 37}$ mutant embryos.

WAVE is required for the migration of RP2 neurons

WAVE is one of the key regulators of actin polymerization mediated by the Arp2/3 complex. It is required for the formation of branched and cross-linked actin cytoskeleton networks in the lamellipodia of migrating cells. In *Drosophila*, the loss of function for WAVE causes embryonic lethality. However the maternal contribution of WAVE may rescue the phenotype of zygotic loss of function for WAVE (Zallen et al., 2002). By generating mosaic animals carrying germline mutant clones, the maternal contribution of WAVE can be completely depleted and thereby the full range phenotype of WAVE mutants can be observed. Since germline clones homozygous for *WAVE*$^{\Delta 37}$ result in developmental arrest during oogenesis, a weaker mutant *WAVE*^{K13811} was used to generate germline clones as described in the materials and methods. The resulted embryos were named as *WAVE*^{mat}. As described before, in wild type embryos (Fig. 1.9A),

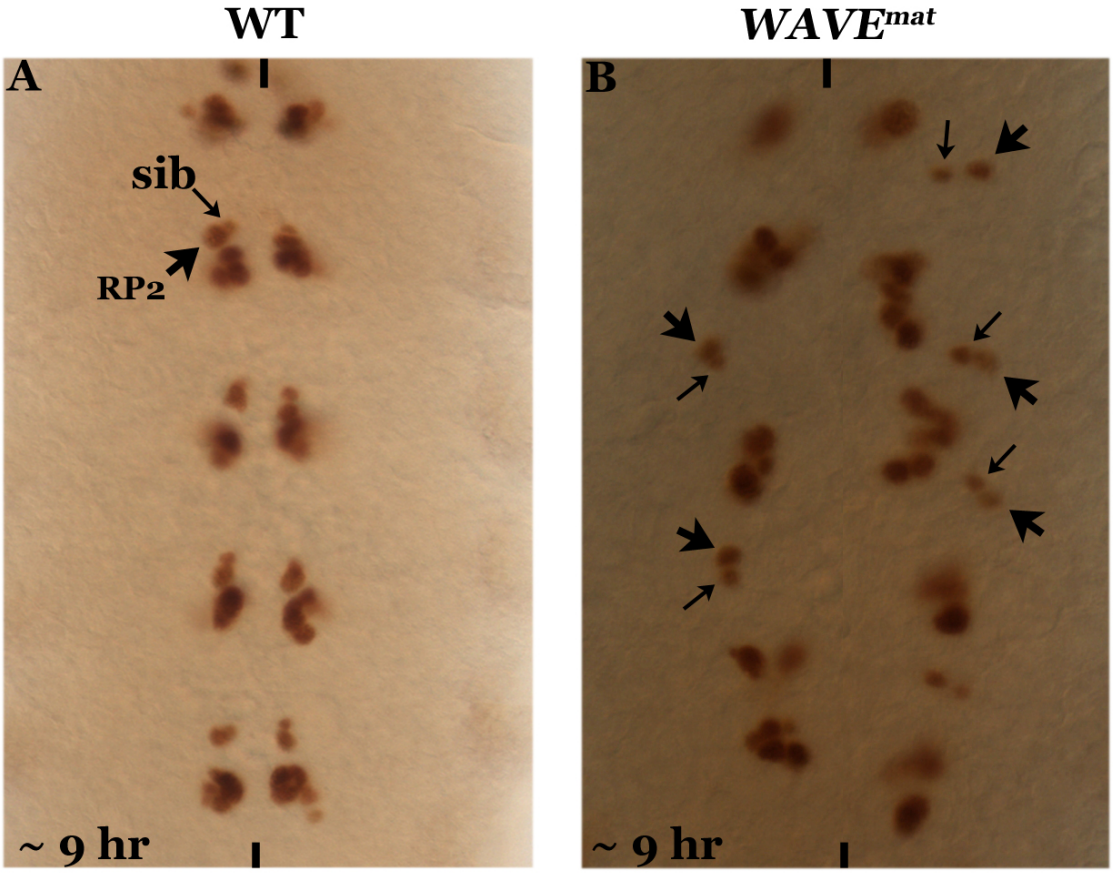


Fig. 1.9 RP2 neurons stop migration when WAVE was completely depleted. Embryos are stained with Eve antibody. A) In wild type embryos, RP2 neurons and sib cells first move toward the midline and then migrate in the posterior direction along the midline. B) In *WAVE*^{mat} embryos in which WAVE is completely depleted, RP2 neurons and sib cells fail to migrate at all. In embryos at ~ 9 hour, they still stay in the position where they were formed from GMC. This phenotype is nearly fully penetrant.

RP2 neurons and sib cells first migrate towards the midline and then in the posterior direction. In contrast, in *WAVE*^{mat} embryos (Fig. 1.9B) where both maternal contribution and zygotic expression of WAVE are depleted, RP2 neurons and sib cells stay in the

place where they were produced from GMC-1, in the GMC-1 location. This indicates that RP2 neurons and sib cells are unable to migrate at all without WAVE. Therefore, WAVE is required for the migration of RP2 neurons and sib cells. I could not generate mosaic flies for *Hem* due to technical difficulty because of close location of *Hem* to the centromere (too close to the FRT insertion). It seems highly likely that the Hem pathway is required for normal migration right from the beginning.

The down regulation of WAVE might cause the migration defect of RP2 neurons in Hem mutants

The same migration defect of RP2 neurons was observed in both *Hem* and *WAVE* mutants. This indicates that they may act together to regulate the migration of RP2 neurons in the VNC (they could also independently regulate the process). WAVE has been shown to be required for the migration of RP2 neurons. Since Hem might be able to regulate the activity of WAVE in the WAVE complex, it is possible that Hem might regulate the migration of RP2 neurons through WAVE. Therefore, the protein level of WAVE was examined in $Hem^{J4\text{-}48}$ mutants by western blotting. At least three bands very close to each other (82kDa, 80 kDa and 78 kDa, respectively) are detected by the WAVE antibody (Fig. 1.10A, represented by 1, 2 and 3). These three bands are all down regulated in WAVE deficiency embryos (Fig. 1.10A, *Df(2L)BSC32*) and are up regulated when the WAVE gene was ectopically expressed from a transgene (Fig. 1.10B). Thus, these three bands must be all originate from WAVE. However, it is not clear if they have distinct functions.

As shown in Fig. 1.10A, the protein level of WAVE is decreased in *Hem*$^{J4\text{-}48}$ mutants compared to wild type embryos in both early stages (6 ~ 10 hour) and late stages (12 ~ 16 hour). The levels of WAVE is also lower in *Hem*$^{J4\text{-}48}$ embryos compared to the *WAVE* deficiency *Df(2L)BSC32* embryos. This might explain why the penetrance of the migration defect in *Hem*$^{J4\text{-}48}$ mutants is higher compared to the WAVE deficiency embryos (12.9% vs 7%). The protein level of WAVE is also lower in *Hem*$^{J4\text{-}48}$ embryos compared to the *Hem* deficiency *Df(3L)ED230* embryos (data not shown). It is consistent with the result that the protein level of Hem is lower in *Hem*$^{J4\text{-}48}$ embryos compared to the *Hem* deficiency *Df(3L)ED230* embryos (Fig. 1.6G) and therefore indicates that Hem is necessary to maintain certain protein level of WAVE.

These immunoblotting results for WAVE are also supported by whole mount immunohistochemistry staining (Fig. 1.10C). In late stage wild type embryos at ~ 13 hour, WAVE protein is accumulated in axons (small arrow), forming a ladder like structure in the CNS (Fig. 1.10C). The RP2 neuron (arrow) is located in each hemi-segment, at the armpit of the anterior commissure. In *Hem*$^{J4\text{-}48}$ mutant embryos with mis-migrating RP2 neuron (arrow), very little of WAVE can be detected (small arrow) in. It seems likely that this down regulation of WAVE (but not its complete loss) is causing the migration defect of RP2 neurons (a complete loss of WAVE, and possibly Hem also, causes loss of migration altogether).

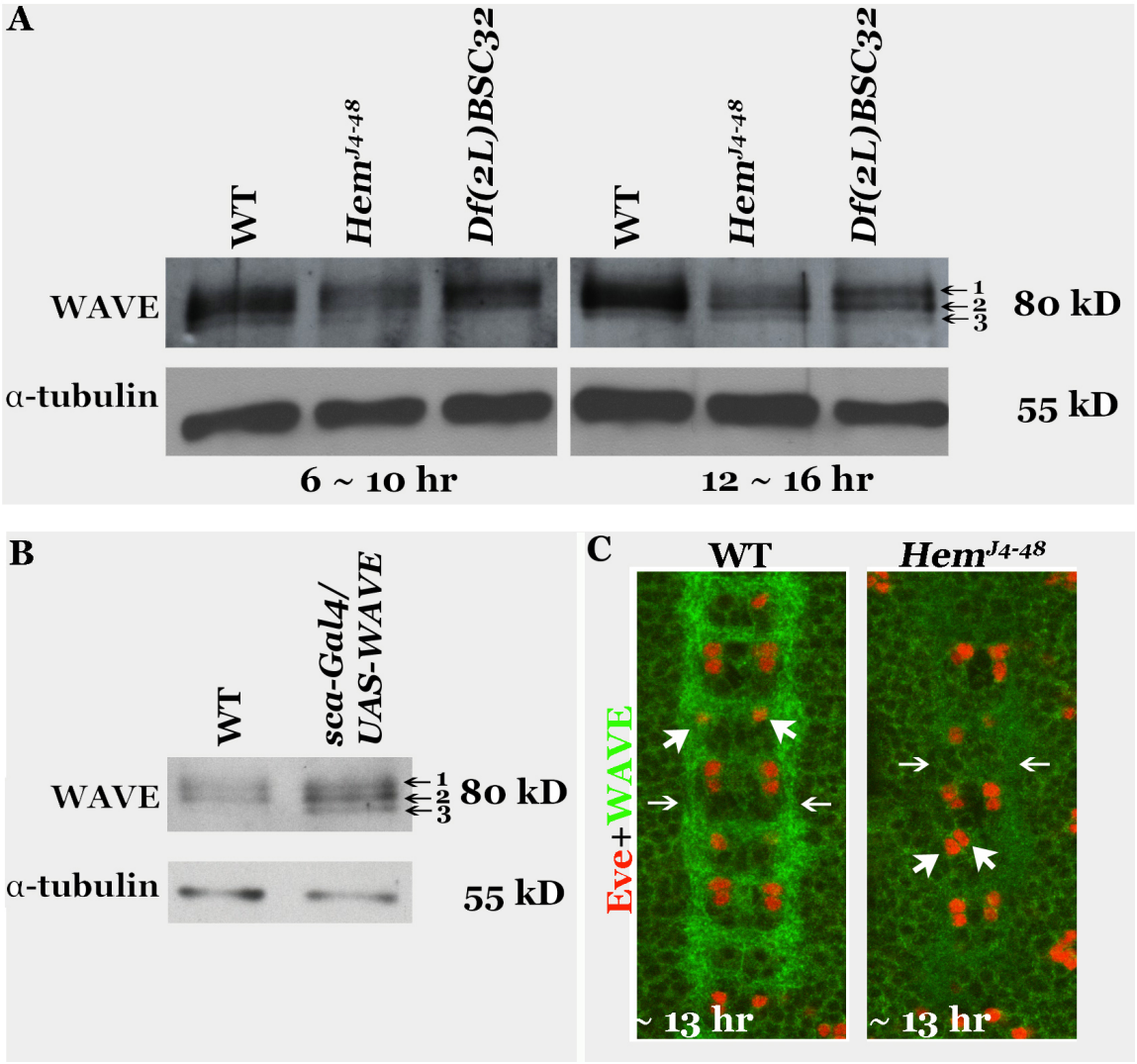


Fig. 1.10 The down regulation of WAVE in *Hem*$^{J4-48}$ mutants. A) The protein level of WAVE in wild type, *Hem*$^{J4-48}$ and *WAVE* deficiency *Df(2L)BSC32* embryos in early stage (6 ~ 10 hour) and late stage (12 ~ 16 hour). WAVE is down regulated in *Hem*$^{J4-48}$ mutants compared with both wild type and *Df(2L)BSC32* embryos. B) The same three bands are present when the cDNA of WAVE is ectopically expressed. C) The immunochemistry result of wild type embryos and *Hem*$^{J4-48}$ embryos. Embryos are stained with Eve and WAVE antibodies. RP2 neurons are shown by arrow and WAVE ladder structure is pointed by small arrow. WAVE accumulates in the axons in the wild type embryos, forming ladder like structure in the CNS but disappears in *Hem*$^{J4-48}$ mutants.

The fact that WAVE is down regulated in *Hem* mutants raises the possibility that the migration defect of RP2 neurons in *Hem* mutants might be caused by this reduction in WAVE. The expressions of WAVE in embryos at different developmental stages were therefore examined. In Fig. 1.11, embryos stained with antibodies against Eve (red) and WAVE (green) are shown. In wild type embryos, at ~7.5 hour, WAVE is ubiquitously expressed in GMC-1 (Fig. 1.11A). After that, GMC-1 divides to generate a sib cell and an RP2 neuron and both of them move toward the midline. At this time, WAVE is localized in the RP2 neuron to the direction of its movement (Fig. 1.11C). The RP2 neuron then moves in the posterior direction and again WAVE is localized to this direction of its movement (Fig. 1.11E). In embryos at ~ 9.5 hr (Fig. 1.11G), the RP2 neuron stops migration and WAVE is observed to be ubiquitously expressed at that point. Since an RP2 in the VNC begins to send out its axon by this time, I could not determine the localization pattern of WAVE in the cell body. In embryos of ~ 13 hour age, WAVE is accumulated in the axons in the VNC, forming a ladder-like structure (Fig. 1.11I, pointed by small arrow) similar to BP102 pattern (Fig. 1.5G).

Next, I examined the localization of WAVE in *Hem* mutant embryos. In $Hem^{J4\text{-}48}$ mutant embryos, WAVE is expressed ubiquitously in GMC-1 (Fig. 1.11B) as in wild type embryos. When the RP2 neuron moves toward the midline (Fig. 1.11D) and in the posterior direction (Fig. 1.11F), WAVE is localized to the direction of movement as observed in wild type embryos. However, in $Hem^{J4\text{-}48}$ mutant embryos at ~ 9.25 hour in which RP2 neuron is observed crossing the midline, WAVE is not ubiquitously expressed in the RP2 neuron but instead is localized to the direction of its movement toward the

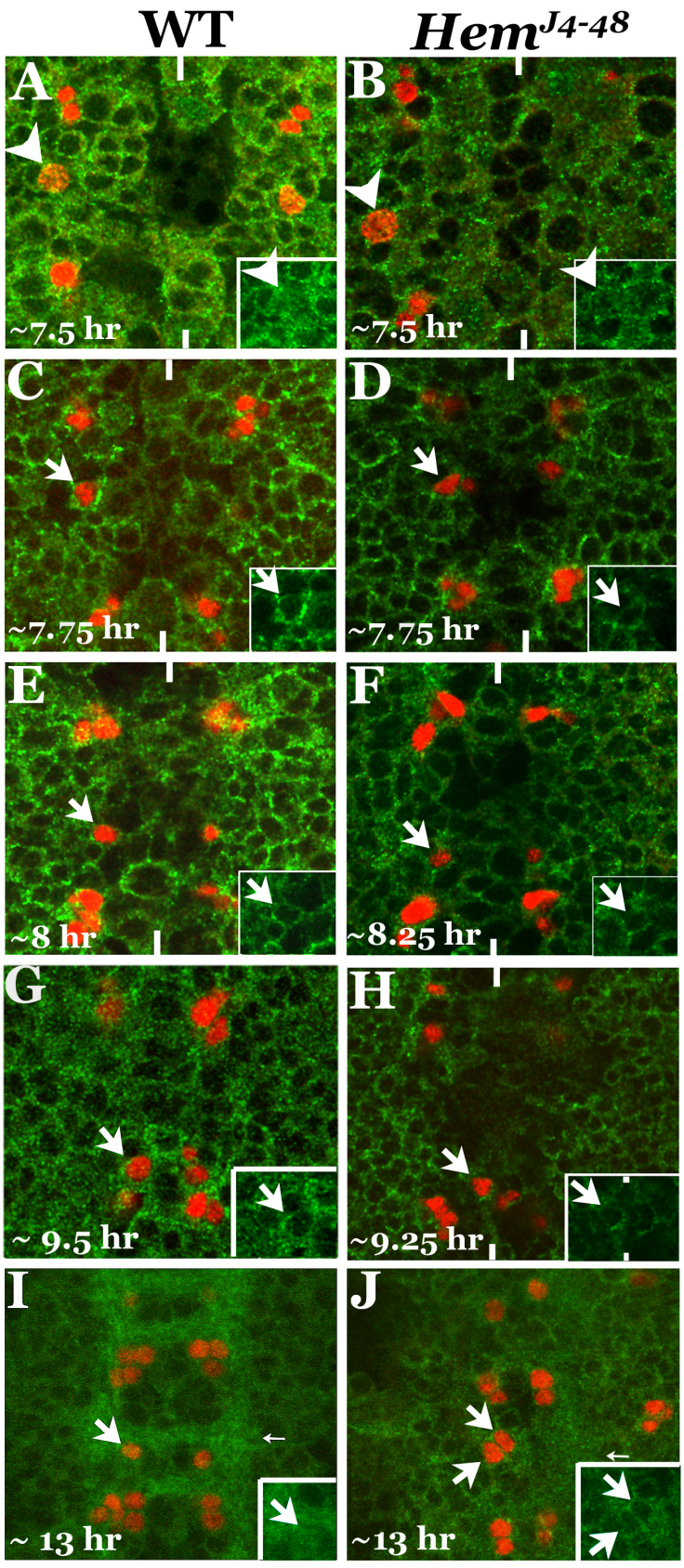

WT
Hem^J4-48
A
B
~7.5 hr
~7.5 hr
C
D
~7.75 hr
~7.75 hr
E
F
~8 hr
~8.25 hr
G
H
~ 9.5 hr
~9.25 hr
I
J
~ 13 hr
~13 hr
Eve+WAVE

Fig. 1.11 WAVE is mis-localized in *Hem^{J4-48}* mutants. The inset pictures in the bottom right corner represent WAVE staining around the RP2 neurons. In wild type embryos, WAVE is ubiquitously expressed in GMC-1 (A) and then becomes localized in the direction of migration in RP2 neurons (C, E). When the RP2 neuron stops migration, WAVE is ubiquitously present in the RP2 neuron (G). In *Hem^{J4-48}* mutant embryos, WAVE is still ubiquitously expressed in GMC-1 (B) and then becomes localized in the direction of its migration in RP2 neurons (D, F). However, in embryos at ~ 9.25 hour, instead of being ubiquitously expressed in the RP2 neuron, WAVE is localized in the direction of its abnormal migration, which might be the reason for the migration defect of RP2 neurons.

midline (Fig. 1.11H). In embryos at ~ 13 hour, RP2 neurons have completely crossed the midline and at this time, the protein level of WAVE is down regulated (Fig. 1.11J). These results indicate that in *Hem^{J4-48}* mutants, WAVE is mis-localized, which might be the reason for the specific migration defect of RP2 neurons.

This data leads to a further question of whether the down regulation and mis-localization of WAVE in RP2 neurons in *Hem* mutants lead to the migration defect or whether some other mechanism is responsible for the migration defect and, consequently causes the mis-localization of WAVE in RP2 neurons to the direction of mis-migration. To answer this question, WAVE was ectopically expressed by *sca*-Gal4 in *Hem* mutants. As described in detail in the methods, by crossing sca-Gal4/+;*UAS-WAVE, Hem^{J4-48}*/+ flies to *UAS-WAVE,Hem^{J4-48}*/+ flies (homozygous *Hem^{J4-48}* flies are lethal), embryos of several genotypes were obtained : +/+;+/+ (wild type), +/+; *UAS-WAVE,Hem^{J4-48}*/ *UAS-WAVE, Hem^{J4-48}* (*Hem^{J4-48}* mutants), sca-Gal4/+; *UAS-WAVE,Hem^{J4-48}*/+ (Heterozygous embryos with ectopic expression one copy of WAVE) and *sca*-Gal4/+; *UAS-WAVE,Hem^{J4-48}*/ *UAS-WAVE,Hem^{J4-48}* (*Hem^{J4-48}* mutants with ectopic expression of two

copies of WAVE). Embryos of different genotypes could be identified by the expression levels of WAVE (Fig. 1.12). As shown in Fig. 1.12A in wild type embryos, WAVE accumulates in the axons, forming a ladder like structure in the VNC and there is only one RP2 neuron in each hemi-segment, located at the armpit of anterior commissure (Fig. 1.12B). In *Hem* mutants, WAVE is down regulated (Fig. 1.12C), and two RP2 neurons are in the same hemi-segment due to the migration defect (Fig. 1.12D). The ectopic expression of one copy of WAVE in heterozygous $Hem^{J4\text{-}48}$/+ embryos (Fig. 1.12E) increases the level of WAVE compared with the wild type embryos (Fig. 1.12A and B). It however does not affect the normal migration pattern of RP2 neurons (Fig. 1.12F). The ectopic expression of two copies of WAVE in homozygous $Hem^{J4\text{-}48}$/ $Hem^{J4\text{-}48}$ mutant embryos strongly increases the level of WAVE (Fig. 1.12G) and only one RP2 neuron is there in each hemi-segment (Fig. 1.12H). This indicates that the ectopic expression of WAVE suppresses or rescues the RP2 migration defect in *Hem* mutant embryos. Therefore I conclude that down regulation and mis-localization of WAVE in RP2 neurons in *Hem* mutants causes the migration defect of RP2 neurons.

WAVE is phosphorylated in vivo

As shown above, three bands of approximate 82 kDa, 80 kDa and 78 kDa are detected in western blotting by using antibodies against WAVE (Fig. 1.10A and B). Since ectopic expression of WAVE from a WAVE transgene also shows the same three bands (Fig. 1.10B), it seems unlikely that they are coming from different isoforms. One possibility is that post-translational modifications such as phosphorylation are responsible for these three bands. To test this possibility, protein extracts from *Drosophila* embryos

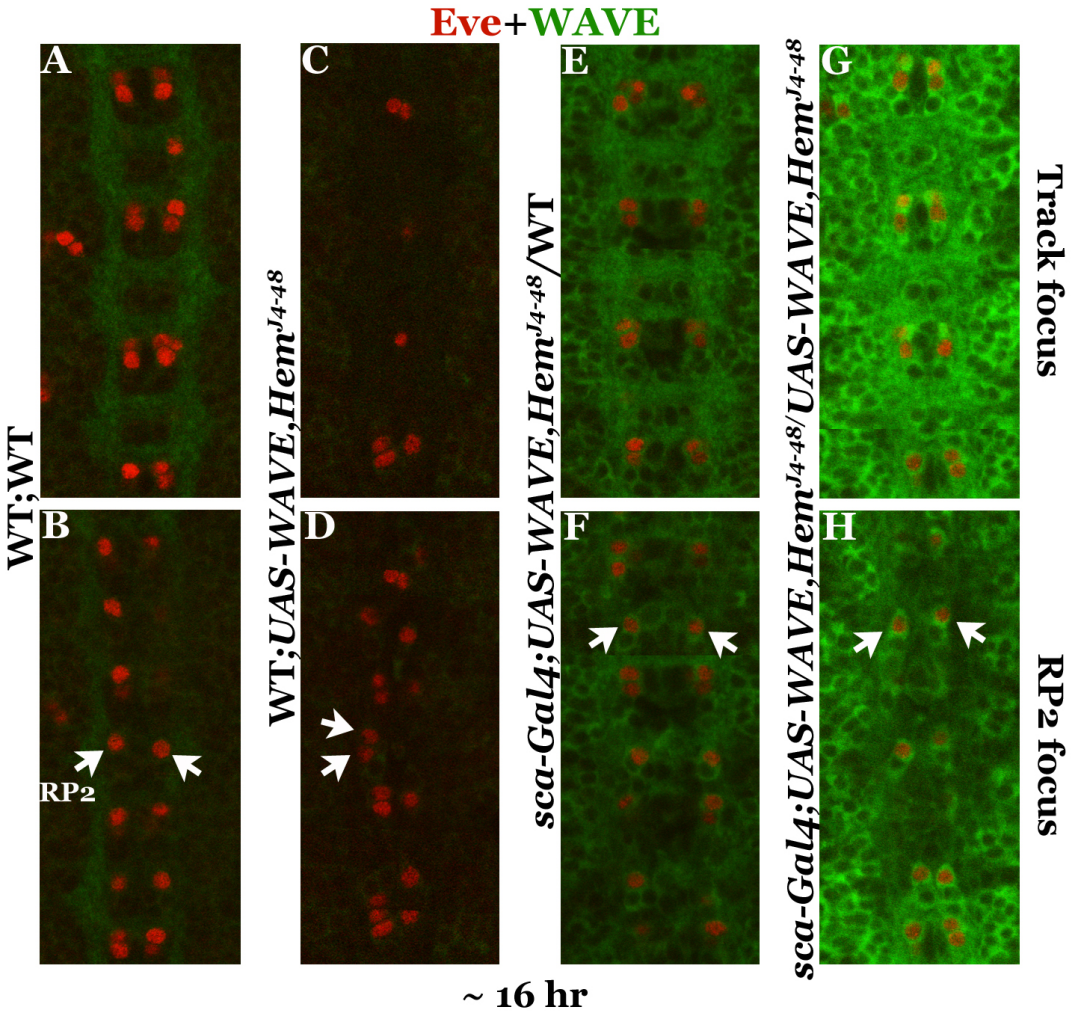


Fig. 1.12 Ectopic expression of WAVE in *Hem*$^{J4\text{-}48}$ mutants rescues the migration defect of RP2 neurons. Imaging was done in two different focal planes to visualize both the RP2 neurons and the axon tracts. A and B) In wild type embryos, WAVE is accumulated in the axons, forming a ladder structure. Only one RP2 neuron is present in each hemi-segment. C and D) In *Hem*$^{J4\text{-}48}$ mutants, WAVE is down regulated. Two RP2 neurons were observed in one hemi-segment caused by the migration defect. E and F) By ectopic expression of one copy of WAVE using *sca*-Gal4 in heterozygous *UAS-WAVE, Hem*$^{J4\text{-}48}$/+ embryos, WAVE is accumulated both in the axons and in the cell body of RP2 neuron. No migration defect was observed. G and H) By ectopic expression of two copies of WAVE using *sca*-Gal4 in homozygous *UAS-WAVE,Hem*$^{J4\text{-}48}$/*UAS-WAVE,Hem*$^{J4\text{-}48}$ mutant embryos, WAVE is accumulated in the axons and cell body of the RP2 neurons. The migration defect of RP2 neurons is suppressed by ectopic expression of WAVE.

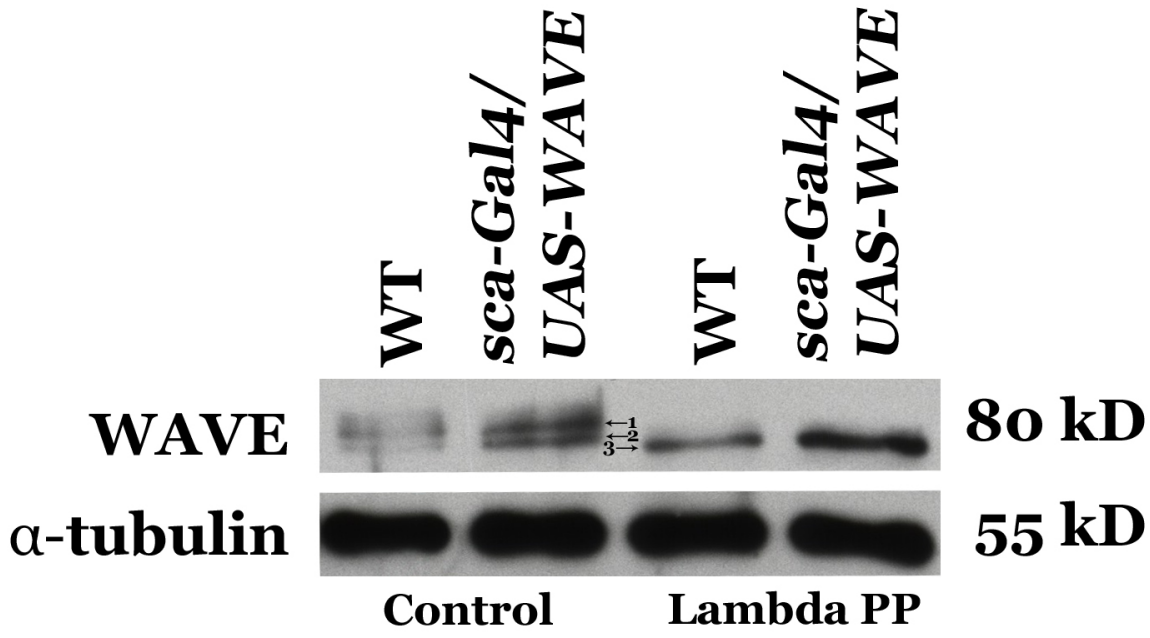


Fig. 1.13 Treatment of Lambda protein phosphatase (Lambda PP) dephosphorylates WAVE. WAVE was detected as two bands of ~82 kDa and ~80 kDa in wild type embryos and embryos with ectopic expression of WAVE. (There should be three bands as shown in Fig. 1.10A and Fig. 1.10B. The reason why the third band of ~78 kDa is missing here is unknown). After the treatment with Lamdba PP, WAVE was detected as only one band of ~78 kDa, indicating that the higher molecular weight WAVE are phophorylated WAVE.

were treated with Lambda protein phosphatase (Lambda PP) at 30 ºC for 30 minutes. As shown in Fig. 1.13, in the control group (without the PP treatment), two bands of ~ 82 kDa and ~80 kDa can be detected in both wild type embryos (WT) and embryos with ectopic expression of WAVE (*sca*-Gal4/*UAS-WAVE*). The third band of ~ 78 kDa shown in Fig. 1.10A and B, cannot be detected for some unknown reason. It is uncertain if this may have been caused by the incubation at 30 ºC for 30 minutes. After treatment with Lambda PP, the two bands of ~ 82 kDa and ~80 kDa that are detected in the control group have collapsed into a single band of ~ 78 kDa. The same result is observed in both

the wild type embryos and embryos with ectopic expression of WAVE (Fig. 1.13). Therefore, two bands of ~ 82 kDa and ~80 kDa represent phosphorylated WAVE and the third band of ~78 kDa is non-phosphorylated WAVE. The functional significance of this phosphorylation remains unknown at this moment.

Abl negatively regulates the levels of WAVE protein

The same $Hem^{J4\text{-}48}$ and $WAVE^{\Delta 37}$ migration defect of RP2 neuron was also observed in Abl^2 mutants. Since the protein expression level of WAVE is down regulated in $Hem^{J4\text{-}48}$ mutants and WAVE is localized to the leading edge of the mis-migrating RP2 neurons, it seems likely that in Abl^2 mutants, the expression of WAVE might also be down regulated. Therefore, I examined first the protein levels of WAVE in Abl^2 mutants. Since Abl might regulate WAVE through Hem, the protein level of Hem was also examined in *Abl* mutants. In contrast to the decreased levels of WAVE in $Hem^{J4\text{-}48}$ mutants and *Df(2L)BSC32*, an increase in the protein level of WAVE was observed in Abl^2 mutants (Fig. 1.14A). This increase of WAVE in Abl^2 was further supported by the whole mount embryo immunohistochemistry (Fig. 1.14B). As shown in Fig. 1.14B, Abl^2 mutants expresses more WAVE (small arrow) while very little WAVE is detected in $Hem^{J4\text{-}48}$ mutants, although the same migration defect of RP2 neurons (arrow) is observed in both of them. It is unlikely that the increase of WAVE in Abl^2 mutants is mediated by Hem because there is no significant change of Hem in Abl^2 mutants (Fig. 1.14A).

At the same time, ectopic expression of Abl (*sca*-Gal4/*UAS-Abl.F)* results in a decrease of the protein level of WAVE compared to in the wild type embryos; the protein

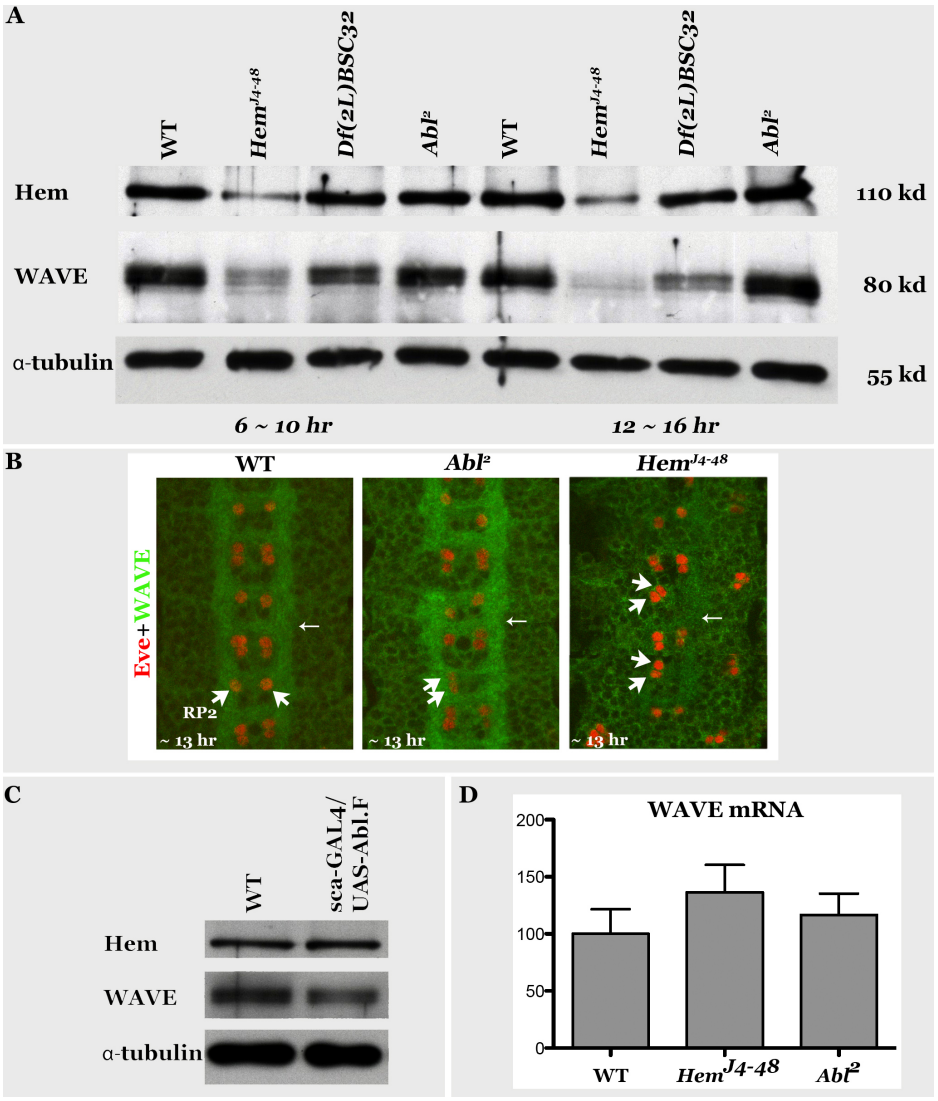


Fig. 1.14 Abl negatively regulates the protein level of WAVE. A) The protein level of WAVE and Hem in wild type, *Hem*$^{J4-48}$, WAVE deficiency *Df(2l)BSC32* and *Abl*^{2} mutant embryos. α-tubulin is used as the loading control. Compared to wild type embryos, both Hem and WAVE decreases in *Hem*$^{J4-48}$ mutants; In *Df(2l)BSC32*, Hem remains unchanged and WAVE decreases; In *Abl*^{2}, Hem remains unchanged but WAVE increases. B) Whole mount embryo immunohistochemistry. RP2 neurons are represented by arrow and WAVE is presented by small arrow. Compared to wild type embryos, WAVE increases in *Abl*^{2} mutants but decreases in *Hem*$^{J4-48}$ mutants. C) The protein level of WAVE and Hem in embryos with ectopic expression of Abl. Compared to wild type embryos, when Abl is ectopically expressed, Hem remains unchanged while WAVE decreases. D) mRNA levels of *WAVE* is detected in *Hem*$^{J4-48}$ mutants and *Abl*^{2} mutants by real-time PCR.

level of Hem remains unchanged in these embryos (Fig. 1.14C). Therefore Abl appears to negatively control the protein level of WAVE by a different mechanism or/and a different player and not via Hem. The regulation of the protein level of WAVE by Abl and Hem is unlikely at the transcriptional level but at the post-translational level since the mRNA level of WAVE does not show a significant change in both Abl^{2} and $Hem^{J4\text{-}48}$ mutants compared to wild type embryos as judged by real-time PCR (Fig.1.14D).

Abl and Hem may regulate the activity and or level of WAVE in different ways

The same migration defect of RP2 neurons is observed in both Abl^{2} and $Hem^{J4\text{-}48}$ mutants. In *Hem* mutants, the protein level of WAVE is decreased as well as it is mis-localized in RP2 neurons that show the migration defect. In Abl^{2} mutants however, although the same migration defect is detected, the protein level of WAVE is increased. In view of the fact that Abl is unlikely to regulate the protein level of WAVE through Hem, it may instead control the protein level as well as the activity of WAVE by a separate pathway. Since *Abl* and *Hem* are on the same chromosome, I made a double mutant of Abl^{2}, $Hem^{J4\text{-}48}$ though genetic recombination. The same migration defect is observed in the double mutant of *Abl, Hem* embryos as well (Fig 1.15D), however, the penetrance of the defect is enhanced (16.5%) compared to $Hem^{J4\text{-}48}$ single mutants (Fig 1.15B, 12.9%) and Abl^{2} single mutants (Fig 1.15C, 9%). Therefore, it seems more likely that Abl and Hem regulate migration of RP2 neurons mediate by WAVE via distinct pathways. If on the other hand both of them act via regulating the protein level of WAVE, in the double mutant embryos the penetrance should decrease. In $Hem^{J4\text{-}48}$ mutants, the protein level of WAVE is down regulated as well as mis-localized, which

may cause the migration defect of RP2 neurons. On the other hand, Abl negatively regulates the protein level of WAVE. In *Abl*^{2} mutants, the protein level of WAVE is increased. The ectopic expression of WAVE in wild type embryos does not cause any migration defect of RP2 neurons (Fig. 1.12E and F). It is therefore unlikely that the enhanced levels of WAVE in *Abl* mutants are responsible for the migration defect. Thus, it seems likely that Abl may be responsible for the inactivation of WAVE after it is activated. The degradation or inactivation of activated WAVE is necessary because a continuously activated WAVE could cause uncontrolled actin polymerization. In *Abl*^{2} mutants, due to an inefficient inactivation of activated WAVE, the enhanced activity of WAVE may result in the migration defect of RP2 neurons.

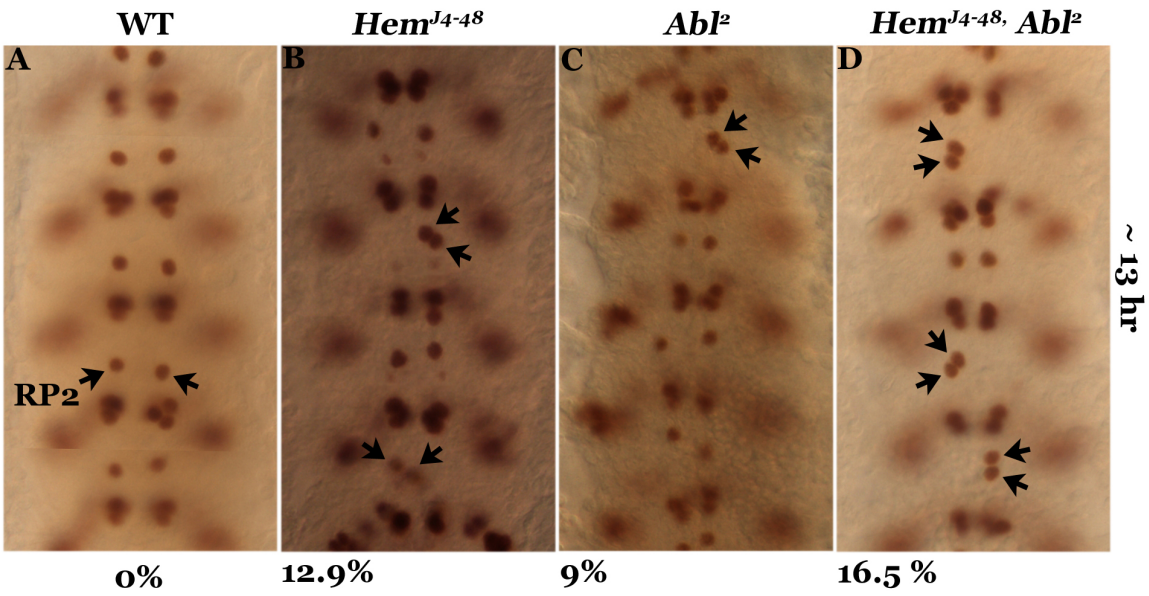


Fig. 1.15 Migration defect of RP2 neurons is observed in *Hem*$^{J4-48}$,*Abl*^{2} double mutant embryos. A) Wild type embryos. B) Migration defect of RP2 neurons in *Hem*$^{J4-48}$ mutants and the penetrance is 12.9%. C) Migration defect of RP2 neurons in *Hem*$^{J4-48}$ mutants and the penetrance is 9%. D) Migration defect of RP2 neurons in double mutants of *Hem*$^{J4-48}$,*Abl*^{2} and the penetrance is 16.5%, higher than in *Hem*$^{J4-48}$ and *Abl*^{2} mutants.

Discussion

During development of the nervous system, neurons and their precursor cells migrate from their initial locations to various positions, where they make connections with other neurons or muscle cells to form functional neuronal circuits. Failure to migrate or migrating in the wrong direction could harm development of the nervous system and thus affect its proper function. In this work I used *Drosophila* as a model system to study regulation of neuronal migration during development of the VNC. My aim is to elucidate the molecules involved in neuronal migration and their interaction with each other to regulate this neuronal migration during development. Many studies carried out on cell migration have used models of cells migrating on 2D substratum *in vitro,* which have provided important information on the characteristics of cell migration, molecules involved in cell migration and their regulation etc. Our model however, provides an opportunity to study neuronal migration *in vivo* during development of the nervous system. It hence allows for a better understanding of events occurring during neuronal migration. I investigated the migration pattern of a typical neuronal cell lineage GMC-1 →RP2/sib in the *Drosophila* VNC. Previously we have found that GMC-1→RP2/sib undergoes a 3-step migration during development of the VNC (Bhat, 2007). This 3-step migration is necessary for the proper synaptic formation. RP2s that fail to undertake a normal migration in *wg* mutants fail to project their axon tracts correctly (Bhat, 2007). However, the detailed mechanisms of this migration remain unclear. In my study, I found that in *Hem* mutants, RP2 neurons migrate from the hemi-segments where they were produced (initial hemi-segment) to the opposite hemi-segments in the same segments during development of the VNC. This results in two RP2 neurons in one hemi-segment

and none in the opposite hemi-segment (Fig. 1.2B). (We could not visualize the migration of RP2/sib cells in live embryos using the GFP since it takes ~ 3 hours for the GFP to become fluorescent after it is expressed. It is therefore too late to investigate the migration of RP2/sib cells since they start migrating immediately after they were born.)

During their migration in the wild type embryos, RP2 neurons remain in their initial hemi-segments and never cross the midline. In *Hem* mutants, at ~ 9 hour of embryonic development, RP2 neurons start to migrate toward the opposite hemi-segments, cross the midline and remain in the opposite hemi-segments at ~ 11 hour (Fig. 1.3). This data reveals that Hem is required for the proper migration of RP2 neurons in wild type embryos. It ensures that RP2 neurons not only migrate in their initial hemi-segments but never cross the midline. Of the RP2 neurons and sib cells produced from GMC1s, it is only RP2 neurons that are observed with this migration defect (Fig. 1.3 and Fig. 1.4). The reason for this difference/specificity remains unknown. It however indicates that different cells have unique pathways and mechanisms that regulate their migration pattern. The migration defect of RP2 neurons is not caused by disruption of midline glial cells, cell identity changes or the disruption of the VNC structure (Fig. 1.5). Thus, it is unlikely due to being passively moved but is an active migration process. The fact that only RP2s cross the midline but not sibs also argues against a passive event. An interesting phenotype that I observed is that even though dislocated RP2 neurons left their initial hemi-segments, they still sent out their axons to fasciculate with axons of the aCC neurons in their initial hemisegments (Fig. 1.5F). This indicates that RP2 neurons might send out their axons during their migration but before they cross the midline.

The penetrance of the migration defect in *Hem* mutants however, is not very high, with 12.9% in $Hem^{J4\text{-}48}$, 8.3% in $Hem^{C3\text{-}20}$and 8.9% in *Df(3L)ED230*. The existence of other redundant genetic pathways can result in this low penetrance. However, since Hem is maternally deposited in the embryos from their parents (maternal effect), it is more likely that the maternally contributed wild type Hem rescues the migration defect of RP2 neurons in *Hem* mutants thus causes this incomplete penetrance. In *Hem* deficiency embryos *Df(3L)ED230,* the whole *Hem* gene is deleted and there should be no zygotic expression of Hem, Hem was surprisingly still detected by western blotting, which can only be from the maternal contribution (Fig. 1.6G). Also, the method by which I calculated the penetrance of the migration defect – number of hemi-segments with 2 RP2 neurons divided by the total number of hemi-segments – has to be take into account since the penetrance that can be obtained is a maximum of 50%. Furthermore, it is also possible that I might have missed a situation where two RP2 neurons in the same segment exchange their positions by migrating to the opposite hemi-segments. I have observed that in early stage embryos two RP2 neurons migrating toward the midline (data not shown), which is consistent with this possibility.

Interestingly the penetrance 12.9% in $Hem^{J4\text{-}48}$ mutants is higher than 8.9% in *Df(3L)ED230* mutants and 8.3% in $Hem^{C3\text{-}20}$ mutants. Therefore, $\Delta Hem^{J4\text{-}48}$ might function in a dominant negative manner, interfering with the maternally contributed wild type Hem, leading to a higher penetrance of the defect. Ectopic expression of $\Delta Hem^{J4\text{-}48}$ by using a maternally expressed Gal4 driver *P{GAL4::VP16-nos.UTR}MVD2* in

Df(3L)ED230 background (*UAS-Hem^{J4-48}/ P{GAL4::VP16-nos.UTR}MVD2; Df(3L)ED230)* leads to an increase in the penetrance of the migration defect from 8.8% in *Df(3L)ED230* to 17.2% in *UAS-ΔHem^{J4-48}/ P{GAL4::VP16-nos.UTR}MVD2; Df(3L)ED230* embryos. This result further supports the idea that ΔHem^{J4-48} could interfere with maternally contributed wild type Hem and result in higher penetrance of the migration defect of RP2 neurons. My data shows that the protein level of Hem is less in *Hem^{J4-48}* mutants compared to *Df(3L)ED230* embryos, suggesting that ΔHem^{J4-48} may cause the degradation of maternally contributed wild type Hem. In *Hem^{J4-48}* mutants, a premature stop at 490 amino acids produces a truncated Hem (ΔHem^{J4-48}, 489 aa). In *Hem^{C3-20}* mutants, a premature stop at 256 amino acids produces a truncated Hem of smaller size (255 aa). Hem is part of the WAVE complex, which has four additional proteins. It is possible that ΔHem^{J4-48} might compete with the maternally contributed wild type Hem during the formation of the WAVE complex, thus the wild type Hem that fails to form the WAVE complex might be degraded, resulting in the lower protein level of maternal Hem in *ΔHem^{J4-48}* mutants. It is also possible that there is a reduction in the maternal Hem itself in this allele, which we have not yet determined. Moreover, when I generated mosaic animals for WAVE, the migration defect became stronger in the sense that the cells failed to undergo any migration at all, and that the penetrance was nearly complete. Therefore, a complete loss of Hem function is likely to cause the same arrest in migration as WAVE.

How could a down regulation of Hem lead to the migration defect of RP2 neurons in the VNC? Hem in *Drosophila* and it homologues in other organisms have been shown

to participate in many biological processes that require dynamic regulation of actin cytoskeleton networks such as in axon pathfinding (Baumgartner et al., 1995; Bogdan and Klambt, 2003; Hummel et al., 2000), myoblast fusion (Richardson et al., 2007; Schäfer et al., 2007; Schroter et al., 2004), neuromuscular junction maturation (Schenck et al., 2004), and cell migration (Nakao et al., 2008). At the same time, the dynamic regulation of actin cytoskeleton networks is observed in migrating cells *in vitro* and it is believed to provide the driving force for cell migration. Hem forms the WAVE complex in the cell with four other proteins: WAVE/SCAR, Sra-1/PIR121/CYFIP, Abi and HSPC300. The WAVE complex is able to promote actin polymerization through activation of Arp2/3 complex, which is able to initiate actin polymerization or actin filament branching in the cell. Another regulator of Arp2/3 complex, WASp, can be activated by Hem (Bogdan and Klambt, 2003). Hem may also mediate the transduction of external signals to the cytoskeleton by binding to an adaptor protein Dock/Nck of several RTKs (Kitamura et al., 1996; Li et al., 2001). With the expectation that Hem may regulate the migration of RP2 neurons together with or mediated by these molecules, a mini-screen with all the alleles and deficiencies of the *Hem* partner genes that are available (Table 1.2) was executed. The same Hem-migration defect of RP2 neurons is observed in $WAVE^{\Delta 37}$ and Abl^{2} mutants as well. This indicates that both $WAVE^{\Delta 37}$ and Abl^{2} mutants may be involved in the same pathway as Hem to regulate the migration of RP2 neurons. However, the possibilities of other genes however cannot be excluded even though no migration defect is observed in the mutants examined. All of these genes have maternal deposition of their products into the embryo and therefore maternally

contributed wild type gene products might rescue the zygotic loss of function phenotype for these genes.

As mentioned before, WAVE is able to promote actin polymerization, which is required for the actin skeleton networks in the leading edge of migrating cells. Regulation of its activity in the WAVE complex has been the subject of intense research. In my study, in *Hem* mutants the protein level of WAVE is decreased (Fig 1.8A) while the mRNA level remains unchanged (Fig. 1.14C). The protein level of WAVE in $Hem^{J4\text{-}48}$ mutants is even less than in *Df(2L)BSC32* embryos - the WAVE deficiency in which the whole WAVE gene is completely deleted. This indicates that Hem is necessary to maintain the protein level of WAVE (Fig. 1.10A). In $WAVE^{mat}$ embryos where both the maternally contributed and zygotically expressed WAVE is eliminated, there is a complete arrest of migration (Fig. 1.9B), indicating that WAVE is required for cell migration. The regulation of migration of RP2 neurons by Hem therefore, might be mediated by WAVE. In $Hem^{J4\text{-}48}$ mutants, the activity or the localization of WAVE might be affected as well. As a result, the migration pattern of RP2 neurons is disrupted.

As show in Fig. 1.11, in wild type embryos, WAVE is localized to the direction of the migration of RP2 neurons (Fig. 1.11 C, E). When RP2 neuron is not migrating (at ~9 hour of development), WAVE is not localized in RP2 (Fig. 1.11G), indicating that the localization of WAVE to the direction of migration is required for the proper migration of RP2 neurons. During development of the nervous system, RP2 neurons follow a migration route to their final positions and localized distribution of WAVE appears to be

an important factor in this. In *Hem* mutants since WAVE is not completely eliminated, RP2 neurons are therefore still able to migrate within the VNC. And the early stages of migration – step 1 and step 2 migration – of RP2 neurons are not affected (Fig. 1.11 D and F). In addition, the localization of WAVE to the direction of the migration of RP2 neurons remains unaffected. In embryos at ~ 9 hour, RP2 neurons in *Hem* mutants start to migrate toward the opposite hemi-segments, while RP2 neurons in wild type embryos remain stationary. However, in *Hem* mutants, WAVE is aberrantly localized to the direction of the migration of RP2 neurons (Fig. 1.11H). This indicates that sufficient WAVE is required for the correct migration of RP2 neurons during development of the VNC. A decrease in the protein level can result in the mis-localization of WAVE within RP2 neurons. Our result is further supported by data for the over-expression of WAVE in *Hem* mutant background (Fig. 1.12H), in which situation the migration defect of RP2 neurons is rescued. Thus, a decrease in the levels of WAVE appears to mediate the migration defect of RP2 neurons.

Three bands of WAVE that are close to each other (~ 82 kDa, ~ 80 kd and ~ 78 kDa) are detected by western blotting against WAVE antibody (Fig. 1.10A, Fig. 1.14A). These three bands are also detected when the single transgene of WAVE is expressed by using *sca*-Gal4 driver (Fig. 1.10B). It is therefore unlikely that these bands are three different isoforms, but more likely they arise from the post-translational modifications. When treated with lambda protein phosphatase (Lambda PP), these bands collapsed into a single band of ~ 78 kDa (Fig. 1.13), indicating that they arise from phosphorylation of WAVE. In $Hem^{J4\text{-}48}$ mutants the protein level of the first band appears to be less than that

of the second and third bands, although all three of them show a decrease (Fig. 1.10A and Fig. 1.14A). The significance of the phosphorylation and the functional difference of these three bands *in vivo,* remain unclear.

The same migration defect of RP2 neurons is observed in Abl^2 mutants (Fig. 1.8C). Abl is a tyrosine kinase and is able to phosphorylate many proteins in the cell. It is recruited to the WAVE complex by Abi after cell stimulation, and is required for the activation of WAVE *in vitro*. Mutation of tyrosine residue Y150, the major site of phosphorylation by Abl in WAVE2, abrogated WAVE2 driven actin polymerization, indicating that the phosphorylation of WAVE2 by Abl is required for the activation of WAVE complex (Leng et al., 2005; Stuart et al., 2006). It is yet to be shown if Abl is required for the activation of WAVE *in vivo*. Also, besides Abl, WAVE is also phosphorylated by other kinases. As there is a decrease in the protein levels of WAVE in *Hem* mutants, I expected Abl^2 mutants to show a similar decrease in the protein level of WAVE. Surprisingly, the protein level of WAVE is increased in Abl^2 mutants (Fig. 1.14A). And by ectopic expression of Abl, the protein level of WAVE is decreased. Therefore, in contrast to Hem, Abl negatively regulates the protein levels of WAVE. Abl may be directly or indirectly involved in the degradation of WAVE. Consistent with this, the protein levels of Hem remains unchanged in Abl^2 mutants (Fig. 1.14A), making it unlikely that Abl regulates the protein level of WAVE through Hem.

As shown in Fig. 1.15D, in the $Hem^{J4\text{-}48}$, Abl^2 double mutant embryos, the penetrance of the migration defect (16.5%) is higher than in $Hem^{J4\text{-}48}$ or Abl^2 mutants

alone. Based on this result, $Hem^{J4\text{-}48}$ and Abl^{2} seem to regulate the migration of RP2 neurons at different levels, otherwise in the double mutant, the penetrance should have been lower. At the same time, ectopic expression of WAVE does not result in any migration defect. It thus seems unlikely that the increased amount of WAVE in Abl^{2} mutants can cause the migration defect. The migration defect of RP2 neurons crossing the midline was also not observed (data not shown) when Abl is ectopically expressed by *sca*-Gal4, although the protein level of WAVE is down regulated (Fig. 1.14C). It further indicates that Abl regulates the activity of WAVE at a different level other than Hem. In *Hem* mutants, the down regulation of WAVE leads to the migration defect of RP2 neurons crossing the midline. A more probable explanation is that Abl is necessary for the inactivation of WAVE after it is activated. In Abl^{2} mutants therefore, WAVE is hyper-activated, causing the migration defect. It has been shown in *in vitro* experiments that Abl is required for the activation of WAVE2 and WAVE3 (not clear for WAVE1) (Sossey-Alaoui et al., 2007; Stuart et al., 2006). When stimulated by growth factors, Abl is recruited to the WAVE complex and it acts together with other components to activate WAVE. The formation of lamellipodia is decreased when the phosphorylation of WAVE by Abl is interrupted. So far, no *in vivo* evidence is available to support the idea that Abl is required for the activation of WAVE. However, if it is true, Abl may provide a negative feedback mechanism to regulate the activity of WAVE: Abl is required for the activation of WAVE. When WAVE is activated, Abl is then involved in the inactivation of WAVE. The activity of WAVE therefore is dynamically regulated. Since we cannot currently distinguish between an activate WAVE and an inactive WAVE, finding direct evidence to support this hypothesis is difficult.

To summarize, Hem, WAVE and Abl act together to regulate the migration of RP2 neurons during development of the *Drosophila* VNC. A migration defect of RP2 neurons is observed in these mutants: the RP2 neurons cross the midline and migrate from the initial hemi-segment to the opposite hemi-segment. Hem is needed for maintaining a certain level of WAVE in cells and its proper localization to the leading edge of a migrating RP2. In *Hem* mutants, the protein level of WAVE is down regulated and is not properly localized causing the migration defect of the RP2 neuron. In *Abl* mutants, the protein level of WAVE is increased and ectopic expression of Abl leads to down regulation of WAVE. Since the ectopic expression of WAVE does not result in any migration defect, Abl appears to be in part involved in the inactivation of activated WAVE. In *Abl* mutants, the continuously activated WAVE may cause the migration defect of RP2 neurons. My study uses *Drosophila* VNC as the model system and describes how cell migration is dynamically regulated in *vivo* during development of the VNC. It will not only help us understand the development of the *Drosophila* VNC better but may also improve our general understanding of genetic regulation of cell migration in the CNS.

Chapter II

HEM-protein regulates asymmetric cell division during development of the ventral nerve cord in *Drosophila melanogaster*

Abstract

Asymmetric cell division produces two daughter cells of different cell fates and is used by multi-cellular organisms to generate cell diversity. The nervous system consists of the most diverse cell types. Understanding the mechanisms responsible for asymmetric cell division in the nervous system could help us to learn how a variety of different cell types are generated, and to better understand the organization and function of the nervous system. Studies using the *Drosophila* VNC as a model system have revealed many molecules and their interaction in asymmetric cell division, yet many questions remain unanswered. In my study, two types of embryos are observed in $Hem^{J4\text{-}48}$ mutants: a "weak phenotype embryo" and a "strong phenotype embryo". Examining a typical and widely used NB4-2→GMC-1→RP2/sib lineage, I have found that the asymmetric division of GMC-1 is disrupted in the strong phenotype embryos of $Hem^{J4\text{-}48}$ mutants but not in the weak phenotype embryos of $Hem^{J4\text{-}48}$ mutants, $Hem^{C3\text{-}20}$ or *Hem* deficiency *Df(3L)ED230* embryos, despite that migration defect of RP2 neurons is observed in all of them. In the strong phenotype embryo of $Hem^{J4\text{-}48}$ mutants, instead of producing an RP2 neuron and a sib cell, GMC-1s symmetrically divides to produce two RP2 neurons. The symmetric division of GMC-1 may be caused by the truncated Hem protein ($\Delta Hem^{J4\text{-}48}$) in

Hem$^{J4\text{-}48}$ behaving as a neomorphic protein. I find that Insc, a polarity protein, which is localized in the apical cortex of GMC-1 in wild type, is distributed non-asymmetrically in the GMC-1 in the strong phenotype embryo of *Hem*$^{J4\text{-}48}$ mutants. The same symmetric division of GMC-1s is also observed in *Abl*^{2} mutants but not in *WAVE*$^{\Delta 37}$ mutants. It indicates that Abl may act together with Hem to regulate the asymmetric division of GMC-1s. Our results indicate that the actin cytoskeleton proteins Hem and Abl might be involved in the regulation of asymmetric localization of Insc, thereby regulating the asymmetric cell division of GMC-1.

Introduction

Asymmetric Cell Division

Cell division is the process by which a parent cell divides to produce two daughter cells. For simple unicellular organisms, cell division produces a new organism. For more complex multi-cellular organisms, cell division not only increases cell numbers but also generates a variety of cell types. Two daughter cells, produced by cell division, could be of the same cell fate by symmetric cell division. Alternatively, they could be of distinct cell types by asymmetric cell division. Asymmetric cell division is a mechanism widely adopted by multi-cellular organisms to generate cellular diversity during development, since it generates cells of different fates with distinct functions (Tajbakhsh et al., 2009). For example, stem cells that undergo self-renewing asymmetric cell divisions generate stem cells and progeny committed to a differentiation pathway, so that the stem cell

population is maintained and simultaneously different types of differentiated cells are produced (Knoblich, 2008; Tanaka et al., 2002).

It is clear that asymmetric cell division to produce daughter cells of distinct cell fates is regulated via both extrinsic and intrinsic pathways (Horvitz and Herskowitz, 1992; Tajbakhsh et al., 2009). In the extrinsic pathway, two daughter cells are identical to each other at the time of cell division. Through environmental cues or interaction with neighbor cells, they then adopt different cell fates. In the intrinsic pathway, two daughter cells inherit different cell fate determinants from the parent cell by preferential segregation during cell division and hence are different from each other at the time of cell division. Both of these strategies are employed by multi-cellular organisms to generate cell diversity. The specific cell lineage determines which one to choose. On the other hand, both in intrinsic and extrinsic pathways, intracellular genetic circuitry and extracellular circuitry interact (cell-cell communication), thus, these divisions are not very strict or exclusive.

The eukaryotic nervous system, of both vertebrates and invertebrates, consists of the most diverse cell types. For instance, the mammalian brain is estimated to contain several thousand different types of neurons (Stevens, 1998). It is believed that multi-potential neural progenitor cells may undergo a series of asymmetric cell divisions, either through an extrinsic pathway or an intrinsic pathway to self-renew and to generate several rounds of neurons of distinct identities. In living slices of developing ferret cerebral cortexes, neural precursor cells have been shown to divide asymmetrically to

produce different daughter cells. The basal daughter cell behave like a migratory neurons while the apical daughter cell is a neural precursor cell (Chenn and McConnell, 1995). Most of the molecules and mechanisms known to be involved in asymmetric cell division are conserved in both invertebrates and vertebrates. For example, Numb is shown to localize asymmetrically in dividing neural precursor cells both in mammals and *Drosophila*, indicating that an evolutionarily conserved network controls asymmetric cell division in many different cell types and organisms (Chenn and McConnell, 1995; Wakamatsu et al., 1999; Zhong et al., 1996). Therefore, mechanisms revealed by studies using model systems such as *S. cerevisiae*, *C. elegans* and *D. melanogaster* could also apply to humans.

Asymmetric cell division in Drosophila VNC

Much of our knowledge about the role of asymmetric cell division during the development of the nervous system has come from the study of the *Drosophila* nervous system. In *Drosophila,* asymmetric cell division is used to produce cell diversity in both the central nervous system (CNS, including the brain and the VNC) and the peripheral nervous system (PNS) (Jan and Jan, 2001). Most neurons are derived from their precursor cells by a stereotyped pattern of asymmetric cell division. During development of the *Drosophila* VNC, neural precursor cells called neuroblasts (NBs) delaminate from the neuroectoderm epithelium. Each NB divides asymmetrically to produce an apical parent-like NB and a basal ganglion mother cell (GMC). The GMC then divides asymmetrically again to produce two final stage daughter cells, which could be neurons or glial cells. By repeating this stereotyped pattern of asymmetric cell division, the

Drosophila VNC is built with all the neurons and glial cells (Bate, 1976; Bhat, 1999; Campos-Ortega and Jan, 1991; Gaziova and Bhat, 2007).

In general, there are three steps in asymmetric cell division: 1) Establishment of cell polarity; 2) Localization of cell fate determinants (could be proteins or mRNAs) in response to cell polarity to the opposite poles of dividing cells; 3) Orientation of mitotic spindle along the axis of polarity during mitosis. Thus, after cell division, cell fate determinants are segregated into two daughter cells of different cell fates. These three steps are repeated during development of the *Drosophila* nervous system, producing the variety of cell types needed (Broadus and Doe, 1997a; Knoblich, 2008; Matsuzaki, 2005; Uemura, 1994; Wodarz and Huttner, 2003; Yamashita and Fuller, 2008; Zhong and Chia, 2008).

Establishment of cell polarity

The first step in asymmetric cell division is to establish cell polarity in coordination with the body axis. It could be apical-basal polarity in NBs (Doe, 1996; Lin and Schagat, 1997) in the VNC or planar polarity in sensor organ precursors (SOPs) in the PNS (Orgogozo et al., 2002; Posakony, 1994). Despite this difference, NBs and SOPs use similar machinery to establish cell polarity (Jan and Jan, 2001).

The apical-basal polarity of NBs is established when they are delaminated from the neuroectoderm epithelium, inheriting the polarity of epithelial cells. The polarity of epithelial cells in turn is set up very early during embryogenesis by the apical localization

of the evolutionary conserved PAR complex consisting of Bazooka (Baz/Par-3), Par-6 and atypical protein kinase C (aPKC) (Muller and Wieschaus, 1996; Ohno, 2001; Wodarz, 2002). During the delamination, NBs move into the interior of the embryo, however leaving an apical stalk that remains in contact with other epithelial cells. During this time, Inscuteable (Insc) is recruited to the PAR complex via direct interaction with Baz, which leads to the apical localization of Insc. In embryos lacking both maternal and zygotic *bazooka* function, Insc no longer localizes asymmetrically in NBs and is instead uniformly distributed in the cytoplasm (Schober et al., 1999; Yu et al., 2000). Insc forms another complex with Partner of Insc (Pins) and heterotrimeric G-protein αi subunit (Gαi) during mitosis and thus mediates their localization in the apical region (Schaefer et al., 2000). In addition, the complex seems necessary to maintain the apical localization of Insc since in the absence of Pins, Insc becomes cytoplasmic (Yu et al., 2000). These proteins themselves do not influence cell fates of the daughter cells directly. Instead, they mediate the localization of the cell fate determinants to the apical or basal cortex (Li et al., 1997; Tio et al., 1999). They also help to orient the mitotic spindle during mitosis to ensure that cell fate determinants are segregated into the daughter cells, in this case NBs and GMCs (Kaltschmidt et al., 2000). In the absence of polarity proteins, cells have randomly distributed cell fate determinants or randomly positioned mitotic spindles In the late stages of mitosis - anaphase and telophase - Insc is degraded and becomes undetectable (Kraut et al., 1996). In GMCs however, Insc become localized to the apical cortex, serving again as a polarity determinant protein for the asymmetric division of GMCs.

The localization of these cell polarity proteins might be regulated by an apical/basal cytoskeleton structure established in cells. Actin filaments have been indicated in this structure since the apical localization of Insc is completely destroyed when actin filament formation is inhibited (Broadus and Doe, 1997b). This suggests that these polarity proteins depend on the organization of actin filaments to be apically localized. In contrast, when microtubules are destroyed, Insc remains in its normal apical localization, which indicates that microtubules may not be involved (Kraut et al., 1996). It is still unknown how the structure of actin filament functions to control the localization of polarity proteins.

Localization of cell fate determinants

Cell fate determinants in *Drosophila*, Numb, Prospero (Pros), Brat and their adaptor proteins Partner of Numb (PON) and Miranda (Mira), become localized to the basal cortex of the NB as it enters mitosis (Hirata et al., 1995; Knoblich et al., 1995; Shen et al., 1997; Spana and Doe, 1995; Spana et al., 1995). Thus, after division the determinants are segregated predominantly into the basal daughter cells (the GMCs). Pros is a transcription factor (Spana and Doe, 1995). In GMCs, upon degradation of Mira, Pros is translocated to the nucleus, activating genes that specify the GMC identity as well as inhibiting those that block GMCs differentiation. It may act as a switch between self-renew and differentiation (Choksi et al., 2006; Srinivasan et al., 1998). Numb is a membrane-associated protein that functions not only in NBs but also in many other binary cell fate decisions (Bhalerao et al., 2005; Uemura et al., 1989). Numb at least in part acts by antagonizing Notch activity to make two daughter cells that are different

from each other (Frise et al., 1996; Guo et al., 1996; Spana and Doe, 1996; Wai et al., 1999), although its function in the GMC is not clear. Its two mammalian homologues, m-Numb and Numb-like are also involved in cell fate determination (Zhong et al., 1997). Evidence shows that polarity proteins control the localization of cell fate determinants. The apical localization of PAR complex and Insc/Pins/Gαi complex regulates the basal localization of Pros and Numb. In the mutants for polarity components, such as in *baz*, *insc* or *pins* mutants, Pros and Numb as well as their adapter proteins, all lose their asymmetric localization pattern in NBs, which will have a uniform Numb/Pros distribution or randomly positioned Numb/Pros crescents (Li et al., 1997; Schaefer et al., 2000; Tio et al., 1999).

What is the mechanism used by apically localized proteins to control the basal localization of cell fate determinants in NBs? Since they are localized in the opposite cortex, a direct interaction between them seems to be very unlikely. The complex with three tumor suppressors Lethal giant larvae (Lgl), Disc Large (Dlg) and Scribble (Scrib) may function to fill this gap (Albertson and Doe, 2003; Ohshiro et al., 2000). Different from PAR complex that is localized only in the apical cortex, the Lgl/Dlg/Scrib complex is present ubiquitously in the whole cortex and thus, could directly interact with both polarity proteins and cell fate determinants. Studies have shown that Lgl protein directly binds to PAR-6 and aPKC of PAR complex and could be phosphorylated by aPKC (Betschinger et al., 2003). The phosphorylation of Lgl releases Lgl from the PAR complex and subsequently recruits PAR-3/Baz to the PAR complex (Plant et al., 2003; Yamanaka et al., 2003). At the same time, the phosphorylation of Lgl leads to its

conformational change and its ability to promote the cortical localization of cell fate determinants (Betschinger et al., 2005; Gaziova and Bhat, 2007; Hutterer et al., 2004; Smith et al., 2007). As a result, cell fate determinants could be only in the basal cortex, where aPKC is absent and Lgl is active. Then how does active Lgl promote the cortical localization of cell fate determinants in the basal cortex? Since an intact actin cytoskeleton but not microtubule is required for the basal localization of cell fate determinants (Knoblich et al., 1997), an actin-based motor may be responsible for transporting cell fate determinants to the basal cortex along actin filaments. The non-muscle myosin II, which is found to be in a complex with Lgl, may mediate the function of Lgl by an actin-dependent transport process (Strand et al., 1994). During the mitosis of NBs, myosin II first localizes at the apical cortex and then moves towards the site of cytokinesis. It may prevent binding of the cell fate determinants to actin filament and thus push the cell fate determinants to the basal cortex along actin filaments (Barros et al., 2003). Myosin VI Jaguar (Jar) has been shown to bind directly to the adapter protein Mira localized with it. In *jar* mutant NBs, Mira is mis-localized (Petritsch et al., 2003). Therefore, myosin VI may function as a motor to transport cell fate determinants to the basal cortex. However, this hypothesis is challenged by a quantitative live analysis of GFP-Pon and Numb-GFP fluorescence and fluorescence recovery after photo bleaching (FRAP) during SOP division. A directional lateral mobility of cell fate determinants was not detected. Instead, they investigated the rapid exchange of cell fate determinants between cytoplasm and cortex. This led to the conclusion that the change of their binding affinity to cytoplasm and cortex is responsible for the segregation of cell fate determinants (Mayer et al., 2005).

Orientation of the mitotic spindle

To ensure the asymmetric segregation of cell fate determinants into the daughter cells, mitotic spindle needs to be oriented along the axis of cell polarity in the NBs (Kraut et al., 1996). In addition, the shape of the mitotic spindle is essential for creating daughter cells of different sizes in asymmetric cell division (Kaltschmidt et al., 2000). During cell division, the centrosome replicates: one centrosome remains apical and the other moves 180º to the basal cortex of the cell, forming the mitotic spindle aligned with the plane of the epithelium during prophase. Then at metaphase, the mitotic spindle rotates 90º, in alignment with the apical-basal axis (Kaltschmidt et al., 2000; Spana and Doe, 1995). Cell polarity proteins are considered to be responsible for determining the orientation of the mitotic spindle. In *insc* mutants, spindle rotation is stopped. Thus NBs divide randomly instead of in the apical-basal manner (Kraut et al., 1996). Furthermore, ectopic expression of Insc in epithelial cells, which normally divide in alignment with the epithelium, causes a rotation of the mitotic spindle by 90º. This leads to an apical-basal cell division (Kraut et al., 1996). Insc is therefore required for the correct orientation of the mitotic spindle in NBs. The mechanism however, remains unclear. It has been suggested that Insc might interact with astral microtubules, causing rotation of the mitotic spindle (Giansanti et al., 2001). However there is no evidence that Insc directly binds to microtubules. Gαi, another polarity protein forming a complex with Insc at the apical cortex has also been indicated to be responsible for orientating the mitotic spindle (Betschinger and Knoblich, 2004). The protein Mushroom Body Defect (Mud, the fly homologue of human NuMA) has been shown to bind to Gαi, and enhance the

microtubule polymerization. In *mud* mutants, the mitotic spindle orientation is disrupted, leading to mis-segregation of cell fate determinants (Bowman et al., 2006). Gαi may thus act through Mud to control the orientation of the mitotic spindle.

The orientation of the mitotic spindle is required not only for the segregation of cell fate determinants, but also for producing two cells of difference sizes at the NB → GMC level. In the *Drosophila* VNC, NBs divide to produce a larger NB and a smaller GMC. This means that during the mitosis of NBs, an asymmetric positioning of the cleavage plane is required. The mitotic spindle therefore, should be asymmetric during mitosis: the apical half of the mitotic spindle should be longer than the basal half during mitosis (Kaltschmidt et al., 2000). In addition, the two centrosomes also differ in size and composition (Albertson and Doe, 2003; Giansanti et al., 2001). Studies have shown that both the PAR complex and the Insc/Gαi/Pins complex are probably involved in regulating the size asymmetry in two redundant pathways. No single mutants of any components of the PAR complex or the Insc/Gαi/Pins complex result in a high penetrance of loss of cell size asymmetry between the NB and GMC. It indicates that components of each pathway are sufficient to regulate the size asymmetry when the components of the other pathway are mutated. Instead, in any double mutant combinations of components of the PAR complex and the Insc/Gαi/Pins complex, two daughter cells of equal size are formed (Cai et al., 2003). Whether these two complexes target the same or different molecules remains unknown.

Asymmetric cell division in NB4-2→GMC-1→RP2/sib lineage

In our lab, we utilize NB4-2→GMC-1→RP2/sib to study asymmetric cell division during development of the VNC in *Drosophila*. It is a typical and well-studied NB lineage in *Drosophila* VNC (Bhat, 1996, 1999; Bhat and Apsel, 2004; Bhat et al., 1995; Bhat and Schedl, 1994, 1997; Bhat et al., 2000; Buescher et al., 1998; Gaziova and Bhat, 2007; Lear et al., 1999; Mehta and Bhat, 2001; Wai et al., 1999; Yedvobnick et al., 2004). A wealth of information is available in terms of genetic regulation of asymmetric cell division obtained by studying this lineage. Cells in this lineage can be distinguished by specific gene expression pattern, size differences and their position within the hemisegments in the VNC (Bhat, 1999; Doe, 1992). As mentioned before, after delamination from the neuroectoderm, NB4-2 divides asymmetrically to produce another NB and its first GMC (GMC-1). GMC-1 then divides asymmetrically, generating an RP2 motor neuron and a sib cell. The NB4-2, GMC-1, RP2 and sib differ in both cell size and nuclear size: GMC-1 is ~7.5 μm, RP2 is ~5 μm and sib is ~3 μm; the nuclear size of GMC-1 is ~6.5 μm, RP2 is ~4 μm and sib is ~2.5 μm (Gaziova and Bhat, 2007). There is also a level difference in marker gene expression between an RP2 and a sib. Even-skipped (Eve) was expressed both in the RP2 and sib. However the expression of Eve in the sib cell shows a gradual down regulation. By ~ 14 hour of embryonic development, expression of Eve is completely lost in the sib. MAP1B (Mab 22C10, stains the axon membrane) and Zfh-1 (Zn finger homeodomain 1) are also only detected in mature RP2. By using these genetic markers, as well as their position in the para-segment, we can very easily identify GMC-1, RP2 neuron and sib cell in the VNC (Buescher et al., 1998; Gaziova and Bhat, 2007).

During cell division of GMC-1, Insc is localized apically, which results in the basal localization of Numb (Buescher et al., 1998). After cell division of GMC-1, Numb is segregated into the basal daughter cell. Then the trafficking of the intracellular part of Notch into the nucleus is inhibited by Numb in the basal daughter cell, which finally adopts the RP2 neuron fate (Buescher et al., 1998; Schuldt and Brand, 1999; Skeath and Doe, 1998). In contrast, the apical daughter cell does not inherit Numb, thus the Notch signaling is not inhibited. As a result, the apical daughter cell becomes a sib cell (Fig. 2.1). In the loss of function for *Insc* mutants, Numb is distributed universally in the GMC-1. After cell division, both of the two daughter cells will have Numb and will thus become RP2 neurons. On the other hand, in the loss of function for *Numb* the two daughter cells will become two sib cells (Buescher et al., 1998).

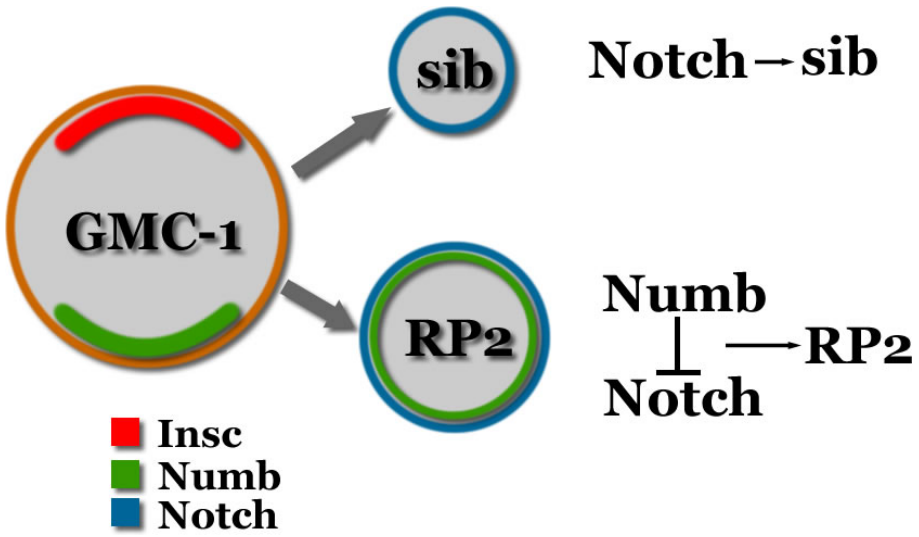


Fig. 2.1 Asymmetric division of GMC-1 generates an RP2 and a sib. Insc is apically localized in GMC-1. It leads to the basal localization of Numb. After cell division, Notch is present on the membrane of both daughter cells. In the cell that has Numb, Notch signaling is inhibited. It adopts an RP2 fate. In the cell that does not have Numb, it adopts a sib fate.

During our studies on Hem, I have found that loss of function for *Hem,* in addition to the migration defect, causes a symmetric division of GMC-1. Instead of producing an RP2 neuron and a sib cell, GMC-1 in $Hem^{J4\text{-}48}$ mutants divides symmetrically to produce two RP2 neurons. This symmetric division of GMC-1 is only observed in $Hem^{J4\text{-}48}$ mutants but not in another *Hem* allele $Hem^{C3\text{-}20}$ or *Hem* deficiency *Df(3L)ED230* embryos. The truncated Hem protein ($\Delta Hem^{J4\text{-}48}$) in $Hem^{J4\text{-}48}$ alleles may behave as a neomorphic protein, causing the symmetric division of GMC-1. The apical localization of Insc is disrupted in $Hem^{J4\text{-}48}$ mutants, indicating that Hem regulates the asymmetric division of GMC-1 mediated by Insc. The same symmetric division of GMC-1 is also observed in Abl^{2} mutants but not in $WAVE^{\Delta 37}$ mutants. Our results indicate that the actin cytoskeletons protein Hem and Abl may mediate the asymmetric cell division of GMC-1 by regulating the localization of Insc in the apical cortex.

Materials and Methods

Refer to the Materials and Methods in Chapter I.

Results

Two types of phenotypic embryos – weak and strong – are observed among $Hem^{J4\text{-}48}$ mutant embryos

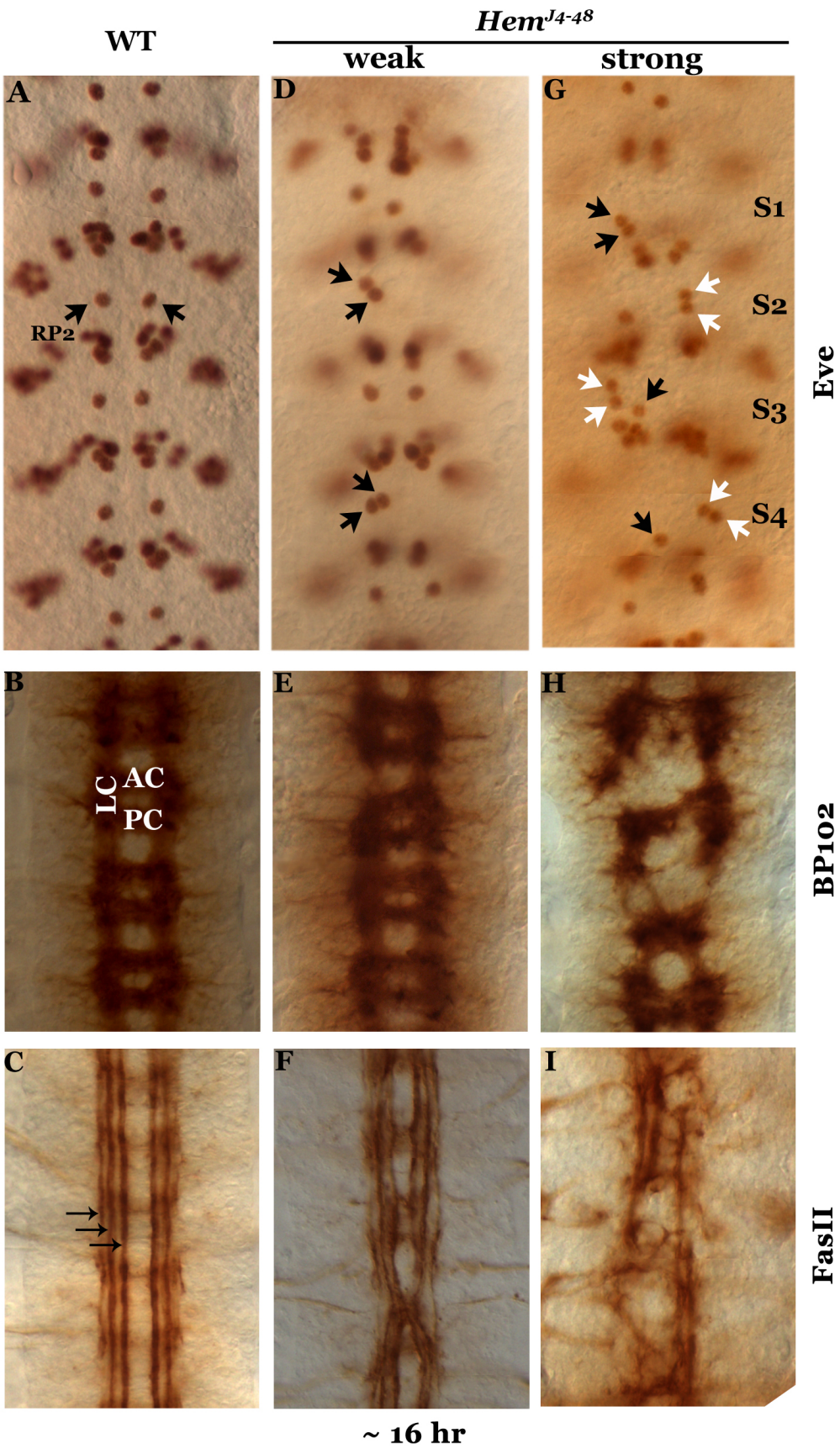
Two types of embryos are observed among the *Hem*$^{J4\text{-}48}$ mutant embryos. In the first type, the migration defect of RP2 neurons is the main mutant phenotype observed (Fig. 1.2B, Fig. 2.2D). In these embryos, the structure of the VNC by immunostaining with anti-BP102 (Fig. 2.2E), which stains the commissural and connectives structure of the nerve cord, compared to wild type embryos is marginally affected (Fig. 2.2B). However, a weak axon guidance defect is observed by the more sensitive immunostaining with anti-Fas II (Fig. 2.2F). In wild type, Fas II staining displays three longitudinal tracts on either side of the midline in the embryo (Fig. 2.2C). In *Hem*$^{J4\text{-}48}$ mutants, occasionally inappropriate midline crossing of the tracts is observed, indicating weak axon guidance defects (Fig. 2.2F).

In the second type of mutant embryos however, the VNC structure is significantly disrupted as shown by both the BP102 staining (Fig. 2.2H) and Fas II staining (Fig. 2.2I). It is observed in about 1 out of 4 *Hem*$^{J4\text{-}48}$ mutant embryos. I have named the first type of embryos as "weak phenotype embryo" and the second type of embryos as "strong phenotype embryo".

Symmetric division of GMC-1s is observed in strong phenotype embryo of Hem$^{J4\text{-}48}$ mutants

The migration defect of RP2 neurons is observed in both of the two types of embryo of *Hem*$^{J4\text{-}48}$ mutants (Fig. 2.2D and G, Table 2.1). The penetrance of the migration defect of RP2 neurons is 14.1% in the strong phenotype embryo, slightly higher than 12.9% in the weak phenotype embryo. Interestingly in the strong phenotype

embryos of the *Hem*$^{J4\text{-}48}$ mutant, besides the migration defect of RP2 neurons, another phenotype is observed: hemisegments had two RP2 neurons but this is not caused by the migration of an RP2 from the contralateral hemi-segment (Fig. 2.2G, white arrow). In the first segment (S1) of the nerve cord shown in this photomicrograph, two RP2 neurons (black arrow) are observed in one hemi-segment and none in the opposite hemi-segment (Fig. 2.2G). This is caused by the migration defect as described in Chapter I (Fig. 1.2B). In the second segment (S2) two RP2 neurons (white arrow) are observed in one hemi-segment and one RP2 neuron is observed in the opposite hemi-segment. As described in the introduction, in wild type embryos GMC-1 divides asymmetrically to produce an RP2 neuron and a sib cell in each hemi-segment. Hence there is only one RP2 neuron and one sib cell in each hemi-segment (Fig. 2.2A). In these hemisegments of *Hem*$^{J4\text{-}48}$ mutants that have duplicated RP2s without any missing RP2, it appears likely that the GMC-1 divides symmetrically, producing two identical daughter cells, both of which adopt an RP2 neuron fate. I did not see a sib cells in such hemisegments, which is consistent with the idea that the GMC-1 divided symmetrically in these hemisegments. I also point out that the symmetric division of GMC-1 and the migration defect of RP2 neuron are observed in the same embryo and even in the same segment. As shown in the third and the fourth segment (Fig. 2.2G), duplicated RP2 neurons (white arrow) are produced from the symmetric division of GMC-1, while an RP2 neuron (black arrow) crosses the midline and is found in the opposite hemi-segment.


WT
Hem^J4-48
weak
strong
A
D
G
RP2
S1
S2
S3
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H
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AC
PC
BP102
C
F
I
FasII
~ 16 hr

Fig. 2.2 Two types of embryos – weak phenotype embryo and strong phenotype embryo - are observed in *Hem^{J4-48}* mutants. A) Wild type embryo stained with anti-Eve, each hemisegment has only one RP2. B) Wild type embryo stained with anti-BP102, showing the VNC structure. AC: Anterior commissure; PC: Posterior commissure; LC: Longitudinal connective. C) Wild type embryo stained with anti-Fas II. Three longitudinal tracts can be seen on either side of the midline. D) Weak phenotype embryo of *Hem^{J4-48}* mutant stained with anti-Eve. The migration defect of RP2 neuron results in two RP2 neurons in one hemi-segment and none in the opposite hemi-segment. E) Weak phenotype embryo of *Hem^{J4-48}* mutants stained with anti-BP102. The VNC structure remains more or less normal. F) Weak phenotype embryo of *Hem^{J4-48}* mutants stained with anti-Fas II. A weak axon guidance phenotype was observed, showing tracts crossing the midline. G) Strong phenotype embryo of *Hem^{J4-48}* mutants stained with anti-Eve. In the S1 segment, the RP2 neuron in one hemi-segment crosses the midline and stays in the opposite hemi-segment (black arrow). In S2 segment, GMC-1 divides symmetrically, producing two RP2 neurons in one hemi-segment (white arrow). In S3 and S4 hemi-segments, GMC-1s divide symmetrically to produce two RP2 neurons (white arrow). At the same time, one RP2 neuron (black arrow) crosses the midline and moves to the opposite hemi-segment. H) Strong phenotype embryo of *Hem^{J4-48}* mutants stained with anti-BP102. The VNC structure was significantly disrupted. I) Strong phenotype embryo of *Hem^{J4-48}* mutants stained with anti-Fas II. Significant axon guidance defect was observed. That these embryos are indeed *Hem^{J4-48}* homozygous embryos were confirmed by sequencing the *Hem* gene in these embryos.

Symmetric division of GMC-1 is observed in Hem^{J4-48} mutants but not in Hem^{C3-30} or in Hem deficiency Df(3L)ED230 embryos.

As described in Chapter I, the migration defect of RP2 neurons is observed in all the examined alleles of *Hem* as well as a deficiency for *Hem* (Fig. 1.6B, Table 2.1). However, the strong phenotype embryo as well as the symmetric division of GMC-1 is only observed in *Hem^{J4-48}* mutants but not in *Hem^{C3-20}* or *Df(3L)ED230* mutants (Fig. 2.3, Table 2.1). As shown in Fig. 2.3B, in the weak phenotype embryos of *Hem^{J4-48}* mutants, two RP2 neurons (black arrow) are present in one hemi-segment and none in the opposite

Table 2.1 The penetrance of the migration defect of RP2 neurons and the symmetric division of GMC-1 in *Hem* alleles*

Genotype		Penetrance	
		Migration defect of RP2 neurons	**Symmetric division of GMC-1**
$Hem^{J4\text{-}48}$	Weak phenotype embryo	12.9%	0
	Strong phenotype embryo	14.1%	33%
$Hem^{C3\text{-}20}$		8.3%	0
Df(3L)ED230		8.8%	0
$Hem^{J4\text{-}48}$/ *Df(3L)ED230*	Weak phenotype embryo	9.29%	0
	Strong phenotype embryo	12%	31%

* The VNC of some of the strong phenotype embryos is severely disrupted. It makes impossible to identify RP2 neurons in the VNC in these embryos. The penetrance data in strong phenotype embryos therefore, is only counted in those embryos that the VNC remains relatively better and I can identify RP2 neurons for sure.

hemi-segment (Fig. 2.3A). At the same, in Fig. 2.3C, the strong phenotype embryos with symmetric division of GMC-1s is observed in $Hem^{J4\text{-}48}$ mutants. However, in $Hem^{C3\text{-}30}$ (Fig. 2.3D) and *Df(3L)ED230* (Fig. 2.3 E) mutants, only migration defect of RP2 neurons is observed but never the symmetric division of GMC-1s. Interestingly, in strong phenotype embryos that are trans-heterozygous for $Hem^{J4\text{-}48}$ and the *Hem* deficiency *Df(3L)ED230,* the symmetric cell division of GMC-1s as well as the migration defect of RP2 neurons are observed (Fig. 2.3 F and G). Furthermore, when the truncated Hem protein $\Delta Hem^{J4\text{-}48}$ is ectopically expressed in the background of *Df(3L)ED230*, the symmetric cell division of GMC-1 is observed (Fig. 2.3H). These results suggest that

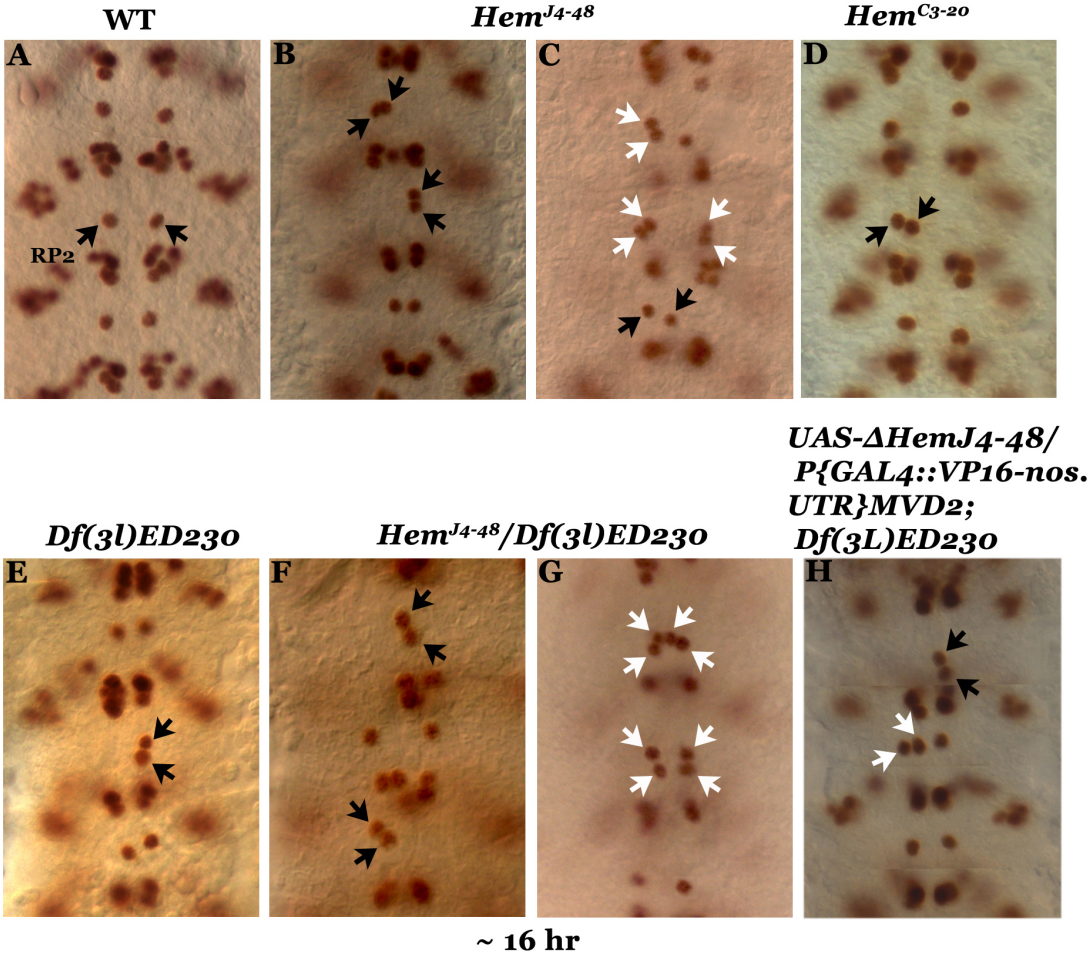

Fig. 2.3 Symmetric division of GMC-1 is only observed in *Hem*$^{J4-48}$ mutants and not *Hem*$^{C3-20}$ or *Df(3L)ED230* mutants. Embryos are stained with Eve antibody. A) Wild type embryos. B) *Hem*$^{J4-48}$ mutants. Migration defect of RP2 neurons (black arrow) can be seen. C) *Hem*$^{J4-48}$ mutants. Symmetric division of GMC-1 is indicated by the duplication of RP2 neurons (white arrow) without the loss of RP2 in the other hemi-segment. D) *Hem*$^{C3-20}$ mutants. Only migration defect of RP2 neurons is observed but not the symmetric division of GMC-1. E) *Df(3L)ED230* mutants. Only the migration defect of RP2 neurons is observed. F) Heterozygous *Hem*$^{J4-48}$*/Df(3L)ED230* embryos (weak phenotype). Only the migration defect of RP2 neurons is observed. G) Heterozygous *Hem*$^{J4-48}$*/Df(3L)ED230* mutants (strong phenotype). Symmetric division of GMC-1s is observed. H) Ectopic expression of *ΔHem*$^{J4-48}$ *in Df(3L)ED230* mutants. Both migration defect and the symmetric division of GMc-1 are observed.

the truncated *Hem*$^{J4\text{-}48}$ protein somehow interacts with other proteins to produce this strong phenotype given that this truncated protein behaves as an antimorphic protein. However, since the deficiency does not suppress this defect (Table 2.1, the penetrance of the symmetric division of GMC-1 is 33% in *Hem*$^{J4\text{-}48}$ and 31% in *Hem*$^{J4\text{-}48}$*/Df(3L)ED230*), it seems more likely that it also carries a neomorphic effect.

Inscuteable (Insc) is mis-localized in Hem$^{J4\text{-}48}$ mutants

Insc is one of the key cell polarity proteins that function in the asymmetric division of GMC-1. Its apical localization leads to the basal localization of Numb and the proper orientation of the mitotic spindle along the cell polarity axis. This ensures the segregation of Numb into the basal daughter cell. After cell division, the basal daughter cell becomes an RP2 neuron while the apical daughter cell becomes a sib cell. In *Insc* mutants, since there is no apical localization of Insc, the basal localization of Numb is disrupted but is expressed around the GMC-1. It causes both daughter cells to inherit Numb and both of them become RP2 neurons.

I next examined if the symmetric division of GMC-1 in *Hem*$^{J4\text{-}48}$ mutants is caused by the disruption of Insc localization. As shown in Fig. 2.4A, in wild type embryos, Insc (arrow) is apically localized in the GMC-1 (arrow head). In *Hem*$^{J4\text{-}48}$ mutants however, Insc is non-localized and is distributed all along the cortex of GMC-1. Hem is therefore, involved in the apical localization of Insc. It seems likely that *Hem*$^{J4\text{-}48}$ mutation leads to non-localization of Insc, resulting in symmetric division of GMC-1.

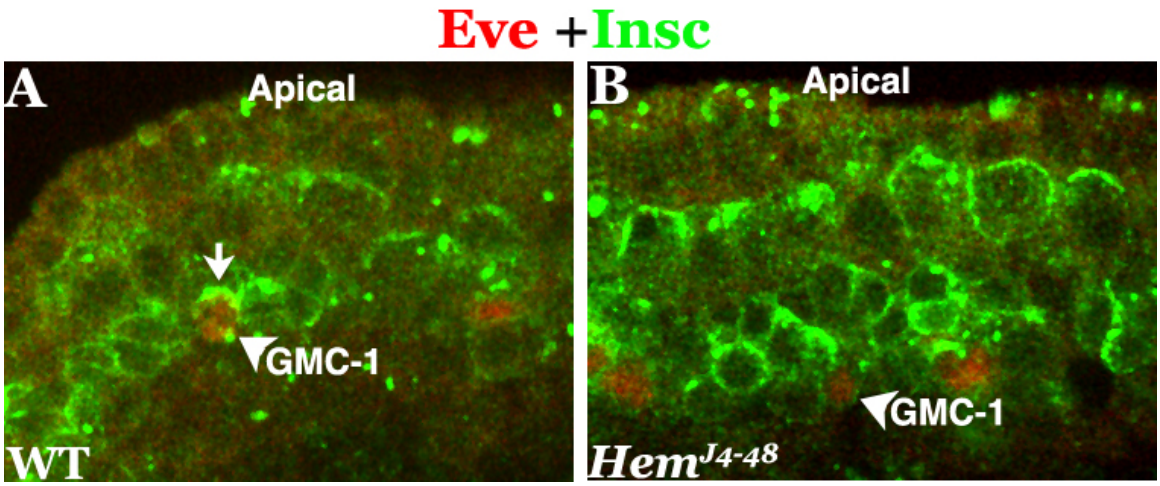

Fig. 2.4 Insc is mis-localized in *Hem*$^{J4-48}$ mutants. A) In wild type embryos, Insc (arrow) is apically localized in GMC-1 (arrowhead). B) In *Hem*$^{J4-48}$ mutants, Insc is uniformly distributed along the cortex of GMC-1.

Symmetric division of GMC-1 is also observed in Abl^2 mutants

Hem forms a complex with four other proteins: WAVE/SCAR, Sra-1/PIR121/CYFIP, Abi and HSPC300. Abl might be recruited to this complex through interaction with Abi (Stuart et al., 2006). Hem has also been shown to activate WASp in the membrane (Bogdan and Klambt, 2003). Dock, the homologue of Nck in human may interact with Hem protein. For that reason, these proteins might function with Hem to regulate the asymmetric division of GMC-1. I examined the alleles and deficiencies for these *Hem* partner genes that I was able to obtained from the Bloomington stock center and private labs (listed in Table 2.2). Of the alleles and genes examined, a similar symmetric division of GMC-1 is observed only in *Abl^2* mutants. All of the other genes examined have maternal effect (so does Abl) and this lack of RP2 duplication might be due to maternal deposition of the wild type product.

Table 2.2 All the alleles and deficiencies of *Hem* partner genes from Bloomington stock center and private labs

Gene	Alleles and deficiencies
WAVE	$SCAR^{\Delta 37}$, $SCAR^{k03107}$, $P\{EP\}SCAR^{G12874}$ and *Df(2L)BSC32*
Sra-1	$P\{EPgy2\}Sra\text{-}1^{EY06562}$ and *Df(3R)Exel6174*
Abi	$P\{EPgy2\}Abi^{EY20423}$ and *Df(3R)Exel7359*
HSPC300	$P\{EP\}HSPC300^{EP506}$， $P\{EP\}HSPC300^{G19021}$ and *Df(2R)Exel6080*
Abl	Abl^{1}, *Abl2*, Abl^{4} and *Df(3L)st7*
WASp	$P\{EPgy2\}WASp^{EY06238}$, wsp^{1}, wsp^{3} and *Df(3R)3450*
dock	$P\{PZ\}dock^{04723}$, $P\{lacW\}dock^{k13421}$ and *Df(2L)ast1*

In *Abl* mutants, due to a migration defect, two RP2 neurons (black arrow) can be found in one hemi-segment and none in the opposite hemi-segment (Fig. 2.5C). At the same time, two RP2 neurons (white arrow) are also observed in a hemi-segment without any loss in the opposite hemi-segment. This indicates that the RP2 neurons are produced by symmetric division of GMC-1 but not by a migration defect. On the other hand, though migration defect of RP2 neurons is observed in $WAVE^{\Delta 37}$ mutants (Fig. 1.8D), no symmetric division of GMC-1 is observed. In $WAVE^{mat}$ embryos in which the maternally contributed wild type WAVE is completely depleted, there is still no symmetric division of GMC-1. Therefore, although *Hem*, *Abl* and *WAVE* are all involved in controlling the migration of RP2 neurons, only *Hem* and *Abl* are involved in regulating the symmetric division of GMC-1 cells. In the double mutant of $Hem^{J4\text{-}48}$, Abl^{2} embryos (Fig. 2.5D), both

the migration defect of RP2 neurons (black arrow) and the symmetric division of GMC-1 (while arrow) could be observed. The penetrance of the migration defect in $Hem^{J4\text{-}48}$, Abl^{2} double mutants is 16.5%, higher than 12.9% in $Hem^{J4\text{-}48}$ mutants and 9% in Abl^{2} mutants. However, the penetrance of the symmetric division of GMC-1 in $Hem^{J4\text{-}48}$, Abl^{2} double mutants is about 29%, higher than 9% in Abl^{2} mutants but it is almost the same compared with 33% in $Hem^{J4\text{-}48}$ mutants. This result indicates that Hem and Abl may regulate the asymmetric cell division of GMC-1 by a different mechanism than the migration of RP2 neurons.

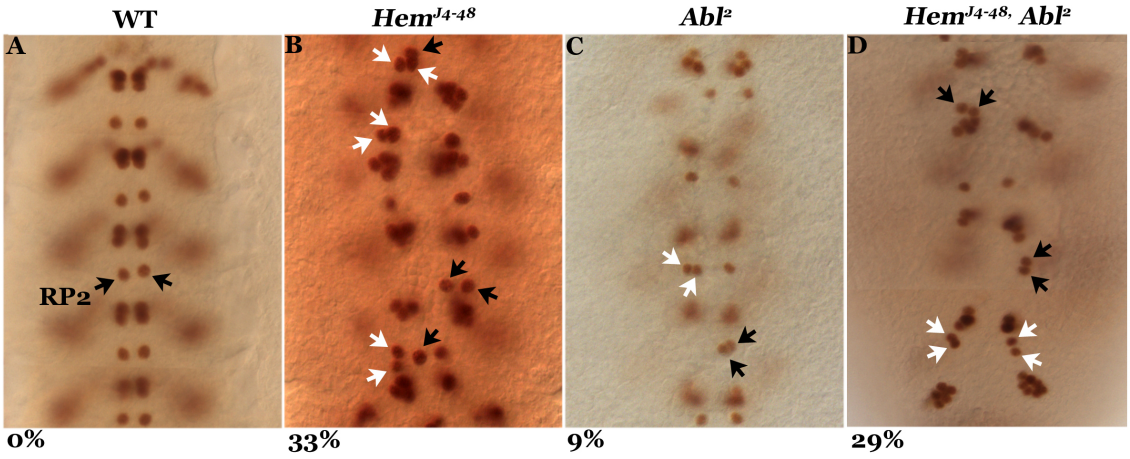

Fig. 2.5 Symmetric cell division in $Hem^{J4\text{-}48}$, Abl^{2} and $Hem^{J4\text{-}48}$,Abl^{2} double mutant embryos. A) In WT embryos, there is only one RP2 produced by the asymmetric division of GMC-1 in each hemi-segment. B) In $Hem^{J4\text{-}48}$ mutants, symmetric division of GMC-1s produces two RP2 neurons (white arrow) in one hemi-segment (33% penetrance). At the same time, migration defect of RP2 neurons (black arrow) is also observed. C) In Abl^{2} mutants, both symmetric division of GMC-1 (penetrance 9%) and the migration defect of RP2 neurons are observed. D) In $Hem^{J4\text{-}48}$, Abl^{2} double mutant embryos, both symmetric division of GMC-1 (penetrance 29%) and migration defect of RP2 neurons are observed.

Discussion

Two types of embryos – "weak phenotype embryo" and "strong phenotype embryo" are observed in *Hem*$^{J4\text{-}48}$ mutants. The weak phenotype embryos had more or less a wild type like morphology (data not shown) and the VNC structure as per the BP102 staining remains mostly unperturbed (Fig. 2.2E) compared to wild type embryos (Fig. 2.2B). When stained with more sensitive anti-Fas II, it shows a weak axon guidance defect (Fig. 2.2F). The longitudinal tracts occasionally crossed the midline. A migration defect of RP2 neurons is observed in weak phenotype embryos as described in detail in Chapter I (Fig. 2.2D). However, the RP2/sib lineage is unaffected. In strong phenotype embryos, however, the overall structure of the VNC is significantly disrupted (Fig. 2.2H). The longitudinal tracts show significant axon guidance defects (Fig. 2.2I). These embryos have the symmetric division of GMC-1 to generate two RP2 neurons in a significant number of hemisegments (penetrance 33%). This division defect as well as the generation of the strong phenotype embryos is not observed in the other *Hem* mutant alleles or a deficiency that removes the *Hem* gene. This makes this class of embryos highly specific to *Hem*$^{J4\text{-}48}$ mutants.

The migration defect of RP2 neurons is observed in both the weak phenotype embryos and the strong phenotype embryos of *Hem*$^{J4\text{-}48}$ mutants (Fig. 2.2D and G). A symmetric cell division however, is observed only in the strong phenotype embryos of *Hem*$^{J4\text{-}48}$ mutants (Fig. 2.2G). In wild type embryos, GMC-1 divides asymmetrically to generate an RP2 neuron and a sib cell. In my study, I have found that in the strong

phenotype embryos of *Hem*$^{J4\text{-}48}$ mutants, the symmetric cell division of GMC-1 produces two RP2 neurons, instead of one RP2 neuron and one sib cell (Fig. 2.2G). This indicates that Hem is involved in regulating the asymmetric cell division of GMC-1s. There is a significant difference between the migration defect of RP2 neurons (described in Chapter I) and the symmetric cell division of GMC-1 (described in Chapter II) although both the migration defect and the symmetric cell division of GMC-1 finally lead to two RP2 neurons in one hemi-segment. In the migration defect of RP2 neurons, one RP2 neuron migrates from its initial hemi-segment, crosses the midline and resides in the opposite hemi-segments. As a result, two RP2 neurons are found in one hemi-segment, while there are none in the opposite hemi-segment (Fig. 2.2G, S1, S3 and S4, black arrow). In the symmetric division of GMC-1, GMC-1 divides symmetrically to produce two RP2 neurons instead of one RP2 neuron and a sib cell. Therefore, there are two RP2 neurons in one hemi-segment at the expense of a sib cell (Fig. 2.2G, S2, S3 and S4 white arrow). The RP2 neuron in the opposite hemi-segment however, is unaffected. The RP2 neurons of the mis-migration and from the symmetric division of GMC-1s could be observed in the same embryos and the same segment (Fig. 2.2G). It indicates that during development of the *Drosophila* embryo, Hem is repeatedly used in different biological events.

Also, symmetric cell division of GMC-1s is not observed in *Hem*$^{C3\text{-}20}$ mutants or *Df(3L)ED230 Hem* deficiency embryos (Fig. 2.3) although the migration defects of RP2 neurons were detected in both *Hem*$^{J4\text{-}48}$, *Hem*$^{C3\text{-}20}$ mutants and *Df(3L)ED230 Hem* deficiency embryos. It seems possible that the truncated Hem protein (ΔHem$^{J4\text{-}48}$) may interfere with the maternally contributed Hem. The interference therefore can disrupt its

normal function and result in the symmetric division of GMC-1s. The *Hem*$^{C3\text{-}20}$ in the other hand produces a smaller truncated Hem protein ΔHem$^{C3\text{-}20}$ of 255 amino acids than ΔHem$^{J4\text{-}48}$ of 489 amino acids. ΔHem$^{C3\text{-}20}$ therefore, may not be able to interfere with the normal function of maternally contributed Hem in the asymmetric cell division of GMC-1s. As a result, GMC-1s still divide asymmetrically in *Hem*$^{C3\text{-}20}$ mutants. However, the penetrance of the migration defect in the strong phenotype embryo of *Hem*$^{J4\text{-}48}$ mutants (14.1%) is not significantly higher than that of the weak phenotype embryos (12.9%). Since the down regulation of Hem results in the migration defect of RP2 neurons (Chapter I), a higher penetrance of migration defect is expected in the strong phenotype embryos if they are caused by stronger interference of ΔHem$^{J4\text{-}48}$ with the maternal contributed Hem.

Therefore, it seems likely that the strong mutant embryos in *Hem*$^{J4\text{-}48}$ allele arising from the possibility that truncated Hem protein ΔHem$^{J4\text{-}48}$ protein also behaves as a neomorphic protein, with certain new activity acquired due to its truncated structure and via interaction with some unknown proteins. This possibility is consistent with the finding that the strong mutant embryos are also observed from a cross between *Hem*$^{J4\text{-}48}$ and *Hem* deficiency. The penetrance in the trans-heterozygous of *Hem*$^{J4\text{-}48}$ and *Hem* deficiency *Df(3L)ED230* is ~31% and is 33% in *Hem*$^{J4\text{-}48}$. That is, this class of embryos did not respond to the *Hem* deficiency.

The results from my studies show that Hem influences the asymmetric localization of Insc. During asymmetric cell division, Insc is a key polarity protein. Its

apical localization in GMC-1s ensures the basal localization of Numb and subsequent segregation only to the basal daughter cells, which finally become RP2 neurons (Fig. 2.1). In *Hem*$^{J4\text{-}48}$ mutants, the apical localization of Insc is disrupted (Fig. 2.5B) and became uniformly distributed around the cortex of GMC-1s. Since the basal localization of Numb is based on the apical localization of Insc, the loss of apical localization of Insc would result in the non-asymmetric distribution of Numb. As a consequence, both daughter cells inherit Numb after cell division of GMC-1 and hence, become two RP2 neurons. An intact actin cytoskeleton network is required for the apical localization of Insc. When actin filaments are inhibited, the apical localization of Insc is completely repressed (Broadus and Doe, 1997b). Hem is an actin cytoskeleton protein that dynamically regulates the actin filaments polymerization. It is involved in many biological processes that require a dynamic regulation of actin networks. Hem may therefore, regulate the apical localization of Insc through the regulation of the actin cytoskeleton network.

Through the mini-screen (Table 2.2), the same symmetric division of GMC-1 is also observed in *Abl*^{2} mutants. As Abl has been identified to regulate the actin polymerization, it may act together with Hem to regulate the actin cytoskeleton network that is responsible for the apical localization of Insc. No symmetric division of GMC-1 is observed in WAVE mutants, deficiency and in *WAVE*^{mat} mutants, in which WAVE is completely depleted. Therefore, WAVE is not involved in the regulation of the apical localization of Insc. It indicates that the asymmetric division of GMC-1 is regulated by Hem by a different mechanism than that of cell migration of RP2 neurons. WASp is

another actin polymerization regulator and has been shown to promote the polymerization of the parallel actin filaments in filopodia (Marchand et al., 2001; Takenawa and Miki, 2001). Though symmetric cell division of GMC-1 was not observed in *WASp* mutants in my mini-screen, this is likely due to maternal deposition. In fact, Ben-Yaacov *et al*. have described duplication of RP2 neurons in *WASp* mutants (Ben-Yaacov et al., 2001). If it is true, Abl and Hem might regulate the apical localization of Insc through WASp. This theory needs further investigation. The same symmetric division of GMC-1 is observed in double mutants of $Hem^{J4\text{-}48}$, Abl^{2}. Different from the higher penetrance of the migration defect in $Hem^{J4\text{-}48}$, Abl^{2} double mutants (16.5%) than in $Hem^{J4\text{-}48}$ (12.9%) mutants and in Abl^{2} mutants (9%), the penetrance of the symmetric division of GMC-1 in $Hem^{J4\text{-}48}$,Abl^{2} double mutants (29%) is almost the same as $Hem^{J4\text{-}48}$ mutants (33%). This result further supports that Hem and Abl may regulate the asymmetric cell division of GMC-1 by a different mechanism than that of the migration of RP2 neurons.

To summarize, there are two types of embryos in $Hem^{J4\text{-}48}$ mutants: the strong phenotype embryos and weak phenotype embryos. Symmetric cell division of GMC-1 is observed only in the strong phenotype embryo of $Hem^{J4\text{-}48}$ mutants although migration defects of RP2 neurons are observed in both of them. The strong phenotype embryo as well as the symmetric division of GMC-1 is not observed in other *Hem* alleles and deficiency. The truncated Hem protein ($\Delta Hem^{J4\text{-}48}$) in $Hem^{J4\text{-}48}$ alleles may behave as a neomorphic protein, resulting in the strong phenotype embryo and the symmetric division of GMC-1. The apical localization of Insc in GMC-1 is disrupted in the strong phenotype

embryos of *Hem*$^{J4\text{-}48}$ mutants and becomes uniformly distributed in the cortex. This indicates that Hem may regulate the asymmetric division of GMC-1 mediated by the localization of Insc. In *Abl* mutants, the same symmetric division of GMC-1 is observed. Since the intact cytoskeleton is required for the localization of Insc, Hem and Abl may regulate the apical localization of Insc by dynamically regulating the actin cytoskeleton and finally regulating the asymmetric division of GMC-1.

Appendix: List of Abbreviations

Abl	Abl tyrosine kinase
Abi	Abelson interacting protein
CNS	central nerve system
Eve	Even-skipped
GMC	Ganglion mother cell
Hem	HEM-protein
Insc	Inscuteable
NB	Neuroblast
VNC	ventral nerve cord
WASp	Wiskott-Aldrich Syndrome protein
WAVE	WASP-family verprolin-homologous protein

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