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miranda localizes staufen and prospero asymmetrically in mitotic neuroblasts and epithelial cells in early *Drosophila* embryogenesis

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SUMMARY

When neuroblasts divide, prospero protein and mRNA segregate asymmetrically into the daughter neuroblast and sibling ganglion mother cell. miranda is known to localize prospero protein to the basal cell cortex of neuroblasts while the staufen RNA-binding protein mediates prospero mRNA localization. Here we show that miranda is required for asymmetric staufen localization in neuroblasts. Analyses using *miranda* mutants reveal that prospero and staufen interact with miranda under the same cell-cycle-dependent control. miranda thus acts to partition both prospero protein and mRNA. Furthermore, miranda localizes prospero and staufen to the basolateral cortex in dividing epithelial cells, which express the three proteins prior to neurogenesis. Our observations suggest that the epithelial cell and neuroblast (both of epithelial origin) share the same molecular machinery for creating cellular asymmetry.

Key words: miranda, prospero, staufen, neuroblast, asymmetric division, epithelial polarity, *Drosophila*

INTRODUCTION

Asymmetric cell division operates in many developmental contexts to create two differently fated cells from a single progenitor cell (reviewed by Horvitz and Herskowitz, 1992). This process can result from extrinsic signals that instruct equivalent daughter cells to take different cell fates, intrinsically from unequal partition of protein determinants to a daughter cell from the progenitor, or from the combination of both mechanisms (reviewed by Jan and Jan, 1998). In intrinsically asymmetric divisions, protein determinants are asymmetrically localized within cells prior to or during cytokinesis for their unequal partition. How they distribute asymmetrically is a fundamental problem to understanding the molecular mechanisms that underlie the asymmetric cell division.

During the embryonic development of the *Drosophila* central nervous system, neural precursor cells called neuroblasts (NB) divide to produce another NB and a smaller ganglion mother cell (GMC), which then divides once into a pair of neurons or glia (Goodman and Doe, 1993). NB division is asymmetric in terms of differences between the daughter cells in morphology, mitotic activity and gene expression. numb and a transcription factor prospero (pros) appear to be asymmetrically distributed cell-fate determinants in NB divisions (Uemura et al., 1989; Doe et al., 1991; Vaessin et al., 1991; Matsuzaki et al., 1992). They are localized to the basal cell cortex in NBs during mitosis and then segregate asymmetrically into the GMC which buds off from the basal side of the NB (Rhyu et al., 1994; Hirata et al., 1995; Knoblich et al., 1995; Spana and Doe, 1995). In the GMC, pros translocates to the nucleus (Vaessin et al., 1991; Matsuzaki et al., 1992) where it establishes differential gene expression between the two sibling cells (Doe et al., 1991; Vaessin et al., 1991). While numb function in the GMC is still unclear, it is known to suppress the Notch signal and act as a fate determinant during the development of the peripheral nervous system (Guo et al., 1996) and for the two daughter neurons of the MP2 precursor in the central nervous system (Spana et al., 1995; Spana and Doe, 1996).

miranda (mira) has been identified as a factor that binds pros to direct it to the GMC (Ikeshima-Kataoka et al., 1997; Shen et al., 1997). At the NB mitosis, mira is basally localized tethering pros protein to the basal cortex; this protein complex is consequently partitioned to the GMC. Shortly after cytokinesis, pros dissociates from mira into the GMC nucleus and mira disappears. Mutational analyses has revealed that the cell-cycle-dependent association of the two proteins is regulated via a domain on mira located adjacent to the pros-binding site (Ikeshima-Kataoka et al., 1997).

In the absence of mira, GMCs are not able to express genes required for correct neuronal identities (Ikeshima-Kataoka et al., 1997), suggesting that the asymmetric segregation of pros is essential for establishing neural diversity. Although numb is

colocalized with the mira/pros complex during the NB mitosis, numb localization appears to be independent of mira function (Ikeshima-Kataoka et al., 1997; Shen et al., 1997).

For such factors to segregate unequally to one daughter cell, the mitotic apparatus must orient along the axis of the polarized protein localization (Chenn and McConnell, 1995; Rhyu and Knoblich, 1995); In the NB, the division axis orients parallel to the apicobasal direction such that the GMC is formed from the basal side of the NB. The localization of protein factors is regulated to the basal or apical side during NB mitosis, resulting in their asymmetric segregation; however, coupling between spindle orientation and the direction of protein localization can be disrupted by reagents affecting cytoskeletal organization (Knoblich et al., 1995; Kraut et al., 1996; Broadus and Doe, 1997). inscuteable protein (insc) (Kraut and Campos-Ortega, 1996) plays essential roles in coordination of the two axes (Kraut et al., 1996). In *insc* mutants, pros and numb are dislocalized or localized in random orientations during mitosis; since the spindle orientation is also randomized, the two axes become uncoordinated (Kraut et al., 1996).

mRNAs encoding protein determinants are also asymmetrically distributed (reviewed by St Johnston, 1995). For example, in budding yeast, the mother cell can switch its mating type but the daughter cell can not because the *HO endonuclease* gene is selectively activated in the mother cell (Bobola et al., 1996; Jansen et al., 1996; Sil and Herskowitz, 1996). This asymmetry is due to the asymmetric partition of *ASH1* mRNA that encodes a repressor protein of the HO endonuclease to the daughter cell (Long et al., 1997; Takizawa et al., 1997). Asymmetric RNA segregation also occurs during *Drosophila* NB divisions; *pros* mRNA is localized in a basal cortical crescent at mitosis to segregate asymmetrically to the GMC (Li et al., 1997; Broadus et al., 1998). This asymmetric *pros* mRNA segregation depends on the double-strand RNA-binding protein staufen (stau). It has been shown to participate in the establishment of the anterior-posterior axis of the embryo by mediating the localization of two RNA species (St Johnston et al., 1991): (1) *oskar* mRNA, which is localized to the posterior pole of the oocyte to define the region where the posterior determinant nanos accumulates (St Johnston and Nusslein-Volhard, 1992), and (2) *bicoid* mRNA, which is restricted by stau to the anterior cortex of the embryo forming a bicoid morphogen gradient (Driever and Nusslein-Volhard, 1988). In the embryonic central nervous system, stau is localized in the basal crescent overlapping with pros/mira in mitotic NBs (Broadus et al., 1998). In addition, stau has been shown to bind with the 3′ untranslated region of *pros* mRNA (Li et al., 1997). Thus stau appears to act to localize and segregate *pros* mRNA asymmetrically during NB divisions; however, the interaction between the stau/*pros* RNA complex and the mira/pros complex is unclear.

In this study, we show that the asymmetric segregation of stau requires mira. To analyze the mode of interaction among the asymmetrically localized proteins, mira, pros and stau, we determined the molecular lesions of *mira* mutants that we previously identified (Ikeshima-Kataoka et al., 1997) and investigated the localization of encoded mutant proteins as well as the pros and stau localization in those *mira* alleles. We are able to divide the mira sequence into three regions; (1) for the basal localization of mira, (2) for association with both pros and stau, and (3) the region that regulates interaction with the two proteins in a cell-cycle-dependent manner. mira thus plays a central role in the basally localized protein/RNA complex at the NB mitosis by determining the subcellular localization and also by mediating cell-cycle-dependent signals. Furthermore, we show that mira localizes pros/stau to the basolateral cortex during epithelial cell division as in NB cell divisions. We discuss possible mechanisms for protein localization that are shared by epithelial cells and NBs.

MATERIALS AND METHODS

Drosophila strains

Six ethylmethanesulphonate-induced *mira* alleles, $mira^{L44}$, $mira^{YY227}$, $mira^{JJ92}$, $mira^{AB78}$, $mira^{ZZ176}$ and $mira^{RR127}$ (Ikeshima-Kataoka et al., 1997) were balanced by TM3 P[ry+, ftz-LacZ] to facilitate the identification of homozygous embryos. $stau^{D3}$ was obtained from Bloomington *Drosophila* Stock Center.

Molecular biology

The sequence of mutant *mira* genes was determined by RT-PCR. First-strand cDNA was synthesized from poly (A)+ RNA prepared from embryos heterozygous for mira alleles using the SuperScript Preamplification system (Gibco) and amplified by four pairs of primers; 5′-CGTTACCAAGTTAAACCGAAAGTCC-3′(226)/5′-CCTGAAGAGCAGAGAGTTGTGC-3′(1090), 5′-CACTGCGT-CTGGCCAACGAGCT-3′(1013)/5′-CTGGCTCTCGGTCAATTGC-TG-3′(1960), 5′-AACGAGCTGATCAAGATCGAGC-3′(1591)/5′-TCCTCGATCTTCTCCAGATCGG-3′(2399) and 5′-TGCAAA-CTCGTTCCAGGAGCGC-3′(2124)/5′-TCGAATCGGATCTTAGCT-AAGG-3′(2785). Numbers in parentheses indicate the base position of the 3′-end of each primer in the full-length cDNA having the coding region from 244 to 2733. This set of primers amplifies cDNA fragments that collectively cover the entire coding region overlapping each other. Because embryos for RT-PCR are heterozygous for *mira* alleles, we sequenced more than seven PCR fragments for each primer pair to detect mutations. Mutations identified in RT-PCR fragments were confirmed by sequencing the corresponding genomic DNA sequence.

Immunohistochemistry

A rabbit antiserum was raised against the N-terminal mira polypeptide, KAKLKRFNDVDVAIC (from amino acid 5 to 19). The anti-N-mira specifically recognizes mira protein based on the two criteria; first, it stains wild-type embryos in the same pattern as two antibodies against an anti-C-terminal polypeptide (Ikeshima-Kataoka et al., 1997). Second, the anti-N-mira does not stain $Df(3R)\ ora^{I9}$ embryos that delete the *mira* gene (Ikeshima-Kataoka et al., 1997). This anti-N-mira was used at 1:2000. Mouse polyclonal anti-stau (a gift from C. Q. Doe) was used at 1:100; rabbit anti-β-galactosidase (Cappel) at 1:4000 and monoclonal anti-β-galactosidase (Promega) at 1:100. Cy-3- or FITC-conjugated secondary antibodies (Jackson Immunoresearch) were used at 1:200. DNA was stained by TOTO-3 as described (Hirata et al. 1995). RNA in situ hybridization was carried out essentially as described (Tautz and Pfeifle, 1989). Digoxigenin-labeled single-strand RNA probe (Boehringer-Mannheim) was made from the 5′ 4.2 kb *prospero* cDNA fragment. RNA localization was detected in whole-mount embryos by mouse anti-digoxigenin antibody and Cy-3-conjugated anti-mouse antibody. Stained embryos were examined using a confocal microscope, MRC1024 (BioRad).

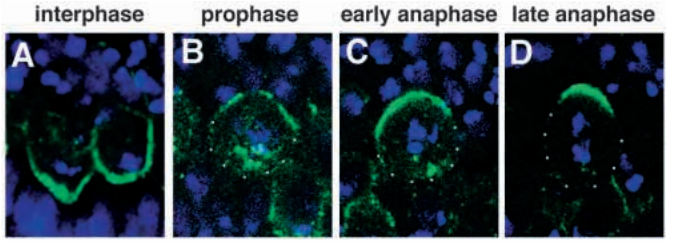

Fig. 1. The localization of wild-type mira protein during the NB cell cycle is detected by an antibody against a N-terminal mira polypeptide, anti-N-mira. (A) At late interphase, mira is concentrated to the apical cell cortex. (B) At prophase, mira begins to translocate basally while a proportion of mira persists in apical patches. (C,D) By anaphase, most mira has been localized in the basal cortex leaving a small fraction of mira apically. Anti-N-mira staining is shown in green, DNA staining in blue. In this and following figures, apical is toward the bottom and white dots outline cell borders.

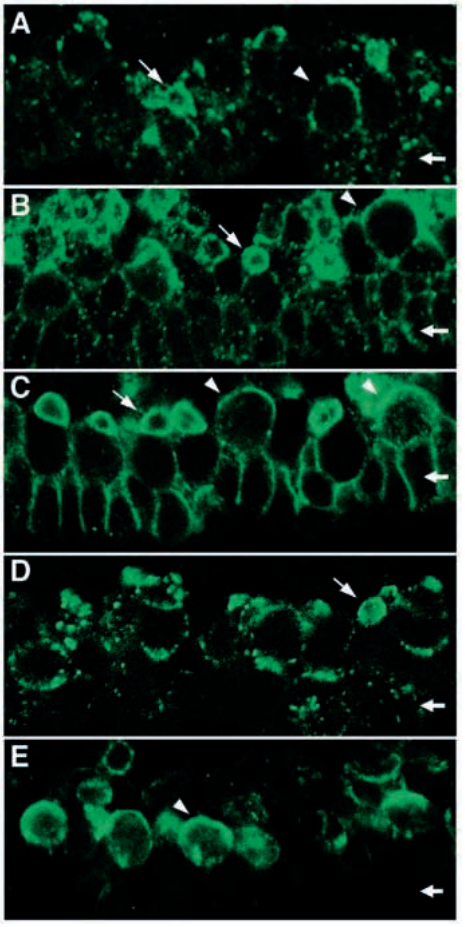

Fig. 2. The distribution of mutant mira proteins is detected by anti-N-mira in neuroblasts, GMCs and neuroectodermal cells in embryos homozygous for *mira* alleles, (A) $mira^{YY227}$, (B) $mira^{JJ92}$, (C) $mira^{AB78}$ and (D) $mira^{RR127}$ as well as for (E) the wild type. Arrowheads indicate mitotic neuroblasts with the basal crescent of mutant mira proteins; arrows indicate GMCs retaining mutant mira proteins cortically; thick arrows at the right side of each panel indicate the layer of the neuroectoderm. (A) $mira^{YY227}$, (B) $mira^{JJ92}$ and (C) $mira^{AB78}$ proteins are basally localized at mitosis in NBs (arrowheads). Note the loss of apical localization of mutant mira proteins in (C) interphase except for (D) $mira^{RR127}$. $mira^{JJ92}$ and $mira^{AB78}$ proteins are localized to the basolateral cell cortex also in ectodermal cells. See Fig. 3A,B for $mira^{ZZ176}$ localization.

RESULTS

Molecular mapping of mira mutations

Among six mira alleles that we have identified, previous analyses revealed that two alleles, $mira^{ZZ176}$ *and* $mira^{RR127}$ encode truncated proteins with N-terminal 446 and 727 amino acids, respectively, both of which are followed by unrelated amino acids (Ikeshima-Kataoka et al., 1997). To map functional domains in the mira protein, we determined the sequence of four other alleles, $mira^{L44}$, $mira^{YY227}$, $mira^{JJ92}$ and $mira^{AB78}$; all these alleles encode truncated mira proteins (see Fig. 8). The $mira^{L44}$ gene codes for the wild-type sequence of the N-terminal 103 amino acids that flanks 33 unrelated amino acids due to the insertion of two bases. Three other alleles, $mira^{YY227}$, $mira^{JJ92}$ and $mira^{AB78}$, have a stop codon within the coding sequence at different positions, producing truncated proteins of 290, 329 and 405 amino acids, respectively. Thus the six *mira* alleles provide a series of *mira* mutants expressing truncated proteins with various C-terminal deletions (see Fig. 8).

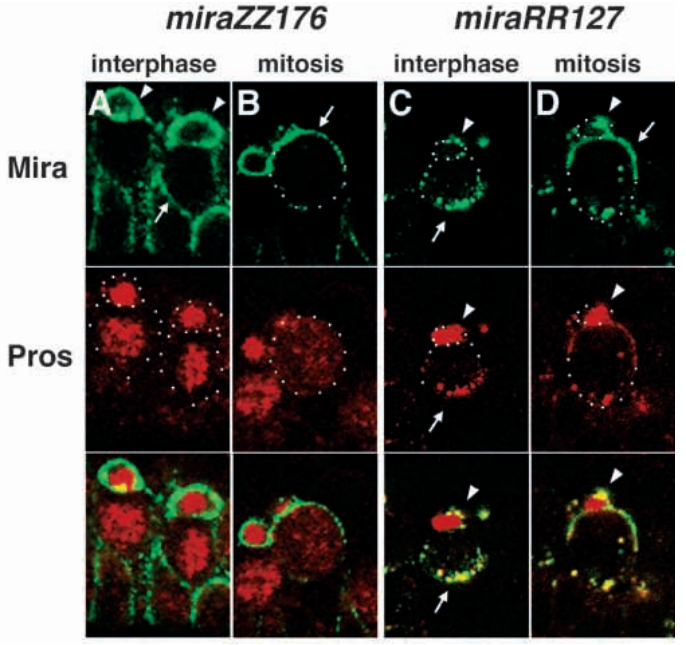

Fig. 3. $mira^{ZZ176}$ and $mira^{RR127}$ proteins are abnormally localized in interphase NBs and GMCs whereas they normally segregate to GMCs. (A) Two pairs of interphase NBs (lower and larger cells) and GMCs (upper and smaller cells) in a $mira^{ZZ176}$ homozygous embryo are shown. $mira^{ZZ176}$ protein (green) is localized throughout cell cortex in interphase NBs (arrow) and GMCs (arrowheads) in contrast to pros (red), which is localized to the nucleus. (B) At mitosis, $mira^{ZZ176}$ protein is normally localized at the basal cortex (arrow) whereas pros (red) distributes to the cytoplasm. (C) In $mira^{RR127}$ NBs (lower and larger cell), $mira^{RR127}$ protein distributes in patches to the apical cortex in interphase (arrows). Cortical patches of $mira^{RR127}$ protein are completely overlapped with those of pros. (D) During the division of $mira^{RR127}$ NBs (lower and larger cell), $mira^{RR127}$ protein normally forms a basal crescent (arrow). After segregation into the GMC, $mira^{RR127}$ remains on the cortex (arrowheads in C,D), where it is again colocalized with pros (arrowheads) in patches; a proportion of pros in the GMC is slowly dissociated from cortical $mira^{RR127}$ protein and translocated to the nucleus (C,D).

The subcellular localization of the wild-type mira protein

The subcellular localization of the wild-type mira protein was examined in the embryonic central nervous system using the anti-N-mira, antiserum against a N-terminal mira polypeptide that is contained in all truncated mira proteins (Fig. 1). The wild-type mira protein predominantly localizes to the cortex in interphase NBs, especially to the apical cortex along with pros at late interphase (Fig. 1A). At the onset of prophase (Fig. 1B), the majority of the wild-type mira becomes localized to the basal cortex as a crescent while a fraction of the protein punctately distributes to the apical region. As the mitotic stage proceeds, an increasing proportion of mira appears to be incorporated in the basal crescent (Fig. 1C,D). While some mira protein is still observed apically during anaphase, most mira protein segregates to the basally budding GMC. This pattern of subcellular localization is equally evident using polyclonal and monoclonal antibodies against a C-terminal polypeptide (Ikeshima-Kataoka et al., 1997; our unpublished data).

The localization of mutant mira proteins that fail to bind pros

The anti-N-mira antibody hardly stains *mira*^{L44} homozygous embryos, which express the shortest mira protein among mutant alleles, although transcripts from the *mira*^{L44} gene are detected in the mutant embryos (data not shown). This may be due to the abnormal tertiary structure of this short form, which may prevent the antibody from recognizing its epitope, or may be due to the abnormal subcellular localization resulting from a lack of domains necessary for correct mira localization; both of these would expose the protein to rapid proteolytic degradation. The anti-N-mira detects all other mutant proteins. Fig. 2 shows the localization of mutant mira proteins, mira^{YY227}, mira^{JJ92}, mira^{AB78} and mira^{RR127}, as well as the wild-type mira. In *mira*^{YY227} mutant embryos, which express the second shortest mutant protein, the antibody stains faintly the cell cortex of GMCs as well as NBs (Fig. 2A). *mira*^{JJ92} embryos are stained more strongly than *mira*^{YY227}. The mira^{JJ92} protein appears to distribute in patches along the cell cortex in interphase NBs (Fig. 2B). Both mira^{AB78} and mira^{ZZ176} protein are clearly detected with the anti-N-mira (Figs 2C, 3A,B). The four mutant proteins that fail to localize pros to the cortex, mira^{YY227}, mira^{JJ92}, mira^{AB78} and mira^{ZZ176}, are localized to the basal cortex in dividing NBs to segregate into the GMC. This indicates that those proteins include the region required for their asymmetric localization during NB mitosis although none of them contains the region essential for binding with pros. A striking difference in the localization is observed between the four mutant proteins and the wild-type protein. The mutant proteins remain on the GMC cortex while wild-type mira disappears in the GMC immediately following cytokinesis. This persistent cortical localization in the GMC is also observed for mira^{RR127} protein as described below; mira^{YY227} protein tends to accumulate to the GMC cortex compared to the wild-type protein despite its weak staining intensity. Thus all mutant proteins detectable by the anti-N-mira antibody are resistant to degradation in the GMC suggesting that the C-terminal region that is missing commonly in those mutant proteins is necessary to promote rapid protein degradation. Interestingly, mira proteins are not confined to the apical side in the interphase NBs of *mira*^{JJ92}, *mira*^{AB78} and *mira*^{ZZ176} mutants, but, instead, tend to distribute throughout the NB cortex (see Discussion).

The localization of mira^{RR127} protein

The *mira*^{RR127} allele shows a distinct phenotype in pros localization from the other five *mira* alleles, which equally partition pros between the NB and GMC, as previously reported (Ikeshima-Kataoka et al., 1997). In *mira*^{RR127} embryos, the basal localization of pros and its subsequent segregation into GMCs appear essentially normal. However, pros fails to translocate to the nucleus persisting at the GMC cortex, or its translocation is greatly delayed. A difference in the subcellular localization between mira^{RR127} and wild-type mira is detected in interphase NBs and GMCs (Fig. 2D,E). mira^{RR127} distributes in patches along the apical cortex during interphase (Fig. 3C) in contrast to the smooth distribution of the wild-type mira. This abnormal distribution of mira^{RR127} completely overlaps with that of pros. Once NBs enter mitosis, mira^{RR127} and pros are localized in a basal crescent as seen in the wild type (Fig. 3D), then segregate to the GMC. In the GMC, mira^{RR127} protein persists at the cortex even after the delayed accumulation of pros in the nucleus. In contrast to the accumulation of mutant mira proteins that do not bind pros, mira^{RR127} is distributed irregularly at the cortex and colocalized with cortically tethered pros protein (Fig. 3C,D). Thus abnormal mira^{RR127} localization accompanies pros throughout the cell cycles of both the NB and GMC. This suggests that mira^{RR127} constitutively associates with pros protein at the cortex without the cell-cycle-dependent regulation in the interaction between wild-type mira and pros, although pros slowly dissociates from mira into the nuclei in the GMC.

mira is required for stau localization

Since stau protein, like pros and mira, is known to be localized during NB divisions to segregate asymmetrically to the GMC (Broadus et al., 1998), we tested whether asymmetric stau localization requires *mira* function (Fig. 4). In wild-type NBs, stau and mira are colocalized during interphase (Fig. 4A). Upon mitosis (Fig. 4B), the colocalization of the two proteins is observed in a basal crescent in approximately 70% of NBs (73/108). stau remains in the GMC cytoplasm following cytokinesis while mira becomes undetectable (Fig. 4A). We found that all *mira* alleles exhibit abnormal stau localization. In five *mira* alleles that partition pros equally to the two NB progenies, the basal localization of stau protein is not detected during NB mitosis; as typically shown in the *mira*^{ZZ176} allele, it distributes evenly in the cytoplasm and segregate equally to both NB and the sister GMC (Fig. 4C,D). mira function is thus required for the normal segregation of stau protein. As expected from the abnormal localization of stau, *pros* mRNA fails to be basally localized in those *mira* alleles (Fig. 5); instead, it distributes throughout the cytoplasm during both interphase and mitosis.

In *mira*^{RR127}, the localization of stau (Fig. 4E,F) is essentially the same as that seen for pros (Ikeshima-Kataoka et al., 1997); it distributes in patches to the apical side in interphase NBs whereas, at mitosis, it normally changes its localization into a basal crescent to segregate to the GMC.

Following cytokinesis, stau persists at the cortex in patches being colocalized with mira^{RR127} protein (Fig. 4E). Thus the two proteins are colocalized throughout the cell cycle of both the NB and GMC as seen for pros and mira^{RR127}. In contrast to the observation that a certain fraction of GMC nuclei slowly accumulates pros that is free from cortical mira^{RR127} protein, we could not detect stau protein that clearly does not overlap with mira^{RR127} deposition in the GMC. Taken together, the dependence of stau localization on the two types of mira alleles is essentially the same as that of pros localization. We infer from these observations that mira localizes stau directly or indirectly at mitosis in wild-type NBs. A region of mira responsible for stau localization is thus defined by the *mira^{RR127}* and *mira^{ZZ176}* alleles, which is the same region as a domain necessary to bind pros protein (see Fig. 8 and Discussion).

mira localizes pros and stau in epithelial cells

The three genes that we have described here are widely expressed in early embryonic stages prior to neurogenesis (Doe et al., 1991; St Johnston et al., 1991; Ikeshima-Kataoka et al., 1997; Shen et al., 1997); till early stage 9 (Campos-Ortega and Hartenstein, 1985), they are essentially coexpressed in all cells except mesodermal and pole cells, and subsequently in both the neuroectoderm and the endoderm until early stage 11. Expression is finally restricted to the neural precursor cells in the central and peripheral nervous system and to the stem-like cells for adult midgut precursors in the endoderm (Ikeshima-Kataoka et al., 1997; Shen et al., 1997). These facts suggest that mira could also localize pros and stau in epithelial cells. In fact, mira is localized at the basolateral cortex of all ectodermal cells until stage 8 after gastrulation (Fig. 6A). Whereas the level of mira expression decreases in epithelial cells as the developmental stage proceeds, its basolateral localization is observable in the neuroectoderm until stage 11; interestingly, the cortical mira localization becomes most evident during mitosis (Fig. 6B). Mutant mira proteins are also localized to the epithelial cell cortex; mira^{JJ92}, mira^{AB78} and mira^{ZZ176} accumulate in the basolateral cortex of interphase epithelial cells more abundantly than wild-type mira (see Fig. 2), while, in dividing cells (Fig. 6D), these mutant mira proteins are most evidently localized to the basolateral cortex, as seen for the wild-type mira. The localization of mira^{YY227} protein is not clearly detected in epithelial cells probably due to its low detectability with the anti-N-mira antibody. These results therefore suggest that mira protein is asymmetrically localized to the epithelial cell cortex via the same mechanisms that act in its localization in the NB.

We examined whether pros and stau are colocalized with mira in the ectodermal cells. During stage 8 and 9, pros is observed in the nucleus but partly colocalized with mira at the cortex of interphase ectodermal cells (Fig. 6B,C). stau is below the level of detection during interphase with the antibody used in this study. We are able to visualize clearly stau and pros proteins that are colocalized with mira at the basolateral cortex during mitosis (Fig. 7A,B). Thus the three proteins are asymmetrically localized at mitosis in epithelial cells. Their basolateral localization, however, results in their equal partition to the two daughter epithelial cells because the division axis orients vertically to the apical-basal axis.

We tested whether mira is required for the basolateral localization of pros and stau in the mitotic ectodermal cells. In *mira* alleles that fail to localize stau or pros basally in the NB, the two proteins are not localized to the cortex during mitosis of the epithelial cell, spreading to the cytoplasm (Fig. 7C). Conversely, in *mira^{RR127}* allele, mira^{RR127} protein and the other two proteins are colocalized to the basolateral cortex in dividing epithelial cells (Fig. 7D). Thus the localization of pros and stau depends on *mira* function in epithelial cells; the association of mira protein with pros/stau and its cell-cycle-dependent regulation in epithelial cells are essentially the same as those in NBs. Our results suggest that the same mechanism acts in the epithelial cell, the NB and GMC to localize the proteins cortically at mitosis.

DISCUSSION

mira function in stau localization

During the asymmetric divisions of NBs, two cell fate determinants, numb and pros protein are unequally partitioned into the daughter GMC from the parental NB (Rhyu et al., 1994; Hirata et al., 1995; Knoblich et al., 1995; Spana and Doe, 1995). The localization of pros is directed by mira protein that binds pros to tether it to the basal cortex during mitosis (Ikeshima-Kataoka et al., 1997; Shen et al., 1997). In addition of these three factors, it was recently shown that *pros* mRNA also segregates asymmetrically to the GMC (Li et al., 1997; Broadus et al., 1998). *pros* RNA is basally localized via the RNA-binding protein stau that is itself localized to the basal cortex at mitosis. In this study, we have shown that *mira* function is necessary for stau to be basally localized during the NB mitosis. The same finding was recently reported independently (Schuldt et al., 1998; Shen et al., 1998).

In *pros*-deficient embryos, stau is localized normally in a basal crescent at mitosis (data not shown). Similarly, the localization of pros protein is not affected by *stau* mutations (Li et al., 1997; Broadus et al., 1998). These observations, together with the requirement of mira for stau localization, indicate that the localization of both stau and pros in the NB requires only mira. In addition, mira is localized normally in pros (Ikeshima-Kataoka et al., 1997; Shen et al., 1997) or stau mutants embryos (Schuldt et al., 1998; F. M., unpublished observation), implying that mira does not require the other two proteins for its localization. This epistatic relationship between the three proteins indicates that mira acts the adapter to tether the other two proteins to the basal cell cortex of the NB. mira thus partitions both *pros* RNA and pros protein from the NB to the GMC.

stau is involved in the localization of *oskar* RNA to the posterior pole of the oocyte as well as in *bicoid* RNA localization to the anterior pole of embryos (St Johnston and Nusslein-Volhard, 1992). Loss of maternal stau results in a defective anterior-posterior embryonic axis. One can speculate that mira plays a role in stau localization during the anterior-posterior axis formation in the oocyte as during neurogenesis. If so, *mira* mutants would affect the localization of stau in oogenesis or in early embryogenesis causing a similar phenotype to *stau* mutants. Germline clones homozygous for *mira* mutants, however, develop normally without any apparent defects (T. O. and F. M., unpublished observation). This

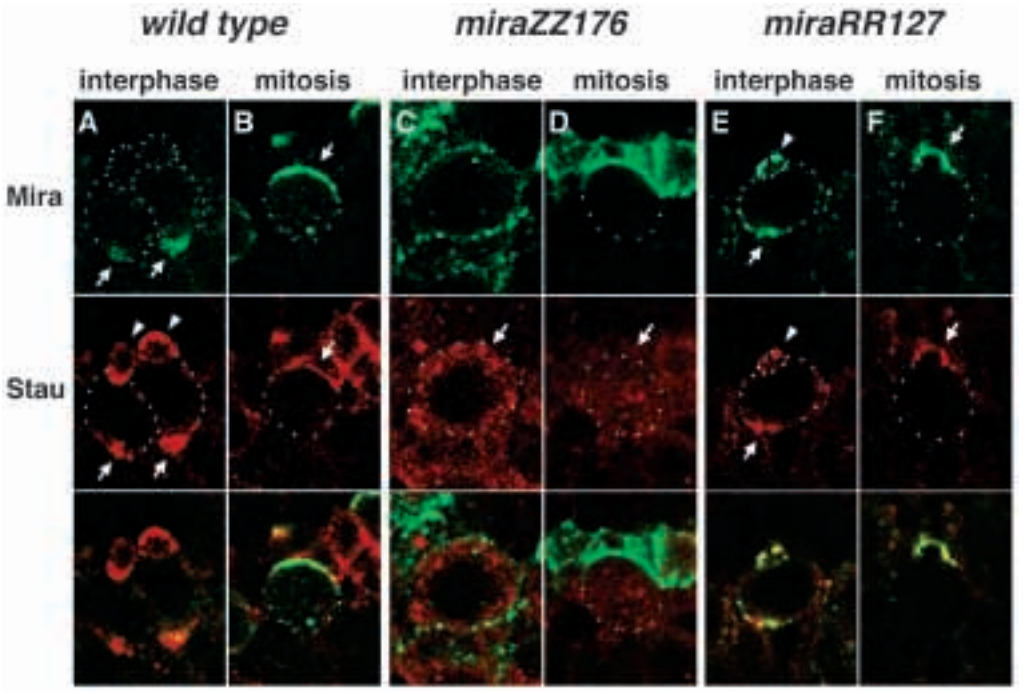

Fig. 4. mira is required for stau localization during NB divisions. (A,B) In wild-type NBs, mira (green) and stau (red) are colocalized in the apical cortex in interphase (arrows in A), then in a basal crescent at mitosis (arrows in B). stau remains in the GMC cytoplasm after the degradation of mira (arrowheads in A). (C,D) In *mira*^{ZZ176} NBs, stau (red) distributes to the cytoplasm during interphase (arrow in C) and mitosis (arrow in D). (E) In *mira*^{RR127} NBs, stau (red) and mira^{RR127} protein (green) are punctately concentrated to the apical cortex (arrows in E) during interphase. Upon mitosis, they normally segregate to the GMC. (F) A telophase NB is shown, where the two proteins are being incorporated into the budding GMC (arrows in F). After cytokinesis, stau and mira^{RR127} protein remain in patches on the GMC cortex (arrowheads in E).

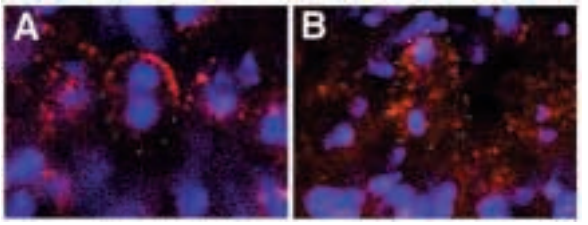

Fig. 5. The localization of *pros* mRNA depends on *mira* function. (A) In wild-type NBs, *pros* mRNA (red) is localized as a basal crescent at mitosis to segregate to the GMC. (B) In the *mira*^{AB78} allele, which fails to localize stau asymmetrically, *pros* mRNA (red) is distributed to the NB cytoplasm at mitosis and partitioned equally to the NB and sibling GMC. DNA staining is shown in blue.

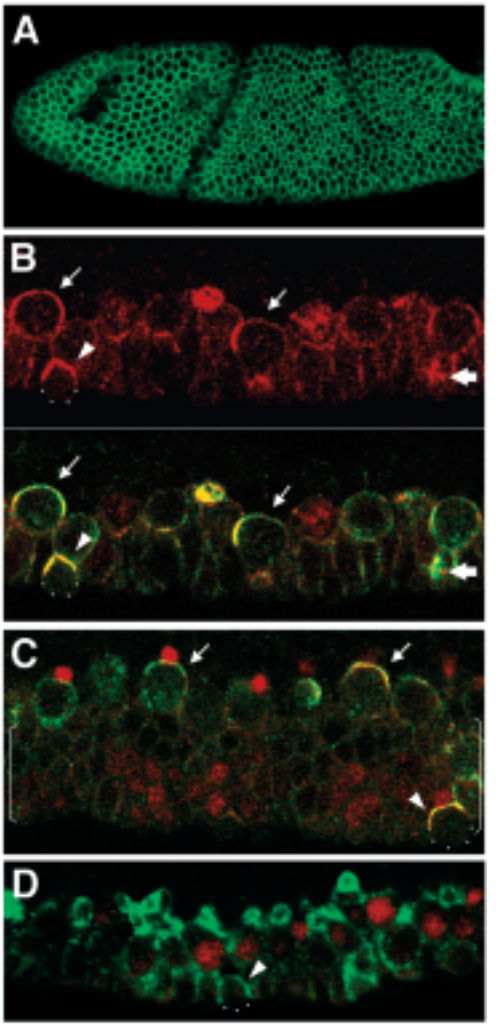

Fig. 6. mira and pros are asymmetrically localized in all mitotic epithelial cells prior to neurogenesis and subsequently in neuroectodermal cells during neurogenesis. mira is shown in green; pros is in red. (A) At stage 8, mira is expressed in all cells in the ectoderm and localized to the cell cortex. Anterior is to the left and dorsal up. (B) At stage 9, mira and pros are colocalized in cells in the neuroectoderm. Whereas interphase epithelial cells localize mira and pros to the basolateral cortex, the basal concentration of the two proteins is most evidently seen at mitosis (arrowheads). The upper panel of B shows only pros distribution; the lower panel shows the merge of pros (red) and mira (green) staining. Thick arrows indicate the neuroectoderm. (C) At sage 9, a proportion of pros (red) is localized to the nucleus in addition to the basolateral cortex in interphase epithelial cells as seen in the region within brackets. At mitosis, pros and mira are, however, colocalized in a basal crescent (arrowhead in C). In C, the embryo is tilted to allow better visualization of the neuroectoderm. In B and C, arrows indicate NBs at mitosis. (D) In *mira*^{JJ92} embryos, mutant mira protein (green), which is localized to the basolateral cortex, is most clearly observed in dividing neuroectodermal cells (arrowhead).

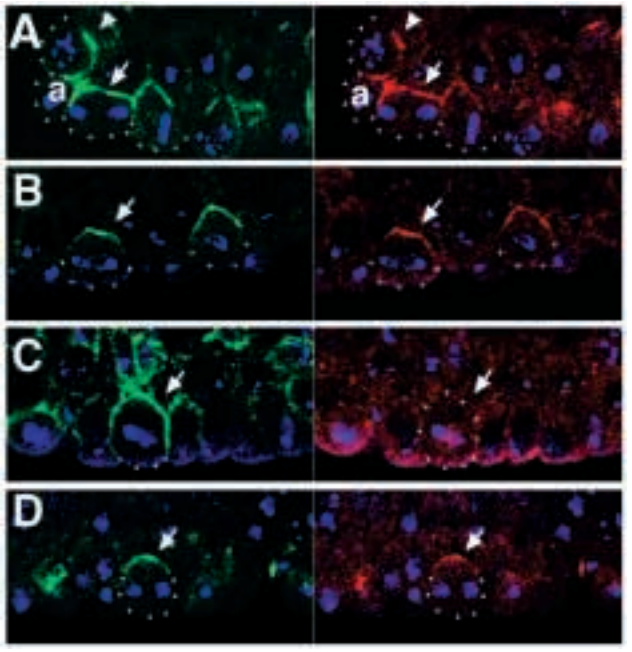

Fig. 7. The localization of pros and stau requires mira in dividing ectoderm cells. (A) The most anterior part of the ventral thorax in a wild-type embryo at stage 9 (Campos-Ortega and Hartenstein, 1985) is laterally viewed. Anterior is to the left and dorsal up. The cell labeled by (a) is located at the corner between the cephalic furrow and the ventral ectoderm. The neighboring anaphase cell, which is dividing parallel to the ventral surface, localizes mira (green in the left panel) and pros (red in the right panel) to the basolateral cortex (arrows). An ectodermal cell facing the cephalic furrow is at prophase and localizing both proteins to the basolateral cortex (arrowheads). (B) In the wild type, mira (green) and stau (red) are colocalized to the basolateral cortex (arrows) in dividing ectodermal cells. (C) In the *mira*^{ZZ176} allele, stau (red in the right panel) is distributed to the cytoplasm at mitosis (arrow) and not colocalized with mira^{ZZ176} protein (green in the left panel) at the basolateral cortex (arrow). In right panels of B and C, staining signals at the apical side of the epithelial cells are artifacts with anti-stau, which are also seen in *stau*-deficient embryos. (D) In the *mira*^{RR127} allele, mira^{RR127} protein (green) and pros (red) are colocalized to the basolateral cortex in dividing ectodermal cells as indicated by arrows. In A-D, DNA staining is shown in blue.

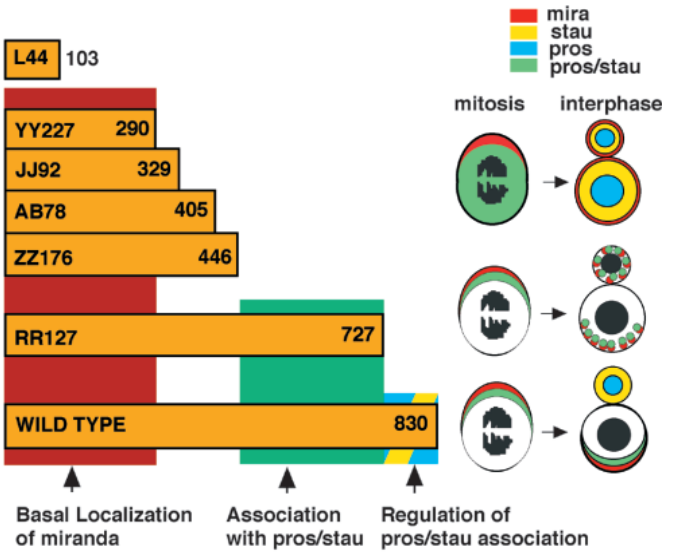

Fig. 8. *mira* mutations define three distinct functional regions along the mira sequence. The N-terminal 290 amino acids region acts in the basal localization of mira at mitosis in the NB and the epithelial cell. The region between amino acid 447 and 727 includes a domain necessary for the binding with pros as well as the domain (s) required for the asymmetric localization of stau in the NB. The C-terminal 103 amino acids region confers the cell cycle dependence on the interaction with pros/stau; the absence of this region results in the prolonged association with pros/stau during interphase without rapid proteolytic degradation in the GMC and NB. See text for details.

suggests that different factors or redundant mechanisms function in stau localization in the oocyte or fertilized eggs, although we do not exclude the possibility of a role for mira in stau localization.

Interaction of mira with pros and stau

Using six *mira* alleles that provide a series of C-terminally truncated mira proteins, we are able to map three functional domains in mira protein sequence as summarized in Fig. 8; (1) the domain for the association with pros and stau, (2) the regulatory domain for the association, and (3) the domain for the basal localization of mira. Our previous analyses (Ikeshima-Kataoka et al., 1997) have shown that pros associates with mira protein via the 281 amino acids region defined by the two *mira* alleles, *mira*^{ZZ176} and *mira*^{RR127}. In this study, we showed that the same region is responsible for stau protein to be basally localized at NB mitosis (Fig. 8). Although our analysis focuses on the in vivo interaction of mira with pros/stau, recent publications (Schuldt et al., 1998; Shen et al., 1998) have shown that mira directly binds to stau in vitro. The binding region of mira to stau has been determined (Schuldt et al., 1998; Shen et al., 1998), which is consistent with our in vivo assignment. It is thus likely that mira associates directly with stau in vivo. It is interesting to know whether the pros-binding domain and the region necessary for stau localization are separately located or overlap with each other within the mira sequence. Further analyses will reveal the mode of the interaction of the three proteins.

After asymmetric segregation, mira, pros and stau take different paths from each other in the wild-type GMC following brief cortical colocalization (Hirata et al., 1995; Knoblich et al., 1995; Spana and Doe, 1995): pros is translocated to the nucleus, stau remains in the cytoplasm and mira is rapidly degraded (Ikeshima-Kataoka et al., 1997; Shen et al., 1997). We do not know whether the degradation of mira causes the release of pros/stau from the GMC cortex. In the *mira*^{RR127} allele, the three proteins remains colocalized to the cell cortex with the punctate contour suggesting that mira fails to dissociate from pros and stau. This characteristic distribution is also seen in interphase NBs. These observations have three implications. First, the interaction of stau with mira is under the same control that acts between mira and pros. Second, this mechanism operates in both NBs and GMCs. Third, as discussed in our previous study (Ikeshima-Kataoka et al., 1997), the C-terminal 103 amino acids region, which is deleted from the mira^{RR127} protein, regulates the interactions between mira and pros/stau (Fig. 8).

In the late interphase of wild-type NBs, pros and stau are colocalized with mira to the apical cortex. Is this colocalization due to the interaction among the three proteins in NB interphase, too? In the previous study, we have demonstrated that the domain in pros protein necessary for the basal localization at NB mitosis is also required for its cytoplasmic retention during NB interphase (Hirata et al., 1995). Since this domain in pros has turned out to be the mira-binding site (Ikeshima-Kataoka et al., 1997), our previous result suggests that the association of pros with mira is necessary for its localization in interphase. This interpretation is consistent with our observations shown in this study using *mira* mutants. In the four *mira* mutants that do not localize pros/stau basally at mitosis (*mira*^{YY227}, *mira*^{JJ92}, *mira*^{AB78} and *mira*^{ZZ176}), those mira proteins, pros and stau exhibit distinct patterns of distribution from one another in interphase NBs: pros is detected only in the nucleus of *mira* mutant NBs even at late interphase, mutant mira shows a cortically uniform distribution and stau distributes evenly in the cytoplasm (newly synthesized stau in a given cell cycle is not distinguishable from stau that has been partitioned into the NB during the previous cell division). The localization of pros and stau during the NB interphase thus appears to depend on their association with mira as seen during mitosis. On the contrary, the distribution of pros/stau in the *mira*^{RR127} allele coincides with the apically punctuate localization of mira^{RR127}. This suggests that, in *mira*^{RR127} NBs, the three proteins associate with each other throughout interphase, which is consistent with the notion that the pros and stau protein localization is dictated by their association with mira. We therefore conclude that mira plays a central role for the localization of the other two proteins during both interphase and mitosis.

Regulation of mira protein localization

The asymmetric localization of mira together with pros and stau proteins is clearly observed starting from the late interphase throughout NB mitosis. mira distribution dramatically changes from the apical side to the basal cortex between interphase and mitotic phase. The N-terminal region of 290 amino acids in mira is sufficient for its basal localization at mitosis because all mutant mira proteins except undetectable mira^{L44} are localized in a basal crescent (Fig. 8). This result is consistent with the study of Shen et al. (1998) who identified functional domains of mira using myc-tagged transgenes. In our study, this region of mira does not appear sufficient for its apical concentration during NB interphase although it is sufficient for the cortical attachment of mira during interphase. This is suggested by the distinct distribution of mutant mira proteins from that of the wild-type mira in interphase NBs; four mutant mira proteins, which fail to associate with pros/stau, do not appear to be concentrated to the apical cortex, whereas, the longest mira protein, mira^{RR127}, which can associate with pros/stau at mitosis, is clearly localized to the apical cortex during interphase despite its highly punctate distribution. These results suggest that a domain necessary for mira apical localization at late interphase lies in part within the region defined by mira^{ZZ176} and mira^{RR127}, the region necessary for the association with pros/stau. However, the association of mira with pros/stau is not required for the apical localization of mira in the interphase NB because mira is normally localized to the apical cortex in *pros*-deficient or *stau*^{D3} embryos (our unpublished observation). These observations suggesting the inability of mutant mira proteins to localize apically in interphase NBs appear to contradict the result obtained by Shen et al. (1998), who showed that an myc-tagged N-terminal mira fragment (298 amino acids) is sufficient for apical localization. At present, we do not have a reasonable explanation for this discrepancy.

How is the mira protein complex localized asymmetrically at the NB cortex at mitosis? One possibility is the involvement of cytoskeletal elements. During *Drosophila* oogenesis, the posterior localization of *oskar* mRNA mediated by stau involves two cytoskeletal elements, microtubules and microfilaments (St Johnston, 1995). In budding yeast, the segregation of *ASH1* mRNA to the daughter cell depends on microfilaments (Long et al., 1997; Takizawa et al., 1997) and an unconventional myosin (Bobola et al., 1996; Jansen et al., 1996). At the first division of *C. elegans*, an unconventional myosin is involved in the asymmetric partition of PAR-1 kinase (Guo and Kemphues, 1996). In *Drosophila* NB divisions, a contribution of microtubules is unlikely because the formation of pros basal crescent is insensitive to microtubule-disrupting drug treatment (Knoblich et al., 1995). However, microfilament-disrupting reagents affect the basal localization of pros and mira (Broadus and Doe, 1997; Knoblich et al., 1997; Shen et al., 1998) suggesting the asymmetric localization of the mira protein complex is actin-dependent. The basal localization of mira is first observed as a wide crescent spreading on the NB cell cortex at prophase. The crescent is gradually condensed into the basal cap during mitosis. The mira complex thus appears to be translocated along the cell cortex from apical side to the basal side. These observations raise the possibility that the NB at mitosis contains a cytoskeletal network underlying the cell membrane, which acts to condense the mira complex unidirectionally in the basal direction.

Asymmetric protein localization common in the epithelial cell and NB

The three proteins described in this study are expressed in all epithelial cells in the two germ layers, the ectoderm and the endoderm in early embryogenesis prior to neurogenesis. Although the roles of their epithelial expression are unknown, we have found that the epithelial cells that express mira have the ability to localize mira to the cortex together with pros/stau at mitosis. As in NBs and GMCs, the localization of the mira protein complex is asymmetric but the division vertical to protein localization leads to the equal partition of the mira complex. In addition, the cell cycle dependence of the protein complex formation in epithelial cells is similar to that in NBs. These observations suggest that the epithelial cell and the NB share a common molecular machinery to tether mira asymmetrically to the cortex underneath the cell membrane.

There is a difference in the localization of the mira complex between epithelial cells and NBs; the mira complex is not observed at the apical cortex during interphase in epithelial cells. Instead the mira/pros complex distributes to the basal-lateral cortex in ectodermal cells, being excluded from the apical side throughout the cell cycle in embryos during stage 8 and 9 (Fig. 6A,B). In addition, some pros is separately localized to the nucleus (Fig. 6C). Epithelial cells thus lack the mechanism that localizes mira or the mira complex apically

even during late interphase, but the three proteins form the complex at the basolateral cortex during mitosis. These observations indicate that apical localization at late interphase is not necessary for the three proteins to form the complex basolaterally in dividing epithelial cells.

insc protein appears to be absent in epithelial cells in the ectoderm or endoderm (Kraut and Campos-Ortega, 1996). It begins to be expressed once a neuroectoderm cell is committed to the NB cell fate, and distributes to the apical cortex of NBs from interphase to metaphase (Kraut et al., 1996). Its apical localization is thought to be essential for insc to coordinate both spindle orientation and the axis of protein localization, such as mira, along the apical-basal axis (Kraut et al., 1996; Shen et al. 1997). When insc is ectopically expressed in epithelia from a transgene, it distributes to the apical cortex in those cells and rotates the division axis parallel to the apicobasal axis (Kraut et al., 1996). The apical localization of ectopically expressed insc in epithelial cells implies that those cells have the ability to localize insc apically. The absence of insc in epithelial cells might be a reason for the absence of apically localized mira complex in interphase epithelial cells. It would be interesting to see where the mira complex is localized in epithelial cells expressing insc ectopically. Apical insc localization in epithelial and NB cells has suggested a similarity in the determination of apical sides in the two cell types. By the same token, the basolateral localization of the mira complex suggests a similarity in the basal cortex between the NB and the epithelial cell. We suggest that epithelial polarity shares common characteristics with the polarity that creates polarized protein localization in the NB. This appears to be reasonable because the NB and the endodermal stem-like cell are generated from the epithelial cell layer. Those cells of epithelial origin may inherit their asymmetric molecular organization from epithelial cells when they are committed within the epithelial cell layer.

Studies using vertebrate systems have revealed that cell-cell interactions play essential roles in the formation of epithelial polarity (reviewed by Drubin and Nelson, 1996); for example, apically localized adherens junctions play critical roles. However, the basal extracellular matrix also plays roles in conferring basal characteristics on cells. Genetic studies using *Drosophila* have revealed several factors required for epithelial cell formation such as E-cadherin (Tepass et al., 1996; Uemura et al. 1996) and crumbs (Knust, 1994). Zygotic mutations of those factors, however, have little effect on the neuroectoderm during stages when NBs delaminate. Consistently, the basolateral localization of the mira complex appears to be normal in dividing epithelial cells of embryos homozygous for those mutations (Matsuzaki, unpublished observation); Maternally supplied gene products may be sufficient for epithelial formation during these stages (Tepass et al., 1996; Uemura et al. 1996). Identification of components that localize the mira complex would open the way to elucidate the common mechanism in the formation of both the NB and epithelial polarity.

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