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The GC kinase Fray and Mo25 regulate *Drosophila* asymmetric divisions

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Abstract

Drosophila neuroblasts provide an excellent model for asymmetric cell divisions, where cell-fate determinants such as Miranda localize at the basal cortex and segregate to one daughter cell. Mechanisms underlying this process, however, remain elusive. We found that Mo25 and the GC kinase Fray act in this regulation. *mo25* and *fray* mutants show an indistinguishable defect in Miranda localization. On the other hand, *Drosophila* Mo25 interacts with the tumor suppressor kinase Lkb1 *in vivo,* as have shown in mammals. Overexpression of Lkb1, which accumulates in the cell cortex, drastically relocalizes both Mo25 and Fray from the cytoplasm to the cortex, causing the same phenotype as *mo25*-mutant neuroblasts. Recovery from this defect caused by Lkb1 overexpression requires simultaneous overexpression of Mo25 and Fray. We suggest from those results that Mo25 and Fray operate together or in the same pathway in *Drosophila* asymmetric processes, and that their function counterbalances Lkb1.

Keywords: Mo25; Fray; Lkb1; GC kinase; Miranda; Prospero; Drosophila; Neuroblast; Asymmetric division; Cell polarity

During *Drosophila* neural development, neuroblasts repeat typical asymmetric divisions into a neuroblast and a ganglion mother cell (GMC), which cleaves once to produce neurons and glia. Neuroblasts have provided an excellent model to study molecular mechanisms of asymmetric divisions [1,2]. During neuroblast divisions, cell-fate determinants (Prospero and Numb) and their adaptor proteins (Miranda and Pon) are localized asymmetrically to the basal cortex and the spindle is oriented along the apical-basal axis, resulting in the asymmetric inheritance of the determinants to the GMC. The localization of the determinants relies on the function of the evolutionarily conserved protein complex aPKC/Par3/Par6 [3–5] and the tumor suppressor Lgl [6,7]. The mechanisms underlying the neuroblast asymmetric division, however, remain elusive.

In this study, we have screen maternal effect lethal mutants to identify genes regulating of the neuroblast asymmetric division, and found two genes *mo25* and *frayed* (*fray*). Mo25 protein is highly conserved in all eukaryotes [9]. Fray is a serine threonine kinase belonging to the germinal center kinase GCK family [10–12]. In mammals, Mo25 interacts with the GCK family pseudokinase STRAD to activate the serine threonine kinase Lkb1, whose mutations are known to cause the Peutz-Jeghers cancer syndrome [13,14]. In fission yeast, Mo25 binds and activates the GCK family authentic kinase Nak1 [15]. In both cases, Mo25 and GCK family proteins regulate the cell polarity. Our study reveals that Mo25 and Fray play indistinguishable roles in regulating asymmetric properties in embryonic neuroblasts, suggesting the conserved partnership between Mo25 and GCK in *Drosophila*. We also show that Lkb1 can affect the subcellular distribution of both Mo25 and Fray in the same manner, while the role of Lkb1 in this regulation remains elusive.

Materials and methods

Fly stocks. Lethal P-insertion lines provided by N. Perrimon [16] were screened by making germ line clone (GLC) embryos, using the FLP-DFS

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technique [17]. The $mo25^{D8\text{-}2}$ allele was generated by imprecise excision of the P element insertion from *l(3)00274*. $lkb1^{4A4\text{–}2}$ and $lkb1^{4B1\text{–}11}$ were provided from D. St. Johnston [18]. *UAS-Lgl3A* [8] was expressed using the maternal GAL4 V32 [19]. Transgenic lines, *UAS-FLAG-Lkb1*, *UAS-FLAG-Lkb1(K201M)*, *UAS-GFP-Lkb1*, *UAS-FLAG-Fray* and *UAS-Myc-Mo25* were generated by germline transformation and crossed with maternal GAL4 V32 or *prospero*-GAL4 [6] to express transgenes.

DNA construction. *pUAST-FLAG-Lkb1* and *pUAST-FLAG-Fray* were constructed by cloning the XhoI–XbaI fragment of *Lkb1* cDNA (GM01569) and XhoI–SpeI fragment of *Fray* cDNA (LD15845) into the XhoI–XbaI sites of pUAST-FLAG vector (derived from pUAST vector [20], containing *MFLAG* sequence), respectively. *pUAST-GFP-Lkb1* was constructed by inserting the EcoRI–XhoI fragment of *GFPS65T* and the XhoI–XbaI fragment of *Lkb1* cDNA in-flame into pUAST vector [20]. For *pUAST-Myc-Mo25*, *Mo25* cDNA (LD09950) was first subcloned into pCMV-Myc vector (BD Biosciences), and the *Myc-Mo25* fragment was cloned into the XhoI–XbaI sites of pUAST vector. The kinase negative form of Lkb1 (K201M) was generated by site directed mutagenesis with QuikChange II kit (Stratagen).

Immunostaining and imaging. Embryos were fixed with 4% of paraformaldehyde for 20 min. Primary antibodies used were: rabbit anti-Miranda [21], mouse anti-Miranda [6], rabbit anti-DmPar6 [22], rabbit anti-Bazooka [6], rabbit anti-PKCζ C20 (Santa Cruz Biotechnology, Inc.), rabbit anti-Lgl [6], rabbit anti-Pins [23], mouse anti-FLAG (Sigma–Aldrich), mouse anti-β galactosidase (Promega, Madison, WI). Rabbit anti-Mo25 and rabbit anti-Fray were generated as described below. All images were collected using confocal microscope (Radiance 2100; Bio-Rad), and processed with Adobe Photoshop.

Immunoprecipitation. Embryos lacking both maternal and zygotic *mo25* were prepared by crossing females harboring $mo25^{D8\text{-}2}$ GLCs with $mo25^{D8\text{-}2}$ homozygous males rescued by its transgene: *y*, *w*, *hsFLP*/ *w*; $mo25^{D8\text{-}2}$, *FRT2A*/ ovo^{D1}, *FRT2A* and *w*; $mo25^{D8\text{-}2}$, *da*-GAL4/ $mo25^{D8\text{-}2}$, *UAS-FLAG-Mo25*. Wild type and *mo25* null mutant embryos were collected and homogenized with a 5-fold volume of the lysis buffer (25 mM Tris–HCl, pH7.5, 150 mM NaCl, 1 mM EDTA and 1% Triton X-100) containing protease inhibitor cocktail (P8340; Sigma). The embryo lysates were centrifuged at 14000 rpm for 30 min. The supernatants were pre-adsorbed for 30 min and used for immunoprecipitation with proteinG sepharose beads (Amersham-Pharmacia) conjugated by rabbit anti-Mo25, rabbit anti-Fray and rabbit anti-Lkb1 antibodies. After incubation at 4 °C for 2 h, beads were washed 6 times with the lysis buffer and mixed with SDS–PAGE sample buffer. Samples were analyzed by SDS–PAGE and Western blots with same antibodies used for immunoprecipitation.

Generation of antibodies. The full-length of *Drosophila Mo25* cDNA, the 1 Kb cDNA fragment encoding the Fray C-terminus, and the 0.5 Kb cDNA fragments encoding N- and C-terminus of *Drosophila* Lkb1 were cloned into pGEX vector (Amersham-Pharmacia, Piscataway, NJ). GST-fusion proteins were expressed in *Escherichia coli*, and used to generate rabbit polyclonal antibodies (MBL, Nagoya, Japan). The GST-fusion protein of Lkb1, which was purified from bacterial cultures, was subjected for generation of rabbit polyclonal antibodies (Takara Bio, Shiga, Japan).

Results

Mo25 and Fray are required for proper localization of Miranda in embryonic neuroblasts

In this study, we have focused lethal P-element insertion lines showing maternal effects, which include those identified by Perrimon et al. [16]. We made germ line clone (GLC) embryos both maternaly and zygotically mutant for those P-insertions, and stained for Miranda to examine the asymmetry of neuroblasts. Two mutants, *l(3)00274* and *l(3)07551,* showed similar defects in the distribution of Miranda. In wild type, metaphase neuroblasts localized Miranda exclusively to the basal cell cortex (Fig. 1A). In neuroblasts in those two mutants, a significant amount of Miranda was distributed to the cytoplasm and concentrated near mitotic spindles, although a proportion of Miranda remains diffusely on the basal cortex (Fig. 1). This defect in Miranda localization is similar to that previously reported for *lgl* mutants [6,7].

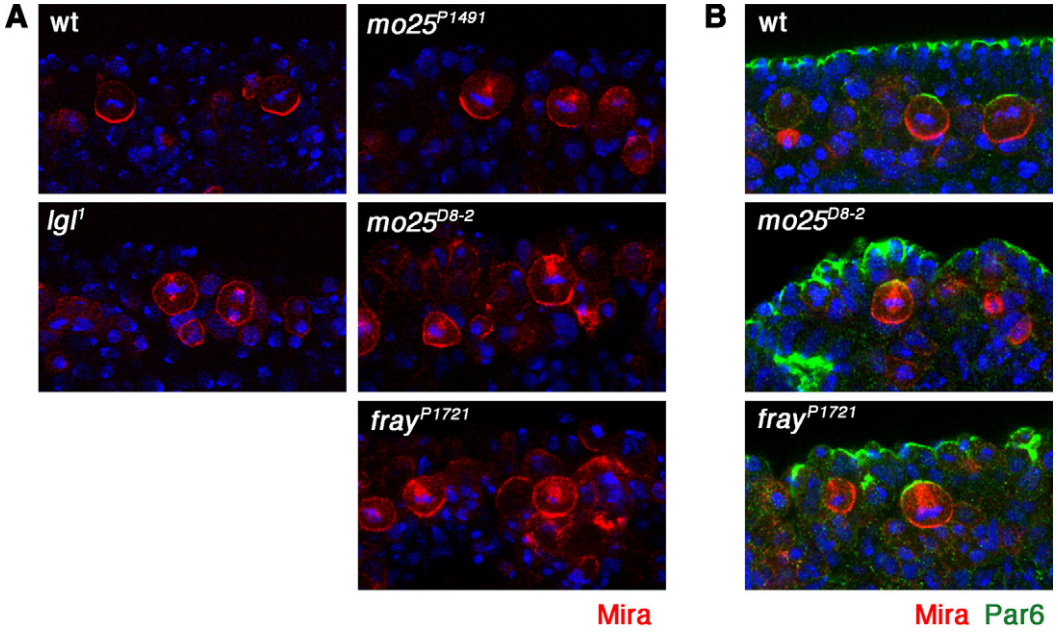

Fig. 1. *mo25* and *fray* mutants show abnormal Miranda localization in embryonic neuroblasts. (A) Neuroblasts stained for Miranda (red) and DNA (blue) in the wild type (wt) embryos, GLC embryos mutant for *l(3)00274* ($mo25^{P1491}$), $mo25^{D8\text{-}2}$, and *l(3)07551* ($fray^{P1721}$) as well as for lgl^{1}. (B) Neuroblasts and epithelial cells stained for Par6 (green), Miranda (red) and DNA (blue) in wild type (wt) embryos, $mo25^{D8\text{-}2}$ GLC embryos and $fray^{P1721}$ GLC embryos. Apical is up in all figures.

The P-element was inserted in the 5′ UTR of the *mo25* gene in *l(3)00274*, and in the first intron of *frayed (fray)* gene in *l(3)07551*, respectively. Antibodies were raised against both Mo25 and Fray to examine their distribution in embryos. Antibody staining showed that those proteins were ubiquitously expressed in embryos. Both distribute uniformly in the cytoplasm of epithelial cells and neuroblasts, with an occasionally slight concentration along the cell cortex (Fig. 3B and see below).

To investigate null phenotypes for *mo25*, we generated new mutant alleles by the imprecise excision of the P-element. The $mo25^{D8\text{-}2}$ allele completely lacks the *mo25* open reading frame. GLC embryos for this allele showed a Miranda phenotype almost identical with that of *l(3)00274* GLC embryos; Miranda is both cytoplasmic and cortical in neuroblasts.

Mo25 and Fray are not required for the apical localization of the Par6/Baz/aPKC complex

Epithelia became bumpy in $mo25^{D8\text{-}2}$ and *fray (l(3)07551)* GLC embryos, and individual epithelial cells sometime showed a round, rather than cuboidal shape, suggesting that the epithelial polarity was affected in those mutants. We, therefore, examined the localization of the apical components (Par6/Baz/aPKC) in those GLC embryos. Par6 (Fig. 1B) as well as Baz and aPKC (data not shown) appeared to normally localize to the apical cortex in both epithelial cells and neuroblasts. Thus Mo25 and Fray are unlikely to be essential for the normal localization of the Par6/Baz/aPKC complex, suggesting that Mo25 and Fray function downstream or independently of the Par6/Baz/aPKC complex to regulate Miranda localization.

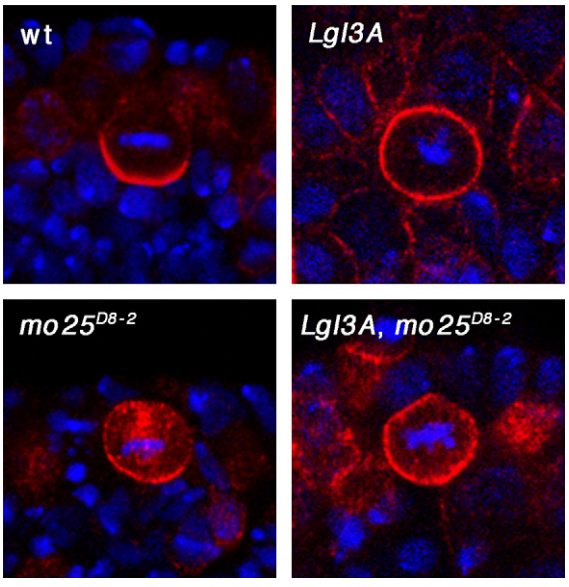

Fig. 2. Mo25 is dispensable for Lgl to localize Miranda cortically. Overexpression of Lgl3A causes Miranda to distribute uniformly along the neuroblast cortex in the wild type (wt). Lgl3A causes a similar phenotype in $mo25^{D8\text{-}2}$ GLC embryos whereas the loss of Mo25 results in the cytoplasmic distribution of Miranda.

Mo25 is not required for Lgl to localize Miranda

The similarity of *mo25* and *lgl* mutants in the Miranda mislocalization phenotype prompted us to investigate the relationship between Mo25 and Lgl. aPKC directly phosphorylates Lgl, allowing it to fall off the cell cortex. Such apical inactivation and basal activation of Lgl are thought to cause basal targeting of Miranda [8]. We first examined the subcellular distribution of Lgl in $mo25^{D8\text{-}2}$ and *fray* GLC embryos. There was no obvious change in the distribution of Lgl between those mutants and wild type, where Lgl was virtually cortical in interphase (on the basal–lateral cortex in epithelial cells and on the entire cortex in neuroblasts), while it significantly spread into the cytoplasm during mitosis (data not shown).

We next examined the effect of overexpression of the non-phosphorylatable form of Lgl, Lgl3A, in wild type and *mo25* null embryos. Lgl3A, in which three phosphorylation sites by aPKC are mutated, functions as a constitutively active form of Lgl in neuroblasts [8]. Lgl3A is resistant to the inactivation by aPKC, and its overexpression causes Miranda to distribute along the entire cortex ([8] and Fig. 2). If Mo25 acts downstream of Lgl, the phenotype caused by overexpressing Lgl3A should change by the loss of mo25. We observed that overexpression of Lgl3A induced the uniformly cortical distribution of Miranda in both $mo25^{D8\text{-}2}$ GLC and wild type embryos (Fig. 2), suggesting that Lgl does not require Mo25 to localize Miranda to the cortex. Thus Mo25 is unlikely to act downstream of Lgl.

Mo25 interacts with Lkb1 in Drosophila embryos

It is known that Mo25 interacts with Lkb1 as well as GCK members in other organisms [13,15]. To investigate the relationship of Mo25 and Fray with Lkb1, we first analyzed interactions between Mo25, Fray and Lkb1 by co-immunoprecipitation assay with embryonic extracts. We found that the endogenous Mo25 and Lkb1 were co-immunoprecipitated reciprocally, strongly suggesting that Lkb1 interacts with Mo25 in *Drosophila* as well (Fig. 3A). We did not obtain clear evidence regarding the interaction of endogenous Fray with Mo25 or Lkb1 by this assay (Fig. 3A), although interactions among those three proteins were observed by similar assays with *Drosophila* S2 cells exogenously expressing them (data not shown).

Lkb1 overexpression phenocopies the mo25 and fray mutant phenotype

To investigate the role of Lkb1 in the regulation of Miranda localization in the neuroblast, we attempted to analyze *lkb1* mutant embryos, using two different alleles, $lkb1^{4A\text{-}4\text{-}2}$ and $lkb1^{4B\text{-}1\text{-}11}$ [18]. Embryos homozygous for those alleles did not show any obvious defect in neuroblast divisions probably due to the maternal contribution of *lkb1*. We also generated *lkb1* GLC embryos lacking *lkb1,*

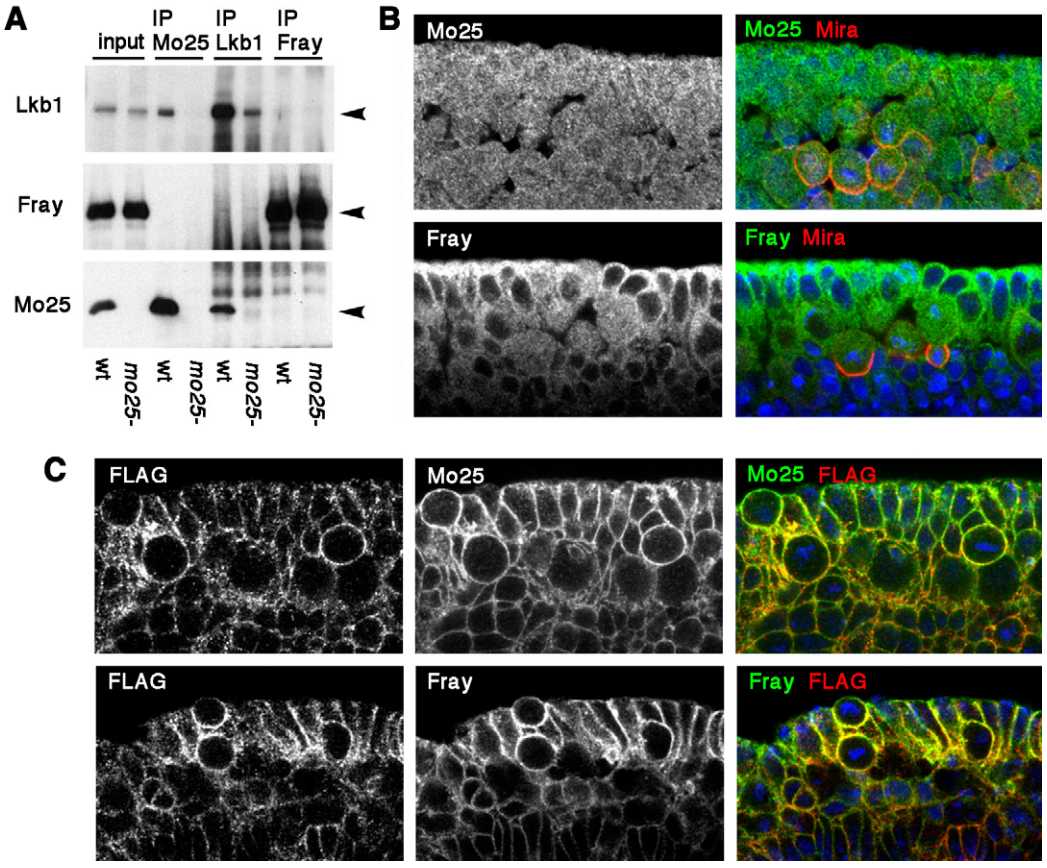

Fig. 3. Mo25 and Fray proteins interact with Lkb1 in *Drosophila* embryos. (A) Immunoprecipitation of embryonic lysates using anti-Mo25, anti-Lkb1 and anti-Fray antibodies. Lysates from wild type (wt) embryos or those lacking both maternal and zygotic Mo25 (mo25-) were analyzed. Mo25 and Lkb1 co-immunoprecipitate reciprocally, whereas neither Mo25 nor Lkb1 co-immunoprecipitate with Fray. (B) Distribution of Mo25 and Fray proteins in wild type embryos. The endogenous Mo25 and Fray proteins diffusely distribute in the cytoplasm in epithelial cells and neuroblasts. Fray but not Mo25 is excluded from the nucleus in interphase. (C) Distribution of Mo25 and Fray in embryos overexpressing Lkb1. The overexpressed Lkb1 localizes to the cell cortex of the epithelial cells and neuroblasts. Mo25 and Fray colocalize with the overexpressed Lkb1 at the cell cortex and are excluded from the cytoplasm in those cells.

but those embryos did not develop beyond cellularization, making it impossible to examine the *lkb1* loss-of-function phenotype in neuroblast divisions.

We then looked at the effect of Lkb1 overexpression in embryos by the GAL4-UAS system [20] with the maternal GAL4 driver [18]. Embryos overexpressing the wild type Lkb1 were abnormally shrunk (Fig. 4B). In those embryos, neuroblasts redistributed Miranda more broadly along the cortex and also to mitotic spindles as observed in *mo25* and *fray* GLC embryos (Fig. 4A). Because the same phenotype in Miranda distribution was observed by the neuroblast-specific overexpression of Lkb1 with the *prospero*-GAL4 driver (data not shown), this neuroblast phenotype was caused independently of the morphological defect of embryos. Strikingly the same defect in Miranda distribution was observed even when Lkb1 lacking its kinase activity was overexpressed (Fig. 4A). This result raises the possibility that the effect of Lkb1 kinase overexpression does not depend on its kinase activity, but on an excess amount of the Lkb1 protein.

We examined the effect of Lkb1 overexpression on the distribution of Mo25 and Fray. Although the endogenous Lkb1 failed to be detected in embryos due to the lack of available antibodies, overexpressed Lkb1 was found along the cell cortex in both epithelial cells and neuroblasts. In embryos overexpressing Lkb1, Mo25 and Fray were clearly excluded from the cytoplasm and co-localized with Lkb1 at the cell cortex (Fig. 3C). Overexpressed Lkb1 thus recruits Mo25 and Fray from the cytoplasm to the cell cortex. This result suggests that Lkb1 is able to interact with Mo25 and Fray directly or indirectly *in vivo*.

Given the possibility that Mo25 and Fray interact with Lkb1, the dosage balance among the three proteins may be important for their normal function. We therefore investigated if a simultaneous overexpression of Mo25 and/or Fray with Lkb1 rescues the defective Miranda localization due to Lkb1 overexpression. When either Mo25 or Fray alone was overxepresssed with Lkb1 in embryos, neuroblasts still showed an abnormal Miranda localization (data not shown). In contrast, when both Mo25 and Fray were simultaneously overexpressed together with Lkb1, neuroblasts recovered the normal cortical crescents of Miranda (Fig. 4B). The same was true for the embryo morphology, which became a normal shape by the triple overexpression

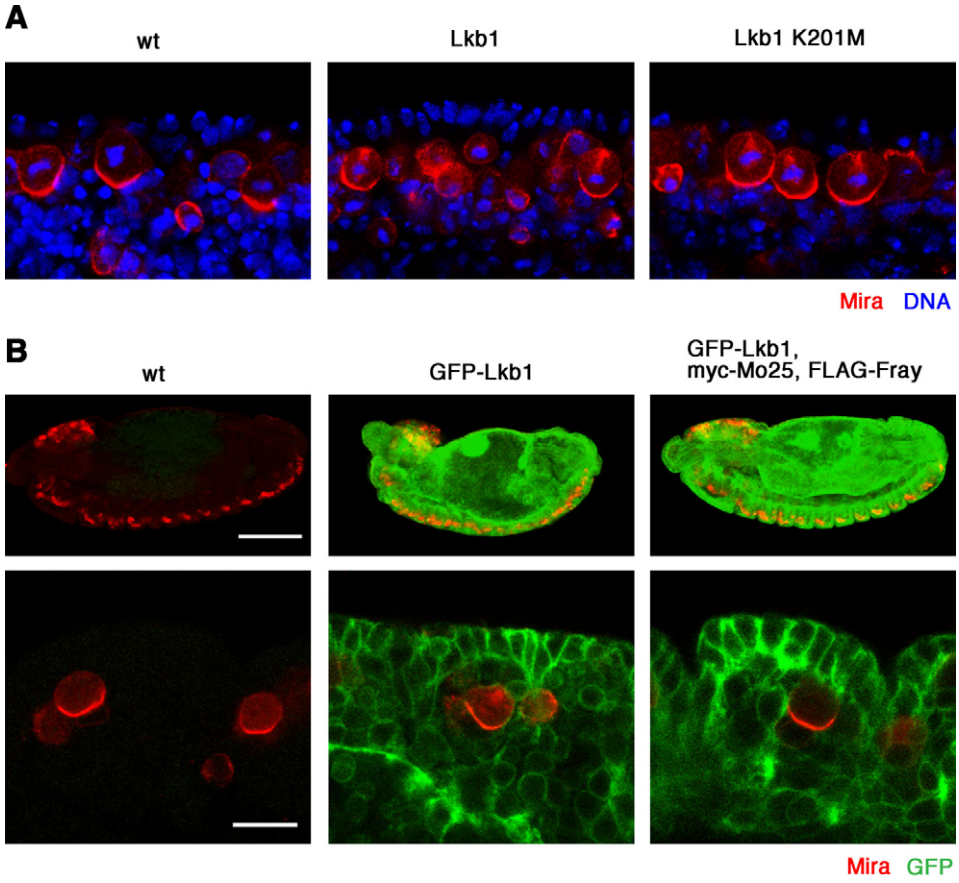

Fig. 4. Overexpression of Lkb1 phenocopies the loss of Mo25 and Fray in embryos. (A) Distribution of Miranda in embryos overexpressing the wild type Lkb1 (Lkb1) or the kinase negative Lkb1 (K201M). (B) Suppression of phenotypes caused by Lkb1 overexpression. A shrunken morphology along the anterior-posterior axis of embryos (upper central panel) and abnormal Miranda distribution in neuroblasts (lower central panel) are observed in embryos overexpressing Lkb1 (GFP-Lkb1), but not in embryos overexpressing myc-Mo25 and Flag-Fray simultaneously with GFP-Lkb1. Embryos are at stage 13. GFP-Lkb1 is in green and Miranda is red. Scale bars: 100 μm (upper) and 10 μm (lower).

(Fig. 4B). These results indicate that the dosage balance among Mo25, Fray and Lkb1 is critical for their functions not only in the normal Miranda localization in neuroblasts but also in other cells.

Discussion

In this study, we have shown that the asymmetric localization of cell fate determinants in *Drosophila* neuroblasts involves two molecules: Mo25, an accessory protein of kinase complexes, and the GCK family kinase Fray. Previous studies on other species have revealed that Mo25 acts through GCK members in two distinct ways. In mammals, Mo25 and the GCK family pseudo-kinase STRAD operate together as cofactors of Lkb1 kinase to activate its activity, and regulate epithelial and neuronal polarity [13,14,24–26]. On the other hand, Mo25 in fission yeast associates with the GCK family authentic kinase Nak1, and directly regulates its kinase activity to activate the downstream kinase Orb6 [15]. The question is how *Drosophila* Mo25 act in neuroblasts, via Lkb1 kinase with an unidentified STRAD ortholog, or through a real kinase belonging to the GCK family?

We demonstrated that *Drosophila* Lkb1 interacts with Mo25 *in vivo* (Fig. 3), suggesting that Mo25 can function with Lkb1 as in mammals. A recent study, using *Drosophila lkb1* GLC embryos, reported that Lkb1 functions upstream of AMP-activated protein kinase AMPK in the regulation of the epithelial polarity [27]. We however failed to observe that GLC embryos for the *lkb1* alleles used in this study (as well as $lkb1^{X5}$ used in ref. [27]) reach the cellularization stage, thereby making it impossible to confirm the *lkb1* neuroblast phenotype in embryos. Another recent work has suggested that the apical components such as Baz, Par6 and aPKC as well as the basal component Miranda mislocalize in *Drosophila* larval neuroblasts mutant for *lkb1* [28]. Nonetheless, we observed relatively normal crescents of Miranda as well as Par6 and Pins in larval neuroblasts of zygotic *lkb1* mutants (data not shown). Thus we have so far failed to obtain evidence supporting that the Miranda localization defect in the *mo25*-null embryos is mediated by Lkb1.

We have found that the overexpression of Lkb1 causes the same Miranda mislocalization phenotype as that in *mo25* (or *fray*) mutants (Figs. 4 and 1). This effect does not appear to require the Lkb1 kinase activity because

the kinase-defective version of Lkb1 causes the same effect (Fig. 4A). The drastic relocalization of Mo25 and Fray to the cell cortex in this situation raises the possibility that the Lkb1 overexpression causes abnormal Miranda localization by sequestering Mo25 and/or Fray to the cortex from the cytoplasm. This model assumes that the cytoplasmic function of Mo25 and/or Fray is important for Miranda to normally localize to the cortex. The absence of those proteins in the cytoplasm will cause abnormal Miranda localization in *mo25* and *fray* mutants, or when Lkb1 is overexpressed. As an alternative scenario, Lkb1 on the cell cortex may have an effect on Miranda localization through its regulatory function of cell polarity. This cortical Lkb1 activity may be negatively regulated by Mo25 and Fray in the wild type situation. If so, an excess Lkb1 or the absence of Mo25 (or Fray) results in enhancing this effect, leading to the abnormal Miranda distribution.

Although there is no solid evidence for the interrelationship between Mo25 and Fray kinase, three lines of evidence suggest that they operate together or in the same pathway. First, both *mo25* and *fray* mutants show the indistinguishable phenotypes in embryos (Fig. 1). Second, the overexpression of Lkb1 relocalizes both Mo25 and Fray to the cell cortex (Fig. 3B and C). Third, the simultaneous overexpression of the two proteins is required to rescue the Miranda mislocalization phenotype upon Lkb1 overexpression (Fig. 4B). This mode of *mo25* function is similar to the case where Mo25 activates the GCK Nak1 in fission yeast [15].

In this study, we have shown that Mo25 and Fray play important roles in polarized cellular processes in *Drosophila* embryos, and proposed the two possible ways where Mo25 operates, one through the association of Lkb1 kinase and another through the Fray kinase. These two kinases appear to be counterbalanced, but all three of Mo25, Fray and Lkb1 may operate together or in the related pathways. To pursue these issues, it is essential to analyze *lkb1* mutant phenotypes in relation to *mo25* and *fray* functions.

Acknowledgments

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