Functional analysis of a new dominant-negative allele of *miranda* during CNS development in *Drosophila*

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1 Introduction

1.1 Generation of cell diversity

Multicellular organisms generate a variety of different cell types during development. Cell diversity can be achieved by asymmetric cell division, which produces two different cell types from one progenitor cell. There are two ways to produce two distinct daughter cells. One is by an extrinsic mechanism, which means that two initially equivalent cells are committed to different fates as a consequence of communication between the cells or exposure to different environments. The other is by an intrinsic mechanism, in which cell fate determinants segregate into only one of the two daughter cells, thus committing one cell to a different fate from the other (Horvitz and Herskowitz, 1992) (figure 1.1).

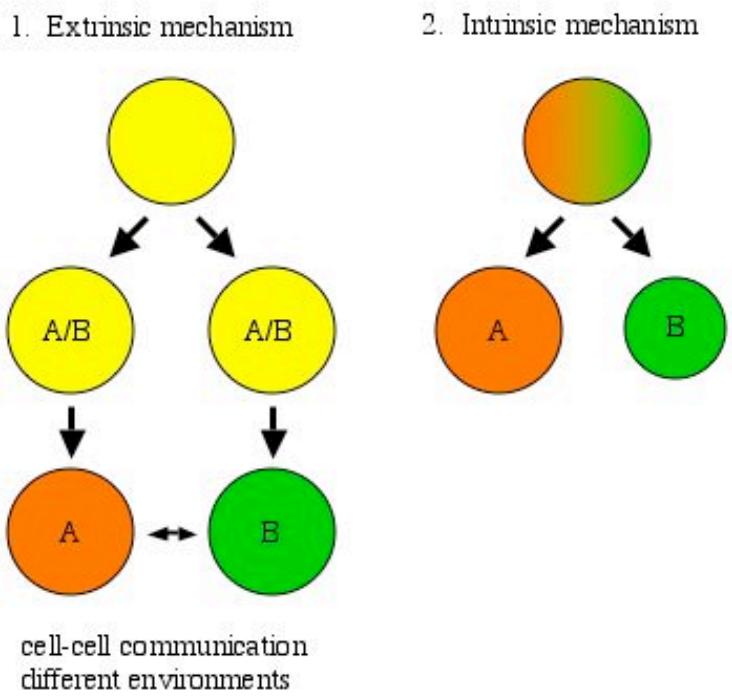


Figure 1.1. Two mechanisms of asymmetric cell division

Modified from Horvitz and Herskowitz, 1992 (Horvitz and Herskowitz, 1992). Two mechanisms of asymmetric cell division, the extrinsic mechanism (a) and the intrinsic mechanism (b), are shown. (a) Two initially equivalent daughter cells (yellow), which can potentially become either an A or a B cell, are committed to distinct fates A (orange) and B (green), due to cell-cell communication between each other

or exposure to a different environment from the other. (b) Cell fate determinants segregate unequally to one of the two daughter cells, B (green), which is committed to a different fate from the other, A (orange).

Drosophila neural progenitor cells, called neuroblasts (NBs), use the intrinsic mechanism to generate cell diversity in the embryonic central nervous system (CNS). Asymmetric cell division is observed in many developmental contexts, such as nervous system development in vertebrates, in the *C.elegans* zygote and in the peripheral nervous system of *Drosophila* (Bardin et al., 2004; Jan and Jan, 2001; Lyczak et al., 2002; Schneider and Bowerman, 2003; Sulston et al., 1983). Thus, asymmetric cell division is a general process to generate divergent cell types during development. I investigated the mechanism of asymmetric cell division by studying the *Drosophila* CNS as a model system.

1.2 Development of the central nervous system in *Drosophila*

The *Drosophila* embryonic CNS consists of the brain and the ventral nerve cord. The ventral nerve cord, which has a segmentally repeated pattern, contains about 300 neurons and 30 glial cells in each hemisegment. Each neuron and glial cell has its own unique characteristics that are represented by its specific gene expression and stereotypical pattern of axon projection. All cells, except for the midline cells in the CNS, originate from approximately 30 neural stem cells, called neuroblasts (NBs). Each NB can be identified by the position, timing of birth and expression of specific genes. Cell lineage trace analyses have also shown that each NB produces specific progeny (Bossing et al., 1996; Schmid et al., 1999). NBs undergo several rounds of asymmetric cell division in order to produce divergent progeny (figure 1.2) (Development of *Drosophila melanogaster*, 1993).

Recently, it has been shown that neurogenesis in the vertebrate nervous system is essentially similar to that of the *Drosophila* CNS. Progenitor cells undergo asymmetric cell divisions to produce divergent cell types (Cayouette and Raff, 2003; Chenn and McConnell, 1995; Haydar et al., 2003; Noctor et al., 2004). The *Drosophila* NB, as well as sensory organ precursor cells in the *Drosophila* peripheral nervous system and the *C. elegans* zygote, has served as a good model system to investigate asymmetric cell division. The advantages of using *Drosophila* are the following: [1] The generation time is short. Especially, embryonic development is completed in 22 hours at 25°C. [2] The

CNS has fewer cells and its structure is much less complicated than those of vertebrates. As mentioned previously, most NBs can be identified by their position and specific gene expression. Cell lineage tracing is possible for some specific lineages. [3] Accumulated genetic tools are available. [4] The Genome has been sequenced, and the sequences of predicted genes are available in databases.

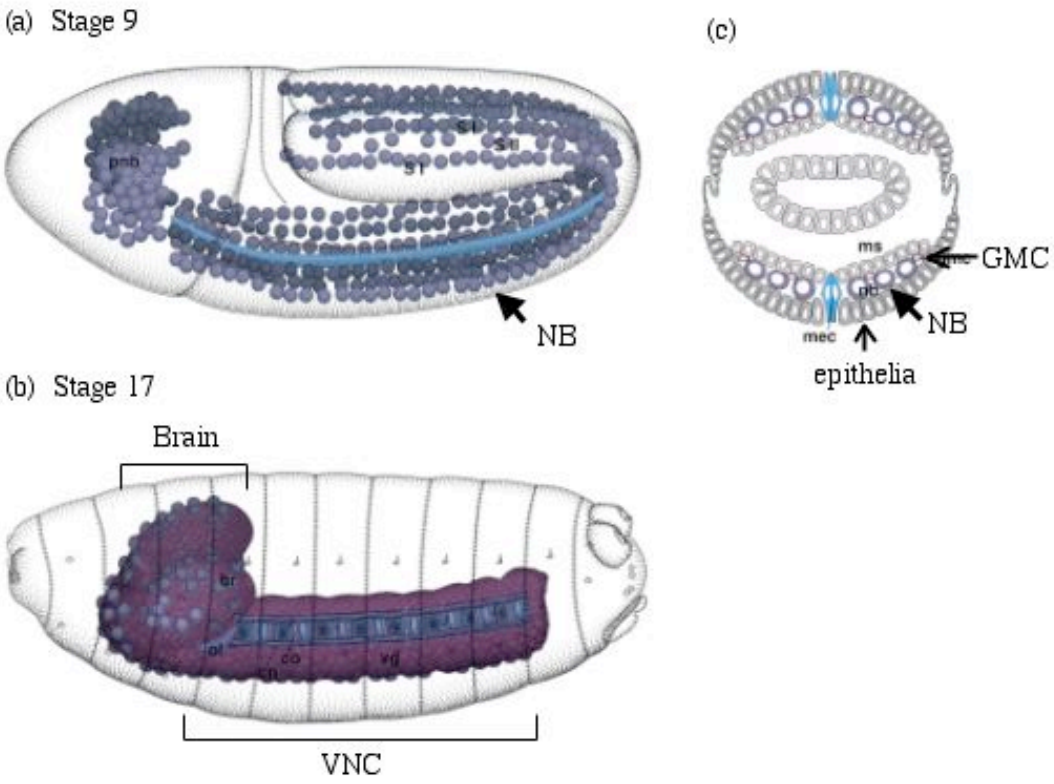


Figure 1.2. Development of the *Drosophila* embryonic central nervous system

Adopted from Hartenstein. Lateral views of stage 9 (a) and stage 17 (b) embryo, (c) Transversal section of a stage 9 embryo. (b) A stage 17 embryo has a completely organized embryonic central nervous system (CNS) (shown in purple), which consists of the brain and the ventral nerve cord (VNC). The VNC has approximately 300 neurons and 30 glial cells with different identities per hemisegment. Most cells in the CNS are derived from neural stem cells called neuroblasts (NBs). (a) (c) NBs (purple) delaminate from the neuroectodermal epithelium. After delamination, NBs undergo several rounds of asymmetric cell division.

1.3 Asymmetric cell division of *Drosophila* neuroblasts

NBs delaminate from the ventral neuroectoderm into the interior of the embryo and lie beneath the epithelial layer, where they start mitosis. The mitotic spindle rotates by 90 degrees in mitotic NBs and aligns parallel to the apical-basal axis in metaphase (Kaltschmidt et al., 2000; Kraut et al., 1996). Subsequently, each NB divides asymmetrically along the apical-basal axis in a stem cell-like fashion. Each cell division gives rise to two distinct daughter cells that differ in size and mitotic potential. The larger apical daughter cell remains as a NB that retains the stem cell characteristics and repeats some more rounds of asymmetric division. The smaller basal daughter cell, called a ganglion mother cell (GMC), undergoes terminal division to produce two post mitotic neurons or glial cells (figure 1.3) (Bossing et al., 1996; Schmid et al., 1999).

The process of asymmetric cell division of NBs can be divided into 4 steps; [1] Apical-basal polarity is established in a NB. Apical-basal polarity is supposed to be inherited from the epithelial cells from which NBs are derived. It is thought that the so-called Par/aPKC complex is involved in apical-basal polarity inheritance in NBs. [2] The mitotic spindle orients along the apical-basal axis. [3] Cell fate determinants are localized in a polarized manner along the apical-basal axis. [4] Cell fate determinants segregate into only one of the two daughter cells. To ensure the correct segregation of cell fate determinants, their localization has to be coordinated with the spindle orientation in NBs.

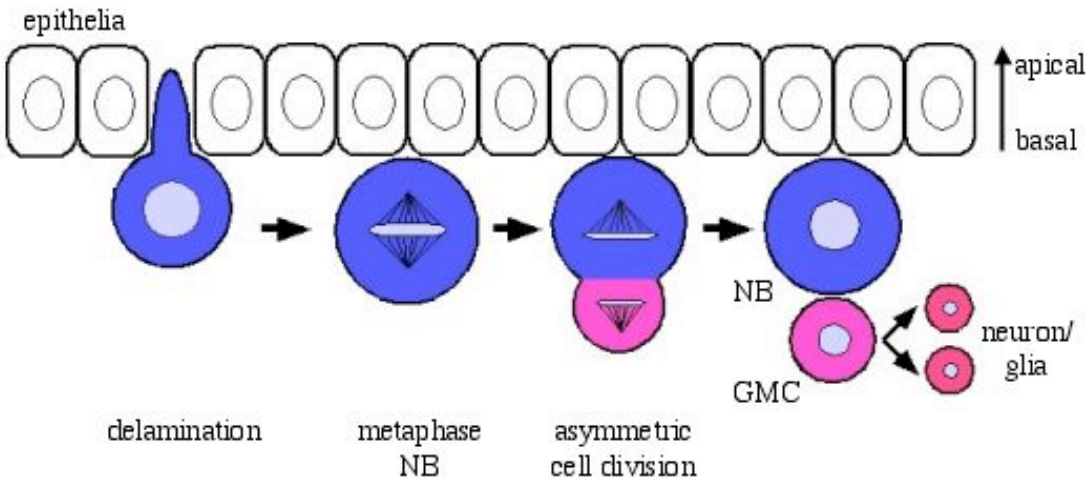


Figure 1.3. Asymmetric cell division of neuroblasts

(1) NBs (blue) delaminate from the epithelium. (2) In metaphase NBs, the mitotic spindle aligns along the apical-basal axis. (3) Subsequently, the NB divides asymmetrically, giving rise to two different daughter cells. (4) The basally located, smaller cell is a ganglion mother cell (GMC) (purple) that divides once more to produce neurons and/or glial cells (pink). The larger one is another NB (blue) that also divides asymmetrically, as its progenitor. Apical is up.

1.4 Proteins involved in the asymmetric cell division of neuroblasts

According to the apical-basal polarity, some proteins and mRNAs are localized in a polarized fashion in NBs. Several protein complexes, which play crucial roles in the asymmetric cell division of NBs, are localized at either the apical or basal cortex in NBs (figure 1.4).

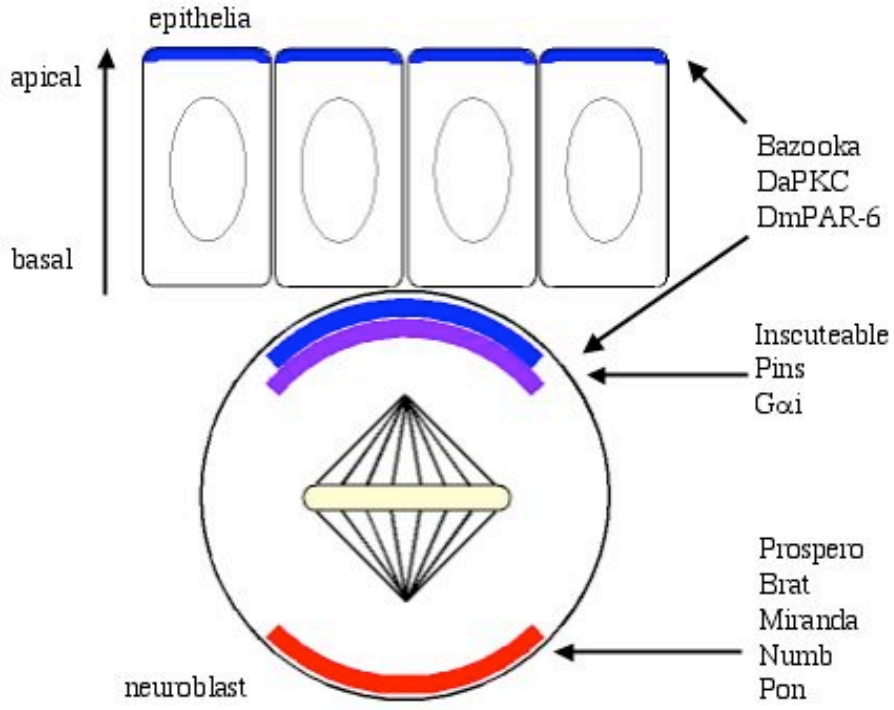


Figure 1.4. Subcellular localization of proteins involved in asymmetric cell division in the neuroblast

In the NB, the Par/aPKC complex (blue) and Inscuteable/Partner of inscuteable (Pins)/Gαi (purple)

proteins are localized at the apical cortex. On the other hand, the cell fate determinants Prospero, Brat and Numb, and their adapter proteins Miranda and Partner of numb (Pon), are localized at the basal cortex (red). The asymmetric localization of cell fate determinants and the apical-basal orientation of the mitotic spindle are essential for the asymmetric cell division of NBs. Apically localized proteins are required for both the mitotic spindle orientation and basal localization of the cell fate determinants.

1.4.1 Basally localized proteins

The cell fate choice between NB and GMC is mediated by the proteins that are localized at the basal cortex in NBs. Among them, Prospero (Pros) (Chu-Lagraff et al., 1991; Doe et al., 1991; Matsuzaki et al., 1992; Vaessin et al., 1991) and Numb (Uemura et al., 1989) are known to function as cell fate determinants. Pros is a homeodomain transcription factor. Numb is a cytoplasmic protein, which acts as an antagonist of Notch signaling. They are anchored to the basal cortex in metaphase NBs via their respective adaptor proteins, Miranda (Mira) (Ikeshima-Kataoka et al., 1997; Schuldt et al., 1998; Shen et al., 1997) and Partner of Numb (Pon) (Lu et al., 1998). In each cell division, the basally localized proteins exclusively segregate into the GMC (Hirata et al., 1995; Knoblich et al., 1995; Rhyu et al., 1994; Spana and Doe, 1995). Pros is thought to determine the cell fate of the GMC by activating GMC-specific gene expression (Doe et al., 1991) and suppressing NB-specific gene expression (Li and Vaessin, 2000; Vaessin et al., 1991).

Quite recently, the tumor suppressor gene, Brain Tumor (Brat) was identified as another cargo for Mira. Brat is localized at the basal cortex of the NBs and exclusively segregates into the GMCs, where it functions as a cell fate determinant (Betschinger et al., 2006; Lee et al., 2006).

1.4.2 Apically localized proteins

The localization of cell fate determinants has to be coordinated with the apical-basal orientation of the mitotic spindle to ensure the exclusive segregation of cell fate determinants into the GMC. A protein complex that is localized at the apical cortex in NBs is required for both the correct orientation of the mitotic spindle and the basal localization of the cell fate determinants. This complex, which is called the Par/aPKC complex, consists of Bazooka (Kuchinke et al., 1998; Schober et al., 1999; Wodarz et al., 1999), atypical protein kinase C (DaPKC) (Wodarz et al., 2000) and DmPAR-6

(Petronczki and Knoblich, 2001) (figure 1.4). Baz and DmPAR-6 have PDZ domains that are involved in protein-protein interactions and, therefore, they are thought to function as scaffolding proteins. The Par/aPKC complex proteins are partially dependent on each other for their apical localization. In loss of function mutants for these proteins, the basal components, such as Mira, fail to be localized at the basal cortex. Instead, they are localized uniformly at the cortex or in the cytoplasm. It should be noted that the Par/aPKC complex is evolutionally conserved and is found in many organisms, such as *C. elegans*, *Drosophila* and vertebrates (Ohno, 2001).

The Par/aPKC complex has another function in NBs. The apical-basal polarity of NBs is inherited from the epithelial cells from which NBs are derived. The Par/aPKC complex is supposed to be responsible for inheriting the polarity. This complex is localized at the subapical region that is just apical to the adherens junction in the epithelial cells and is required for establishing the apical-basal polarity of epithelial cells. In loss of function mutants for the Par/aPKC complex, the apical polarity markers are mislocalized along the entire basolateral membrane (Bilder et al., 2003). When NBs delaminate from the epithelial layer, the Par/aPKC complex is localized at the stalk where the NBs still have contact with the neighboring epithelial cells. When the NBs lose contact with the neighboring cells after delamination, the Par/aPKC complex is localized at the apical cortex in NBs, suggesting that this complex is involved in the inheritance of the polarity cue from epithelial cells to NBs (Schober et al., 1999; Wodarz et al., 1999).

In addition to the Par/aPKC complex, Inscuteable (Insc) (Kraut and Campos-Ortega, 1996; Kraut et al., 1996), Partner of Inscuteable (Pins) (Parmentier et al., 2000; Schaefer et al., 2000; Yu et al., 2000) and Gαi (α subunit of heterotrimeric G protein) (Schaefer et al., 2001) are also localized at the apical cortex of NBs (figure 1.4). These proteins form a complex that associates with the Par/aPKC complex. The apical localization of these proteins at least partially depends on the Par/aPKC complex. When the Par/aPKC complex is localized at the stalk during the delamination of the NBs, Insc, which is not expressed in epithelial cells, starts to be expressed and is recruited to the stalk by the Par/aPKC complex. Insc, in turn, recruits the GoLoco motif protein Pins and the α subunit of the heterotrimeric G protein Gαi to the apical cortex of the NBs. In loss of function mutants for these proteins, while the localization of cell fate determinants remains asymmetric, the spindle orientation becomes randomized,

suggesting that this complex is mainly responsible for the spindle orientation, rather than the localization of cell fate determinants (Cai et al., 2003; Kraut et al., 1996; Schaefer et al., 2000; Yu et al., 2003; Yu et al., 2000). This complex also plays crucial roles in the generation of daughter cell size asymmetry (Cai et al., 2003).

In the classical model, heterotrimeric G proteins are coupled to a seven-pass transmembrane receptor, and the binding of an extracellular ligand triggers the signal transduction cascade. Upon ligand binding, the GDP bound to the Gα subunit is exchanged for GTP, which allows the Gα subunit to dissociate from the Gβγ subunit. Consequently, GTP-Gα initiates downstream signal transduction cascades (Hamm, 1998). However, this classical G-protein signaling pathway is not active in NBs. Gαi in NBs is independent of extracellular signals, since the NBs lack contact with neighboring cells. Instead, the activation of Gαi is regulated by its interaction with Pins. Signaling in NBs is triggered by the production of the free Gβγ subunit that dissociates from Gαi upon Pins binding to GDP-Gαi (Schaefer et al., 2001).

1.4.3 Other proteins involved in asymmetric cell division

It was not clear how the apical components direct cell fate determinants to the opposite side of the cell, the basal cortex. Recently, however, additional proteins that also interact with the Par/aPKC complex and that might serve as links between the apical Par/aPKC complex and the basally localized cell fate determinants have been identified.

The tumor suppressor genes, *lethal giant larvae* (*lgl)*, *discs large (dlg)* (Ohshiro et al., 2000; Peng et al., 2000) and *scribble (scrib)* (Albertson and Doe, 2003) were found to be involved in the basal localization of cell fate determinants and their adaptor proteins. Mutations of these genes do not affect the localization of apical proteins, but the basal localization of cell fate determinants is abnormal. The determinants are uniformly distributed in the cytoplasm in mutant NBs, instead of being localized at the basal cortex. Although Lgl is distributed throughout the cortex in wild type NBs, its activity is differently regulated between the apical cortex and the basal cortex. Lgl is a key target of DaPKC, and phosphorylation of Lgl by DaPKC regulates the basal localization of Mira through Lgl inhibition. In the apical cortex, where DaPKC is localized, Lgl loses its association with the cell cortex due to DaPKC-dependent phosphorylation of Lgl. Consequently, Mira is excluded from the apical cortex, since Lgl is required for the cortical localization of Mira (figure 1.5) (Betschinger et al., 2005; Betschinger et al.,

2003).

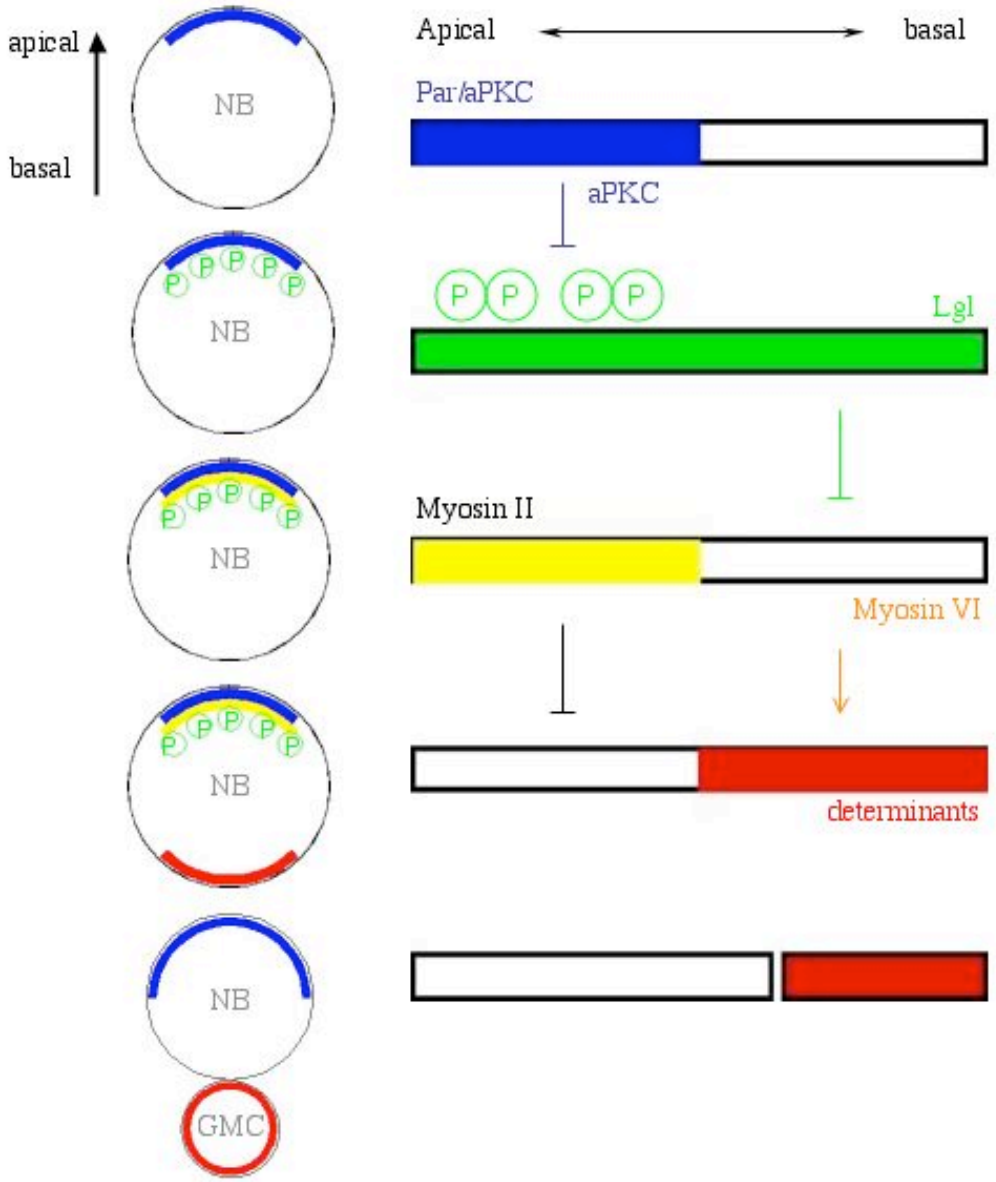


Figure 1.5. Proteins controlling the basal localization of cell fate determinants in the neuroblast

Modified from Betschinger et al., 2004 (Betschinger and Knoblich, 2004). In the apical cortex, Par/aPKC (blue) inactivates uniformly localized Lgl (green) by aPKC-dependent phosphorylation, which results in myosin II filament (yellow) association with the apical cortex. Myosin II excludes cell fate determinants

(red) from the apical cortex and restricts them to the basal cortex. Myosin VI (orange) also promotes the basal localization of cell fate determinants.

Furthermore, it has been shown that when NBs are treated with actin filament depolymerizing drugs, the basally localized proteins are delocalized, suggesting that actin filaments are involved in the localization of cell fate determinants (Broadus and Doe, 1997; Knoblich et al., 1997; Shen et al., 1998). The involvement of actin-based myosin motors in the localization of cell fate determinants was also implied, for the following reasons. Firstly, Lgl binds to non-muscle myosin II (Strand et al., 1994). Secondly, genetic interaction studies revealed that the *lgl* mutant phenotype was suppressed by reducing the gene dosage of *zipper*, the gene that encodes myosin II (Ohshiro et al., 2000; Peng et al., 2000). Barros et al. investigated myosin II function in the asymmetric cell division of NBs. Myosin II and Mira showed a complementary localization pattern in NBs. In metaphase, Mira is localized at the basal cortex, whereas Myosin II is localized at the apical cortex. Myosin II directs basal localization in a unique way: it pushes Mira out from the apical cortex. The inhibition of Lgl at the apical cortex allows Myosin II filaments to associate with the cortex, which exclude Mira from the apical cortex. On the other hand, active Lgl at the basal cortex prevents Myosin II from association with the cortex, which allows the cortical localization of Mira at the basal side of the NBs (Barros et al., 2003) (figure 1.5).

Myosin VI is also involved in the localization of cell fate determinants. Myosin VI, encoded by *jaguar (jar)* in *Drosophila*, is an unconventional myosin that moves towards the minus end of actin filaments. Genetic interaction studies revealed that the phenotype of *lgl* loss of function mutants is enhanced by reducing the *jar* gene dosage (Petritsch et al., 2003). In the *jar* mutant, Mira is distributed throughout the cytoplasm, instead of being localized at the basal cortex. It was also shown that Jar binds to Mira. These data suggested that Jar might transport Mira to the basal cortex (Petritsch et al., 2003) (figure 1.5).

Thus, it had been thought that lateral transport was responsible for the asymmetric localization of cell fate determinants. Quite recently, however, Mayer et al. demonstrated that Numb and Pon rapidly exchange between a cytoplasmic pool and the cell cortex and that direct recruitment from the cytoplasm, rather than lateral transport, is responsible for the asymmetric distribution of the cell fate determinants in SOP cells

(Mayer et al., 2005). It is not known if the same mechanism is used for the asymmetric localization of cell fate determinants in NBs.

1.5 Control of daughter cell size asymmetry

Apically localized proteins are involved not only in the localization of cell fate determinants and the spindle orientation but also in the control of cell size asymmetry. The two daughter cells produced by the asymmetric division of NBs differ not only in cell fate but also in cell size. Cai et al. found that cell size asymmetry was controlled by apically localized protein complexes comprising of two signaling pathways: the Par/aPKC/Insc complex and the Pins/Gαi complex. While NBs divide asymmetrically when any single component of the apically localized proteins is deleted, the division of NBs becomes symmetric in size only when both of the two complexes are simultaneously inactivated, e.g. the *baz/pins* double mutant. These data indicate that the Par/aPKC/Insc and Pins/Gαi complexes have redundant functions in the generation of cell size asymmetry (Cai et al., 2003).

1.6 Asymmetric cell division in other systems: common features and differences

Asymmetric cell division has been intensively studied in the sensory organ precursor (SOP) cell lineage of *Drosophila* and in the *C. elegans* zygote, in addition to the *Drosophila* CNS. The mechanisms of asymmetric cell division (mentioned in chapter 1.3) are essentially similar in these three systems. I will outline the mechanism and the key players in the asymmetric cell division of SOP and the *C. elegans* zygote.

1.6.1 *Drosophila* sensory organ precursor cells

Sensory organ precursor (SOP) cells in the *Drosophila* peripheral nervous system are the cells that produce sensory organs, which consist of four distinct cell types, by a series of asymmetric cell divisions.

The SOP cell, also called the pI cell, is specified in the epithelial layer and undergoes asymmetric cell divisions along the anterior-posterior axis within the plane of the epithelium, which is perpendicular to that of the NBs. pI cell division gives rise to two distinct daughter cells, the anterior pIIb and the posterior pIIa cell. The two daughter cells continue asymmetric cell divisions to produce the four distinct cell types of the

sensory organ.

Although most components involved in the asymmetric cell division of the embryonic CNS are also involved in this process, some differences exist. The cell fate determinant Numb is localized at the anterior cortex in pI and exclusively segregates into the anterior daughter cell, pIIb (Rhyu et al., 1994). The Par/aPKC complex is localized at the posterior cortex and is involved in the localization of cell fate determinants (Roegiers et al., 2001). On the other hand, Pins/Gαi, which is colocalized with the Par/aPKC complex in NBs, is localized at the opposite, anterior cortex in pI (Schaefer et al., 2001). The main difference is that while the Par/aPKC complex establishes the axis of cell division in NBs, planar cell polarity defines the division axis in pI cells (Gho and Schweisguth, 1998). pI cells inherit the posterior localization of Frizzled (Fz) and the anterior localization of Strabismus (Stbm) from the epithelial cell as polarity cues (Bellaiche et al., 2004; Lu et al., 1999). Stbm recruits Pins to the anterior cortex, which restricts the Par/aPKC complex to the opposite, posterior cortex (Bellaiche et al., 2004; Bellaiche et al., 2001) (figure 1.6A). This protein interaction is altered when Insc, which is not expressed in pI cells, is ectopically expressed in pI cells. Pins recruits ectopically expressed Insc to the anterior cortex, which leads to the recruitment of the Par/aPKC complex to the anterior cortex. As a consequence, the cell fate determinant Numb forms a crescent at the opposite, posterior side, which is a similar situation as in NBs. Although the cell polarity is reversed, the spindle orientation is not affected (Bellaiche et al., 2001).

1.6.2 *C. elegans* zygote

In the *C. elegans* zygote, the Par/aPKC complex is involved in the establishment of cell polarity along the anterior-posterior axis. The position of sperm entry defines the posterior pole of the zygote (Goldstein and Hird, 1996). The zygote, which is also called the P0 cell, divides asymmetrically along the anterior-posterior axis to produce two daughter cells, a larger, anterior AB cell and a smaller, posterior P1 cell. The two daughter cells have different size and are committed to distinct cell fates.

P-granules segregate into one of the two daughter cells, the P1 cell. The Par proteins are involved in both the P-granule localization and the spindle orientation. Cell polarity in the *C. elegans* zygote is established by an interaction between the centrosome derived from the sperm and the cell cortex, which initiate the actin-myosin-dependent cortical

flow. This cortical flow is essential for the polarized localization of Par proteins. The posterior cell cortex that overlies the sperm derived centrosome is depleted of the Par-3/6 complex, which allows the posterior accumulation of Par-2. After the cortical flow stops, the localization of Par-3/6 and Par-2 becomes mutually dependent (figure 1.6B).

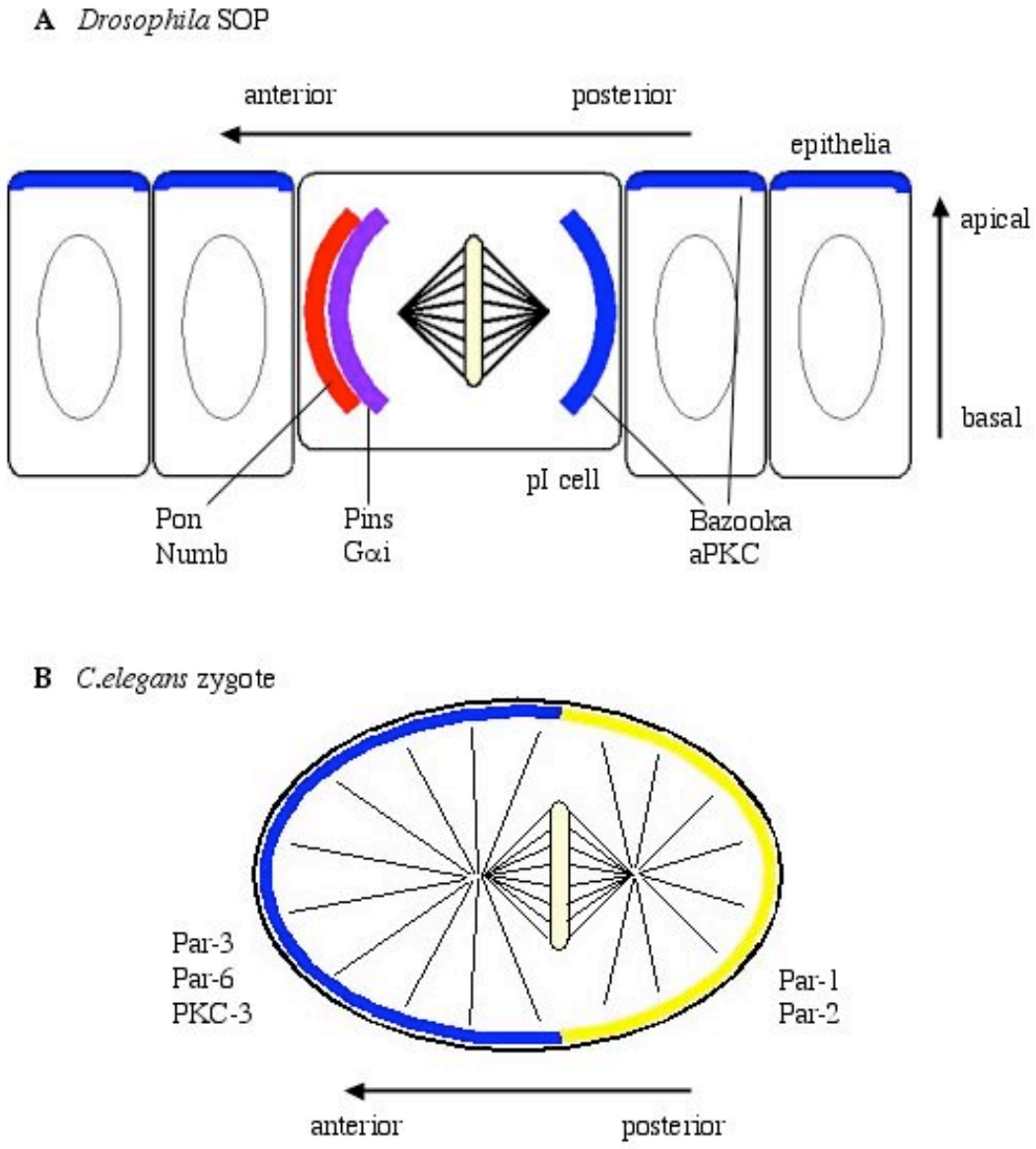


Figure 1.6. Asymmetric cell division of SOP cells in *Drosophila* and the *C. elegans* zygote

Modified from Wodarz, 2001; Betschinger et al., 2004; Wodarz 2002 (Betschinger and Knoblich, 2004; Wodarz, 2001; Wodarz, 2002). A: *Drosophila* SOP cell. The pI cell divides asymmetrically along the

anterior-posterior axis within the epithelial layer. The mitotic spindle aligns along the anterior-posterior axis. Bazooka and aPKC (blue) are localized at the posterior cortex, whereas Numb/Pon (red) and Pins/Gαi (purple) are localized at the anterior cortex. Pins/Gαi are colocalized with Numb in pI, while they are localized at the opposite side in NBs. Insc and Pros, which are expressed in NBs, are not expressed in the pI cell. Par-6 and Mira expression were not examined in pI. B: *C.elegans* zygote. PAR-3 (Bazooka homolog), PAR-6 and aPKC isoform PKC-3 (blue) are localized at the anterior cortex, whereas PAR-1 and PAR-2 (yellow) are localized at the posterior cortex.

1.7 Evolutionary conservation of the Par/aPKC complex

The Par/aPKC complex is evolutionarily conserved from *C. elegans* through vertebrates. The *partitioning-defective (par)* mutants were originally isolated in a genetic screen for mutants in which the first cell division in *C. elegans* was abnormal (Kemphues et al., 1988). Six complementation groups, *par-1* to *par-6*, which encode various proteins, such as PDZ domain proteins and Ser/Thr-kinases, were isolated in the screen. Additionally, *pkc-3* (homolog of *aPKC*) was later found to be essential for the asymmetric cell division of the *C. elegans* zygote (Tabuse et al., 1998). All of these proteins, except for Par-2, are conserved in evolution. These proteins are divided into three groups according to their localization: Par-3, Par-6 and aPKC are localized at the anterior cortex, and Par-1 and Par-2 are at the posterior cortex. Par-4 and Par-5 are uniformly distributed at the cortex. Among them, only Par-3, Par-6 and aPKC, which form a protein complex and are localized at the anterior cortex in the *C. elegans* zygote, have a conserved function in asymmetric cell division, as mentioned in the previous chapter. The Par/aPKC complex is also involved in the establishment of epithelial cell polarity in *Drosophila* and vertebrates.

1.8 Miranda

Finally, I will outline what is known about *miranda*, as a mutation in this gene was analyzed in this study.

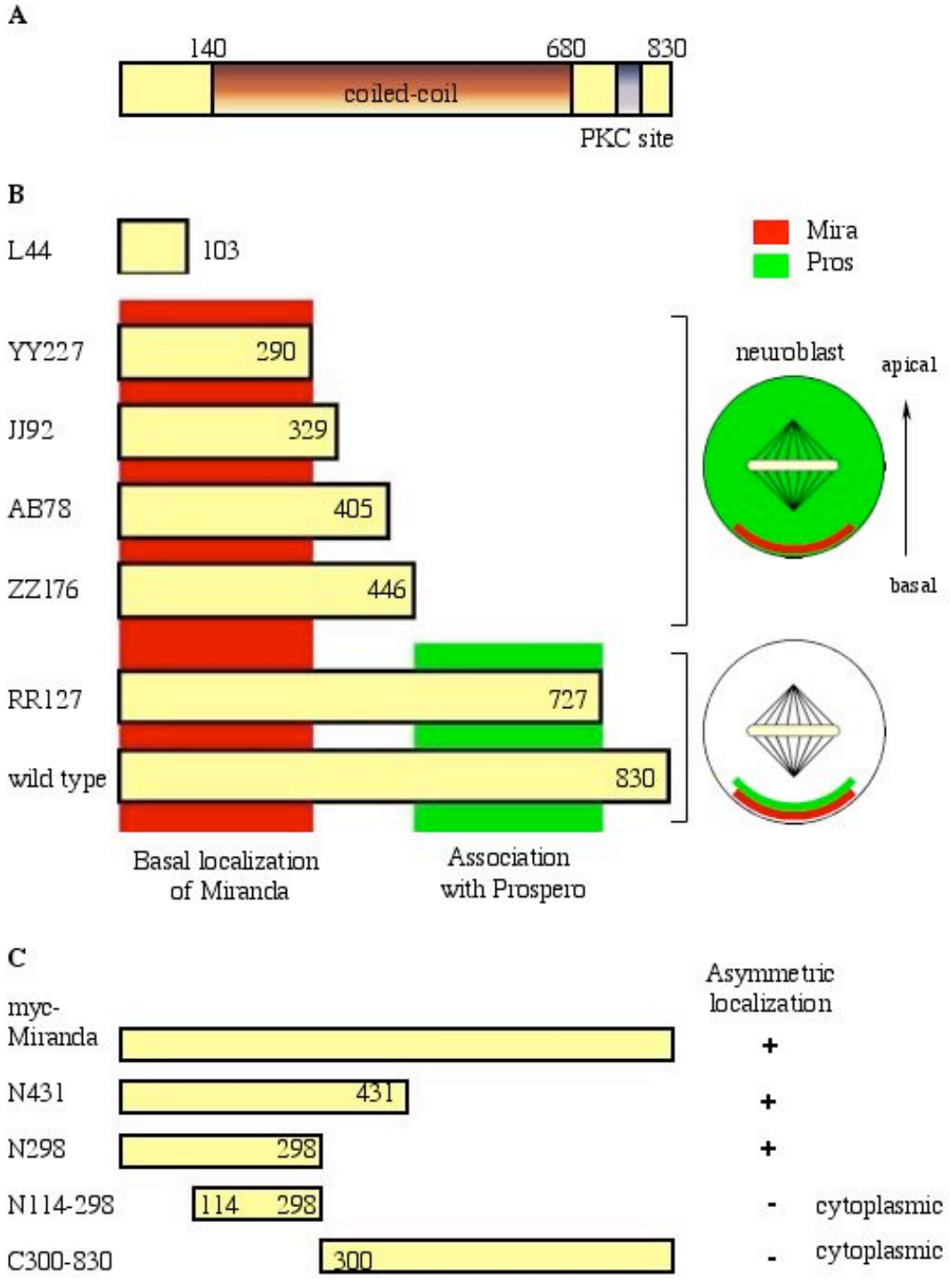


Figure 1.7. Mutant Miranda proteins produced in *miranda* alleles and subcellular localization in NBs

A: Modified from Fuerstenberg et al., 1998 (Fuerstenberg et al., 1998). Schematic drawing of the

Miranda domain structure. The central region of Mira (brown) forms a coiled-coil structure that is similar to the myosin rod. PKC phosphorylation sites (grey) are located within the C-terminal 100 amino acids. B: Modified from Matsuzaki et al., 1998 (Matsuzaki et al., 1998). Seven *mira* mutant alleles have been isolated. The primary structures of the mutant Mira proteins produced in six alleles ($mira^{L44}$, $mira^{YY227}$, $mira^{JJ92}$, $mira^{AB78}$, $mira^{ZZ176}$ and $mira^{RR127}$) and the subcellular localization of the mutant Mira (red) and Pros (green) in the NB are schematically drawn. The mutant Mira protein is not detected with the available antibody in two of the seven alleles, $mira^{L44}$and $mira^{DEB}$ (not shown). The other five alleles produce C-terminally truncated Mira proteins. Mutant Mira proteins longer than 290 amino acid residues are localized at the basal cortex, as in the wild type, indicating that amino acids 1-290 (red box) are sufficient for the basal localization of Mira. Although Pros fails to be localized at the basal cortex in $mira^{YY227}$, $mira^{JJ92}$, $mira^{AB78}$ and $mira^{ZZ176}$, in spite of the fact that Mira is correctly localized, it is localized at the basal cortex in $mira^{YY127}$, indicating that amino acids 446-727 of Mira (green box) are responsible for the association with Pros. C: Adopted from Shen et al., 1998 (Shen et al., 1998). Scheme of the *mira* constructs used in the experiments performed by Shen et al. They ectopically expressed myc-tagged full length and truncated Mira proteins in NBs. When the full length, the N-terminal 431 amino acids and the 298 amino acids of Mira are expressed, they are localized at the basal cortex, as in the wild type. In contrast, when amino acids 114-298 and amino acids 300-830 of Mira are expressed, they fail to be localized at the basal cortex. Instead, they are distributed into the cytoplasm.

1.8.1 Function of Miranda

Mira plays a critical role in the determination of daughter cell fate during the asymmetric cell division of NBs, since Mira is an adaptor protein for Pros which functions as a cell fate determinant in one of the two daughter cells, the GMC. Like other components involved in the asymmetric cell division of NBs, Mira localization is regulated in a cell cycle-dependent manner in mitotic NBs. In interphase, Mira is localized at the apical cortex. In metaphase, Mira switches its localization to the basal cortex, where it recruits Pros. After cytokinesis, these proteins segregate exclusively into the GMC, where Mira is degraded and Pros, after its release from Mira, enters the nucleus. Pros, which is a homeodomain transcription factor, is thought to function as a cell fate determinant by activating GMC-specific gene expression (Doe et al., 1991) and repressing NB-specific gene expression (Vaessin et al., 1991). In amorphic *mira* mutants, Pros fails to be localized at the basal cortex. Instead it is distributed into the cytoplasm, indicating that Mira is required for the basal localization of Pros.

When I was preparing this manuscript, two new papers were published describing that the tumor suppressor protein, Brat was identified as an additional cargo for Mira. Brat segregates exclusively into the GMCs and functions as a cell fate determinant together with Pros (Betschinger et al., 2006; Lee et al., 2006).

1.8.2 Protein structure

mira produces at least two transcripts, due to alternative splicing. The larger product encodes 829 amino acid residues and the smaller product encodes 799 residues. Although Mira homologues have not been isolated from other organisms, Mira has some known protein motifs. The middle part of Mira is homologous to the myosin rod that forms the coiled-coil structure, and the C-terminal region contains two leucine-zippers. Eight consensus sites for PKC phosphorylation are located in the C-terminal 100 amino acids (figure 1.7A) (Ikeshima-Kataoka et al., 1997; Schuldt et al., 1998; Shen et al., 1997).

1.8.3 *miranda* mutant alleles

Seven mutant alleles had been isolated so far. One is $mira^{DEB}$ from the Jan lab (Shen et al., 1998) and six are from the Matsuzaki lab (Matsuzaki et al., 1998). Matsuzaki et al. sequenced the mutant *mira* genes to identify the mutations and investigated the subcellular localization of these mutant Mira proteins and the localization of Pros in NBs of these mutant *mira* alleles. The proteins produced in $mira^{DEB}$ and $mira^{L44}$ are not detectable by an anti-Mira antibody. The other five alleles produce C-terminally truncated Mira proteins (figure 1.7B). Three functional domains of Mira were identified by an analysis of the subcellular localization of the mutant Mira proteins and Pros (Matsuzaki et al., 1998). Firstly, the N-terminal 290 amino acid residues are sufficient for the basal localization of Mira, since a mutant Mira protein longer than 290 residues is localized at the basal cortex, as in the wild type. Secondly, amino acids 446-727 of Mira are responsible for the association with Pros. As Mira tethers Pros to the cortex, Pros is colocalized with Mira at the basal cortex in wild type mitotic NBs. Pros is not localized at the basal cortex in the alleles producing Mira proteins shorter than 446 amino acids, in spite of the correct localization of the mutant Mira. In contrast, Pros is localized at the basal cortex, as in the wild type, when the mutant Mira protein is longer than 727 amino acids. Thirdly, the C-terminal 100 amino acids of Mira are responsible

for the release of Pros in the GMC, since in *mira*^{RR127}, which produces a mutant Mira lacking the C-terminal 100 amino acids, the release of Pros is delayed. Since the PKC phosphorylation sites are located in this region, PKC-dependent phosphorylation of Mira might be involved in the release of Pros in the GMC (Matsuzaki et al., 1998). Shen et al. ectopically expressed truncated Mira proteins in NBs (figure 1.7C). When a C-terminally truncated Mira longer than 298 amino acids was expressed, it is localized at the basal cortex, as in the wild type. This indicates that the N-terminal 298 amino acids are sufficient for the basal localization of Mira, which is consistent with the proposal from Matsuzaki et al. When truncated Mira proteins containing either amino acids 115-298 or 300-830 were ectopically expressed in NBs, they are distributed throughout the cytoplasm, suggesting that the N-terminal 114 amino acids are involved in the cortical localization of Mira (Shen et al., 1998).

How Mira is localized at the basal cortex is not clear yet, although the involvement of Myosin VI, Myosin II and Insc is implied, as mentioned in chapter 1.4. The mechanism that localizes Mira to the basal cortex still needs to be investigated.

1.9 Aim of my work

As mentioned above, many genes involved in the asymmetric cell division of NBs have been identified. However, it is likely that additional proteins are involved in this process. For example, one of the still missing players is a component that anchors the Par/aPKC complex to the cortex. The aim of my work was to identify novel genes involved in the asymmetric cell divisions of NBs in the *Drosophila* CNS. To this end, I conducted two genetic screens. In this thesis, I would like to discuss the strategy and the results of the screens, as well as my characterization of a novel *mira* mutant allele found in the screen.

2 Materials and methods

2.1 Genetic screens

Two genetic screens, a maternal screen and a zygotic screen, were performed in order to look for genes involved in asymmetric cell division of neuroblasts.

2.1.1 Maternal screen

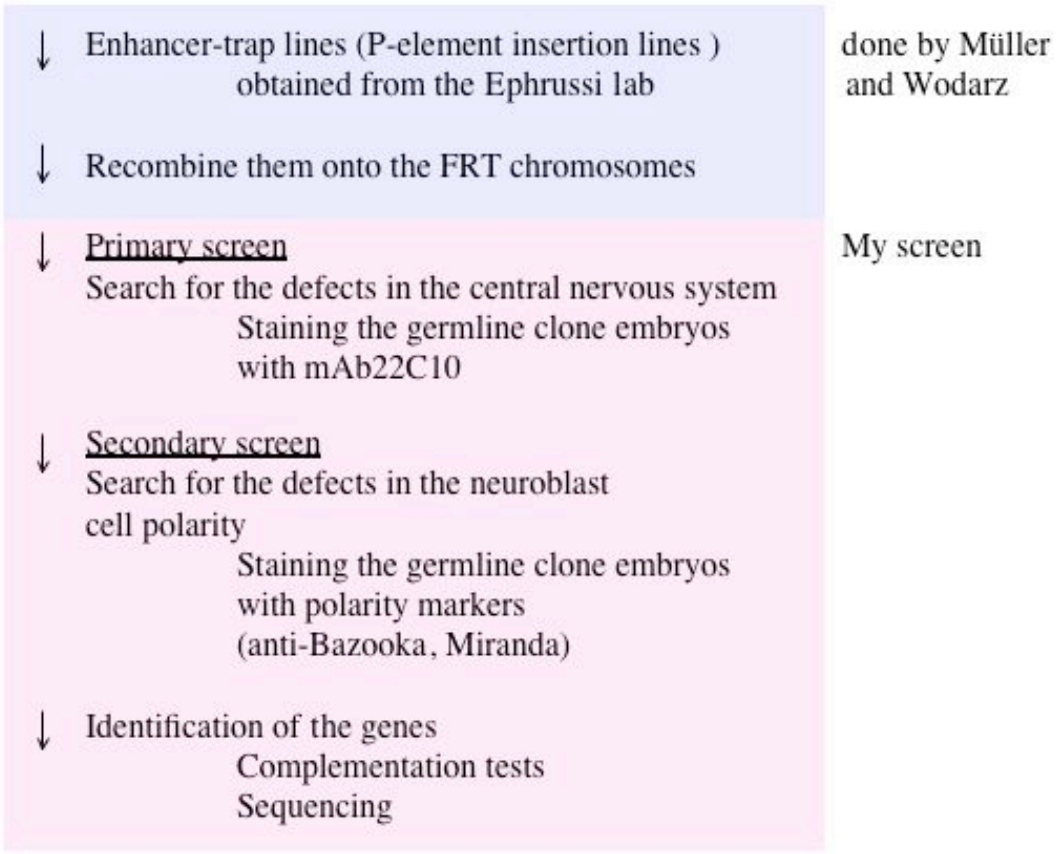


Figure 2.1. Strategy of the maternal screen

Maternal screen was performed in two steps.

Primary screen

- Stain germline clone (glc) embryos with neural marker, mAb 22C10
- Search for the glc embryos that have defects in organization of the CNS

Secondary screen

- Stain glc embryos with polarity markers

- Search for the glc embryos in which subcellular localization of the polarity markers is abnormal

2.1.2 Fly stocks

genotype		Bloomington stock#
FRT stocks		
w; P[hs neo; ry^+; FRT]$^{2L\text{-}40A}$ P[mini w^+; FRT]$^{2R\text{-}G13}$/CyO	FRT on the left and right arms of the second chromosome	
P[mini w^+; FRT]$^{3L\text{-}2A}$ P[hs neo; ry^+; FRT]$^{3R\text{-}82B}$	FRT on the left and right arms of the third chromosome	
Flipase stocks		
y w P[ry; FLP]; Sco/CyO	FLP stock for generating glcs on the second chromosome	#1929
y w P[ry; FLP]; TM3, Sb/CxD	FLP stock for generating glcs on the third chromosome	#1970
DFS stocks		
w; P[mini w^+; ovo^{D1}]^{2L} P[hs neo; ry^+; FRT]$^{2L\text{-}40A}$/ S Sp Ms(2) M bw^D/ CyO	DFS on the left arm of the second chromosome	#2121
w; P[mini w^+; FRT]$^{2R\text{-}G13}$ P[mini w^+; ovo^{D1}]^{2R}/ S Sp Ms(2) M bw^D/ CyO	DFS on the right arm of the second chromosome	#2125
w; P[mini w^+; ovo^{D1}]^{3L} P[mini w^+; FRT]$^{3L\text{-}2A}$/ ru h st βTub85D^D ss e^s/ TM3, Sb	DFS on the left arm of the third chromosome	#2139
w; P[hs neo; ry^+; FRT]^{3R82B} P[mini w^+; ovo^{D1}]^{3R}/ ru h st βTub85D^D ss e^s/ TM3, Sb	DFS on the right arm of the third chromosome	#2149

All stocks listed above, except for the FRT socks, were obtained from Bloomington stock center (Chou and Perrimon, 1996).

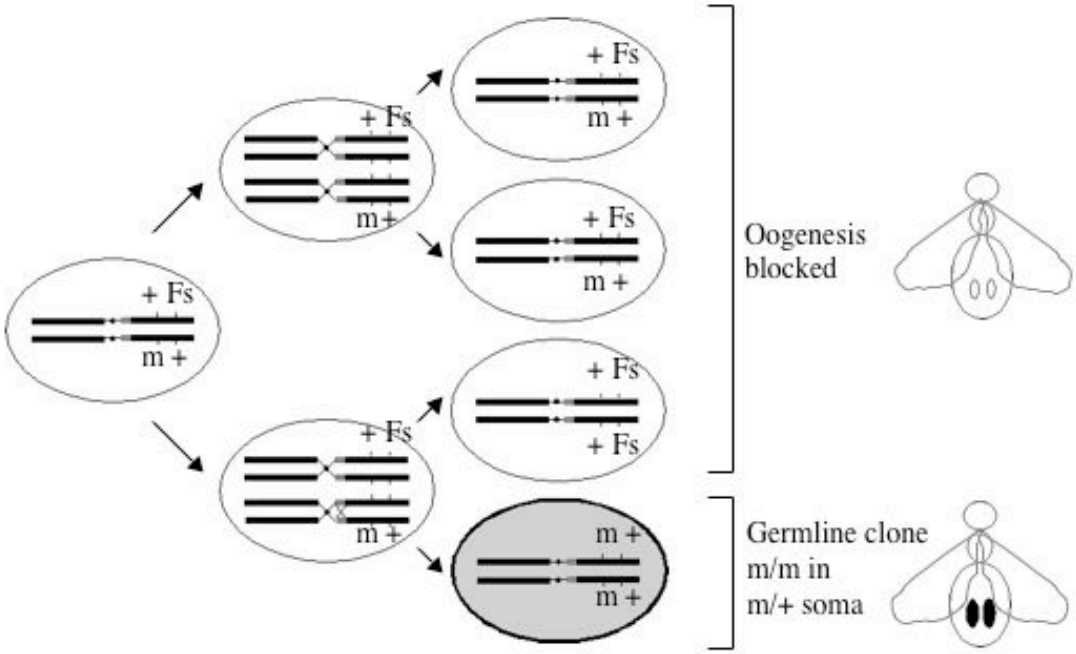
Mutant stocks containing mutations on the FRT chromosomes

Original mutant stocks, which were gifts from the Ephrussi Lab, were

recombined onto FRT chromosomes.

2.1.3 Generation of germline clones

To generate germline clones, I employed the FLP-DFS (flipase-dominant female sterile) technique (Chou and Perrimon, 1996). The ovaries harboring DFS mutation are not matured and the eggs are not laid down. Site-specific recombination at the position of the FRT sequences is catalyzed by the FLP-recombinase. Germ cells that have eliminated the DFS mutation, as a consequence of the FLP-FRT mediated recombination, and thus homozygous for the mutation, will lead to formation of eggs.



Fly cross (in case of the left arm of the second chromosome)

[1] w FLP; Sco/CyO x P[ovoD]2L FRT2L/CyO

[2] w/w; m [FRT2L, FRT2R]/CyO x w FLP/Y; P[ovoD]2L FRT2L/CyO

Heat shock the progeny at 37°C for 2hrs

[3] w/w FLP; m [FRT2L, FRT2R]/P[ovoD]2L FRT2L x

w/Y; m [FRT2L, FRT2R]/P[ovoD]2L FRT2L

Collect embryos

Staining

Figure 2.2. Generation of germline clones

- Generation of flies containing FLP and DFS (ovoD)

  FLP flies were crossed with ovoD flies (figure 2.2 [1])
- Collect male progeny with genotype: w FLP/Y; P[ovoD]2L FRT2L/CyO

  This genotype is an example with which glcs for the mutation located on the left arm of the second chromosome are generated.
- Flies containing FLP and ovoD were crossed with mutant stocks (figure 2.2 [2])
- Heat shock the progeny at 37°C for 2 hrs when they are in third instar larval stage
- Collect female flies containing germline clones:

  w/w FLP; P[ovoD]2L FRT2L/m [FRT2L, FRT2R]
- Cross female flies containing germline clones with males containing the mutation (figure 2.2 [3])
- Collect embryos and stain them

2.1.4 Zygotic screen

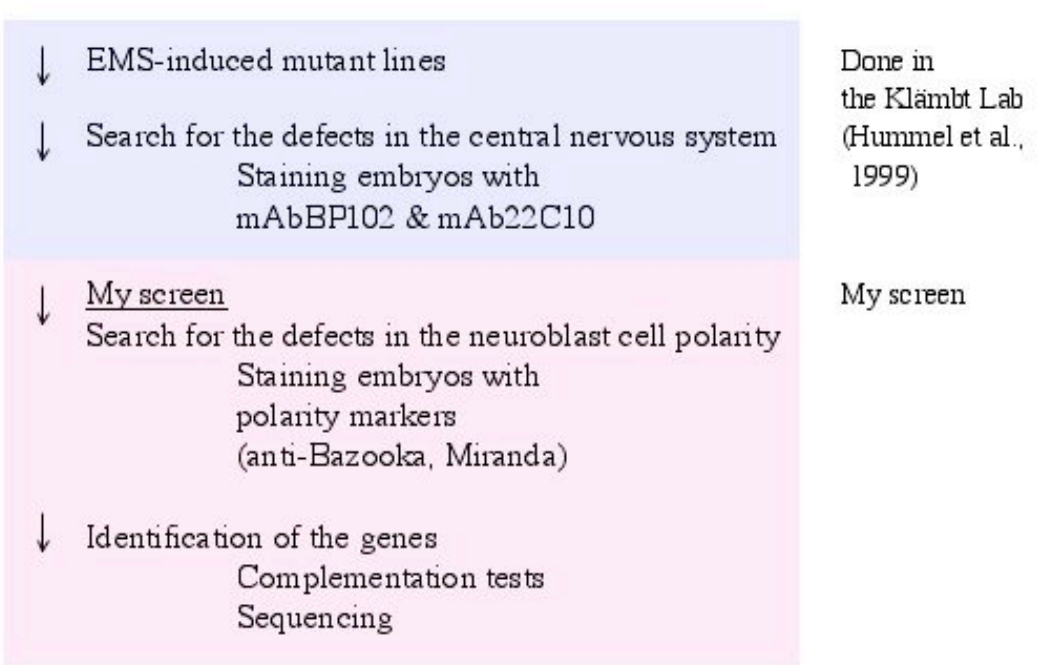


Figure 2.3. Strategy of the zygotic screen

A saturated screen was performed in the Klämbt lab. In the screen, 18,000 lines were stained with neural marker mAb BP102 to examine the defect in the CNS, and 570 homozygous lethal mutant lines that have defects in the CNS were isolated. I got these 570 lines and stained with polarity markers to search for mutants in which subcellular localization of the polarity markers are abnormal. Homozygous mutant embryos were identified by the absence of blue balancers that are marked with anti-β-Gal staining.

2.2 Immunocytochemistry

2.2.1 Embryo collection and fixation

For antibody staining, embryos were fixed in 4 % Formaldehyde.

- Let flies lay eggs on agar plate containing apple juice for 15 hrs at 25°C
- Dechorionate embryos for 5 min in 50 % Na-hypochlorite
- Wash embryos with dH_2O
- Transfer the embryos into scintilation vials that contain 4 ml 4 % Formaldehyde/1x PBS+ 4 ml Heptane
- Fix embryos for 25 min on the shaker at R.T.
- Remove the lower phase (fixative) and add 4 ml methanol
- Strongly shake the vial for 30 sec
- Collect the embryos that sank on the bottom and transfer them to 1.5 ml tube
- Wash embryos 3 times with Methanol
- Fixed embryos can be stored at -20°C

Solution

PBS: phosphate buffer saline	1.86 mM	NaH_2PO_4
	8.41 mM	Na_2HPO_4
	175 mM	NaCl

2.2.2 Antibody staining (immunofluorescence)

- Wash embryos 3x 10 min with 1x PBT

- Block 1 h at R.T. in 10 % NHS/1x PBT
- Incubate with primary antibody in 10 % NHS/1x PBT, O/N at 4°C
- Wash 4x 10 min with 1x PBT
- Incubate with fluorescently labeled secondary antibody for 2 h at R.T. in 10 % NHS/1x PBT
- Wash 4x 15 min with 1x PBT
- Embed the embryos in Mowiol+DABCO

Solution

PBT: PBS, 0.1% Tween 20

Mowiol: 5 g Mowiol/Elvanol
20 ml PBS
10 ml Glycerol

2.2.3 Antibody staining (enzyme-conjugated secondary antibody)

Enzyme conjugated secondary antibody (Alkali phosphatase for blue color, biotin for brown), instead of fluorescently labeled secondary antibody, was used for Even-skipped staining.

- Incubate with enzyme conjugated secondary antibody for 2 h at R.T. in 10% NHS/1x PBT
- Wash 4x 15 min with 1xPBT
- Signal amplification of DAB staining (Biotin-Avidin-HRP)
- Prepare 0.5 ml PBS containing 10 µl solution A (soln A) and 10 µl soln B of the Vectastain ABC kit (Vector Laboratories).
- Add PBS/soln A/soln B to embryos and incubate 30 min.
- Wash 3x 10 min with 1xPBT
- Coloring reaction of DAB staining
- Embryos are soaked in DAB/PBS for 10 min
- Add 10 % H_2O_2 2.5 µl
- Stop reaction by washing embryos with PBT
- Coloring reaction of AP staining

- Incubate embryos in 1.7 µl BCIP/1.5 µl NBT/0.5 ml AP staining buffer
- Stop reaction by washing embryos with PBT
- Mount embryos in 90 % glycerol/PBS

Kit and buffer

Vectastain ABC kit (*Vector Laboratories*)

AP staining buffer: 100 mM Tris-HCl, pH 9.5
100 mM NaCl
10 mM $MgCl_2$
0.1% Tween 20

Detection system

Fluorescence: confocal microscope Leica TCS NT, *Leica*, Heidelberg
confocal microscope Zeiss LSM510 META, *Zeiss*, Oberkochen

Light microscopy: Zeiss Axiophot2, *Zeiss*, Oberkochen
Quantix CCD camera (Photometrics)

2.2.5 Antibodies

Primary antibody

antigen	raised in	name	dilution	origin
Polyclonal antibody				
Bazooka	rabbit		1:500	A. Wodarz
Miranda	guinea pig		1:1000	A. Wodarz, F. Matsuzaki
β-Galactosidase	rabbit		1:5000	Promega
Monoclonal antibody				
Prospero	mouse	mAb MR2A	1:10	DSHB Spana and Doe, 1995
Even-skipped	mouse	mAb 2B8	1:20	DSHB
Neurotactin	mouse	mAb BP106	1:5	DSHB

Futsch	mouse	mAb 22C10	1:100	DSHB
β-Galactosidase	mouse	mAb 40-1A	1:100	DSHB

Secondary antibody

antigen	raised in	dilution	conjugated	origin
Fluorescent-conjugated				
rabbit	goat	1:200	Alexa 647	Dianova
guinea pig	donkey	1:200	Cy3	Dianova
mouse	donkey	1:200	Cy2	Dianova
mouse	donkey	1:200	Cy3	Dianova
Enzyme-conjugated				
mouse	goat	1:200	Alkali phosphatase	Dianova
rabbit	goat	1:200	biotin	Dianova

2.2.6 DNA label with YoYo-1

To label DNA, fluorescent marker YoYo-1 and DNase-free RNase were added when embryos are incubated with secondary antibody.

Reagent

YoYo-1 (*Molecular Probes*, Eugene, OR) dilution 1:10000

DNase-free RNase final concentration 50 µg/ml

2.2.7 Cuticle preparation

Collect embryos of the particular genotype after 15 h of egg laying and store them for additional 48 h at 25°C to make sure that all embryos developed cuticle. Collect them and wash them with dH_2O and dechorionate for 5 min using 50 % Na-Hypochlorite (bleach). Wash them again with dH_2O and put them on a slide in one drop of Hoyer's medium: lactic acid (1:1).

2.3 Genetics

2.3.1 Fly stocks

stocks		**references**
***mira* alleles**		
miranda^{E326}, P[AA142]/ TM3	EMS-induced mutant *mira* allele, P[*AA142*] is a marker for the midline cells	C. Klämbt
miranda^{E326}/ TM3		C. Klämbt
miranda^{L44}/ TM3	EMS-induced mutant *mira* allele	Ikeshima-Kataoka et al., 1997
miranda^{YY227}/ TM3	EMS-induced mutant *mira* allele	Ikeshima-Kataoka et al., 1997
miranda^{JJ92}/ TM3	EMS-induced mutant *mira* allele	Ikeshima-Kataoka et al., 1997
miranda^{ZZ176} / TM3	EMS-induced mutant *mira* allele	Ikeshima-Kataoka et al., 1997
miranda^{RR127}/ TM3	EMS-induced mutant *mira* allele	Ikeshima-Kataoka et al., 1997
w; Sr Df(3R) ora^{I9} e/ TM6C, Sb, e, Tb	Deficiency line lacking entire *mira* locus	Ikeshima-Kataoka et al., 1997
miranda^{E326}, P[AA142]/ TM3	EMS-induced mutant *mira* allele, P[*AA142*] is a marker for the midline cells	C. Klämbt
***prospero* alleles**		
prospero^{10419}/ TM3		Bloomington stock center
w; prospero^{17}/ TM6B, Tb		Bloomington stock center #BL-5458
prospero^{A63}/ TM3	P-element induced mutant *pros* allele	Matsuzaki et al., 1992
prospero^{C7}/ TM3	Imprecise excision line	Matsuzaki et al., 1992
prospero^{C43}/ TM3	Imprecise excision line	Matsuzaki et al., 1992
Df(3R) T-32 cu sr e/ MRS	Deficiency line lacking entire *pros* locus	Bloomington stock center
Df(3R) M-Kx1/ TM3, Sb Ser	Deficiency line lacking entire *pros* locus	Bloomington stock center
Df(3R) T-61, e/ TM6B, Tb	Deficiency line lacking entire *pros* locus	Bloomington stock center

***lgl* alleles**		
l(2)gl4 a px or/ SM1, al2 Cy cn2 sp2		E. Gateff
l(2)glD150/Cy		M. Golubovski
w; P[GT1]CG14722^{BG01876}	P-element insertion line, used for the recombination	Bloomington stock center #12850
E305/TM3	EMS-induced line, used as a control of sequencing	C. Klämbt
Oregon R	wild type	

2.3.2 Complementation test

E326 flies were crossed with *mira* lethal alleles. When *E326* has a mutation in the *mira* locus, transheterozygote for *E326* and the *mira* allele would be lethal, since no functional Mira is produced, which is defined as "no complementation". In contrast, when *E326* does not have a mutation in the *mira* locus, transheterozygote would be viable, which is defined as "complementation".

2.3.3 Recombination

To get recombinant chromosome, $mira^{E326}$/*TM3* and $pros^{+}$ w^{+}/*TM3* flies were crossed. Although in $mira^{E326}$ chromosome it is not clear if pros locus is intact or not (indicated as *pros?*), in $pros^{+}$ w^{+} chromosome *pros* locus is intact (+). When recombination occurs between w^{+} and *mira* locus in the $mira^{E326}$/$pros^{+}$ w^{+} female flies in the next generation, $pros^{+}$ w^{+} $mira^{E326}$ chromosome is obtained. In addition to w^{+}, the genotypes were assured by the fact that transherterozygote for $mira^{E326}$ and another *mira* alleles or a deficiency line that deplete *mira* locus is lethal.

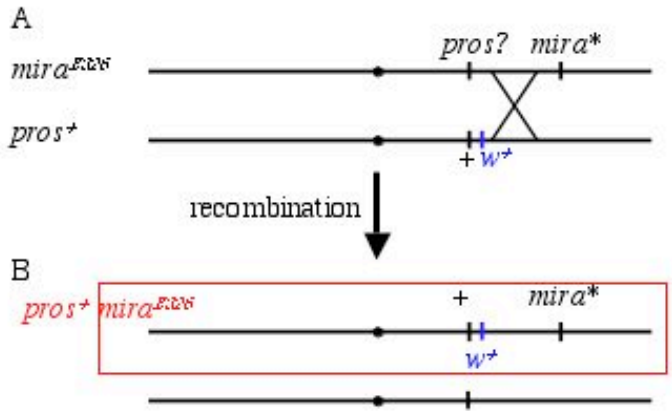


Figure 2.4. **Recombination between the *prospero* and *miranda* loci in *miranda*^{E326}**

2.4 Molecular biology

2.4.1 Sequencing of the *mira*^{E326} genomic DNA

mira genomic DNA sequence was divided into three parts and three pairs of PCR primers to amplify these three fragments were generated. Each fragment was amplified by PCR reaction using genomic DNA isolated from *mira*^{E326}*/TM3* flies as a template. Each fragment was sequenced to identify the mutation in *mira* locus of *mira*^{E326}. Sequencing was done in the company SEQLAB in Göttingen.

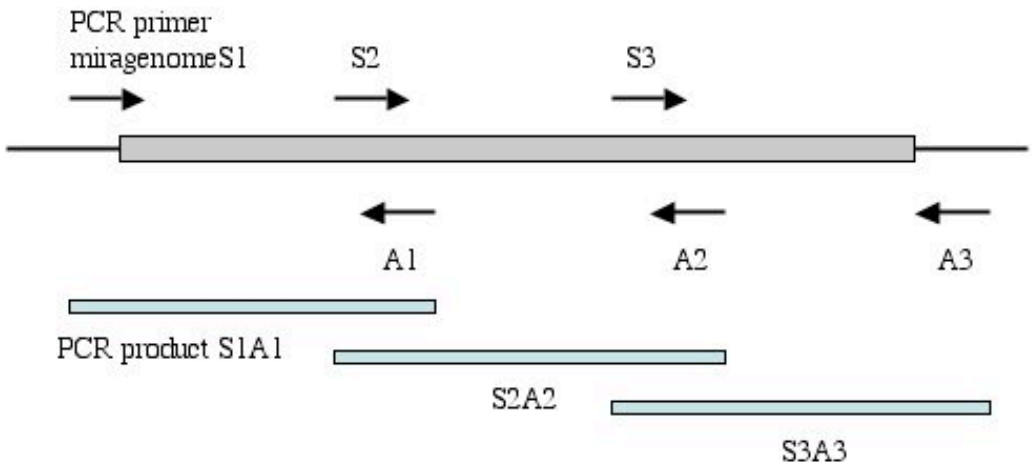


Figure 2.5. **Sequencing of the *mira*^{E326} genomic DNA**

Primers for amplification of the *mira*^{E326} genomic fragment and sequencing

name	sequence
miragenomeS1	5' GGGTCTATCTCGTTTCCACACTCT 3'
miragenomeA1	5' AACGCCTCCACTTGCCTTTCA 3'
miragenomeS2	5' TTATCGCACAAAGCTCTGAACG 3'
miragenomeA2	5' GCTCCGCTCGGTACTGCTCCACA 3'
miragenomeS3	5' GAAGCAGGACATGGCCAAGACGAT 3'
miragenomeA3	5' CCAGAACACAAACGCGAAAGATAG 3'

e.g. the PCR fragment amplified with the primer pair, miragenomeS1 and A1, was sequenced with either the S1primer or A1 primer. Sequencing was done from the both ends and these two sequences were assembled.

2.4.1.1 Genomic DNA isolation

- Collect 30 adult flies of particular genotype and put them in 1.5 ml tube.
- Freeze the 1.5 ml tube in liquid N_2 for 10 min
- Place the 1.5 ml tube on ice
- Add 400 µl of Buffer A and homogenize
- Incubate at 65°C for 30 min
- Add 575 µl of 6M LiCl and 230 µl of 5M KAc
- Incubate on ice for 30 min
- Centrifuge at 13000 rpm at R.T. for 30 min
- Transfer supernatant into a new tube
- Add 600 µl of Isopropanol
- Centrifuge at 13000 rpm for 15 min
- Discard supernatant and dry.
- Dissolve in 150 µl H_2O

Solution

Buffer A: 100 mM Tris-HCl, pH7.5

100 mM	EDTA
100 mM	NaCl
0.5 %	SDS

LiCl/ KAc solution:Mix 1 part 5M KAc: 2.5 parts 6M LiCl stock

2.4.1.2 PCR (polymerase chain reaction)

Total volume per single amplification reaction was 50 µl.

1 µl	template DNA (10 ng)
1 µl	primer 5' (10 µM)
1 µl	primer 3' (10 µM)
5 µl	10x react. Buffer
2 µl	dNTP mixture (25 mM per nucleotide)
1 µl	Taq polymerase
39 µl	dH_2O

Standard parameters that were programmed to the thermal cycler are summarized in the table:

Standard PCR programm

	Duration	Temp	Functional meaning of the step
	5 min	95°C	Starting denaturation of the DNA
	30 sec	95°C	Denaturation step within the cycles
35x	30 sec	60°C*	Annealing of the primers to the template
	60 sec	72°C	Elongation-Polymerase synthesises novel DNA chain
	5 min	72°C	Final elongation
		4°C	End of the reaction

* Annealing temperature needs to be optimal for the set of the primers

2.4.2 Cloning of the *jaguar* cDNA

jar cDNA was cloned for recombinant protein expression. EST-clone RE25996, which was available in BDGP (Berkeley Drosophila genome project), contains full-length jar

cDNA with a point mutation, which results in frame shift. Therefore, I cloned *jar* cDNA by PCR reaction. DNA fragment between ApaI site and 3' end of the *jar* cDNA was amplified by a PCR reaction using cDNA library as a template. On the other hand, RE25996, which was re-cloned into pGBKT7 vector (PCR cloning with externally induced EcoRI/SmaI site), was digested with ApaI and SmaI (3' end) to cut out the sequence correspond to the PCR fragment. The PCR fragment was ligated to the digested RE25996. As a consequence, *jar* cDNA cloned in pGBKT7 was obtained. The insert was amplified by PCR reaction and re-cloned into pBluescript KSII+ vector.

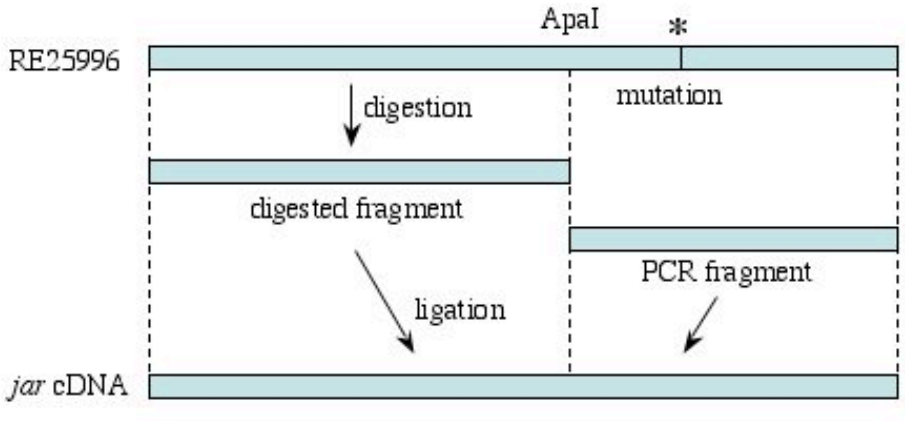


Figure 2.6. **Cloning of the *jaguar* cDNA**

PCR primers for cloning of the *jaguar* cDNA

name	**sequence**	**R.site**
EcoRI-Jar-full For	CGGAATTCGTCGAGAGCGGAG	EcoRI
XhoI-Jar-full Rev	CCGCTCGAGCTACTGTTGTTTCTGCATTG	XhoI

The sequence of the restriction sites is underlined.

Vector

pBluescript KSII+: vector for *in vitro* transcription, sequencing, subcloning.
(*Stratagene*, Heidelberg); ampicilin resistant.

2.4.2.1 Cloning of PCR fragments into plasmids

In this work, several constructs were produced by cloning PCR products into the vectors of choice. Cloning procedure is a three step process and includes: Separate digestion of PCR fragment and vector with suitable restriction enzymes, ligation reaction during which the fragment is incorporated into plasmid with the help of T4-DNA ligase and finally selection of the right clone by transformation in *E.coli*.

Kit and Enzyme

T4-DNA ligase (*Roche*)

Nucleobond gel extraction, PCR Purification, *Machery Nagel*, Düren

2.4.2.2 Transformation of electrocompetent cells

- Electroporation of electrocompetent cells was performed in Gene Pulser, Biorad. Pulses magnitude V=1.8kV
- Concentration of plasmid adjust to up to 100 ng/µl by dilution in dH_2O
- Thaw 50 µl of Xl-1 electrocompetent cells on ice and add 1 µl of DNA
- Transfer mixture into the electroporation cuvette (*Biorad*) and proceed with pulse transformation
- Resuspend bacteria in LB growth medium and incubate 60 min at 37°C
- Plate bacteria on selective surface (for mini preps) and let them grow O/N

Bacteria strain

Electrocompetent XL-1 Blue MRF

Solution

LB medium: 1 % Bactotrypton,
0.5 % Bactoyeast,
1 % NaCl

LB agar plate: 1.2 % Agar, LB medium

For selection, always use antibiotic determined by the vector resistance e.g. ampicillin, kanamycin, chloramphenicol etc.

2.4.2.3 Isolation of plasmid DNA

Small scale DNA isolation (Mini Prep)

To screen a plate for a presence of a colony that contains a wanted plasmid a mini prep was performed. Single colonies were propagated O/N at 37°C in 3 ml LB medium with selective antibiotics.

Plasmid extraction:

- Spin down overnight culture for 1min at 13000 rpm
- Discard supernatant and resuspend pellet in 300 µl S1
- Add 300 µl S2 lysis buffer, and gently invert reaction tube several times
- Add 300 µl S3 neutralization buffer and invert several times
- Centrifuge for 10 min at 4°C at 13000 rpm
- Take supernatant in the new reaction tube and add 630µl Isopropanol
- Centrifuge for 30 min at 4°C at 13000 rpm
- Wash pellet 5 min with ice cold 70 % ethanol and centrifuge for 5 min at 13000 rpm
- Dry the pellet on air and resuspend in 20 µl dH2O

For quantitative isolation of highly concentrated DNA transformed cells were grown in the 40 ml LB medium with antibiotic O/N. In medium scale DNA isolation, so-called "midi prep" procedure, DNA was extracted by *Machery Nagel midi kit*.

Solution

S1:	50 mM	Tris-HCl, pH 8.0;
	10 mM	EDTA;
	100 mg/ml	RNaseA
S2:	200 mM	NaOH;
	1 %	SDS
S3:	3.0M	Kaliumacetate, pH 5.5

2.4.3 Constructs

To express GST-fusion proteins, complete coding region of a cDNA or fragments of interest were cloned 3' downstream of the glutathione-S-transferase (GST) coding sequence in pGEX expression vectors.

		vector	5'	3'	For Primer	Rev Primer
GST fusion						
MirandaN298WT	1-298	pGEX4T-1	BamHI	XhoI	pGEX-Mira For	pGEX-Mira N298 Rev
MirandaN298M	1-298	pGEX4T-1	BamHI	XhoI	pGEX-Mira For	pGEX-Mira N298 Rev
MBP fusion						
Inscuteable	full length	pIVEX-MBP	NotI	SalI	NotI-Insc For	SalI-Insc Rev
Jaguar full length	1-1253	pIVEX-MBP	NotI	XhoI	MBP-NotI-Jar For	XhoI-Jar Rev
Jaguar N-term	1-790	pIVEX-MBP	NotI	XhoI	MBP-NotI-Jar For	MBP-XhoI-Jar N Rev
Jaguar C-term	730-1253	pIVEX-MBP	NotI	XhoI	NotI-JarC-For	XhoI-Jar Rev

Primers for generating constructs for protein expression

name	sequence	R.site
pGEX-MIRA FOR	GCGGATCCATGTCTTTCTCCAAGGCC	BamHI
pGEX-MIRA N298REV	GCCTCGAGCAGGCTGCAGTGCTCGCG	XhoI
NotI-Insc For	AAGGAAAAAAGCGGCCGCATGTCCTTTCAGCGCAGC	NotI
SalI-Insc Rev	ACGCGTCGACTCTAGACGAAACTCTCCTG	SalI
MBP-NotI-Jar For	AAGGAAAAAAGCGGCCGCATGTTGGAGGACACCCAAC	NotI
XhoI-Jar Rev	CCGCTCGAGCTACTGTTGTTTCTGCATTG	XhoI
MBP-XhoI-JarN Rev	CCGCTCGAGCTTGACCCAACGCGATCG	XhoI
NotI-JarC-For	AAGGAAAAAAGCGGCCGCGAAGCCATGTTCCAGTCC	NotI

The sequence of the restriction sites is underlined.

Vectors

pGEX-4T-1: For production of GST fusion proteins in *E.coli*, *Amersham Pharmacia Biotech*, Buckinghamshire, UK; ampicillin resistance

pIVEX-MBP: For production of MBP fusion proteins in *E.coli*, *Roche*

2.5 Yeast two-hybrid assay

2.5.1 Constructs

Complete coding region of a cDNA or fragments of interest were cloned into the vectors designed for the yeast two hybrid assay.

		Vector	5'	3'	For Primer	Rev Primer
pGBKT7						
MirandaN298WT	1-298	pGBKT7	SmaI	BamHI	pACT2-N298 FOR	pACT2-N298 REV
MirandaN298M	1-298	pGBKT7	SmaI	BamHI	pACT2-N298 FOR	pACT2-N298 REV
Inscuteable	full length	pGBT9				
Jaguar full length	1-1253		EcoRI	SmaI	pGBKT7-E-Jar For	pGBKT7-SmaI-Jar Rev2
Jaguar N-term	1-790		EcoRI	SmaI	pGBKT7-E-Jar For	pGBKT7-S-Jar N Rev
Jaguar C-term	730-1253	pGBKT7	SmaI	BamHI	SmaI-JarC For	BamHI Jar-C Rev2
pACT2						
MirandaN298WT	1-298	pACT2	SmaI	BamHI	pACT2-N298 FOR	pACT2-N298 REV
MirandaN298M	1-298	pACT2	SmaI	BamHI	pACT2-N298 FOR	pACT2-N298 REV
Inscuteable	full length	pACT2				

Jaguar full length	1-1253	pACT2	EcoRI	EcoRI	pACT2-JAR FOR	pACT2-XhoI-Jar Rev2
Jaguar N-term	1-790	pACT2	EcoRI	XhoI	pACT2-JAR FOR	pACT2-X-JarN Rev
Jaguar C-term	730-1253	pACT2	SmaI	BamHI	SmaI-JarC For	BamHI Jar-C Rev2

Vectors

pGBKT7: Yeast expression plasmid for expression of fusion proteins with DNA binding domain of Gal4 (*Clontech*, Heidelberg); kanamycin resistant.

pGBT9: Yeast expression plasmid for expression of fusion proteins with DNA binding domain of Gal4 (*Clontech*, Heidelberg); ampicilin resistant.

pACT2: Yeast expression plasmid for production of fusion proteins with activating Gal4 domain (*Clontech* Heidelberg); ampicilin resistant.

Primers for generating constructs for yeast two-hybrid assay

name	**sequence**	**R.site**
pACT2-N298 FOR	TCCCCCGGGGATGTCTTTCTCCAAGGCC	SmaI
pACT2-N298 REV	CGGGATCCCCAGGCTGCAGTGCTCGCG	BamHI
pGBKT7-E-Jar For	CGGAATTCATGTTGGAGGACACCCAAC	EcoRI
pGBKT7-SmaI-Jar Rev2	TCCCCCGGGAACTACTGTTGTTTCTGCATTG	SmaI
pGBKT7-S-JarN Rev	TCCCCCGGGAACTTGACCCAACGCGATCG	SmaI
SmaI-JarC For	TCCCCCGGGGGAAGCCATGTTCCAGTCC	SmaI
BamHI Jar-C Rev2	CCGGATCCCCTACTGTTGTTTCTGCAT	BamHI
pACT2-JAR FOR	CGGAATTCGAATGTTGGAGGACACCCAA	EcoRI
pACT2-XhoI-Jar Rev2	CCGCTCGAGCCTACTGTTGTTTCTGCATTG	XhoI
pACT2-X-JarN Rev	CCGCTCGAGCCTTGACCCAACGCGATCG	XhoI

The sequence of the restriction sites is underlined.

2.5.2 Yeast transformation

Introduction of the yeast expression vectors pACT2 and pGBKT7 into the Y190 strain was performed by the Lithium Acetate (LiAc) precipitation.

- Inoculate an overnight culture of YPDA with a colony of Y190 strain
- Dilute the culture up to OD 0.2 and let it grow until it reaches OD 0.6-0.9
- Spin down 20 ml of the culture 10 min at 5000 rpm
- Wash the pellet with 10 ml dH_2O and spin for 10 min at 5000 rpm
- Wash the pellet with 10 ml 0.1M LiAc and spin down for 10 min 5000 rpm
- Resuspend the pellet in 1 ml 0.1M TE/LiAc
- Incubate 15 min at 30°C
- Make a transformation mixture: 50 µl transformation competent yeast + 2 µg denatured herring sperm DNA + 2 µg plasmid DNA + 300 µl PEG LiAc/TE solution
- Briefly vortex and incubate under shaking 30 min at 30°C
- Heat shock 20 min in the water bath at 42°C
- Spin down for 1 min at 7000 rpm
- Resuspend the pellet in 1 ml YPDA and incubate 1 h at 30°C
- Centrifuge for 30 sec at 7000 rpm and discard supernatant
- Resuspend the pellet in 100 µl dH_2O
- Plate 10 µl on the selective SD agar plate and grow for 3 days

Yeast strain:

Y190 *Clontech*, Palo Alto, USA.

Solutions:

YPDA medium:	20 g/l	Difco Peptone,
	10 g/l	Yeast Extract,
	300 mg/l	Adenine sulfate
SD medium:	6.7 g	Difco Yeast Nitrogen Base without amino acids
	dilute in 850 ml dH_2O	
	After autoclaving, add	100 ml Dropout solution,
		50 ml 40 % glucose
		10 ml 100x Histidin

SD agar:	15 g agar/ 1L SD medium	
100x Histidin:	2 g/l	
10x Dropout solution:	300 mg/l	L-Alanine,
	1500 mg/l	L-Valine,
	200 mg/l	L-ArgininHCl,
	300 mg/l	L-Lysin,
	200 mg/l	L-Methionin,
	500 mg/l	Phenyilalanin,
	2 g/l	L-Threonin,
	300 mg/l	L-Tyrosin,
	200 mg/l	L-Uracil
10x TE:	0.1M	Tris-HCl, pH7.5
	10 mM	EDTA
10x LiAc:	1M	Lithium acstate, pH7.5
50 % PEG 4000:	Polyethylene glycol, average MW 3350	

All solutions were autoclaved.

2.5.3 β-galactosidase test

When "bait" and "prey" protein interact, a functional Gal4 transcription factor is formed and drive expression of genes under control of the upstream activating sequence (UAS). The genome of Y190 contains four genes that are expressed under the control of UAS sequence. Therefore test for β-galactosidase activity is a way to confirm existence of the binding of "bait" and "prey". The colony size should be 1-2 mm in diameter before transferring them to a filter.

- Prepare Z-buffer/X-gal solution (2 ml Z-buffer, 5.4 µl Mercaptoethanol, 33.4 µl X-gal)
- Pre-soak sterile Whatman paper with Z-buffer/ X-gal solution (2 ml for 9 cm plates) and place it on a Petri dish
- Place nitrocellulose membrane over the agar plate and leave it for few moments until it completely attaches to the surface
- Lift the membrane, and transfer it to the liquid nitrogen for 10 sec
- Place the membrane, with colonies face up in the Petri dish with Whatman paper and

reaction buffer

- Incubate 4 h at 30°C
- Colonies containing "bait" and "prey" proteins that interact will turn blue.

Buffer

Z-buffer (pH 7.0):	Na_2HPO_4 $7H_2O$	16.1 g/l
	NaH_2PO_4 H_2O	5.5 g/l
	KCl	0.75 g/l
	$MgSO_4$ $7H_2O$	1.246 g/l
X-gal stock solution:	20 mg/ml in DMF	

2.6 Biochemistry

2.6.1 GST-fusion protein

Complete coding region of a cDNA or fragments of interest can be cloned 3'downstream of the glutathione-S-transferase (GST) coding sequence in pGEX expression vectors. For this work only pGEX4T-1 was used. The plasmid can then be transformed to especially designed bacterial strains, like BL21pLysS, that allow high level expression of the fusion protein after addition of 0.1mM final concentration IPTG. Induction was performed for 3-4 hours at 28°C.

Pellet was resuspended in 4 ml 1x PBS containing protease inhibitors (apoprotin, pefabloc, pepstatin, leupeptin) and cells were sonicated for 60 sec (80 Hz). Triton X-100 was added to the extract up to a final concentration of 0.01 %. Extract was rock over and incubated for 30 min at R.T. and centrifuged for 20 min at 4°C to pellet bacterial debris. Supernatants were separated from the pellet and frozen at -20°C.

Purification of GST fusion proteins

GST fusion proteins can be purified from the rest of the extracted proteins by coupling to Glutathione Sepharose 4A beads. The GST part of the fusion protein strongly interacts with the substrate Glutathione and stays attached to the beads.

- Wash 20 µl of beads 2x 5 min with 1xPBS
- Add 100 µl protein extract and incubate 30-45 min at R.T.
- Wash the beads 4x 10 min with PBS

- Add 2x SDS sample buffer and boil the samples
- Confirm binding of GST fusion protein by SDS-PAGE electrophoresis and Coomassie Brilliant Blue staining.

Reagent

PBS To prevent protein degradation during experimental procedure, protease inhibitors were added in 1:500 dilution from original stock solutions.

Protease inhibitors: Aprotinin (stock 10 mg/ml),
Pepstatin (1 mg/ml),
Leupeptin (0.5 mg/ml)
Pefabloc (1 mg/ml)

Glutathione-Sepharose 4A beads (*Amersham Biosciences*)

2.6.2 *in vitro* transcription and translation

Biotin labeled Jar and Insc proteins were produced by *in vitro* transcription and translation. These proteins were used for a GST pull-down assay.

- Mix the following components in a 1.5 ml tube.

25 µl	Rabbit Reticulocyte Lysate
2 µl	TNT Reaction buffer
1 µl	T3- or T7-RNA Polymerase
0.5 µl	AA-mixture (minus Leu)
0.5 µl	AA-mixture (minus Met)
1 µl	RNase Inhibitor (40 U/µl)
2 µl	template DNA (0.5 µg/µl)
2 µl	transcend tRNA
16 µl	H_2O
50 µl	total volume

- Incubate the reaction at 30°C for 90 min.
- Analyze the results of translation by a Western blot analysis.
- For the detection, membrane was incubated with Streptavidin-POD-conjugates, and

followed by Chemiluminescence detection.

Kit and reagent

in vitro transcription and translation:

TNT Coupled Reticulocyte Lysate Systems/Transcend Non-Radioactive Translation Detection System, *Promega*, Madison, USA.

Streptavidin-POD-conjugates (1:1000, *Boehringer/Roche Diagnostics*, Mannheim)

2.6.3 GST pull-down assay

GST-fusion protein A and biotin labeled protein B are mixed in the presence of the glutathione-Sepharose beads and incubated to allow protein associations to occur. GST-fusion protein and associated proteins are collected by centrifugation.

- Wash 20 µl glutathione-Sepharose 4A beads with 1x PBS/0.1 % NP-40 2x 5 min
- Add GST fusion protein (bacteria extract obtained in chapter 2.6.1) to the beads
  - GST alone 50 µl
  - GST-Mira wt/ M 150 µl
- Incubate 2h at 4°C
- Wash the beads 4x 10 min with PBS/0.1 % NP-40
- Add 10 µl of the Reticulocyte lysate containing biotin-labeled Jar or Insc (obtained in chapter 2.6.2)
- Incubate O/N at 4°C
- Spin down 2 min at 5000 rpm and remove the supernatant. Keep aliquot of supernatant for testing
- Wash the beads 4x 10 min in 1x PBS/0.1 % NP-40
- Add 2x SDS buffer and boil the samples

Samples further undergo SDS-PAGE followed by Western Blotting procedures

Buffer

1x PBS/0.1 % NP-40

containing protease inhibitors: Aprotinin, Pepstatin, Leupeptin, Pefabloc

2.6.4 SDS-PAGE and Western blot analysis

Presence of a protein of interest in the total protein extract can be shown by the Western blot method. Proteins are denatured by SDS and afterwards separated in respect to size by SDS-PAGE on polyacrylamide gels (Laemmli, 1972). Complete protein content from the SDS-gel is transferred on the nitrocellulose membrane.

Western blot analysis

Assemble blot in a tray with Western blot transfer buffer. When sandwich is assembled transfer protein for 1 hour at 100V at 4°C. Afterwards, disassemble sandwich, and check for the proteins on the nitrocellulose membrane by adding PonceauS staining solution for 1 min. Block the membrane 1h in blocking solution (TBST + 3 % skin milk powder +1 % BSA). Dilute primary antibody in desired concentration in blocking solution and incubate the membrane O/N at 4°C. Wash the membrane 3-4 times 15 min in TBST. Incubate the membrane with the HRP-conjugated secondary antibody (1:10000 dilution). Wash the membrane 4x 15 min in TBST. Prepare ECL reagent and incubate the membrane for 1 min. Wrap membrane and expose to X-ray film.

Detection systems

X-ray film:	Fuji Super RX, *Fuji*, Tokyo, Japan
Developer:	Tenetal Roentogen, *Tenetal*, Norderstedt
Fixation:	Tenetal Roentogen Superfix, *Tenetal*, Norderstadt

Reagent

Separating gel:	2.5 ml	30 % Acrylamid/Bis-Acrylamid (29:1)
(10 %)	2.8 ml	1M Tris, pH 8.8
	38 µl	20 % SDS
	2.1 ml	H_2O
	30 µl	10 % APS
	8 µl	TEMED
Stacking gel:	310 µl	30 % Acrylamid/Bis-Acrylamid (29:1)
	235 µl	1M Tris, pH 6.8
	10 µl	20 % SDS
	1.3 ml	H_2O

	10 µl	10 % APS
	5 µl	TEMED
1x TBST:	50 mM	Tris, pH 7.5
	150 mM	NaCl
	0.1 %	Tween 20

Blocking solution: 3 % Skim milk powder, 1 % BSA in 1x TBST

2.7 General laboratory equipment

- PCR machine: MiniCycler (*MS Research*, Watertown)
- Centrifuge: Heraeus biofuge fresco/pico (*Kendo lab products*, Langenselbold)
- Electroporation: Gene Pulser II and Puls Controller Plus (*Bio Rad* Munich)
- UV spectral photometer: Gene Quant II (*Pharmacia Biotech*, Cambridge, UK)
- Sonicator: Labsonic U (*Braun Biotech*, Melsungen)
- SDS-PAGE and Western Blot: Miniprotean 3 (*Bio Rad*, Munich)

3 Results

3.1 Maternal screen

3.1.1 Strategy

I conducted a genetic screen to identify novel genes involved in asymmetric cell division in the *Drosophila* CNS. I screened embryos derived from female flies containing germ line clones (glc) that lacked both maternal and zygotic gene products (figure 3.1). The reason why I generated glc embryos was that many genes involved in asymmetric cell division have a strong maternal contribution. In a zygotic mutant for a gene with a strong maternal contribution, the mutant phenotype is often not detected, since the maternally stored products are sufficient to compensate for the absence of the zygotic product. I screened 628 independent homozygous lethal mutant lines that were generated by insertions of P-elements on either the second or third chromosome. When the original line had mutations on more than one chromosome arms, e.g. one mutation is on the left arm of the second chromosome and the other is on the right arm of the second chromosome, glcs of those mutations were separately generated. Therefore, 926 independent glcs were generated from the 628 original mutant stocks.

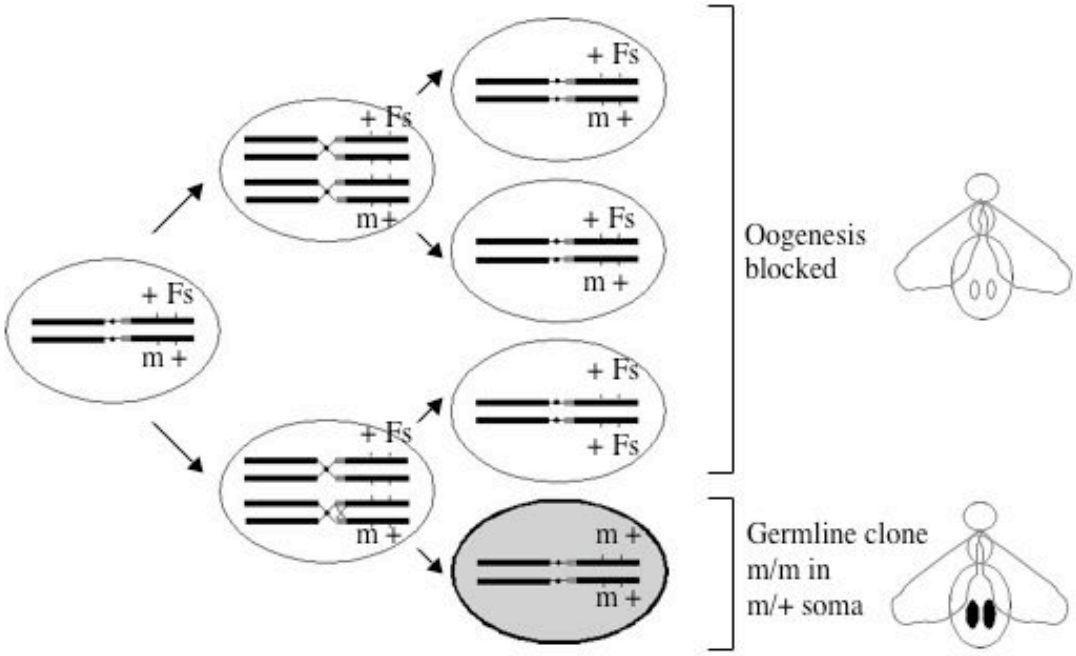


Figure 3.1. Germline clone

Modified from Chou et al., 1996. A: In a fly of the genotype FLP/+; FS m^+ FRT/FS^+ m FRT, when hsp70-flipase (FLP) is activated, site-specific recombination at the point of the FRT sequence is catalyzed. Black ovals: developed ovaries, empty ovals: atrophic ovaries. By this recombination, 100% of the females get germline clones (glc) with the genotype is m/m in m/+ somatic cells. FRT: FLP-recombinase target sequence, FS: dominant female sterile, m: recessive zygotic lethal mutation. B: Embryos derived from a glc female lack maternally provided proteins.

The screen was performed in two steps. In the primary screen, I examined whether glc embryos had defects in the CNS. Embryos were stained with mAb22C10, which labels subsets of the neurons in the CNS and all neurons in the peripheral nervous system (PNS) (figure 3.2). The purpose of the primary screen was to reduce the number of lines that were analyzed further, because it is quite time-consuming to stain all of the embryos with polarity markers and observe them. The strategy of the primary screen was based on the idea that, if asymmetric cell division of NBs does not take place correctly, the CNS would not be organized correctly, resulting in abnormal staining with mAb22C10. I studied glc embryos derived from 926 independent mutations and stained with mAb22C10. From the 926 lines, 83 lines were subjected to the second screen. The majority of these 83 lines showed abnormal staining with mAb22C10 in the glc embryos (figure 3.2). Additionally, I selected some lines that had general defects, such as embryos disorganization, since many proteins involved in asymmetric cell division are required for establishing the polarity of the epithelial cells. In the second screen, I looked for embryos in which the NB cell polarity was abnormal. To this end, the glc embryos were stained with the polarity markers, Baz and Mira. In wild type NBs, Baz is localized at the apical cortex, while Mira is localized at the basal cortex. I investigated the subcellular localization of Baz and Mira in the mutant NBs.

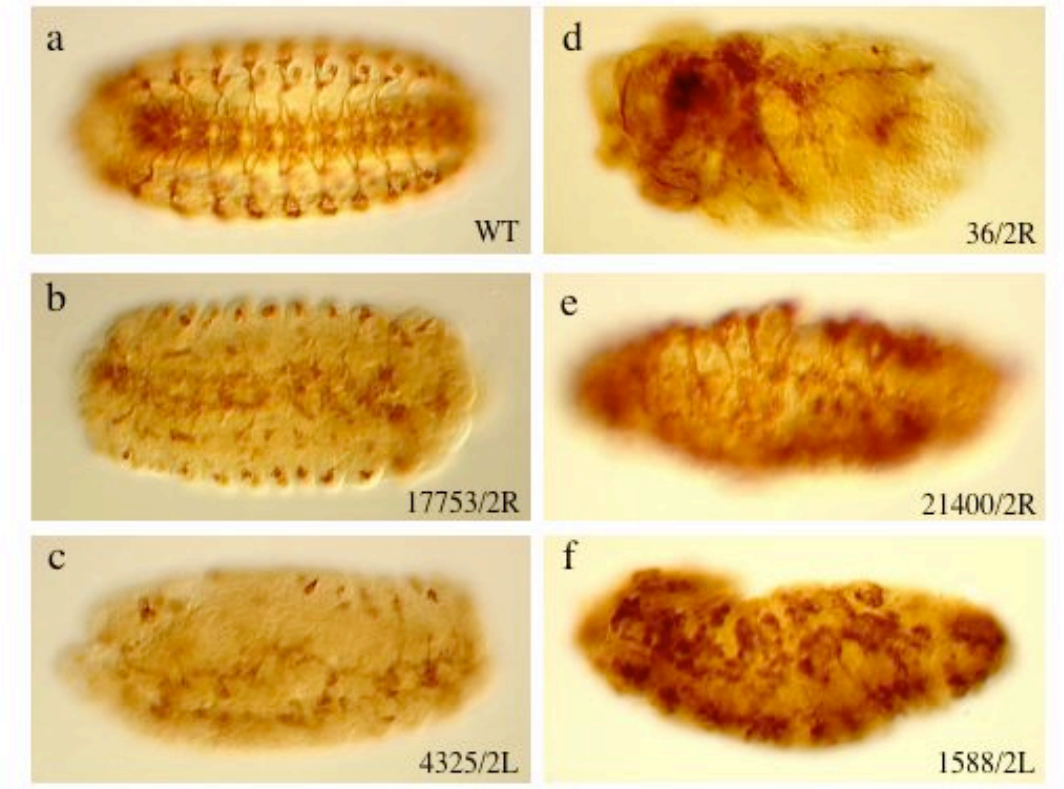


Figure 3.2. Germline clone embryos with defects in the central nervous system

Ventral view of wild-type (a) and germline clone (glc) (b-e) whole mount embryos, and lateral view of glc embryo (f). Optical microscope images. Anterior is to the left. In the wild type embryos (a), mAb22C10 labels subset of the CNS neurons and all neurons in the PNS (peripheral nervous system). In the #17553 glc embryo (b), axon tract is disrupted. Most PNS neurons are missing in addition to defects with axon tract in the #4325 glc embryo (c). Nervous system is severely disorganised in the #36 glc embryo (d). Excess number of neurons is detected in the #1588 glc embryo (e). Hyperplasia is seen in the #21400 glc embryo (f).

3.1.2 Results of the maternal screen

In the screen, I found one line (*#000*) that showed an abnormal distribution of the basal marker Mira in NBs. Mira was distributed into the cytoplasm and also localized at the mitotic spindle (figure 3.3). Since it was previously reported that in *lgl* glc embryos, Mira is mislocalized in a similar manner (Ohshiro et al., 2000; Peng et al., 2000) as in *#000*, I performed complementation tests to examine if this line had a mutation in the

lgl locus (see 3.3.1 for complementation test). This line did not complement mutations in *lgl*, indicating that this line was allelic to *lgl*. Another point of evidence that *#000* was allelic to *lgl* mutants was that this line showed a similar cuticle phenotype as *lgl* mutants (figure 3.4). Since the cuticle is secreted from epithelial cells, the cuticle phenotype is a hallmark for defects in epithelial cell polarity. While structures with a regular pattern, such as the denticle belts and the head skeleton, were observed in wild type embryos, such organized structures were lost in the cuticles of *#000* glcs. Instead, *#000* glcs showed an abnormal cuticle pattern, referred to as "brainish" phenotype, that is characteristic of mutants that secrete too much cuticle and show an expansion of the apical plasma membrane domain.

It should be noted that prior to my screen, another maternal screen using the same mutant stock collection was performed. In the screen, cuticle phenotypes were investigated and 45 lines showed the "brainish" phenotype. As a result of complementation tests, all 45 of these lines turned out to be allelic to *lgl*. In this screen, a new allele of *scrib* (Albertson and Doe, 2003), which is another tumor suppressor gene involved in asymmetric cell division of NBs, was also isolated.

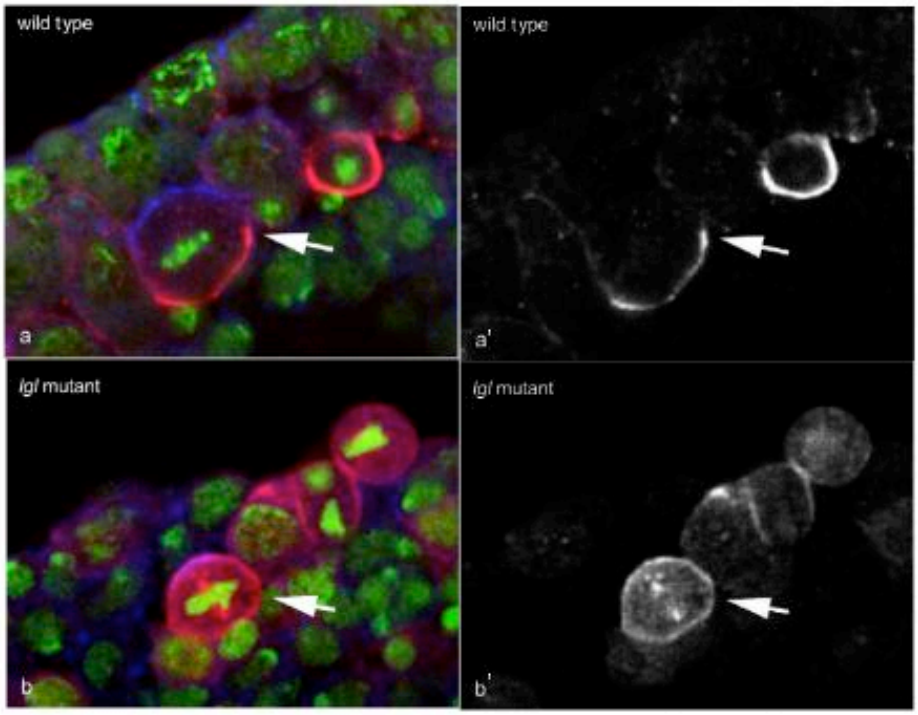


Figure 3.3. Miranda is mislocalized in the NBs in *lgl* germline clone embryos

A: Confocal images of NBs in wild type and *lgl* germline clone embryos at the extended germ band stage. A wild type embryo (a, a') and an *lgl* glc embryo (b, b'), stained with polarity markers. The embryos were triple labeled for Miranda (red), Bazooka (blue) and DNA (YoYo-1, green). The Mira signal aloneis shown in white in a and b. a, a': In the metaphase NB, Baz is localized at the apical cortex, while Mira is localized at the basal cortex (arrow). b, b': Mira is localized throughout the cytoplasm and at the mitotic spindle, although Baz localization is unaffected. Apical is up in all images.

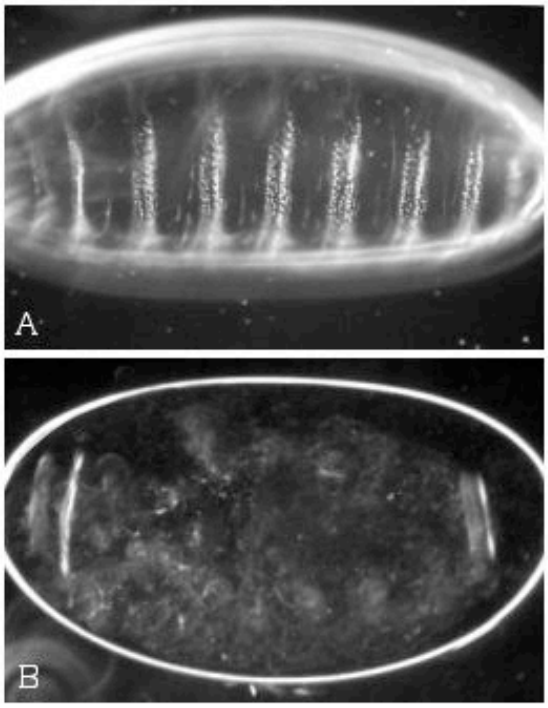


Figure 3.4. Cuticle phenotype in an *lgl* glc embryo

a: Wild type cuticle. Segmentally repeated denticle belts are evident. b: Cuticle of an *lgl* glc embryo. Excess secretion of cuticle is observed. This cuticle phenotype is called the "brainish" phenotype. Anterior is to the left, dorsal is up.

3.2 Zygotic screen

3.2.1 Strategy

Since I could not find any novel gene in the maternal screen, I conducted another

genetic screen to search for novel genes involved in the asymmetric cell division of NBs (figure 3.5). I obtained 570 EMS-induced mutant lines from the Klämbt lab. They were homozygous lethal and showed defects in the CNS. Hummel et al. performed a saturated EMS mutagenesis and screened 18000 lines by staining embryos with the neuronal markers mAb BP102 and 22C10. These 570 lines were found to have defects in the embryonic CNS (Hummel et al., 1999a; Hummel et al., 1999b).

My screen was carried out by immunostaining with polarity markers to examine whether the embryos have defects in the apical-basal polarity of the NBs, which was the same strategy as the second screen of the maternal screen. The difference was that glcs were not generated in this screen. Instead, mutant embryos that lacked only zygotic gene products were screened.

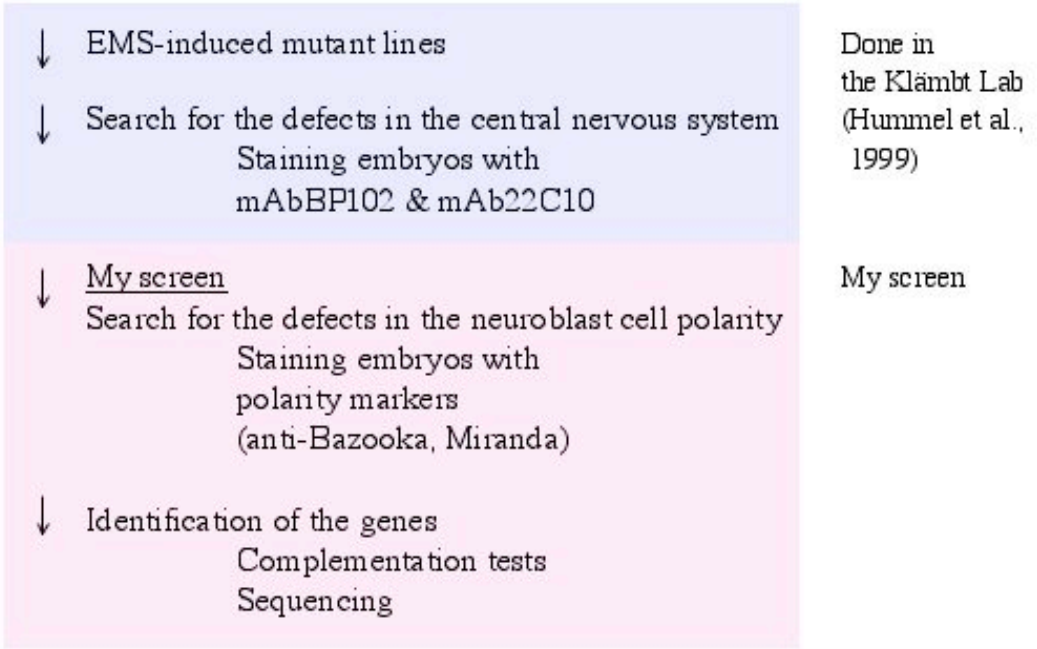


Figure 3.5. Strategy of the zygotic screen

In the Klämbt lab, a saturating EMS mutagenesis was performed to identify all zygotically active genes required for the correct development of the axon pattern in the ventral nerve cord. After screening more than 18,000 lethal lines, they found 757 mutations leading to a specific nervous system defect. If asymmetric cell divisions do not occur properly, the nervous system will be disorganized. Based on this

idea, I screened the mutants with defects in the CNS found in the Klämbt lab.

3.2.2 Results of the zygotic screen

In metaphase, the basal marker Mira forms the basal crescent in wild type NBs. According to the localization of Mira in metaphase NBs, the 570 lines were sorted into various classes as follows (table 1): [1] Mira localization appeared normal, like the wild type, in 355 lines. [2] In 76 lines, Mira formed the crescent, although its orientation of Mira crescent did not appear to be normal. In these lines, it was not clear yet if the orientation of the crescent was normal or abnormal. A closer analysis was required. [3] Mira was localized at the whole cortex in some lines. [4] Mira failed to be localized at the cortex. Instead, Mira was distributed throughout the cytoplasm in one line (*E326*). [4] In 29 lines, no clear Mira crescent was found or the Mira signal was weak. [5] Some other defects were also found. Some lines had defects in cell cycle regulation, resulting in larger NBs with no Mira crescent. Others had NBs with abnormal morphology; e.g. the NBs were not spherical.

I selected one line, *E326*, for the further investigation since the phenotype was fully penetrant and this was the only line in which the cortical localization of Mira in NBs was defective.

Mira localization	**Mira crescent formed?**		
normal			355
abnormal	crescent formed		76
	no crescent/ weak crescent	whole cortex	1
		cytoplasm	1
		cell cycle defect	6
		others	21
others			66
further analysis required			44
total			570

Table 3.1. Results of the zygotic screen

3.3 Identification of the mutation of *E326*

In my work, I analyzed *E326*, in which the basal marker Mira failed to be localized at the cortex. In metaphase, Mira was localized at the basal cortex in the wild type NBs, whereas Mira was distributed throughout the cytoplasm in the *E326* mutant NBs. In telophase NBs, while Mira segregated exclusively into GMCs in the wild type, Mira was still distributed in the cytoplasm in both the presumptive NBs and GMCs in *E326* (figure 3.6). Thus, Mira failed to be localized at the cortex. Baz localization and spindle orientation were normal. The sizes of the two daughter cells were unequal, as in the wild type. In many mutants, even if Mira is mislocalized in metaphase NBs, its localization becomes normal in telophase. This phenomenon is referred to as "telophase rescue" (Cai et al., 2001; Peng et al., 2000). Telophase rescue did not take place in the *E326* mutant NBs (figure 3.6).

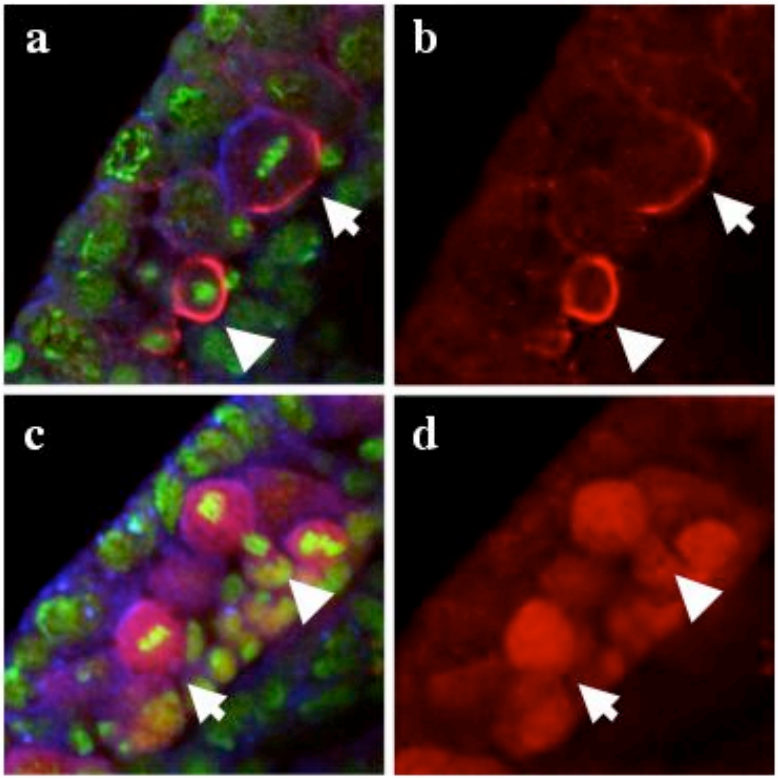


Figure 3.6. **Miranda is mislocalized in *miranda*^{E326} homozygous mutant NBs**

Confocal images of NBs in wild type and *mira*^{E326} embryos at the extended germ band stage. A wild type embryo (a, b) and a *mira*^{E326} homozygous embryo (c, d) are stained with polarity markers. Embryos are triple labeled for Mira (red), Baz (blue) and DNA (YoYo-1, green). a, b: In the metaphase NB, Baz is localized at the apical cortex, while Mira is localized at the basal cortex (arrow). In telophase, Mira segregates exclusively into the GMC (arrowhead). c, d: Although Baz localization is unaffected, Mira is distributed throughout the cytoplasm in *mira*^{E326} NBs (arrow). In the telophase NB, Mira is distributed in both NBs and GMCs (arrowhead). Apical is up in all images.

3.3.1 Complementation test

One possible explanation for the mislocalization of Mira is that *E326* has a mutation in the *mira* locus. It is possible that Mira is mislocalized because the region responsible for the basal localization of Mira is defective. To examine this possibility, I performed complementation tests with other *mira* alleles and with a deficiency with a complete deletion of the *mira* locus (*Df(3R)ora*^{l9}).

The principle of the complementation test is the following: *E326* flies are crossed with a lethal *mira* allele. If *E326* has a mutation in the *mira* locus, the transheterozygotes for *E326* and the *mira* allele would be lethal, since no functional Mira is produced. This case is defined as “no complementation”. In contrast, if *E326* does not have a mutation in the *mira* locus, the transheterozygotes would be viable, which is defined as “complementation”.

E326 did not complement any of the *mira* alleles or the deficiency line, *Df(3R)ora*^{l9}, indicating that *E326* has a mutation in the *mira* locus. Thus, *E326* is the first *mira* allele in which the mutant Mira is mislocalized during mitosis (See chapter 3.5).

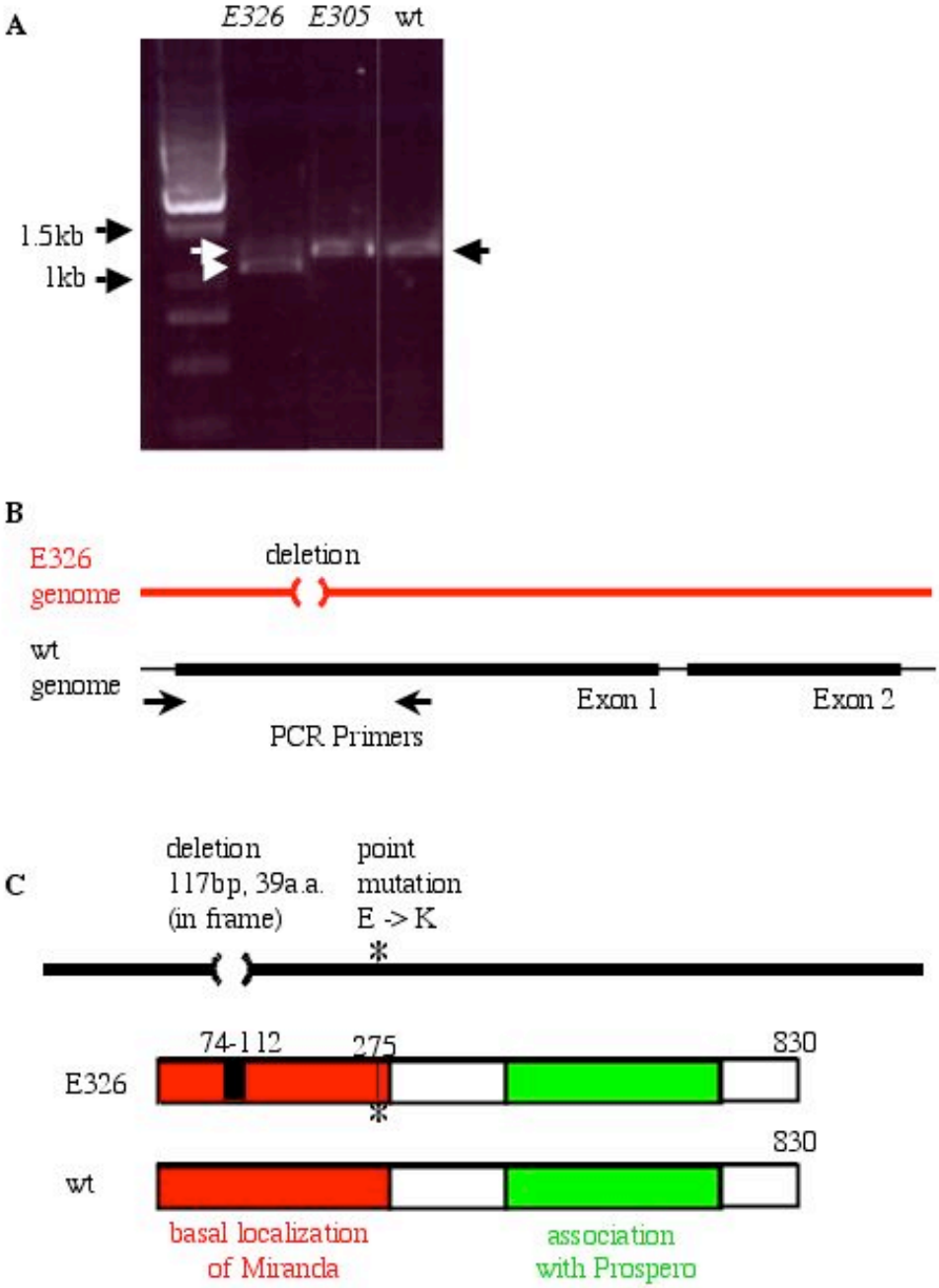
3.3.2 Sequencing of *miranda*^{E326} genomic DNA

I sequenced the *mira* locus in *mira*^{E326} genomic DNA to confirm that *mira*^{E326} has a mutation in the *mira* locus. Since *mira*^{E326} was mutagenized with EMS, a point mutation was expected. However, EMS can also induce small deletions, although such mutations are rather rare.

Since *mira*^{E326} is homozygous lethal and is balanced with a balancer chromosome (TM3), it was difficult to isolate genomic DNA from only the *mira*^{E326} mutant chromosome. Therefore, I used the following strategy for sequencing. The first step was to amplify the *mira* locus by a PCR reaction using genomic DNA isolated from *mira*^{E326}

mutant embryos balanced with TM3 (*mira*^{E326}*/TM3*) as a template. The mixture of the PCR products derived from both the *mira*^{E326} mutant chromosome and the balancer chromosome was then sequenced. If *mira*^{E326} had a point mutation, two different bases would be obtained in the sequence data. Unexpectedly, however, two fragments with different sizes were obtained as a result of the PCR reaction using genomic DNA isolated from *mira*^{E326} heterozygous flies. In a control reaction using genomic DNA isolated from *E305/TM3*, only one fragment was amplified. *E305* was balanced with the same balancer chromosome as *mira*^{E326}, and the *mira* locus is intact on this chromosome. This result suggested that the larger fragment was derived from the balancer chromosome and the smaller fragment was derived from the *mira*^{E326} mutant chromosome (figure 3.7A). In fact, when the smaller PCR product was sequenced, a small deletion was found within the genomic region that encodes the N-terminal part of Mira. The deletion was a 117bp in-frame deletion that corresponds to 39 amino acids, aa 74-113. Additionally, *mira*^{E326} contained a point mutation. The 275th amino acid, Glu, was replaced by Lys (figure 3.7B-D). These sequencing data suggested that although *mira*^{E326} has mutations in the N-terminal region, the C-terminal part of the mutant Mira protein in *mira*^{E326} is intact. Another evidence that the C-terminal part of the mutant Mira in *mira*^{E326} is intact was that the mutant Mira in *mira*^{E326} was recognized by the anti-Mira antibody that was raised against the C-terminal peptide of Mira.

I also tried a Western blot analysis to detect the mutant form of Mira in *mira*^{E326}. An embryo lysate from *mira*^{E326}*/TM3* embryos was prepared and subjected to a Western blot analysis using the anti-Mira antibody. However, I could not detect the smaller form of Mira, corresponding to the mutant protein in the *mira*^{E326}*/TM3* embryo lysate. Instead I detected only one band that had the same size as wild type Mira.


A
E326
E305
wt
1.5kb
1kb
B
E326
genome
deletion
wt
genome
Exon 1
Exon 2
PCR Primers
C
deletion
117bp, 39a.a.
(in frame)
point
mutation
E -> K
74-112
275
830
E326
830
wt
basal localization
of Miranda
association
with Prospero

D

```
WT   MSFSKAKLKRFNDVDVAICGSPAASNSSAGSAGSATPTASSAAAAPPTVQ    50
E326 MSFSKAKLKRFNDVDVAICGSPAASNSSAGSAGSATPTASSAAAAPPTVQ    50

WT   PERKEQIEKFFKDAVRFASSSKEAKEFAIPKEDKKSKGLRLFRTPSLPQR   100
E326 PERKEQIEKFFKDAVRFASSSKEA------------------------    100

WT   LRFRPTPSHTDTATGSGSGASTAASTPLHSAATTPVKEAKSASRLKGKEA   150
E326 -------------TGSGSGASTAASTPLHSAATTPVKEAKSASRLKGKEA   150

WT   LQYEIRHKNELIESQLSQLDVLRRHVDQLKEAEAKLREEHELATSKTDRL   200
E326 LQYEIRHKNELIESQLSQLDVLRRHVDQLKEAEAKLREEHELATSKTDRL   200

WT   IEALTSENLSHKALNEQMGQEHADLLERLAAMEQQLQQQHDEHERQVEAL   250
E326 IEALTSENLSHKALNEQMGQEHADLLERLAAMEQQLQQQHDEHERQVEAL   250

WT   VAESEALRLANELLQTANEDRQKVEEQLQAQLSALQADVAQAREHCSLEQ   300
E326 VAESEALRLANELLQTANEDRQKVKEQLQAQLSALQADVAQAREHCSLEQ   300
                                 *
```

Figure 3.7. Sequencing the *miranda* locus of the *miranda*^{E326} mutant genome

A: PCR products derived from the genomic DNA of *mira*^{E326}*/TM3, E305/TM3* and wild type flies. *E305/TM3* and wild type were used as controls. *E305/TM3* is a fly strain that is balanced with the same balancer chromosome as *mira*^{E326}, in which the *mira* locus is intact. The 5' one-third of *mira* genomic DNA was amplified by a PCR reaction. The positions of PCR primers are indicated by arrows in B. Two PCR products were obtained from *mira*^{E326} genomic DNA, whereas one fragment was amplified from the control DNAs, wild type and *E305*. Since the larger fragment from *mira*^{E326} (white arrow) has the same size as the wild type and *E305* products (black arrow), it corresponds to the intact *mira* fragment derived from the balancer chromosome. On the other hand, the smaller fragment (white arrowhead) is derived from the mutant chromosome, suggesting that *mira*^{E326} has a deletion in the *mira* locus. B: The exon-intron structure of the *mira* gene and the positions of the deletion and the point mutation in the *mira*^{E326} genome are shown. C: A small deletion (black box) and a point mutation (*) were found within the *mira* locus in the region responsible for the basal localization of Mira (red box). Since this deletion is an in-frame deletion, which corresponds to 39 amino acids, the C-terminal part of Mira remains intact. D: Alignment of the amino acid sequences of wild type Mira (blue sequence in the upper row) and mutant Mira (black sequence in the lower row) in *mira*^{E326}. Amino acids 74-113 are missing in Mira^{E326} (-). The 275th amino acid, Glu, is replaced by Lys (*).

3.4 Mislocalization of the mutant Miranda protein in *miranda*^{E326} mutant neuroblasts and epithelial cells

Since it turned out that *E326* was a mutant *mira* allele, I reexamined the subcellular localization of the mutant Mira protein in the *mira*^{E326} mutant.

The subcellular localization of Mira in wild type NBs changes dynamically during mitosis. Mira is localized at the apical cortex in interphase, and it translocates to the basal cortex in metaphase. Mira segregates exclusively into GMCs after cytokinesis. In contrast, in metaphase *mira*^{E326} mutant NBs, Mira was distributed throughout the cytoplasm, instead of forming a basal crescent. In telophase *mira*^{E326} mutant NBs, Mira failed to segregate exclusively into GMCs. Instead, Mira was still distributed in both the presumptive NBs and GMCs in *mira*^{E326} (figure 3.6). In interphase, Mira was uniformly distributed into the cytoplasm and did not form an apical crescent in *mira*^{E326} NBs (data not shown). Thus, Mira failed to be localized at the cortex throughout mitosis. Even in *mira*^{E326} heterozygous embryos, Mira was mislocalized. Mira was distributed into the cytoplasm in addition to the basal cortex (figure 3.8), suggesting that while the wild type Mira was localized at the basal cortex, the mutant Mira was mislocalized in the cytoplasm.

It is known that prior to neurogenesis, Mira is already expressed in epithelial cells and is localized at the baso-lateral cortex in wild type embryos, although a function of Mira in epithelial cells has not been demonstrated (Matsuzaki et al., 1998). In contrast to the wild type, in epithelial cells in *mira*^{E326}, Mira was not localized at the cortex. Instead, it was localized in the cytoplasm (figure 3.9).

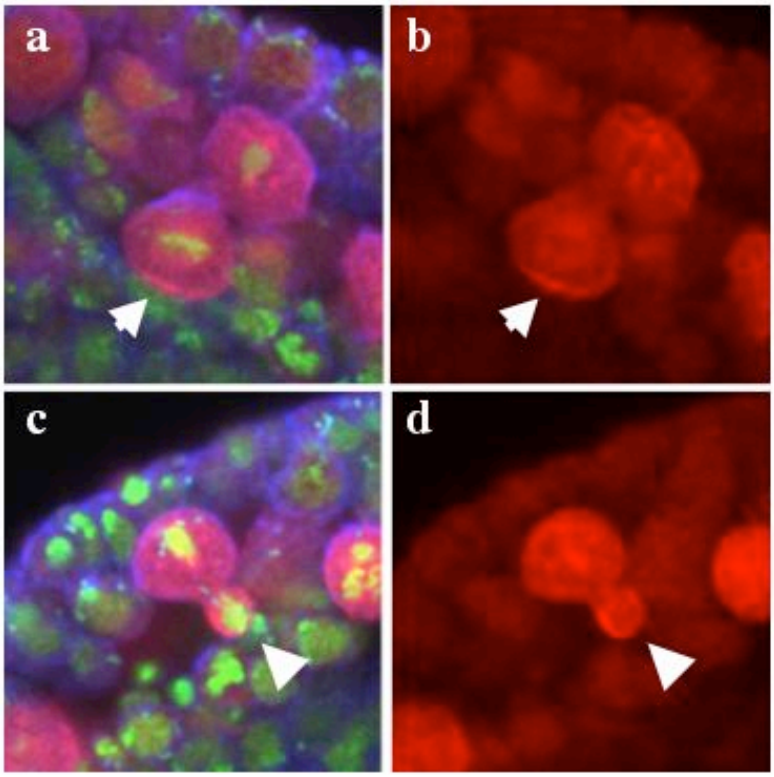


Figure 3.8. Miranda is mislocalized in *miranda*^{E326} heterozygous mutant NBs

Confocal images of NBs in *mira*^{E326} heterozygous mutant embryos at the extended germ band stage. The embryos were triple labeled for Miranda (red), Bazooka (blue) and DNA (YoYo-1, green). a, b: In the metaphase NB, Miranda is distributed throughout the cytoplasm in addition to being correctly localized at the basal cortex (arrow). c, d: In the telophase NB, in addition to the localization at the GMC cortex, Miranda is distributed in both the NBs and GMCs (arrowhead). Apical is up in all images.

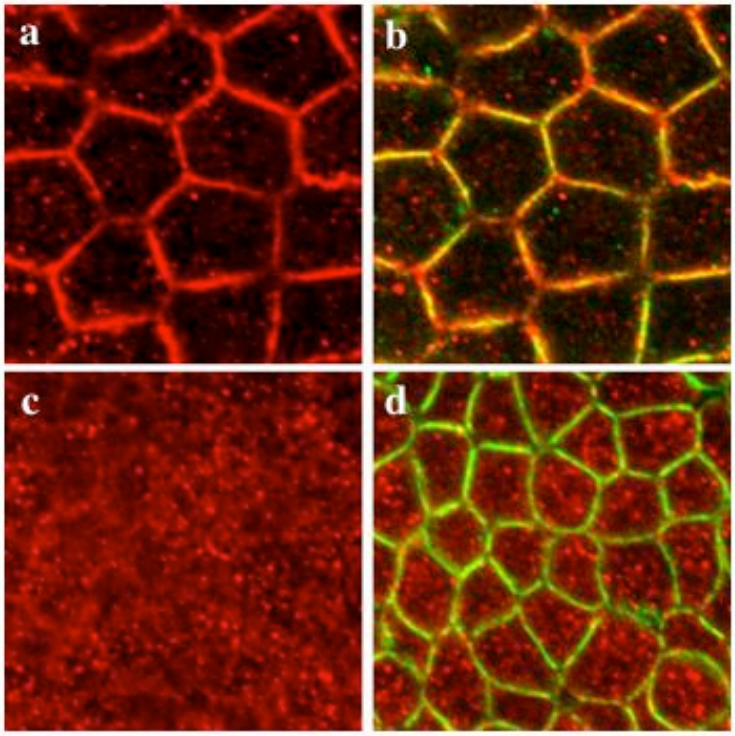
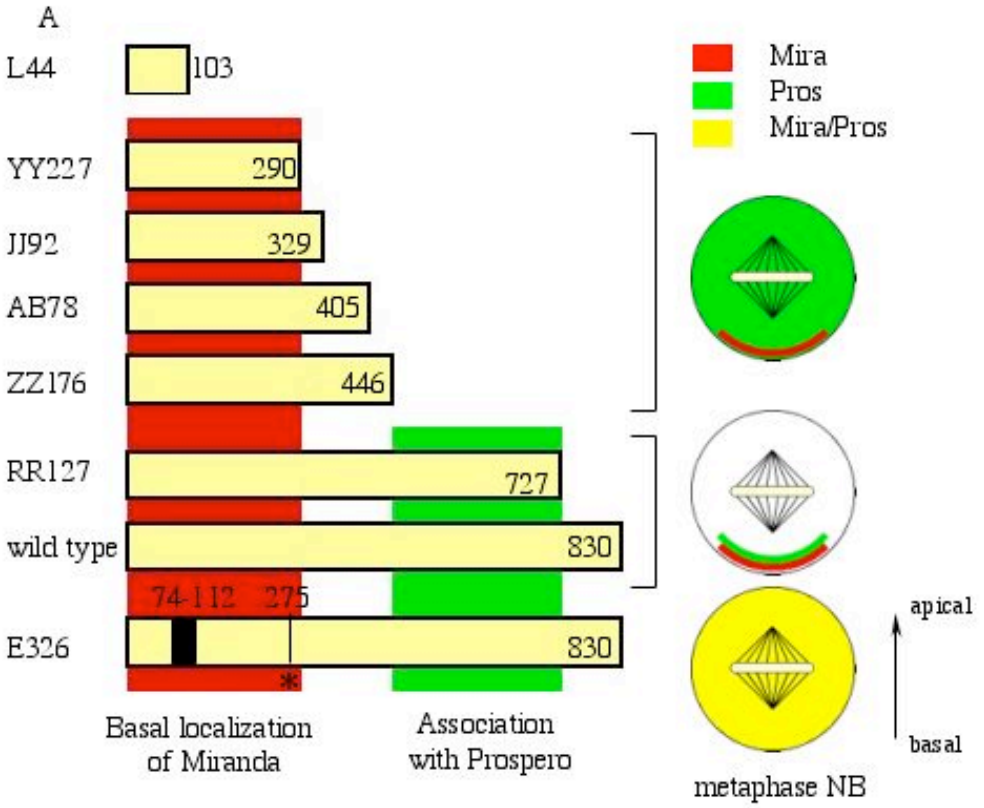


Figure 3.9. Miranda is mislocalized in epithelial cells in *miranda*^{E326} mutants

Confocal images of epithelial cells in wild type and *mira*^{E326} homozygous mutant embryos at the blastoderm stage. The embryos were double labeled for Miranda (red) and Neurotactin (green). Images were taken from the lateral side of the embryo. The focal plane is perpendicular to the apical basal axis. a, b: Mira is cortically localized, giving a "honeycomb-like" staining pattern in wild type epithelial cells. c, d: Mira fails to be localized at the cortex in *mira*^{E326}.



B

mira allele	basal localization of Mira	association with Pros	release of Pros
L44	+	-	
YY227	+	-	
JJ92	+	-	
AB78	+	-	
ZZ176	+	-	
RR127	+	+	-
wild type	+	+	+
E326	-	+	?

Figure 3.10. Comparison of mutant Miranda proteins in *miranda* alleles and their subcellular localization

A: Modified from Matsuzaki et al., 1998 (Matsuzaki et al., 1998). The primary structure of the mutant Mira protein in $mira^{E326}$ is compared with those of other *mira* alleles (See the legend of Figure 1.7). $mira^{E326}$ is a unique mutant *mira* allele in which the mutant protein contains mutations in the N-terminal region. The mutations are located within the domain responsible for the basal localization of Mira (red

box). The mutant Mira fails to be localized at the basal cortex and is distributed throughout the cytoplasm. Pros appears to be colocalized with Mira in the cytoplasm. B: Characteristics of mutant *mira* alleles. The basal localization of the mutant Mira is normal (+) in all alleles except for $mira^{E326}$. The association with Pros is normal (+) in $mira^{RR127}$ and $mira^{E326}$. It is defective (-) in other alleles. The release of Pros is defective (-) in $mira^{RR127}$. It remains unknown in $mira^{E326}$. In other alleles, the mutant Mira does not associate with Pros.

3.5 Comparison with other *miranda* alleles

3.5.1 Mislocalization of the mutant Miranda protein in $miranda^{E326}$ allele

Seven mutant *mira* alleles have been reported so far (Matsuzaki et al., 1998; Shen et al., 1998). In two of these seven alleles, the mutant proteins are not detected by anti-Mira antibody staining. The other five alleles produce C-terminally truncated mutant Mira and the subcellular localization of these mutant Mira proteins in NBs was normal. These data suggested that even the shortest product, which comprises the N-terminal 290 amino acid residues out of a total of 830, is sufficient for the basal localization of Mira (Matsuzaki et al., 1998) (figure 3.10). Another line of evidence that suggests that the N-terminal region is required for the basal localization of Mira has been reported. When a mutant Mira protein lacking the N-terminal 298 residues was expressed in NBs, this protein was distributed in the cytoplasm, instead of being localized at the basal cortex (Shen et al., 1998).

The mutant Mira produced in $mira^{E326}$ had mutations within the N-terminal 290 amino acids and was not localized at the basal cortex. This result is consistent with the earlier prediction that the N-terminal region is responsible for the basal localization of Mira.

3.5.2 Mislocalization of Prospero in $miranda^{E326}$ allele

Mira is an adaptor protein for Pros, which functions as a cell fate determinant in GMCs. In wild type NBs, Mira tethers Pros at the basal cortex, and they segregate exclusively into the GMCs after cell division (Ikeshima-Kataoka et al., 1997; Schuldt et al., 1998; Shen et al., 1997). In the GMC, Pros, which is released from Mira, translocates into the nucleus, where it activates GMC-specific gene expression (Doe et al., 1991) and inhibits NB-specific gene expression (Vaessin et al., 1991).

It was proposed that the C-terminal region of Mira is responsible for the association with Pros because Pros is mislocalized into the cytoplasm, in spite of the normal

localization of mutant Mira in *mira*^{YY227}, *mira*^{JJ92}, *mira*^{AB78} and *mira*^{ZZ176} alleles (Matsuzaki et al., 1998) (figure 3.10). This allowed me to speculate that Pros would be distributed in the cytoplasm in *mira*^{E326}, since although Mira^{E326} binds to Pros this complex is mislocalized in the cytoplasm.

To test this, I investigated the subcellular localization of Pros in *mira*^{E326} mutant NBs. In fact, Pros was distributed in the cytoplasm in *mira*^{E326} homozygous mutant NBs, while it was localized at the basal cortex in wild type NBs. Even in embryos heterozygous for *mira*^{E326} (*mira*^{E326}/*TM3*), Pros was detected in the cytoplasm (figure 3.11). Although I expected Pros to be localized at the basal cortex in addition to the cytoplasm in heterozygous embryos, Pros was not detected in the cortex. This result may be explained by the low quality of the anti-Pros antibody. These results, together with the sequence analysis of the mutant Mira in *mira*^{E326}, suggest that the mutant Mira binds to Pros and that the aberrant Pros localization is caused by mislocalization of the mutant Mira/Pros complex.

After cytokinesis, Pros is released from Mira and translocates into the nucleus in the wild type GMC. It was also reported that the mutant Mira proteins in the *mira*^{YY227}, *mira*^{JJ92}, *mira*^{AB78} *and mira*^{ZZ176} alleles, which cannot bind to Pros, are localized normally, but Pros segregates into both daughter cells and enters the nuclei of both NBs and GMCs (Matsuzaki et al., 1998) (figure 3.10). It would be interesting to determine whether Pros enters the nuclei of NBs and GMCs in *mira*^{E326}. Although I have tried to examine this point, I could not obtain a clear result.

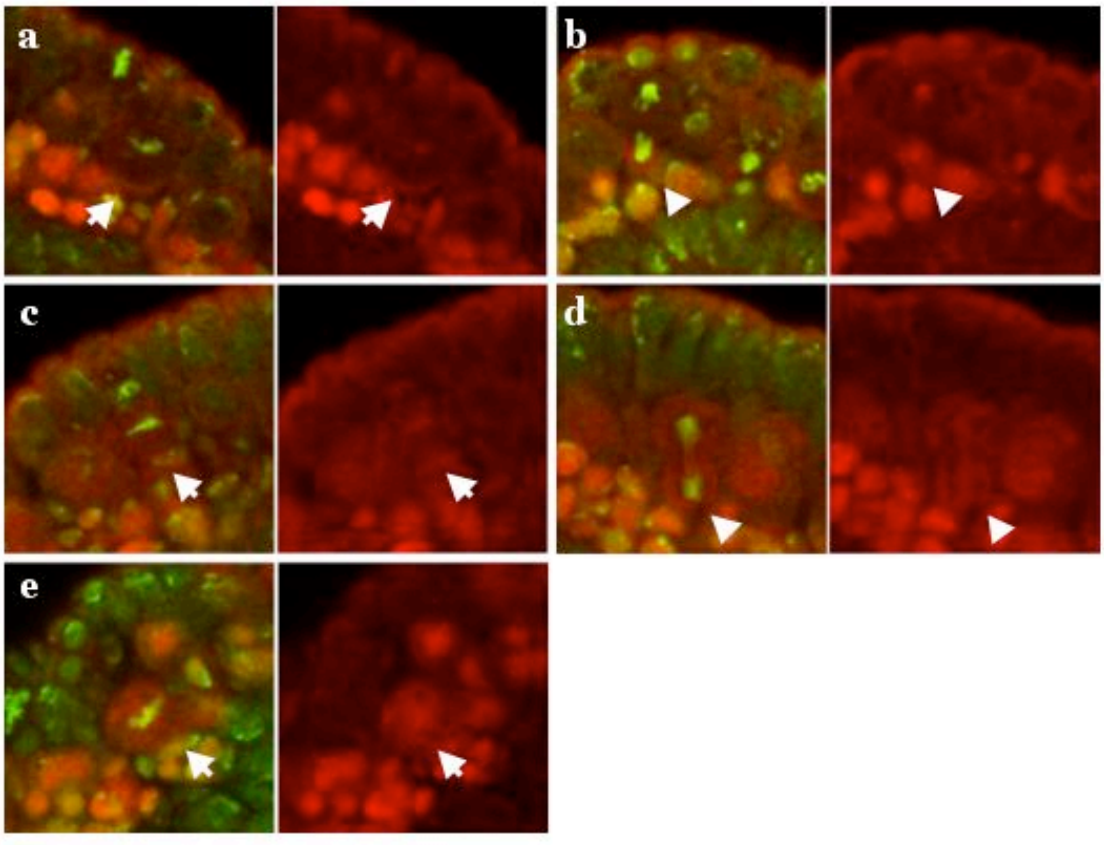


Figure 3.11. Subcellular localization of Prospero in *miranda*^{E326} mutant neuroblasts

Confocal images of NBs in wild type and *mira*^{E326} mutant embryos at the extended germ band stage. A wild type embryo (a, b), a heterozygous *mira*^{E326} embryo (c, d) and a homozygous *mira*^{E326} embryo (e) were double labeled for Prospero (red) and DNA (YoYo-1, green). a: In the metaphase NB, Pros is localized at the basal cortex (arrow). b: In telophase, Pros segregates exclusively into the GMC (arrowhead). c, e: Pros is distributed throughout the cytoplasm in metaphase (arrow). d: In the telophase NB, Pros is distributed in both the NBs and GMCs (arrowhead). Thus, Pros and the mutant Mira show similar localization pattern, suggesting that the mutant Mira binds to Pros. Apical is up in all images.

A

		prospero					
		10419	*A63*	*C7*	*C43*	*17*	*Deficiency*
miranda	*E326*	+	+	+	-	-	-
	L44	+	+	+	+	+	+
	YY227	+	+	+	+	+	+
	JJ92	+	+	+	+	+	+
	ZZ176	+	+	+	+	+	+
	RR127	+	+	+	+	+	+
	Deficiency	+	+	+	+	+	+

B

pros	mutagen	insertion/deletion	class
10419	P-element insertion	P-element in 5' flanking region	
A63	P-element insertion	P-element in 5' flanking region	
C7	P{A63} excision	remove 5' end of transcribed *pros* region	hypomorph
C43	P{A63} excision	remove *pros* transcription unit	hypomorph
17	P-element excision	small deletion within *pros* locus	amorph

Figure 3.12. Genetic interactions between *miranda*^{E326} and amorphic *pros* alleles

A: The *pros* mutant alleles, indicated in the top row (*pros*^{X}), were crossed with *mira* alleles including *mira*^{E326}, as indicated in the left row (*mira*^{Y}). +: Transheterozygote (*pros*^{X}/*mira*^{Y}) was viable. -: Transheterozygotes (*pros*^{X}/*mira*^{Y}) were lethal. When *mira*^{E326} was crossed with strong *pros* alleles, transhererozygotes (*pros*^{X}/*mira*^{E326}) were not obtained. Otherwise, transheterozygotes for *mira* and *pros* were viable. B: *pros* alleles used in the complementation test.

3.6 Genetic interaction between *miranda*^{E326} and *prospero*

When *mira*^{E326} embryos were stained with the neural-specific mAb BP102, they displayed defects similar to those of the *pros* mutants (personal communication, Klämbt lab). Therefore, I performed complementation tests with *pros* alleles to examine if *mira*^{E326} has a mutation in the *pros* locus in addition to the *mira* locus. I used several *pros* alleles for the complementation tests. *pros*^{10419} and *pros*^{A63} are weak *pros* alleles that each have a P-element insertion in the 5'flanking region. *pros*^{C7} and *pros*^{C43} are the progeny of *pros*^{A63}, in which the P-element was imprecisely excised, resulting in removal of the transcription start site. These two alleles are hypomorphic *pros* alleles. On the other hand, *pros*^{17} is an amorphic *pros* allele that has a deletion within the *pros* locus (figure 3.12B). A deficiency line that lacks the entire *pros* locus was also used. All of the *pros* alleles used here are homozygous lethal, and they do not complement the lethality of the deficiency line. When *mira*^{E326} was crossed with the weak *pros* alleles, the transheterozygote flies were viable. In contrast, when *mira*^{E326} was crossed with the strong *pros* alleles or with the deficiency chromosome lacking the *pros* locus, no transheterozygote flies were obtained. This result demonstrated that *mira*^{E326} showed genetic interactions with strong *pros* alleles. When other *mira* alleles were crossed with the strong *pros* alleles, all of the *mira* alleles, except for *mira*^{E326}, complemented the *pros* alleles. Thus, the genetic interaction with *pros* was specific for the *mira*^{E326} allele (figure 3.12A).

Next I examined whether the *pros* locus on the *mira*^{E326} chromosome is really intact. Although it is clear that the *mira*^{E326} chromosome had mutations in the *mira* locus, it was not clear if the *pros* locus was intact. If a recombinant chromosome that has the *mira*^{E326} mutation and a wild type copy of *pros* behaves in a similar manner as the original chromosome, it would be a good hint that the *pros* locus in the original *mira*^{E326} chromosome was intact. To obtain the recombinant, the *mira*^{E326} mutant chromosome was crossed with a chromosome with the visible marker, w^+ *(white*$^+$*)*, due to a P-element insertion between the *mira* and *pros* loci, close to *pros*. By recombination between the marker and *mira*, a recombined chromosome was produced that has both the *mira*^{E326} mutation and a wild type copy of *pros* (*pros*$^+$*mira*^{E326}) (figure 3.13). When this chromosome was crossed with *pros* alleles, it showed essentially the same behavior as the original *mira*^{E326} chromosome; *pros*$^+$*mira*^{E326}/*pros*^{10419}*mira*$^+$ was viable, whereas *pros*$^+$*mira*^{E326}/*pros*^{17}*mira*$^+$ was lethal.

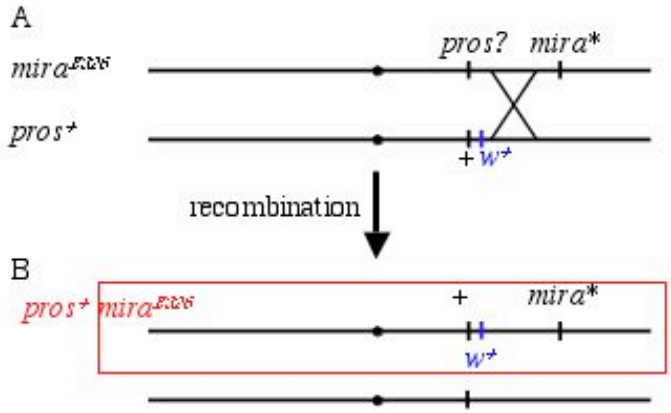


Figure 3.13. Scheme of the recombination between the *prospero* and *miranda* loci in *miranda*^{E326}

To obtain the recombinant chromosome, *mira*^{E326}*/TM3* and *pros*$^{+}$ *w*$^{+}$*/TM3* (Genotype: w; P[GT1]CG14722^{BG01876}, Bloomington stock #12850, P-element insertion line) flies were crossed. When recombination occurs between *w*$^{+}$ and the *mira* locus in the *mira*^{E326}*/pros*$^{+}$ *w*$^{+}$ female flies (shown in A), in the next generation, the *pros*$^{+}$ *w*$^{+}$ *mira*^{E326} chromosome (shown in B) is obtained. A: A pair of chromosomes *mira*^{E326}*/pros*$^{+}$ *w*$^{+}$ is depicted. Although in the *mira*^{E326} chromosome, it is not clear whether the *pros* locus is intact or not (indicated as *pros?*), in the *pros*$^{+}$ *w*$^{+}$ chromosome, the *pros* locus is intact (+). *w*$^{+}$ is the *white*$^{+}$ transgene used as a marker for recombination. B: A pair of chromosomes *pros*$^{+}$ *w*$^{+}$ *mira*^{E326}/+ is depicted.

3.7 Neuronal cell fate determination

The genetic interaction results prompted me to examine if neural cell fate is correctly determined in transheterozygotes for *mira*^{E326} and strong *pros* alleles. Even-skipped (Eve) expression was used as a neuronal cell fate marker. Eve, which is expressed in a subset of neurons, is commonly used as a marker for neuronal cell fate determination (Patel et al., 1989).

3.7.1 Cell fate determination in single mutants for *miranda* or *prospero*

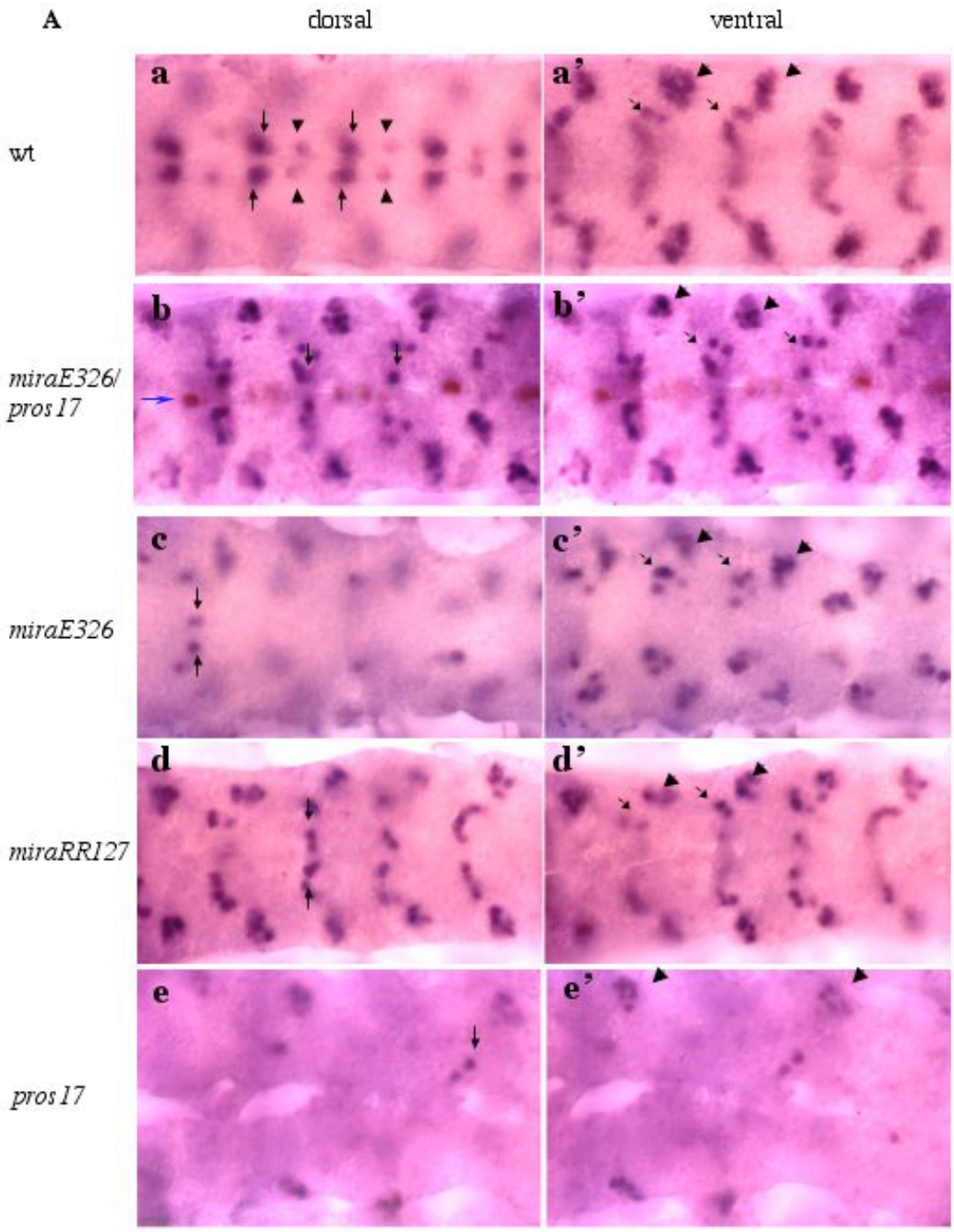
In the CNS of late stage embryos, Eve is expressed in a restricted number of neurons, including aCC/pCC neurons, RP2 neuron, U/CQ neurons and the EL neuron cluster

(Patel et al., 1989) (figure 3.14). The aCC/pCC neurons are the progeny of NB1-1, and the RP2 neuron is one of the progeny of NB4-2 (Bossing et al., 1996; Schmid et al., 1999).

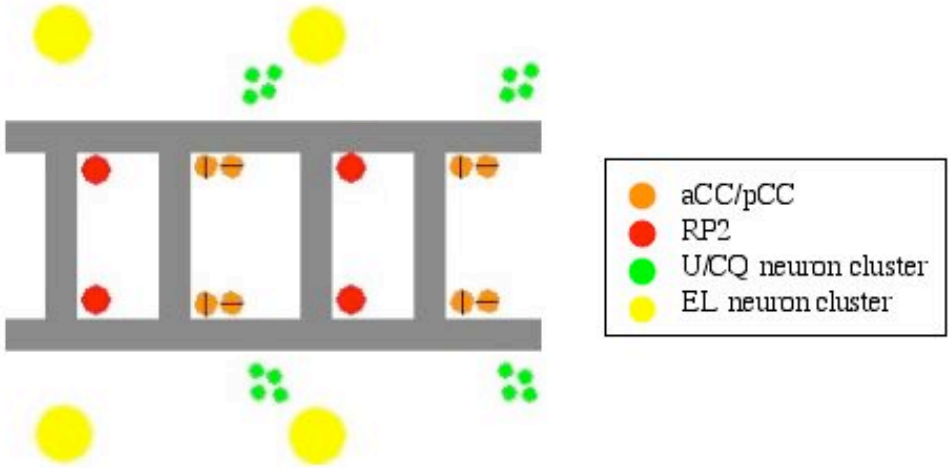
I stained *mira*^{E326} homozygous embryos with an anti-Eve antibody and studied the Eve expression pattern in the late stage embryos. While the EL cluster was not affected, Eve expression in aCC/pCC and RP2 was missing. In the U/CQ cluster, the number of cells appeared to be normal, but its position was abnormal (figure 3.14). This phenotype was more similar to that of *pros* mutants and the *mira*^{RR127} allele than to that of other *mira* alleles. In *mira*^{RR127}, in which the release of Pros from Mira in the GMC is defective, few RP2 cells expressed Eve, and the number of aCC/pCC cells expressing Eve was also reduced. In contrast, the U/CQ and EL neurons were not affected (figure 3.14) (Ikeshima-Kataoka et al., 1997). In other *mira* alleles, Eve expressing cells were reduced to 50% in all cell types (data not shown) (Ikeshima-Kataoka et al., 1997). In strong hypomorphic or amorphic *pros* alleles, the EL neuron cluster was not affected, whereas Eve expression in the aCC/pCC and RP2 neurons was lost (figure 3.14) (Ikeshima-Kataoka et al., 1997). These data suggest that, firstly, the requirement of Pros for Eve expression, which is a marker for neural cell fate determination, is higher in the aCC/pCC and RP2 neurons than in the EL neurons and, secondly, Eve expression in these cell types was lost in *mira*^{E326}, because the amount of Pros that enters the nucleus is not sufficient for neural cell fate determination.

3.7.2 Cell fate determination in *miranda*^{E326} and *prospero* transheterozygotes

In transheterozygotes for *mira*^{E326} and a strong *pros* allele (*mira*^{E326}/*pros*^{17}), 50% of the RP2 neurons lacked Eve expression (figure 3.14). Although this phenotype was milder than those of *mira*^{E326} or *pros*^{17} single mutants, it indicates that the genetic interaction between *mira*^{E326} and strong *pros* alleles not only results in lethality but also causes defects in cell fate determination.


A
dorsal
ventral
wt
a
a'
miraE326/
pros17
b
b'
miraE326
c
c'
miraRR127
d
d'
pros17
e
e'

B



C

	aCC/pCC	RP2	U/CQ	EL
pros amorph	0%	0%	reduction	100%
mira alleles	50%	50%	50%	50%
miraRR127	38%	12%	100%	100%
miraE326	0%	0%		100%
miraE326/pros amorph	100%	50%	100%	100%

Figure 3.14. Neural fate marker Even-skipped expression in the *miranda*^{E326} mutant embryo

A: Two focal planes (dorsal side and ventral side of the ventral nerve cord) of four consecutive segments in late stage (stage 16) embryos. Embryos of wild type (a, a'), *mira*^{E326}/*pros*^{17} (b, b'), *mira*^{E326} (c, c'), *mira*^{RR172} (d, d') and *pros*^{17} (e, e') were labeled with mAb 2B8 (anti Even-skipped, purple) and anti β-galactosidase (brown). Anterior is left. Arrows and arrowheads mark the aCC/pCC and RP2 neurons, respectively in the left panels. Arrows and arrowhead mark the U/CQ neuron cluster and the EL neuron cluster, respectively, in the right panels. a, a': aCC/pCC and RP2, which are located along the axon bundle (not marked), and the U/CQ neuron cluster and the EL neuron cluster, which are located more ventrally and laterally, express Eve in the wild type. b, b': In transheterozygotes for *mira*^{E326} and *pros*^{17} *(mira*^{E326}/*pros*^{17}*),* Eve expression is lost in 50% of the RP2 neurons (arrow). Eve expression in other neurons is not affected. Midline cells are labeled with an anti β-Gal antibody, because the *mira*^{E326}

chromosome has a P-element insertion derived from the enhancer trap line P {AA142} (brown, blue arrow). c, c': Eve expression is lost in aCC/pCC (arrowhead) and RP2 (arrow) neurons in the *mira*^{E326}*/TM3* embryo. Eve expression in the U/CQ neuron cluster and the EL neuron cluster is not affected. d, d': In *mira*^{RR172}, Eve expression in RP2 neurons is lost. The number of Eve positive aCC/pCC neurons is reduced to 50%. The U/CQ neuron cluster and the EL neuron cluster are not affected. e, e': In the *pros* amorphic allele, *pros*^{17}, Eve is not expressed in RP2 neurons. Few aCC/pCC neurons express Eve. The EL neuron cluster is not affected. The number of U/CQ clusters expressing Eve is reduced. B: Schematic drawing of the Eve positive cells in the wild type CNS. Anterior is left. The axon bundle is depicted in gray. Eve is expressed in some neurons including aCC/pCC (orange), RP2 (red), the U/CQ neuron cluster (green) and the EL neuron cluster (Yellow). C: The percentage of cells expressing Eve in comparison to the wild type is shown.

3.8 Interactions between Miranda and its binding partners

3.8.1 Yeast two-hybrid assay

mira^{E326} is a mutant *mira* allele in which the mutant Mira is mislocalized in the cytoplasm. The reason why the mutant Mira in *mira*^{E326} failed to be localized at the basal cortex might be that it is not able to bind to its interaction partners. To test this, I examined the interaction between mutant Mira and two known binding partners of Mira. These two proteins are Jaguar (Jar) and Insc, which are known to interact with the N-terminal 298 amino acids of wild type Mira (Petritsch et al., 2003; Shen et al., 1998).
To examine if the mutant Mira in *mira*^{E326} binds to Jar and Insc, I conducted yeast two-hybrid assays. In this assay, two plasmids that encode protein-X fused to the GAL4-DNA binding domain and protein-Y fused to the GAL4 activating domain are co-transfected into yeast cells containing a *lacZ* reporter gene, which can be activated on in a GAL4-dependent manner. When these two proteins interact, functional GAL4 protein is produced, resulting in expression of β-galactosidase (β-Gal) in the yeast cells. Therefore, if X-gal, which is the substrate for β-Gal, is provided, the yeast colonies will turn blue. If the two proteins do not interact, the yeast colonies remain white. Thus, blue colonies indicate an interaction between the two proteins, and white colonies indicate no interaction.

3.8.1.1 Miranda and Inscuteable

Mira and Insc are colocalized at the apical cortex in interphase NBs. Insc might anchor

Mira to the apical cortex and function as a scaffold where Mira is loaded onto the motor protein that transports Mira to the basal side of the NB (Shen et al., 1998). I speculated that the mutant Mira in *mira*^{E326} failed to be localized at the cortex because it does not bind to Insc.

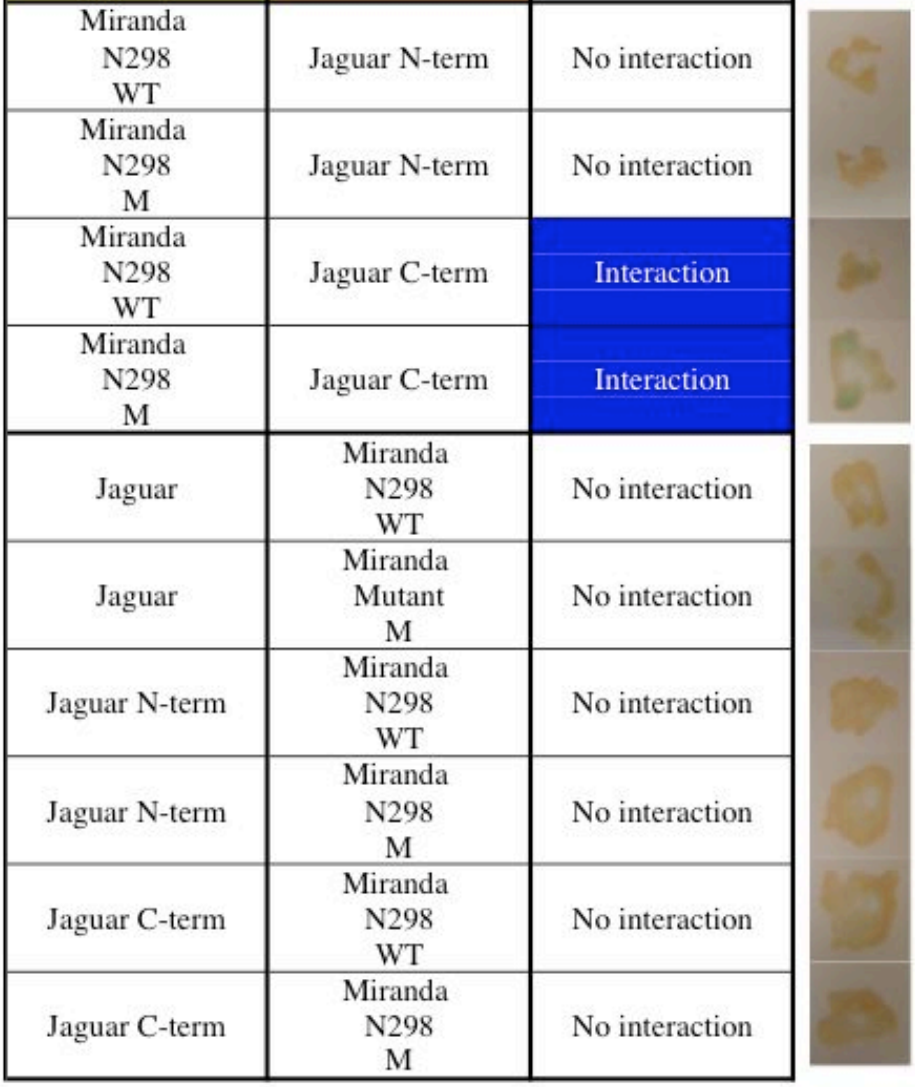
Yeast expressing the N-terminal region of wild type Mira (MiraN298 wt) and Insc turned blue, indicating that MiraN298 wt bound to Insc. In contrast, yeast expressing the N-terminal region of mutant Mira (MiraN298 M) and Insc remained white, which indicates that MiraN298 M did not bind to Insc (figure 3.15A). This result suggested that the N-terminal region of the mutant Mira in *mira*^{E326} lost its ability to bind with Insc.

A

AD	BD	Result of X-gal test
Miranda N298 WT	Inscuteable	interaction
Miranda N298 M	Inscuteable	No interaction
Inscuteable	Miranda N298 WT	No interaction
Inscuteable	Miranda N298 M	No interaction

B

AD	BD	Result of X-gal test
Miranda N298 WT	Jaguar N-term	No interaction
Miranda N298 M	Jaguar N-term	No interaction
Miranda N298 WT	Jaguar C-term	Interaction
Miranda N298 M	Jaguar C-term	Interaction
Jaguar	Miranda N298 WT	No interaction
Jaguar	Miranda Mutant M	No interaction
Jaguar N-term	Miranda N298 WT	No interaction
Jaguar N-term	Miranda N298 M	No interaction
Jaguar C-term	Miranda N298 WT	No interaction
Jaguar C-term	Miranda N298 M	No interaction



C

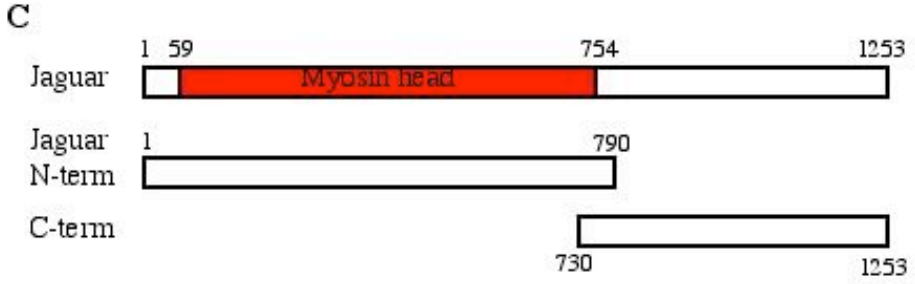


Figure 3.15. **Yeast two-hybrid assay**

A: Results of the two-hybrid assay to examine the interaction between Insc and Mira. cDNAs encoding the N-terminal 298 amino acids of wild type Mira and Mira^{E326} and Insc were cloned into vectors containing sequences encoding either the GAL4 activating domain (AD, left column) or the GAL4 DNA binding domain (BD, middle column). Yeast colonies expressing the N-terminal 298 amino acids of wild type Mira and Insc turned blue in the X-gal test, indicating that these two proteins interact. In contrast, in the case of the N-terminal 298 amino acids of Mira^{E326}, the colonies remained white, indicating that these two proteins do not interact. When bait and prey were exchanged, no interaction was detected. B: The N-terminal 298 amino acids of both wild type Mira and Mira^{E326} interacted with the C-terminal region of Jar (third and fourth columns from the top). No interaction was detected with the N-terminal region of Jar. These results suggest that Mira binds to the C-terminal part of Jar, and that this interaction is not affected by the mutations in Mira^{E326}. When bait and prey were exchanged no interaction was detected. C: Scheme of the "Jar N-term" and the "Jar C-term" constructs. The N-terminal region contains the motor domain. The C-terminal region contains the globular domain that interacts with cargo.

3.8.1.2 Miranda and Myosin VI

In interphase and early prophase, Mira is localized at the apical cortex in wild type NBs. In late prophase, Mira translocates to the basal cortex. Jar is supposed to be involved in Mira transport from the apical to the basal cortex (Petritsch et al., 2003).

jar encodes the unconventional Myosin VI, which moves towards the minus ends of actin filaments. Petritsch et al. reported that Jar co-immunoprecipitated with Mira. The N-terminal part of Mira directly bound to Jar in a GST-pull-down assay. In *jar* mutant NBs, Mira was distributed into the cytoplasm, instead of being localized at the basal cortex. From these data, the authors proposed that Mira might be a cargo for the motor protein Jar (Petritsch et al., 2003). My speculation was that the mutant Mira in *mira^{E326}* was not localized at the basal cortex because it may not be able to bind to Jar, which is required to transport Mira to the basal cortex.

Three different constructs comprising full-length Jar and the N-terminal and C-terminal regions of Jar, were used for yeast two-hybrid assays. Jar has its motor domain in the N-terminal part, while the globular domain, which associates with the cargo, is in the C-terminal part. Although the N-terminal region of Jar showed no interaction with Mira, the C-terminal part of Jar interacted with both the wild type and mutant Mira proteins. In both cases, the colonies turned blue (figure 3.15B). These data indicated that the mutations in the N-terminal part of Mira^{E326} did not influence the ability of Mira to bind

Jar.

I could not detect an interaction of Mira with either Insc or Jar, when Mira proteins fused with the GAL4-binding domain were expressed in yeast cells. One reason for this could be that these Mira proteins were not expressed or folded correctly in the yeast cells. Another possibility could be that the proteins could not interact because of the abnormal topology of the fusion proteins.

3.8.2 GST pull-down assay

Next I tried a GST pull-down assay to confirm the results from the yeast two-hybrid assays. I prepared biotin labeled-Jar and Insc by an *in vitro* transcription and translation reaction. I also prepared GST-fusion proteins for the N-terminal regions of the mutant and wild type Mira proteins, respectively. In the pull-down assays, biotin labeled Jar and Insc were each incubated with GST-Mira fusion proteins coupled with glutathione-Sepharose beads. If Jar or Insc interacted with GST-Mira, it should be precipitated with the beads, and the biotin-labeled protein should be detected in the following Western blot analysis. Although the GST-fusion Mira was expressed successfully (figure 3.16), I did not get a clear result in the pull-down assay.

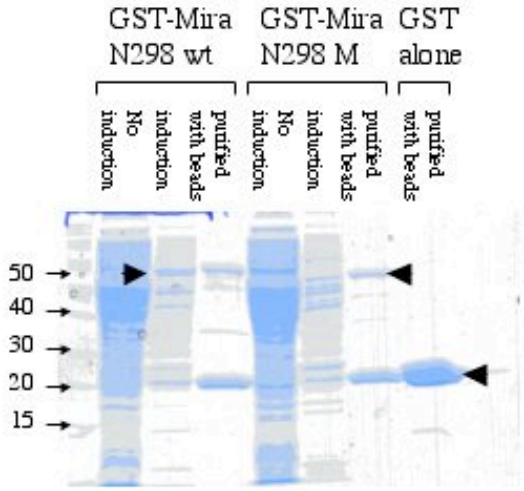


Figure 3.16. GST fusion protein expression

Coomassie Brilliant Blue-stained SDS-PAGE gel to detect GST-fusion proteins. The N-terminal 298

amino acids of wild type Mira and Mira^{E326} and the GST part alone are shown. Both fusion proteins have the sizes expected from the primary sequence.

Since Insc was not labeled efficiently with biotin, I had to use the anti-Insc antibody to detect Insc in the pull-down assays. However, the anti-Insc antibody was not suitable for this assay, since it was raised against an Insc GST-fusion protein. The antibody not only recognized Insc but also GST alone and the Mira GST-fusion protein, resulting in too many extra bands in the Western blot analysis.

The problem I had in the GST pull-down assays between Mira and Jar was that Jar bound non-specifically to the beads, and this problem has not been solved.

4 Discussion

4.1 Maternal screen

In the maternal screen, I isolated mutant alleles of *lgl* and *scrib*. Both genes were already known to be involved in the asymmetric cell division of NBs. This indicates that the screen strategy was appropriate for my purpose.

However, novel genes involved in the asymmetric division of NBs were not identified. There were three disadvantages in my screen. Firstly, not all of the mutants that affect asymmetric cell division are detectable with mAb22C10 staining, although I confirmed that most of the mutants affecting the asymmetric cell division of NBs have an abnormal mAb22C10 staining pattern. Since mAb22C10 labels subsets of CNS neurons, the mAb22C10 signal remains normal when only 22C10 negative cell types are affected. One such example was the *insc* mutant. Although asymmetric cell division of NBs does not take place correctly in *insc* mutants, the mAb22C10 staining pattern appeared normal. Secondly, the screen was not saturated. For the maternal screen, each P-element insertion line had to be recombined onto an FRT chromosome. This recombination is quite time-consuming work. In addition, the collection of P-element insertion lines we obtained from Anne Ephrussi's lab was not saturated. One possible solution would have been to do an EMS-mutagenesis on FRT chromosomes. In this strategy, recombination is not required, which facilitates saturated mutagenesis. Thirdly, the penetrance of the mutant phenotype was low in practice, although, theoretically, germline clones should be generated with 100% efficiency. There might have been some problems with the chromosome carrying the dominant female sterile (DFS) mutation.

4.2 Zygotic screen

The strategy of this screen was essentially similar to that of the maternal screen. This screen was also performed in two steps, and the same antibodies were used for the staining. One difference was that only zygotic phenotypes can be detected in this screen, meaning that if the zygotic phenotype is masked by a maternally provided gene product, the phenotype cannot be detected. Another difference was that the original screen, which was done by Hummel et al., was saturated, whereas my maternal screen was not saturated.

In the screen, several classes of defects, concerning the subcellular localization of the basal marker Mira, were observed. Firstly, mislocalization of Mira was classified into

two classes. One was that the cortical localization of Mira in NBs was disrupted, and the other was that Mira was associated with the cortex, although it was not localized at the basal side. *E326* was the only line that belonged to the former class. The latter class was subdivided further into two categories. One was the orientation of Mira crescent was abnormal, and the other was Mira is uniformly localized at the cortex. However, the lines that belonged to this class need closer observation. Additionally, in some lines, NBs with abnormal cell morphology were observed.

4.3 *E326* is allelic to mutations in *miranda*

I selected the mutant line *E326*, in which the basal marker Mira is uniformly distributed in the cytoplasm, for further analysis. When *E326* was crossed with *mira* alleles, it did not complement the lethality of the *mira* alleles, indicating that *E326* is allelic to *mira*. By sequencing the *mira* locus in the $mira^{E326}$ genome, an in-frame deletion that corresponds to 39 amino acid residues and a point mutation were found in the N-terminal part of *mira*. This result suggests that the C-terminal part of mutant Mira is intact, which is consistent with the fact that mutant Mira in $mira^{E326}$ was detected by the antibody that was raised against the C-terminal peptide of Mira.

The "multicoil" coiled-coil domain structures prediction program predicted that amino acids 140-680 (aa 140-680) form a coiled-coil structure. According to the domain structure, Mira is divided into three regions: an N-terminal part, a central coiled-coil domain and a C-terminal part. The deletion in $Mira^{E326}$ was within the N-terminal region that resides outside of the coiled-coil domain.

Mira is an adaptor protein for Pros, which functions as a cell fate determinant in GMCs. Mira tethers Pros at the basal cortex of the NBs, and they segregate exclusively into the GMCs after cell division (Ikeshima-Kataoka et al., 1997; Schuldt et al., 1998; Shen et al., 1997). In the GMC, Pros, which is released from Mira, translocates into the nucleus, where it activates GMC-specific gene expression (Doe et al., 1991) and inhibits NB-specific gene expression (Vaessin et al., 1991). Thus, the asymmetric localization of Mira in NBs is critical for the cell fate determination of the daughter cells.

Mira was localized at the basal cortex in wild type NBs, whereas the mutant Mira was distributed in the cytoplasm in the $mira^{E326}$ homozygous embryos. In NBs in $mira^{E326}$ heterozygotes ($mira^{E326}$/*TM3*), Mira was distributed in the cytoplasm as well as at the basal cortex, indicating that the Mira immunofluorescence signal in the cytoplasm

corresponds to the mutant Mira, whereas the signal at the cortex corresponds to the wild type Mira localization.

In my further analysis of *mira*^{E326}, I studied two main topics, [1] the effect of the *mira*^{E326} mutation on the development of the embryonic CNS, especially focusing on its effect on Pros localization and function, and [2] the mechanism that controls the subcellular localization of Mira. These two topics are discussed in chapters 4.4 and 4.5, respectively.

4.4 Effect of the *miranda*^{E326} mutation on neural development

4.4.1 Subcellular localization of the cell fate determinant Prospero in neuroblasts

Six mutant *mira* alleles were previously isolated in the Matsuzaki lab, and sequence analyses revealed that all six alleles produce C-terminally truncated mutant Mira proteins (Matsuzaki et al., 1998). The subcellular localization of the mutant Mira and Pros proteins in these mutant *mira* alleles was also investigated. This study identified the three functional domains of Mira involved in [1] the basal localization of Mira, [2] the association with Pros and [3] the regulation of Pros dissociation in GMCs (Matsuzaki et al., 1998).

4.4.1.1 Association with Prospero and Staufen

Matsuzaki et al. showed that Pros binds to the C-terminal part of Mira. In mutant *mira* alleles producing C-terminally truncated Mira proteins longer than 290 residues and shorter than 446 residues, although the mutant Mira localization is normal, Pros is mislocalized in the cytoplasm, indicating that the Pros binding domain is missing in these mutant *mira* alleles (Matsuzaki et al., 1998). According to these data, the mutant Mira produced in *mira*^{E326}, in which the region responsible for Pros binding is intact, should bind to Pros. I showed that Pros had a similar distribution to that of the mutant Mira in *mira*^{E326} mutant NBs; both proteins were distributed into the cytoplasm. From these data, it is likely that the mutant Mira binds to Pros in *mira*^{E326}, which results in the mislocalization of Pros. Due to this unique characteristic, the mutant Mira in *mira*^{E326} seems to have a dominant negative effect on Pros localization (See 4.4.2). However, the immunostaining data could not distinguish between whether Mira and Pros are both mislocalized because they form a complex or Mira and Pros are separately localized.

The binding between the mutant Mira in *mira*^{E326} and Pros has not been confirmed yet. It can be determined by a co-immunoprecipitation analysis.

It is also known that amino acids 290-446 of Mira, which correspond to the region required for Pros binding, are sufficient for the association with Stau. Stau is a double-strand RNA binding protein that shows a similar localization pattern as Mira and Pros in NBs. Stau is responsible for the basal localization of *pros* mRNA in NBs and for its segregation into GMCs. It is thought that the asymmetric inheritance of *pros* mRNA is a back-up system for the unequal segregation of Pros into the GMCs (Broadus and Doe, 1997). Although I did not examine the localization of either Stau or *pros* mRNA, Stau is expected to behave similar to Pros in NBs and GMCs in *mira*^{E326}; it should be distributed in the cytoplasm due to its association with the mutant Mira. However, it is unlikely that this mislocalization of Stau/*pros* mRNA would influence the cell fate determination between the two daughter cells since it is known that the loss of function of *stau* has no effect on the asymmetric distribution of cell fate determinants (Broadus and Doe, 1997).

4.4.1.2 Dissociation from Prospero

It has been proposed that the C-terminal 100 amino acid residues are responsible for the dissociation of Pros from Mira in the GMC. Pros is released from Mira, which degrades after it segregates into the GMC in the wild type. In contrast, the mutant Mira remains at the GMC cortex in *mira*^{RR127}, which produces a truncated Mira protein lacking the C-terminal 100 amino acids. Consequently, the translocation of Pros into the nucleus of the GMC is delayed, although the Pros localization is normal in NBs, unlike the other alleles. Since there are consensus sequences for PKC phosphorylation in the C-terminal part of Mira, PKC-dependent phosphorylation might regulate the dissociation of Mira and Pros (Matsuzaki et al., 1998).

It is not clear whether the Pros/mutant Mira complex dissociates after it segregates into the GMC in *mira*^{E326}. A sequence analysis revealed that the C-terminal part of the mutant Mira was intact, suggesting that the mutant Mira in *mira*^{E326} is potentially able to release Pros in the GMC. It should be examined if Mira is able to receive the signal to release Pros when Mira is not localized correctly; cortical localization might be required for Mira function to release Pros.

It is not clear if Pros enters the nucleus in NBs and GMCs in *mira*^{E326}, although I tried to

investigate it. It is known that Pros enters the nuclei of both NBs and GMCs in *mira* alleles in which the mutant Mira did not bind to Pros although the mutant Mira was normally localized (Matsuzaki et al., 1998). It will be quite important to determine whether Pros enters the daughter cell nuclei in $mira^{E326}$.

4.4.2 Genetic interaction between *miranda*^{E326} and *prospero*

$mira^{E326}$ showed genetic interactions with amorphic and hypomorphic *pros* alleles. I sobtained two lines of evidence for the genetic interaction between $mira^{E326}$ and *pros*. Firstly, animals transheterozygous for $mira^{E326}$ and strong *pros* alleles died in the pupal stage, whereas $mira^{E326}$/weak *pros* heterozygous animals were viable. Secondly, the neural cell fate was not determined correctly in transheterozygotes for $mira^{E326}$ and a strong *pros* allele.

The mutant Mira in $mira^{E326}$ has a unique characteristic: although it binds to Pros, the mutant Mira is not cortically localized, which results in mislocalization of the mutant Mira/Pros complex. Due to this characteristic, the mutant Mira seems to have a dominant negative effect on the subcellular localization of Pros in NBs.

I propose a model that might explain the genetic interaction (figure 4.1). The speculation is that there is a threshold of the amount of Pros that is cortically localized in the NB and segregates into the GMC. It should be noted that in my model, the Pros that binds to the mutant Mira in the cytoplasm and segregates into the GMC accidentally, is not taken into account. If the amount of Pros is more than the threshold, it would be sufficient for viability, and less Pros than the threshold amount would lead to lethality. In a transheterozygote for $mira^{E326}$ and a strong *pros* allele, half of the amount of Pros is expressed in the NB and the mutant Mira titrates half of the amount of Pros expressed in the NB. As a consequence, one quarter of the Pros segregates into the GMC, which results in lethality. In contrast, in a transheterozygote for $mira^{E326}$ and a weak *pros* allele, more Pros than that in the transheterozygote for $mira^{E326}$ and strong *pros* allele segregates into the GMC. More than one-quarter of the Pros segregates into the GMC, which leads to viability. Thus, the threshold must be between one-quarter of the amount of Pros in the wild type and the amount in a $mira^{E326}$/weak *pros* heterozygote.

A

Genotype	Prospero in GMC	viability	
WT	1	viable	(a)
miraE326 hetero	1/2	viable	(b)
pros+ miraE326/ pros10419 mira+	>1/4	viable	(e)
pros+ miraE326/ pros17 mira+	1/4	lethal	(d)
miraE326 homo	0	lethal	(c)

B

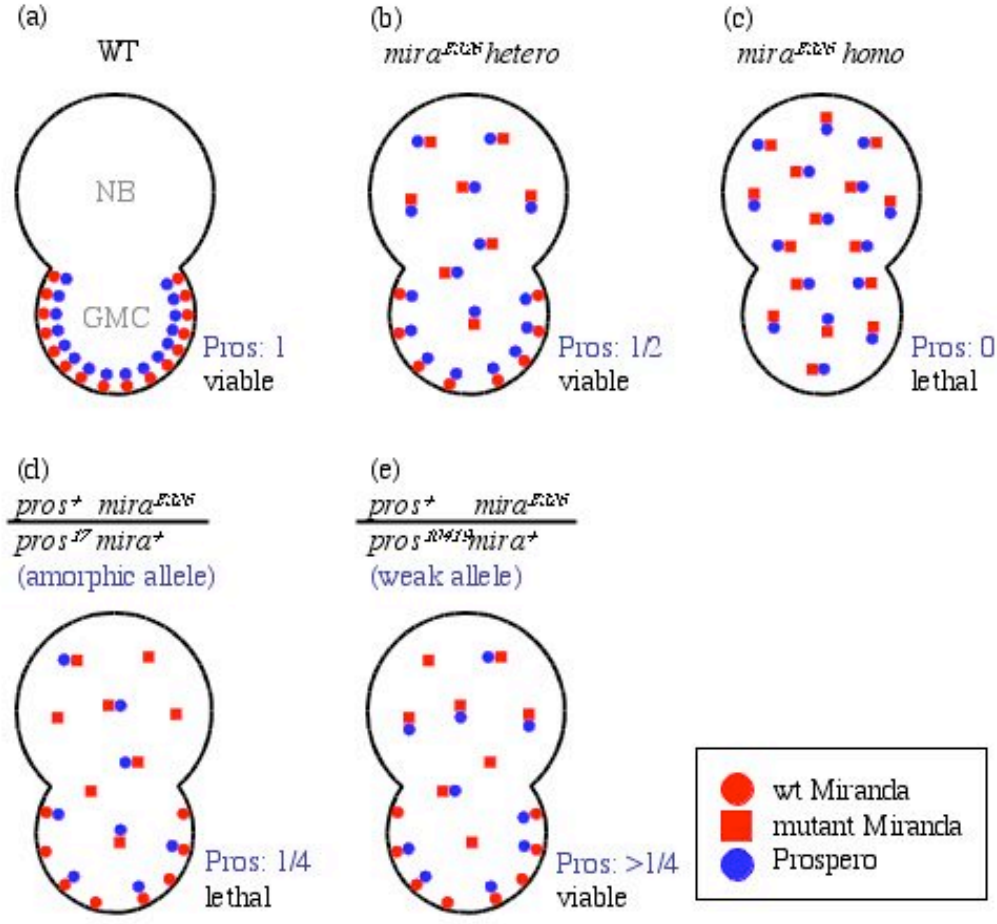


Figure 4.1. Genetic interaction between *miranda*^{E326} and *prospero*

The "threshold model" to explain the genetic interaction between *mira*^{E326} and *pros*. A: My speculation is

that there is a threshold for the amount of Pros segregated into GMCs that is required for viability and normal neural development. More Pros than the threshold amount segregates into GMCs, which leads to viability (blue column), and less Pros than the threshold amount leads to lethality (pink column). The threshold is between the pink and blue columns. The first column from the left indicates the genotype, and the second column indicates the amount of Pros segregated into GMCs. B: Schematic drawing of the subcellular localization of wild type Mira protein (red circles), mutant Miranda (red squares) and Prospero (blue circles). This model fits not only for the *mira*^{E326} and *pros* transheterozygotes, but also for the *mira*^{E326} homozygote and heterozygote. (a) Wild type NB. (b) In the *mira*^{E326} heterozygote, because half of the Pros is titrated out by binding to the mutant Mira mislocalized in the cytoplasm, only half of the Pros is correctly segregated into GMCs. However, half of the amount of Pros is sufficient for viability. (c) A *mira*^{E326} homozygote is lethal because it has no Pros in the GMCs. (d) In the transheterozygote for *mira*^{E326} and the *pros* amorphic allele, a half of the amount of Pros is expressed in the NBs since there is only one copy of Pros. Half of the Pros is titrated out by binding with mutant Mira in the cytoplasm. Only a quarter segregates correctly into GMCs, which is insufficient for viability. (e) In contrast, in the transheterozygote for *mira*^{E326} and a weak *pros* allele, more Pros than the *mira*^{E326}/amorphic *pros* allele is expressed in the NBs. More than a quarter of the Pros segregates into the GMCs, which is sufficient for viability.

The situations of the *mira*^{E326} heterozygotes and homozygotes also fit this model. In the NBs of the *mira*^{E326} homozygote, no Pros is correctly segregated into the GMC, because all of the Pros is titrated out due to binding to the mutant Mira protein, which results in lethality. In *mira*^{E326} heterozygotes, half of the amount of Pros segregates into the GMC, although half of the amount is trapped by the mutant Mira protein in the cytoplasm. Consistent with this, *mira*^{E326} heterozygotes are viable because half of the amount is above the threshold.

4.4.3 Neural cell fate determination in a transheterozygote for *miranda*^{E326} and *prospero*

The genetic interaction results prompted me to analyze the effect of mutant Mira mislocalization in *mira*^{E326} NBs on neural cell fate determination in the CNS. I investigated Even-skipped (Eve) expression, which is commonly used as a neural fate marker. Eve is expressed in four clusters of neurons: aCC/pCC, RP2, the U/CQ neurons and the EL neuron clusters (Patel et al., 1989).

All of the aCC/pCC and RP2 neurons failed to express Eve in *pros* loss of function alleles. In contrast, in *mira* alleles in which the mutant Mira protein does not bind to Pros, the number of aCC/pCC and RP2 neurons that expressed Eve decreaseed to 50% (Ikeshima-Kataoka et al., 1997). In $mira^{E326}$, no aCC/pCC and RP2 neurons expressed Eve, which was more similar to the situation with *pros* mutants than other *mira* alleles. On the other hand, it has also been reported that Eve expression in EL neurons was not affected in *pros* mutants and $mira^{RR127}$, whereas in other *mira* alleles, the number of EL neurons expressing Eve was reduced to 50% (Ikeshima-Kataoka et al., 1997). Eve expression in EL neurons was not affected in $mira^{E326}$, which was similar to the *pros* mutants as well. These data suggest that the requirement for Pros function in cell fate determination is higher in aCC/pCC and RP2 neurons than in the EL neuron cluster. The reason why Eve expression was lost in the aCC/pCC and RP2 neurons in $mira^{E326}$ might be that Pros does not efficiently translocate into the nucleus in the GMC, which leads to a failure of cell fate determination. The efficiency of Pros entry into the nucleus in $mira^{E326}$ should be examined.

In transheterozygotes for $mira^{E326}$ and a strong *pros* allele, the number of Eve positive RP2 cells was reduced to 50%, although other neuron clusters were not affected. This points to a genetic interaction between $mira^{E326}$ and strong *pros* alleles. It is known that RP2 is a progeny of NB4-2, and this cell lineage has been well characterized (Bossing et al., 1996; Schmid et al., 1999). It would be interesting to trace the neural cell fate determination of the NB4-2 cell lineage in $mira^{E326}$ mutant embryos.

4.4.4 Involvement of Brain Tumor in cell fate determination

When I was preparing this manuscript, two new papers were published describing that tumor suppressor protein Brain Tumor (Brat) requires Mira as an adaptor protein for its asymmetric segregation into the GMCs and functions as a cell fate determinant in GMCs (Betschinger et al., 2006; Lee et al., 2006). Brat and Pros function redundantly to specify cell fate in the embryonic CNS.

Betschinger et al. identified Brat as an interaction partner of Mira. And Lee et al. isolated a *brat* mutant allele in a genetic screen for mutations that affect the number of NBs in the larval brain. In the central brain of loss of *brat* function mutant, the cells expressing NB-markers are increased, whereas the cells expressing GMC-markers are reduced, suggesting that the GMCs are transformed into the mitotic NBs, which results

in overproliferation of NBs.

Brat binds to the cargo-binding domain of Mira, where Pros and Stau also interact (Betschinger et al., 2006; Lee et al., 2006). From this data, it is expected that the mutant Mira in *mira*^{E326} has the same effect on the subcellular localization of Brat in NBs as that of Pros: although Brat binds to the mutant Mira in *mira*^{E326}, the Brat/mutant Mira complex fails to be localized at the basal cortex. It is important to examine the effect of mutant Mira in *mira*^{E326} on function of Brat in specifying cell fate.

4.5 Subcellular localization and functional domains of Miranda

Mira localization dynamically changes during mitosis. Wild type Mira is localized at the apical cortex in interphase and early prophase. Subsequently, it translocates to the basal cortex and forms a basal crescent in metaphase. After cytokinesis, it segregates preferentially into one of the two daughter cells, the GMC.

4.5.1 Comparison of the subcellular localization of Miranda^{E326} with those of other *miranda* alleles

4.5.1.1 Apical localization of Miranda in interphase NBs

Not only the basal localization in metaphase but also the apical localization of mutant Mira was defective in *mira*^{E326}. In wild type NBs, Mira is localized at the apical cortex during interphase and then translocates to the basal cortex, whereas Mira^{E326} was never localized at the cortex throughout mitosis in *mira*^{E326}.

There were conflicting reports on the apical localization of Mira. Fuerstenberg et al. proposed that a central region of Mira (aa 446-727) is necessary for its apical localization, since a mutant Mira that lacks this region fails to be localized at the apical cortex in interphase (Fuerstenberg et al., 1998). On the other hand, Shen et al. proposed that when the N-terminal 298 amino acids are expressed in NBs, this form of Mira was localized at the apical cortex, suggesting that this region is sufficient for apical localization (Shen et al., 1998).

My results showed that the N-terminal region of Mira is required for cortical localization. When this region is missing, Mira cannot be localized at either the apical or basal cortex.

4.5.1.2 Basal localization of Miranda in metaphase NBs

Mira has two independent functions in the asymmetric segregation of the cell fate determinant Pros, and distinct domains are thought to be involved in these two processes. One is to tether Pros at the basal cortex in the NB, so that Pros is segregated exclusively into the GMC after cytokinesis. The other function is to release Pros from the cortex in the GMC so that Pros can translocate into the nucleus (Matsuzaki et al., 1998).

It has been proposed that the N-terminal 290 amino acid residues are sufficient for the basal localization of Mira, since all of the C-terminally truncated proteins longer than 290 amino acid residues were normally localized at the basal cortex in metaphase NBs (Matsuzaki et al., 1998). The mutant Mira produced in *mira*^{E326} had mutations in the N-terminal part, within the region responsible for basal localization, and it failed to be localized at the basal cortex. This result was consistent with the proposal from Matsuzaki et al. (Matsuzaki et al., 1998).

Since Mira has been used as a polarity marker of NBs, Mira localization has been investigated in many polarity mutants. Two states of mislocalization have been observed so far. One is localization at the whole cortex and the other is distribution into the cytoplasm. Mira is localized at the whole cortex in loss of function mutants of apically localized proteins, such as *DaPKC*. Such localization was also reported in NBs overexpressing a non-phosphorylatable form of *lgl* (Betschinger et al., 2003). On the other hand, Mira is distributed in the cytoplasm in *lgl* or *jar* mutant NBs (Ohshiro et al., 2000; Peng et al., 2000; Petritsch et al., 2003). From these data, I propose that the mechanism that localizes Mira at the basal cortex is divided into two steps. Firstly, Mira associates with the cell cortex, which is a prerequisite for the polarized localization of Mira. Secondly, Mira localization is restricted to the basal region of the cortex. The first mechanism is defective in *mira*^{E326}. In other words, when the mechanism for cortical localization is defective, Mira cannot be asymmetrically localized.

4.5.1.3 Basal localization of Miranda in epithelial cells

In addition to NBs, Mira is expressed in epithelial cells prior to neurogenesis (Matsuzaki et al., 1998). Mira was localized at the basolateral cortex of epithelial cells in the wild type, whereas Mira^{E326} failed to be localized at the cortex. Instead, it was distributed in the cytoplasm in *mira*^{E326}. Studies by Matsuzaki et al. revealed that

truncated Mira proteins longer than 290 amino acids were normally localized at the basal cortex, suggesting that aa 1-290 are sufficient for basal localization in epithelial cells as well (Matsuzaki et al., 1998). The mislocalization of Mira in *mira*^{E326} epithelia was consistent with this previous proposal.

It is likely that the cortical localization of Mira is directed by a common mechanism in epithelial cells and NBs. At least, the same region in Mira is responsible for the cortical localization of Mira in both NBs and epithelial cells.

4.5.1.4 Telophase rescue

In many mutants, even if Mira is mislocalized in metaphase NBs, its localization becomes normal in telophase. This phenomenon is referred to as "telophase rescue" (Cai et al., 2001; Peng et al., 2000). However, the mechanism by which telophase rescue occurs is unknown.

The observation that telophase rescue did not take place in *mira*^{E326} shed some light on the mechanism of telophase rescue. It might occur only when Mira is tethered to the cortex. Alternatively, a common component may be required for the basal localization of Mira and for telophase rescue. The third possibility is that although the components involved in basal localization and telophase rescue are independent, both mechanisms are defective because of the mutations in the N-terminal region.

4.5.2 Proteins controlling the cortical localization of Miranda

Since *mira*^{E326} is the only allele in which the mutant Mira is not cortically localized, it is a good tool to look for a protein with which the wild type Mira interacts and the mutant Mira in *mira*^{E326} does not, which would help to uncover the mechanism that anchors Mira to the cortex.

4.5.2.1 Involvement of Inscuteable

It has been shown that amino acids 1-298 bind to Insc in a GST pull-down assay (Shen et al., 1998). While Mira was colocalized with Insc at the apical cortex in wild type NBs at interphase, Mira^{E326} was distributed in the cytoplasm. The reason why Mira was not localized at the apical cortex might be that Mira^{E326} cannot bind to Insc. Therefore, I examined the interaction between Mira^{E326} and Insc.

I showed by yeast two-hybrid assay that wild type Mira interacted with Insc, whereas

the mutant Mira in *mira*^{E326} (Mira^{E326}) did not. This result suggests that Mira^{E326} cannot be localized at the apical cortex because Mira^{E326} does not bind to Insc.

However, it is not clear if Insc is involved in the cortical localization of Mira in NBs. Firstly, it is likely that a protein expressed in both epithelial cells and NBs is involved in anchoring Mira to the cortex, since Mira was already mislocalized in epithelial cells prior to the delamination of NBs from the epithelial layer in *mira*^{E326}. However, Insc is not expressed in epithelial cells. Secondly, the Mira subcellular localization in *insc* mutants is different from that in *mira*^{E326}. While the mutant Mira failed to associate with the cortex in *mira*^{E326}, Mira is localized at the whole cortex in *insc* mutants, suggesting that Mira in *insc* loss of function mutants is able to associate with the cortex. It is unlikely that Insc is the key component for the cortical localization of Mira. Additional components are probably involved in anchoring Mira to the cortex, and their binding to Mira may be defective in *mira*^{E326}.

4.5.2.2 Involvement of MyosinVI: Jaguar

It was previously reported that Jar co-immunoprecipitated with Mira from embryo lysates, and that it binds to the N-terminal 298 amino acid residues of Mira in a GST-pull down assay (Petritsch et al., 2003). My working hypothesis was that Mira is mislocalized, because the mutant Mira in *mira*^{E326} fails to interact with the motor protein Jar, which may transport Mira from the apical to the basal cortex (figure 4.2). The results of my yeast two-hybrid assay suggested that this was not the case. I showed that the N-terminal part of wild type Mira was able to directly interact with the C-terminal part of Jar in a yeast two-hybrid assay. However, in contradiction to my speculation, the yeast two-hybrid assay revealed that the mutant Mira in *mira*^{E326} interacted with the C-terminal part of Jar as well. This result suggests that the mutant Mira did not lose its ability to bind Jar.

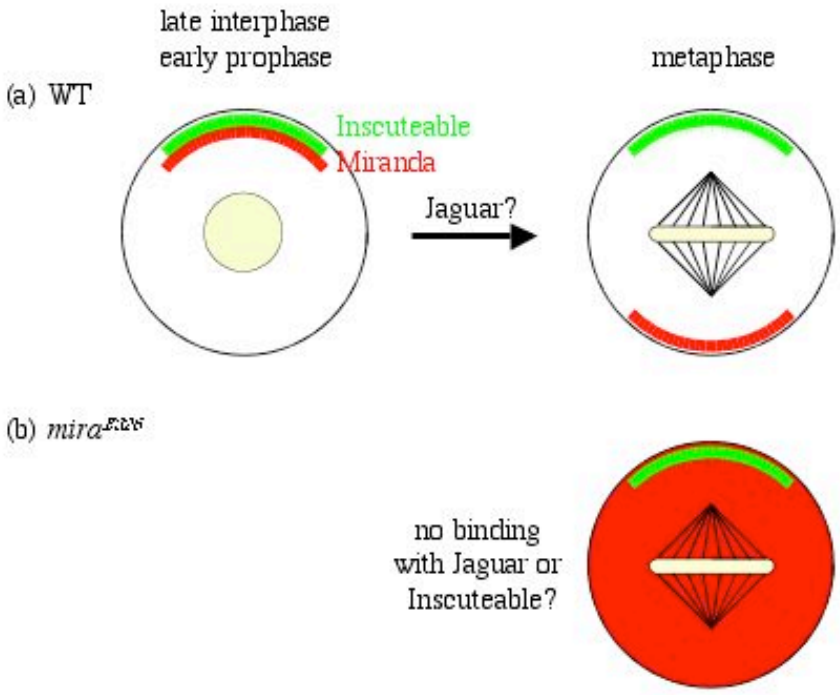


Figure 4.2. Working hypothesis - Why is the mutant Miranda mislocalized in *miranda*^{E326} allele? -

(a) In the wild type NB, Mira (red) is colocalized with Insc (green) at the apical cortex in interphase and translocates to the basal cortex in metaphase. Jar (Myosin VI) is thought to be involved in transporting Mira to the basal cortex. (b) Working hypothesis: the reason why the mutant Mira in *mira*^{E326} fails to be localized at the basal cortex might be that it does not bind to Insc or Jar.

However, I have to investigate whether the mutant Mira interacts with Jar *in vivo* by co-immunoprecipitation, since the possibility that the mutant Mira fails to interact with Jar *in vivo* cannot be excluded. Shen et al. proposed that in interphase, Insc might function as a scaffold where Mira is loaded onto a motor protein that transports Mira from the apical cortex to the basal cortex (Shen et al., 1998). Since the mutant Mira in *mira*^{E326} was not able to bind to Insc, it is possible that the mutant Mira is not loaded onto Jar, even if the mutant Mira is capable of binding to Jar.

The result that Jar binding was not defective in Mira^{E326} is reasonable, since the speculated function of Jar is to transport Mira from the apical cortex to the basal cortex

along the actin filaments: that is, Jar might function in transporting cell fate determinants, rather than tethering determinants to the cortex. It is unlikely that Jar is involved in tethering Mira to the cortex, since the turnover rate of Jar is high, and thus it would not be able to fix Mira at the basal cortex.

The results of the yeast two-hybrid assay also suggested that Mira interacts with the C-terminal part of Jar, not the N-terminal part. This is reasonable because the motor domain is located in the N-terminal region, while the cargo-binding domain is in the C-terminal part of Jar. Additionally, whether the Mira/Pros complex is transported in vesicles or directly associates with motor proteins remains to be determined.

When the "prey" and "bait" were exchanged in the yeast two-hybrid assay, no consistent results were obtained. When constructs in which the N-terminal part of Mira (MiraN298^{wt}, MiraN298^{E326}) was fused with the GAL4 DNA-binding domain were used for the assay, no interaction was observed with either Jar or Insc. One possible reason could be that these proteins were not expressed in yeast cells. This should be examined by a Western blot analysis. Another possibility would be that although these proteins were expressed and Mira and its interaction partner (Jar or Insc) interacted, GAL4AD and GAL4BD were not able to interact with each other, because of the conformation of the fusion proteins.

4.5.2.3 Other possible candidates responsible for the cortical localization of Miranda

One of the candidates that might be involved in the cortical localization of Mira is Lgl. Lgl seems to be involved in the docking of vesicles at the plasma membrane (Larsson et al., 1998; Lehman et al., 1999). It is possible that Lgl allows the docking of vesicles containing cell fate determinants to the basal cortex.

5 Summary

I performed two genetic screens to search for novel genes involved in the asymmetric cell division of *Drosophila* NBs. In the second screen, I isolated a novel mutant *mira* allele named *mira*^{E326}. Since Mira is an adaptor protein for Pros, which exclusively segregates into one of the daughter cells (GMC) and functions as neural cell fate determinant, Mira is essential for the basal localization of Pros.

The mutant Mira protein produced in *mira*^{E326} had an in-frame deletion and a point mutation in the N-terminal region. Since all of the mutant *mira* alleles that have been isolated so far produce C-terminally truncated Mira proteins, Mira^{E326} has unique characteristics. Consistent with previous studies proposing that the N-terminal region of the Mira protein is responsible for the basal localization, Mira^{E326} failed to be localized at the basal cortex. Instead it was distributed in the cytoplasm. However, Mira^{E326} appeared to bind to Pros, since the C-terminal region of Mira^{E326}, which is responsible for binding Pros, was intact. Consequently, the Mira^{E326} protein recruits Pros into the cytoplasm in *mira*^{E326} mutant NBs, which leads to mislocalization of the cell fate determinant Pros in the cytoplasm.

Taking advantage of the unique behavior of the Mira^{E326} protein, I examined the effect of Pros mislocalization in *mira*^{E326} mutant NBs on animal development and CNS development. Firstly, transheterozygous flies for *mira*^{E326} and the amorphic *pros* allele do not survive to adulthood. A possible explanation is that because the amount of Pros segregated into GMCs in the transheterozygote for *mira*^{E326} and amorphic *pros* allele is one-quarter, in comparison with that in the wild type, and this amount is insufficient for viability. Secondly, I examined cell fate determination in the CNS in transheterozygous flies for *mira*^{E326} and the amorphic *pros* allele. Fifty percent of the RP2 neurons did not express Eve, suggesting that RP2 neurons failed in cell fate determination, because only a quarter of the Pros is correctly segregated into the GMC.

I also examined the protein-protein interactions between Mira^{E326} and its interaction partners Insc and Jar, to investigate which protein is responsible for the cortical localization of Mira. The results of the yeast two-hybrid assay revealed that the N-terminal part of Mira^{E326} lost its binding ability with Insc, whereas it retained its interaction with Jar.

Recently, it has been shown that the neurogenesis of the vertebrate nervous system is essentially similar to that of the *Drosophila* CNS, in which progenitor cells undergo

asymmetric cell division to produce divergent cell types. Although a Mira homologue has not been found so far in other organisms, it is possible that a component functionally homologous to Mira is involved in asymmetric cell division in vertebrates. It will be interesting to look for such a molecule and to investigate its function in this process.

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7 Supplementary data

7.1 Results of the maternal screen

No.	Arm	penetrance (%)	CNS		PNS	general defects	variable	Comments
				axon	disorganised				
000	**2L**	100	x	x	x	x		x	same defects were observed on both arms. variable
000	**2R**	2 out of 12	x	x	x	x			
0002	**2R**	50	x	x	x	x		x	
36	**2R**	10	x	x	x	x	x		
127	**2R**	10	x	x	x	x	x	x	
422	**2R**	10	x	x	x	x		x	
476	**2R**	10	x	x	x	x	x	x	
881	**2L**	10	x	x	x	x			
893	**2L**	1 out of 20				x			increased cell number, abnormal pattern
1026	**2L**	10	x	x	x				
1239	**2L**	10	x	x	x	x			
1314	**2R**	10	x	x	x	x	x	x	
1498	**2L**	100	x	x		x	x		pattern formation defects
1588	**2L**	more than 50	x	x		x			more than two classes of phenotypes, increased cell number in the CNS
1624	**2R**	100					x		disintegrated embryos
1738	**2L**	10	x	x	x	x			segmentation, abnormal cell shape
1904	**2L**	100					x		disintegrated embryos
3491	**2R**	2 embryos	x	x		x	x		abnormal cell position, disintegrated embryos
3781	**2R**	10					x		only one embryo has CNS specific defects. Also disintegrated embryos
4325	**2L**	more than 50	x		x	x	x		variable, many disintegrated
4325	**2R**	50					x		disintegrated
5236	**2R**	10	x	x	x	x	x	x	variable, segmentation, abnormal cell position
6105	**2R**	10	x		x	x	x		disorganized nervous system
6168	**2R**	10	x	x	x	x	x		
6224	**2R**	10	x	x	x	x	x		segmentation, cells missing
6301	**2R**	10	x			x			
6380	**2L**	2 embryos	x		x	x		x	only two embryos showed phenotypes.
6730	**2R**	2 out of 30	x		x	x	x		only a few embryos showed phenotypes.
6749	**2R**	10	x		x		x		
6752	**2R**	10	x	x	x	x	x		segmentation

No.	Arm	penetrance (%)	CNS			PNS	general defects	variable	Comments
				axon	disorganised				
14728	**2L**	10	x	x	x	x		x	
15047	**2L**	10	x			x	x		segmentation
15048	**2R**	10	x		x	x	x		
15055	**2R**	10	x	x	x	x			
15186	**2R**	50	x	x	x	x			
15342	**2R**	10	x			x		x	
15384	**2R**	10	x	x	x	x	x		
16630	**2R**	10					x	x	disintegrated embryos
16669	**2R**	10	x		x	x		x	variable
16744	**2R**	10	x	x	x	x			variable
16793	**2R**	10	x	x	x	x	x		
16797	**2R**	10	x			x		x	variable
17050	**2R**	50	x	x		x	x		
17052	**2R**	10				x		x	PNS defects
17068	**2R**	10	x	x	x	x		x	variable
17137	**2R**	10	x		x	x		x	variable. many segmentation defects.
17459	**2R**	10	x		x	x			variable
17641	**2R**	10	x			x	x	x	variable
17753	**2R**	50	x	x	x	x	x		nervous system specific phenotype, increased cell number
18126	**2R**	10	x		x	x			
18483	**2L**	some					x	x	disintegrated embryos
18709	**2R**	10	x		x	x		x	
18832	**2R**	50	x	x	x	x	x		nervous system specific
19010	**2R**	10	x	x	x	x	x		
21400	**2L**	10	x		x	x			same defects were observed on both arms. variable, increased cell number
21400	**2R**	50	x		x	x			neurogenic? CNS abnormal
21486	**2R**	2 out of 26	x		x	x	x		morphology abnormal
22134	**2L**	10	x	x	x	x			cells missing? Same as 19010, weak signal in the nervous system

Lines that were selected for the second screen are listed in this table.

No.	Arm	penetrance (%)	CNS			PNS	general defects	variable	Comments
				axon	disorganised				
389	**3R**	10	x	x		x	x	x	twisted embryos. general defects. disintegrated embryos.
506	**3R**	2 embryos	x	x	x	x			pattern farmation defects.
746	**3R**	100					x		disintegrated embryos
1742	**3R**	100					x		disintegrated embryos
3087	**3L**	10	x	x	x	x	x		pattern formation. segmentation
3124	**3R**	10	x			x	x	x	variable. segmentation defects.
4179	**3L**	50	x	x	x	x			many embryos show same phenotypes, abnormal pattern
4179	**3R**	100					x		disintegrated embryos
4197	**3L**	10	x	x		x	x	x	
4242	**3R**	10	x	x		x		x	cell position defects, nervous system specific, disintegrated embryos
4467	**3R**	10	x	x	x	x	x	x	variable, pattern formation defects
4534	**3L**		x	x	x	x	x	x	variable
4534	**3R**	10	x	x	x	x	x	x	variable, nervous system specific defects, also disintegrated embryos
5761	**3R**	10	x	x	x	x	x	x	variable. only a few embryos showed defects.
5952	**3R**	50	x	x	x	x	x	x	variable, disintegrated embryos, hyperplasia
9835	**3R**	10	x	x	x	x	x	x	variable, disintegrated embryos
15384	**3R**	50	x	x	x	x	x	x	variable. nervous system: diffuse
16775	**3R**	50	x			x	x		mainly PNS defects, extention, abnormal cell position
17251	**3R**	50	x	x	x	x	x	x	variable, nervous system specific defects
17676	**3R**	50	x	x	x	x	x	x	variable
17682	**3R**	10	x	x	x	x	x	x	variable
19008	**3L**	10	x	x	x	x	x		
19282	**3L**	10	x			x		x	variable
20326	**3L**	10	x			x		x	variable

7.2 Results of the zygotic screen

Stock No	Miranda			NB			general defects		bad slide	Other comments
	normal	crescent	orientation	shape	position	cell number	disintegrated	morphology		
E361	x									
E362	x									
E364	x									
E366	x									
E367	x									
E369	x								x	
E370	x									
E371	x									
E372	x									
E373	x									
E374										cell morphology defect, protrusion
E376	x									
E377	x									
E378	x									
E121	x									
E124	x									
E125	x									
E126	x									
E127	x									
E128										few embryos
E129		x						x		crescent formed
E130	x									
E132	x									
E133	x							x		
E136		x				increased?				
E137	x									
E138	x									
E140	x									
E141		x						x		CNS disorganized
E142	x									
E143	x									
E144	x?	x	?					x		
E145		no								Mira cortex, but no crescent
E146		x								disorganized CNS
E147	x									
E148										
E149	x									
E150							x			
E151		x					x			1 embryo Mira crescent and cytoplasm
E152	x									

Stock No	Miranda			NB			general defects		bad slide	Other comments
	normal	crescent	orientation	shape	position	cell number	disintegrated	morphology		
E154		x								
E155										
E157									x	
E158		x								
E159										
E160		x	?		x (1)					
E161										few embryos
E163		x	?	x	x					
E164										
E165										
E166									x	
E167		no?							x	
E168	x									
E170							x			lgl-like?, NB dying?, protrusion
E171		no?								Mira cytoplasmic, severely disorganized CNS
E172										
E173	x?	x								
E174	x									
E175		no		x						
E176										protrusion
E177			?						x	
E178		x		abnormal	abnormal	incresed				2 distinct phenotypes?
E179	x								x	
E180	x									
E181					x					
E182			?			increased				
E183		x	x?	x						
E184									x	
E185						increased				
E186		x	?	x	x					
E187				smaller		decreased				NB dying?, Mira cytoplasmic
E188		x/no				decreased		x		NB too much permeabilized?, Mira cytoplasmic
E189					x		x			
E190		x		x						
E191		x		x	x					
E194				x	x					
E195	x									
E196	x									
E197		x			x					
E198	x									

Stock No	Miranda			NB			general defects		bad slide	Other comments
	normal	crescent	orientation	shape	position	cell number	disintegrated	morphology		
E200	x									
E201	x									
E202	x									
E204		x	?		x					
E205									x	
E206									x	
E207	x									
E208	x									
E209										
E213		x	x							
E379	x									
E380	x									
E383	x									
E384		x			abnormal			x		
E386	x									
E387	x									
E388	x									
E389		x								
E390	x									
E391		x			abnormal	decraesed				
E395	x									
E396	x									
E397	x									
E398										cell morphlogy defect, protrusion
E399										
E400										
E401	x									
E402										
E403	x									
E404										
E405	x									
E407										
E408										
E409										
E410	x									
E413										
E414		no?	?		abnormal			x		Mira polarized in cytoplasm, background?
E418										
E419		x								
E420	x									

Stock No	Miranda			NB			general defects		bad slide	Other comments
	normal	crescent	orientation	shape	position	cell number	disintegrated	morphology		
E421	x									
E422										
E424	x									
E425	x									
E426	x									Mira asymmetric in the cytoplasm?
E427	x									Mira at the spindle?,spot in the cytoplasm(1embryo)
E428	x									Mira asymmetric in the cytoplasm?
E429	x									spots in the cytoplasm(2 embryo)
E432	x?	weak?								spots, spindle, NB not spherical, weak crescent
E433		weak?	?							NB smaller?
E434		?	?							spindle orientation?, crescent formed?, cytoplasmic?
E436										
E437	x									
E438	x							x (1 embryo)		1 embryo no crescent
E439	x									
E440		x	?					x		disorganized neuromere
E441	x?									Mira at the spindle? (balancer homo or hetero)
E442	?	?								NB smaller? increased nunber?
E443								x		weak crescent, NB size position abnormal
E447	x									
E449	x									
E450	x?								x	
E451	x?									
E452										
E454	?	?			abnormal			x		morphology defects, small Mira positive cells
E455				x						protrusion
E456										small Mira positive cells
E460		x	?		abnormal			x		variable, large NBs, NB position abnormal
E464	x?								x	
E469	x									
E470				x						protrusion
E472	x									NB smaller?
E473	?								x	
E474		x	?					x (a few)	x	
E476	x									
E477		?	?					x	x?	bad staining?, few clear crescent, cytoplasm?
E481	x									
E482	x									
E486	x									spot and spindle, soe disintegrated embryos
E488										

Stock No	Miranda			NB			general defects		bad slide	Other comments
	normal	crescent	orientation	shape	position	cell number	disintegrated	morphology		
E489	x									
E490		x	?					x		
E491	x			x						
E492	x									
E493	x									
E494		?	?		abnormal	decreased		x		protrusion
E495										
E496		x?	?			decreased		x (twisted)		
E497										
E499	x									
E500	x?									
E501	x?									
E502	x?									
E503	x									
E504	x									
E505	x									
E506	x									
E507	x									
E508	x									
E382	x									
E509	x?								x	
E510	x									
E511				x (GMC)						
E512	x								x	
E513				x (GMC)					x	
E514	x									
E515	x									
E516	x									
E519		x	normal?			decreased				
E521							x			
E523	x									
E524	x									
E525		?	?					x		
E526	x									
E527										
E528	x									
E529	x									
E530	x									
E531	x									
E532										

Stock No	Miranda			NB			general defects		bad slide	Other comments
	normal	crescent	orientation	shape	position	cell number	disintegrated	morphology		
E533	x									
E534	x									
E535	x									
E536										early stage embryo: variable cell size
E537							x			
E539	x									
E542	x									
E543	x									
E242	x									
E245	x									
E246					abnormal	decreased		x		
E247	x									
E249	x									
E250	x?	x?				decreased		x		
E251	x?							x		
E252	x	x	normal?					x		
E254								x		
E255	x									
E257								x		
E258										
E260	x									
E261								x		no clear crescent
E263		x								
E265		x	abnormal					x		
E266								x		
E267	x									
E268	x?									
E270	x									
E271		x	abnormal					x		
E272	x?									
E273	x									
E275					x					no clear crescent, protrusion
E276	x									
E277	x									
E278					x					no clear crescent
E279	x									
E281										
E283										
E284	x									
E286	x									

Stock No	Miranda			NB			general defects		bad slide	Other comments
	normal	crescent	orientation	shape	position	cell number	disintegrated	morphology		
E289	x?	?								
E290	x									
E291	x									
E292	x									
E293	x									
E295	x									
E296	x									
E297	x									
E298	x									
E300										
E303	x							x		
E304		no		x						*string*, large cells, abormal shape, whole cortex
E305	x									
E309	x									
E310	x									
E311										whole cortex, spindle
E312										
E315		x	?	abnormal		decreased				
E316										
E318	x									
E319	x									
E320										
E321	x									
E322										
E323	x									
E324								x		
E325			increased							
E326		no								cytoplasm
E327		no		x						*string*
E328		no			abnormal			x?		
E821										
E822	x									
E823		x	?	abnormal	abnormal	decreased				
E824	x									
E825										
E826	x									
E827										
E828	x									
E829	x									
E839	x?									

Stock No	Miranda			NB			general defects		bad slide	Other comments
	normal	crescent	orientation	shape	position	cell number	disintegrated	morphology		
E830	x?									
E831		x	random?							
E832	x									
E833	x									
E834		x	random			increased				2 class, 1.random crescent, 2.NBs increased
E835	x									
E836		x	random	larger		decreased				cell cycle defect?
E837	x									
E838	x									
E840										
E841	x								x	
E842	x									
E843	x								x	
E844	x							x	x	
E845		x		larger						
E846		x	random?	smaller		increased				
E847	x									
E848		no				increased				VNC thicker ?
E850	?									protrusion
E851	?			smaller		increased				
E852	x									
E853	x									
E854										
E855	x									
E856				larger	abnormal	increased				
E857	x									
E858	x									
E859	x									
E860	x									
E861										
E862										shape of the neuromere: strange
E863	x									
E864	x									
E865	?	x	?							
E866	x?									hyperplasia?
E867	x									
E868	x									
E869	x									
E870		?			abnormal	increased				
E871	x									

Stock No	Miranda			NB			general defects		bad slide	Other comments
	normal	crescent	orientation	shape	position	cell number	disintegrated	morphology		
E872		x	random?					x		2 classes, 1.VNC abnormal shape, 2.normal
E884	x									
E885	x									
E886		x	normal?	larger	abnormal	less				
E887	x									
E888		no								no signal
E889	x					more				
E890	x									
E891	x?									
E892	x									
E893	x									
E894			normal?	larger	abnormal					
E876								x		
E329	x									
E330								x		
E331	x?									
E332										
E334	x									
E701	x									
E702	x									
E703	x?									
E704	x?									
E705	x							x		
E706								x		
E707	x									
E708							x			
E709										
E710	x									
E712	x									
E713							x			
E715	x									
E716		x	random?			more?				low level expression
E717								x		
E718	x									
E719										
E720	x									
E721	x									
E722	x									
E723	x									
E724	x									

Stock No	Miranda			NB			general defects		bad slide	Other comments
	normal	crescent	orientation	shape	position	cell number	disintegrated	morphology		
E192										
E193	x	x		small						
E199										
E247	x									
E253	x									
E259	x?									
E264		x	abnormal			more		x		variable, VNS: splited?
E269	x									
E301	x									
E306	x?									
E306	x?									
E306			abnormal				x			
E307		no		larger						*string*
E317	x									
E385	x									
E415				abnormal						VNS: splited?
E446										
E480	x									
E484	x									
E874	x									
E711	x									
E727		x		larger						
E728	x									
E731	x									
E734	x									
E735										
E736										
E738	x									
E739	x									
E740	x									
E714	x									
E725	x									
E726	x									
E729		weak						x		weak Mira signal
E730		no?								
E732	x									
E733										
E737		x?	x?							
E741	x									
E742							x			

Stock No	Miranda			NB			general defects		bad slide	Other comments
	normal	crescent	orientation	shape	position	cell number	disintegrated	morphology		
E743							x			
E744	x									
E745	x									
E746										protrusion
E747	x									
E748	x									
E749	x									
E750		x	?			fewer		x		
E751		x	?					x		
E752										
E753	x									
E754	x									
E755										
E756	x									
E757	x									
E758										
E759	x									
E760	?									
E761										
E762										
E763	x									
E764										
E765	x									
E766										
E767										
E768										
E769										
E770	x									
E771	x									
E772	x									
E775	x?									
E776	x?									
E777	x									
E778	x									
E779	x									
E782	x									
E783	x									
E784								x?		
E785								x?		
E786										

Stock No	Miranda			NB			general defects		bad slide	Other comments
	normal	crescent	orientation	shape	position	cell number	disintegrated	morphology		
E788	x									
E789										
E790	x									
E791	x									
E792	?									
E793	x									
E794		x	abnormal							
E795							x			
E796	x									
E797	x									
E798	x									
E799		no?								
E800										
E801	x							x		
E802										
E803										
E804		no		x						
E805	x?									
E806	x									
E807	x					less				
E809		x	random?					x		
E810	x									
E812										
E813	x							x		
E814	x									
E815	x									
E816								x		
E817	x									
E818	x									
E819	x									
E820	x									
E003	x									
E021	x									
E018	x									
E026										
E027								x		
E028								x		
E029	x									
E078		x?	normal?							
E105										

Stock No	Miranda			NB			general defects		bad slide	Other comments
	normal	crescent	orientation	shape	position	cell number	disintegrated	morphology		
E001										too few embryo
E002	x									too few embryo
E004	x									
E005	x									
E007	x									
E009	x									
E010										too few embryo
E011							x			too few embryo
E012										
E013								x?		
E014	x									
E015										
E022										too few embryo
E023										
E032								x		
E033	x									
E034	x									
E035	x						x			
E036										too few embryo
E037										
E038								x		
E039							x			
E040										too few embryo
E041							x			
E042	x									
E045		x	random				x			
E047	x									
E048	x									
E049	x									
E050										
E051		x	random?	smaller	abnormal	more				
E052	x									
E053								x		
E054		x	normal?				x	x		
E055	x									
E056		x	normal?							
E057										too few embryo
E058	x?									too few embryo
E060	x						x			
E065		x	normal?							

Stock No	Miranda			NB			general defects		bad slide	Other comments
	normal	crescent	orientation	shape	position	cell number	disintegrated	morphology		
E062										
E063	x?						x			
E064										no signal
E061	x?						x			
E066										
E067		x	normal		abnormal		x			
E068		x	random?							
E070	x									
E071	x									
E072	x									
E073	x						x	x		
E074							x	x		
E075	x							x		
E076		x	?					x		
E077	x									
E079	x?									
E080	x							x		
E081								x		
E082	x						x			
E083	x						x			
E084	x?							x		
E085	x									
E086										
E087										
E088	x									
E089	x									
E090	x									
E091										
E092		x	random?							
E094							x			
E095	x									
E096	x									
E097	x									
E101	x									
E102								x		
E103		x	random?							
E104	x									
E107		x	?		abnormal					
E107	x									
E108	x									

Stock No	Miranda			NB			general defects		bad slide	Other comments
	normal	crescent	orientation	shape	position	cell number	disintegrated	morphology		
E110	x									
E114		x	normal							
E117	x									
E118	x?									
E120	x						x	x		
E248										
E416	x									
E483	x									
E517	x									
E078								x?		

Abbreviations

aa	amino acid
AP	alkali phosphatase
aPKC	*atypical protein kinase C*
baz	*bazooka*
β–Gal	β–Galactosidase
brat	*brain tumor*
BSA	bovine serum albumin
cDNA	complementary DNA
CNS	central nervous system
C-term	C-terminal
C-terminal	Carboxy-terminal
Df	deficiency
DFS	dominant female sterile
dlg	*discs large*
DmPAR-6	*Drosophila melanogaster partioning defective-6*
DSHB	Developmental Studies Hybridoma Bank
E	glutamate
e.g.	exempli gratia, for example
eve	*even-skipped*
FLP	flipase
GAL4AD	GAL4 activating domain
GAL4BD	GAL4 DNA binding domain
Gα	α-subunit of heterotrimeric G protein
glc	germline clone
Glu	glutamate
GMC	ganglion mother cell
GST	glutathione-S-transferase
h	hour
HRP	horse radish peroxidase
insc	*inscuteable*
jar	*jaguar*

K	Lysine
lgl	*lethal (2) giant larvae*
Lys	Lysine
mAb	monoclonal antibody
MBP	maltose binding protein
min	minute
mira	*miranda*
NB	neuroblast
NHS	normal horse serum
No.	number
N-term	N-Terminal
N-terminal	Amino-terminal
OD	optical density
O/N	over night
PAGE	polyacrylamid gel electrophoresis
par	*partioning defective*
PBS	phosphate buffer saline
PCR	polymerase chain reaction
pins	*partner of inscuteable*
PKC	protein kinase C
PNS	peripheral nervous system
pon	*partner of numb*
pros	*prospero*
rpm	rounds per minute
R.T.	room temperature
scrib	*scribble*
sec	second
soln	solution
SOP	sensory organ precursor
UAS	upstream activating sequence
VNC	ventral nerve cord
w	*white*
wt	wild type

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Erklärung

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