Microtubule-Induced Pins/Gap Cortical Polarity in Drosophila Neuroblasts

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SUMMARY

Cortical polarity regulates cell division, migration, and differentiation. Microtubules induce cortical polarity in yeast, but few examples are known in metazoans. We show that astral microtubules, kinesin Khc-73, and Discs large (Dlg) induce cortical polarization of Pins/Gap in Drosophila neuroblasts; this cortical domain is functional for generating spindle asymmetry, daughter-cell-size asymmetry, and distinct sibling fates. Khc-73 localizes to astral microtubule plus ends, and Dlg/Khc-73 and Dlg/Pins co-immunoprecipitate, suggesting that microtubules induce Pins/Gap cortical polarity through Dlg/Khc-73 interactions. The microtubule/Khc-73/Dlg pathway acts in parallel to the well-characterized Inscutable/Par pathway, but each provides unique spatial and temporal information: The Inscutable/Par pathway initiates at prophase to coordinate neuroblast cortical polarity with CNS tissue polarity, whereas the microtubule/Khc-73/Dlg pathway functions at metaphase to coordinate neuroblast cortical polarity with the mitotic spindle axis. These results identify a role for microtubules in polarizing the neuroblast cortex, a fundamental step for generating cell diversity through asymmetric cell division.

INTRODUCTION

Cell polarity is essential for asymmetric cell division, cell migration, and the proper function of many differentiated cell types. We are interested in how cell polarity is generated in Drosophila neuroblasts, a model system for studying cortical polarity and asymmetric cell division (reviewed in Betschinger and Knoblich, 2004). Embryonic neuroblasts delaminate from an apical/basal polarized epithelium and divide asymmetrically along the apical/basal axis. Mitotic neuroblasts form distinct apical/basal cortical domains and assemble a morphologically asymmetric mitotic spindle, which aligns with the apical/basal axis to generate daughter cells with different sizes and cell fates. The apical neuroblast is larger and proliferative, whereas the basal ganglion mother cell (GMC) is smaller and only divides once before differentiating into neurons or glia.

Neuroblast cortical polarity regulates all known aspects of neuroblast asymmetric cell division, and thus it is essential to understand how cortical polarity is established. Cortical polarity is first visible at late interphase, when a large number of proteins are targeted to the apical cortex. These include Bazooka (Baz; Par-3 in worms and mammals), aPKC (atypical protein kinase C), and Par-6 proteins (hereafter called the Par complex) as well as the Inscutable (Insc), Partner of Inscutable (Pins), and Gai proteins (reviewed in Betschinger and Knoblich, 2004). In addition, during metaphase there is a transient apical enrichment of nonmuscle myosin II (Barros et al., 2003) and the cortical tumor suppressor proteins Disks large (Dlg), Scribble (Scrib), and Lethal giant larvae (Lgl) (Albertson and Doe, 2003).

Recent work has highlighted the central role played by the two apical cortical proteins, Pins and Gai, in regulating asymmetric cell division and spindle-cortex interactions from worms to mammals (Cai et al., 2003; Colombo et al., 2003; Du and Macara, 2004; Du et al., 2001; Fuse et al., 2003; Gotta et al., 2003; Parmentier et al., 2000; Schaefer et al., 2000, 2001; Srinivasan et al., 2003; Yu et al., 2000, 2003). Gai can form a heterotrimer with Gβ/γ proteins, while Pins has three Gαi binding GoLoco domains as well as seven tetracontapeptide repeats (TPPRs) that directly bind Insc. In all animals studied to date, Pins is proposed to activate both Gαi and Gβ/γ in a receptor-independent fashion by binding Gai and disrupting the inactive Gαi/Gβ/γ heterotrimer (reviewed in Willard et al., 2004).

Despite the importance of Pins/Gai cortical polarity in regulating Drosophila asymmetric cell division, it remains unclear how Pins/Gai cortical localization is regulated. In neuroblasts, Insc was initially reported as essential for Pins/Gai cortical localization (Schaefer et al., 2000, 2001; Yu et al., 2000); however, several recent papers have suggested otherwise (Cai et al., 2003; Yu et al., 2003; Yu et al., 2002). In adult sensory organ precursors (SOPs), Insc is not expressed, yet SOPs undergo asymmetric cell division mediated by asymmetrically
polarized Pins/Gαi (Bellaiche et al., 2001; Schaefer et al., 2001).

Here we describe a novel astral microtubule-dependent pathway for inducing Pins/Gαi cortical polarity that includes the plus-end-directed microtubule motor protein, kinesin heavy chain 73 (Khc-73), and the membrane-associated guanylate kinase (MAGUK) protein,Dlg. We find that the microtubule-dependent Khc-73/Dlg pathway and the cortical Insc/Par polarity pathway are partially redundant for inducing Pins/Gαi cortical polarity in neuroblasts yet have unique functions: The Insc/Par pathway is active at late interphase and coordinates neuroblast cortical polarity with CNS tissue polarity, whereas the Khc-73/Dlg pathway is active at metaphase and coordinates neuroblast cortical polarity with the mitotic spindle axis. 

RESULTS

A current model for the establishment of neuroblast cortical polarity is that an unknown cue recruits Baz, aPKC, Par-6, and Insc to the apical cortex of the neuroblast just prior to prophase, which is closely followed by the apical recruitment of Pins/Gαi proteins, presumably via Insc-Pins direct interactions (reviewed in Betschinger and Knoblich, 2004). We term this cortical “Insc/Par pathway” of Pins/Gαi localization to distinguish it from the Insc-independent “microtubule-based pathway” of Pins/Gαi localization that is the focus of this paper.

Astral Microtubules Can Induce Pins/Gαi/Dlg Cortical Polarity in Metaphase Neuroblasts

We confirmed that insc−/− null mutant embryos (insc mutants) lack apical localization of the Insc/Par complex proteins (Insc, Baz, aPKC, and Par-6; Figures 1H–1J, data not shown) (Petronczki and Knoblich, 2001; Wodarz et al., 1999, 2000), but interestingly we found that Pins, Gαi, and Dlg still form robust crescents in the majority of insc mutant metaphase neuroblasts (Figures 1B–1E and 1O). Similar results were observed in mitotic neuroblasts from embryos homozygous for the TE35 deficiency in which insc is not transcribed (Figure S1A) (Cai et al., 2001). Although Pins/Gαi/Dlg crescents form in insc mutants, the timing and position of crescent formation differed from wild-type. First, in wild-type neuroblasts Pins/Gαi/Dlg crescents always formed at the apical surface adjacent to the overlying ectoderm, whereas in insc mutant neuroblasts Pins/Gαi/Dlg crescents were found at all positions around the cortex (compare crescent position in Figure 1A with those in Figures 1B–1E and S1A). Second, in wild-type neuroblasts Pins/Gαi/Dlg crescents formed by early prophase (94%, n = 50; data not shown), whereas in insc mutants Pins/Gαi crescents were not detected at prophase (0%, n = 54; data not shown) but only at metaphase (78%, n = 100; Figure 1O). These results suggest that there is an Insc/Par-independent pathway that is active at metaphase to induce formation of Pins/Gαi/Dlg cortical crescents.

A clue to the identity of the Insc/Par-independent pathway was the observation that Pins/Gαi/Dlg crescents were always localized over one spindle pole, which can be mispositioned relative to the overlying ectoderm in insc mutants (Figures 1D, 1E, and S1A). This observation suggested that either spindle microtubules induced cortical polarity, or cortical polarity formed spontaneously at a nonapical position and induced spindle alignment. To distinguish between these mechanisms, we depolymerized microtubules in insc mutant neuroblasts with Colcemid and scored for Pins/Gαi/Dlg cortical crescents. We found that Colcemid treatment of insc mutant neuroblasts resulted in a nearly complete loss of Pins/Gαi/Dlg crescents: Pins is mostly cytoplasmic and Gαi/Dlg are uniform cortical (Figures 1F and 1O; data not shown). In contrast, Colcemid treatment of wild-type neuroblasts had no effect on Pins/Gαi/Dlg crescent formation (Figures 1G and 1O; data not shown), likely due to the association of Pins/Gαi/Dlg with the apical Insc/Par complex. In fact, the Insc/Par pathway of Pins/Gαi/Dlg localization requires only Insc and Baz proteins, because aPKC mutants that lack aPKC/Par-6 protein localization but retain Baz/Insc localization still formed Pins/Gαi/Dlg crescents in the absence of microtubules (Figures 1O, S1B, and S1C). We conclude that spindle microtubules have the ability to induce Pins/Gαi/Dlg cortical crescents over one spindle pole in the absence of an Insc/Par pathway.

We next tested whether astral microtubules were required for inducing Pins/Gαi/Dlg cortical polarity using both pharmacological and genetic methods. First, we selectively abolished astral microtubules with low concentrations of nocodazole. Wild-type nocodazole-treated neuroblasts had an intact central spindle but lacked detectable astral microtubules as judged by tubulin staining (Figure S2B) and failed to align their spindle with cortical polarity markers (Figure 1K and Table S1), similar to cnn mutants lacking astral microtubules (Megraw et al., 2001). Despite loss of astral microtubules, wild-type nocodazole-treated neuroblasts progressed through mitosis and showed normal colocalization of Pins/Gαi/Dlg with Insc/Par complex proteins at the cortex (Figures 1K and 1O; data not shown). In contrast, insc mutant neuroblasts treated with nocodazole showed a dose-dependent loss of Pins/Gαi/Dlg crescent formation (Figures 1L and 1O, and Supplemental Figures 2C and 2D), suggesting that astral microtubules were required to induce Pins/Gαi/Dlg crescent formation. In addition, we abolished astral microtubules genetically using the fizzy5 mutation. fizzy encodes an essential component of the anaphase-promoting complex, and fizzy mutant neuroblasts show a delay in cell cycle as well as short barrel shaped spindles that lack astral microtubules (Dawson et al., 1995) (Supplemental Figure 2E). fizzy single mutant neuroblasts formed normal Pins/Gαi/Dlg crescents colocalized with Insc/Par, but insc fizzy double mutant neuroblasts showed a loss of Pins/Gαi/Dlg crescents: Pins was delocalized from the cortex, and Gαi/Dlg were uniform cortical (Figures 1M and 1N; data not shown). Thus, both microtubule inhibitor and genetic data allow us to conclude that astral microtubules are required to induce Pins/Gαi/Dlg crescents in the absence of apical Insc/Par complex proteins, revealing a “microtubule-to-cortex” signaling pathway that is sufficient to generate cortical cell polarity.

Importantly, the microtubule-induced Pins/Gαi/Dlg cortical crescents are functional because insc mutant neuroblasts...
have a normal asymmetric mitotic spindle morphology, cell size asymmetry, and neuroblast/GMC identity (Table S1; data not shown) (Kaltschmidt et al., 2000; Kraut et al., 1996).

**Dlg Is Required for Microtubule-Induced Pins/Gαi/Dlg Cortical Polarity**

How do astral microtubules induce Pins/Gαi/Dlg cortical polarity? We first tested the role of Dlg because it is the best candidate to link microtubules to cortical Pins/Gαi proteins: Dlg and Pins are reported to directly interact (Bellaiche et al., 2001), and mammalian Dlg orthologs associate with the microtubule binding proteins GAKIN, MAP1a, CRIPT, APC, or KIF1B (Breiman et al., 1998; Hanada et al., 2000; Matsu-mine et al., 1996; Mok et al., 2002; Passafaro et al., 1999). We assayed dig^{md2} insc double mutant embryos, where there is no zygotic Dlg protein (dig^{md2} is a genetic null allele).
but maternal Dlg protein keeps embryonic Dlg levels at about 50% of wild-type (Figures 2B and 2C) (Woods et al., 1996). We found that \( {\text{dlg}}^{m52} \) insc double mutant embryos showed severe defects in Pins/Ga\( i \) cortical polarity: Pins was mostly cytoplasmic (80%, \( n = 58 \)), and Ga\( i \) was uniformly distributed at the cortex (73%, \( n = 30 \)) (Figure 2C; quantified in Figure 2F, white bars). This is not due to loss of astral microtubules (Figure S2F) but rather to an inability of astral microtubules to induce Pins/Ga\( i \) cortical polarity. In contrast, when the Insc/Par pathway is functional, Pins/Ga\( i \) crescents can form without Dlg or without both Dlg and microtubules (Figures 2F, S1D, S1E, S3D, and S3H; data not shown). We conclude that Dlg is required specifically for microtubule-induced Pins/Ga\( i \) cortical polarity.

To test whether Dlg is required for inducing basal cortical polarity, we assayed Miranda cortical localization. Wild-type neuroblasts show basal Miranda crescents at metaphase and telophase (Ishihama-Kataoka et al., 1997; Shen et al., 1998). insc mutant neuroblasts often failed to localize Miranda at metaphase but exhibited an Insc/Par-independent “telophase rescue” of basal Miranda localization (Kraut et al., 1996) (Figure S4A). Likewise, neuroblasts from \( {\text{dlg}}^{m52} \) germine clone embryos also failed to form basal Miranda crescents at metaphase yet exhibited “telophase rescue” (Figure S4B). We found that \( {\text{dlg}}^{m52} \) insc double mutants lack “telophase rescue” of Miranda localization (Figure S4C), showing that the microtubule/Dlg pathway can directly or indirectly induce basal cortical polarity in the absence of the Insc/Par pathway.

Next we were interested in determining which Dlg domain is required for microtubule-induced Pins/Ga\( i \) polarity. Dlg contains multiple protein–protein interaction domains, including three PDZs, an atypical SH3, and an inactive guanylate kinase (GK) domain (Figure 2A) (reviewed in Funke et al., 2005). We scored for Pins/Ga\( i \) crescents in \( {\text{dlg}}^{\text{m30, insc}} \) mutants that selectively affect the SH3 domain (\( {\text{dlg}}^{m30} \)) or GK domain (\( {\text{dlg}}^{1P20} \)) (diagrammed in Figure 2A). Normal levels of Dlg protein are made in both mutants (Figures 2B–2E) (Woods et al., 1996). We found that the majority of mitotic neuroblasts from both mutant backgrounds (\( {\text{dlg}}^{m30, \text{insc}} \) and \( {\text{dlg}}^{1P20, \text{insc}} \)) showed uniform cortical mutant Dlg, mostly cytoplasmic Pins, and uniform cortical Ga\( i \) (Figures 2D and 2E; data not shown; quantified in Figure 2F, white bars). Thus, both the SH3 and GK domains are required for reliable microtubule-induced Dlg crescent formation and the subsequent establishment of Pins/Ga\( i \) cortical polarity.

**Figure 2. Dlg Is Required for Microtubule-Induced Pins/Ga\( i \) Cortical Polarity**

(A) Dlg domain structure and mutant lesions. 
(B–E) Lateral view of stage 10 metaphase neuroblasts. Genotypes are listed above panels; markers listed within each panel. Images in the top row were taken at the same confocal settings to allow comparison of Dlg protein levels. 
(F) Quantitation of the Pins/Ga\( i \) crescents formed in each genotype: solid black/gray bars, normal to weaker cortical crescents; white bars, no cortical asymmetry (Ga\( i \) uniform cortical, Pins mostly cytoplasmic); number of neuroblasts scored indicated within each bar.
The Dlg GK Domain Is Required to Maintain Dlg-Microtubule Alignment

Although the Dlg SH3 and GK domains are both required for fully penetrant Dlg crescent formation, when we analyzed the small pool of \textit{dlg} \textit{insc} neuroblasts that form weak Pins/Gai crescents (10%–45%, gray/black bars in Figure 2F), we were able to assign the GK domain a distinct function in aligning the mitotic spindle with the Pins/Gai cortical crescent. As expected, the mitotic spindle was tightly aligned with the Pins/Gai/Dlg crescent in both wild-type and \textit{insc} mutant neuroblasts (Figures 3A and 3B). In contrast, the spindle showed poor alignment with the weak Pins/Gai/Dlg crescent in the \textit{dlg}^{m52} \textit{insc} double mutant, indicating that the low levels of maternal Dlg were insufficient for spindle orientation (Figure 3C). Interestingly, the \textit{dlg}^{m30} \textit{insc} neuroblasts that had a Dlg SH3 domain mutation maintained excellent spindle alignment with the Pins/Gai/Dlg crescent (Figure 3D), whereas the \textit{dlg}^{1P20} \textit{insc} mutant neuroblasts that had a premature Dlg GK domain truncation failed to maintain mitotic spindle alignment with the Pins/Gai/Dlg crescent (Figure 3E). Thus, the ability of the mitotic spindle to tightly align with the Pins/Gai/Dlg crescent requires the Dlg GK domain.

Figure 3. Dlg GK Domain Is Required to Maintain Dlg-Microtubule Alignment

(A–E) Lateral view of stage 10 metaphase neuroblasts. Genotypes are listed above panels. Top row, Pins staining with or without PH3. Neuroblasts in (B)–(E) are from the pool represented by solid gray/black bars in Figure 2F (i.e., the minority of \textit{dlg} \textit{insc} neuroblasts that form Pins/Gai crescents). Second row, same neuroblast with \alpha-tubulin staining to show spindle position. Third row, double-labeled neuroblasts to show position of the mitotic spindle relative to cortical polarity. White arrowheads indicate center of the crescent; yellow arrows point to spindle poles, along the long axis of the mitotic spindle. Bottom row, quantitation of spindle orientation relative to Pins/Gai crescents. Each red line indicates the angle between the spindle axis and the center of the Pins/ Gai crescent for each metaphase neuroblast scored, represented as a dashed arc in (C) and (E).
Khc-73 Coimmunoprecipitates with Dlg and Is Required for Microtubule-Induced Pins/Gaz/Dlg Crescent Formation

Because the Dlg GK domain regulates Dlg-spindle alignment, we searched the literature for proteins capable of binding both microtubules and the GK domain. Mammalian MAP1A and GAKIN/Kif13b have these properties (Brenman et al., 1998; Hanada et al., 2000), but only GAKIN has a clear ortholog in Drosophila. GAKIN has two microtubule binding domains (a canonical motor domain and a C-terminal CAP-Gly domain) and a central 224 aa “MAGUK binding stalk” (MBS) that binds the GK domain of human Dlg (Asaba et al., 2003; Hanada et al., 2000). Drosophila Kinesin heavy chain 73 (Khc-73) protein is 50% identical, has the same domain composition as mammalian GAKIN, and is a member of the Kinesin 3 family (formerly Unc-104/Kif1A family). In Drosophila embryos, Khc-73 transcripts are maternally deposited and become enriched in the developing CNS (data not shown and Li et al., 1997). We generated Khc-73 transgenes to drive embryonic expression of a hemagglutinin (HA) epitope-tagged full-length Khc-73 (HA:Khc-73FL) or a shorter form containing just the putative Dlg binding MBS domain (HA:Khc-73MBS). We found that both HA:Khc-73FL and HA:Khc-73MBS proteins were able to immunoprecipitate endogenous Dlg protein from embryonic lysates but not Pins protein, whereas HA antibodies failed to immunoprecipitate Dlg from control lysates lacking HA:Khc-73 protein (Figures 4A and 4B; data not shown). In addition, Dlg and Pins proteins can be coimmunoprecipitated specifically from embryonic CNS tissue (Figure 4C). We conclude that Khc-73/Dlg and Dlg/Pins associate within embryonic neuroblasts and might associate within Drosophila embryos but find no evidence for a stable Khc-73/Dlg/Pins complex (see Discussion).

Next we tested whether Khc-73 is required for microtubule-induced formation of Pins/Gaz/Dlg cortical crescents. Because there are no existing mutations in Khc-73, we used RNA interference (RNAi) to reduce Khc-73 levels. Khc-73 RNAi treatment alone gave the expected strong Pins crescents colocalized with apical Ins/c/Par proteins (Figure 4E; quantified in Figure 4I). However, when we induced the expression of a Khc-73 hairpin dsRNA in insc mutant neuroblasts or performed insc Khc-73 double RNAi, we observed delocalization of Pins into the cytoplasm and uniform Dlg enrichment around the cortex in metaphase neuroblasts (Figures 4D and 4F; quantified in Figure 4I). This phenotype is not due to loss of astral microtubules (Figure S2G) but rather to an inability of astral microtubules to induce Pins/Gaz/Dlg cortical polarity in the absence of Khc-73. We conclude that Khc-73 and Dlg can interact in vivo and that Khc-73 is required for microtubule-induced Pins/Gaz cortical polarity in neuroblasts.

To determine the importance of the Khc-73/Dlg interaction for Khc-73 function, we overexpressed the putative Dlg binding MBS domain of Khc-73. Overexpression of the Khc-73 MBS in insc RNAi-treated neuroblasts resulted in mostly cytoplasmic Pins and uniform Dlg in the majority of metaphase neuroblasts (Figure 4H; data not shown; quantified in Figure 4I). MBS overexpression alone did not alter Pins or Dlg apical enrichment (Figure 4G; quantified in Figure 4I). Thus, Khc-73/Dlg interactions appear to be important for Khc-73-induced cortical polarity.

Khc-73 Is Localized to Microtubule Plus Ends in Mitotic Neuroblasts

Khc-73 has two microtubule binding domains, a motor domain and a CAP-Gly domain, commonly found in microtubule plus-end binding proteins. To determine the subcellular localization of Khc-73, we expressed low levels of HA:Khc-73 in neuroblasts. We found HA:Khc-73 localized in puncta at microtubule plus ends, which were readily apparent in Taxol-stabilized mitotic asters (Figures 5A–5C and Movie S1). HA:Khc-73 was localized to plus ends near the cortex (presumptive astral microtubules) and plus ends associated with condensed DNA (presumptive kinetochore microtubules). We did not detect Dlg in the HA:Khc-73 puncta, suggesting that Khc-73 first contacts Dlg at the cell cortex. Taken together, Khc-73 localization and biochemical data are consistent with a model in which Khc-73 associates with astral microtubule plus ends, contacts cortical Dlg, and induces Dlg cortical clustering; this promotes the subsequent recruitment of Pins/Gaz (see Discussion).

The Microtubule/Khc-73/Dlg Pathway Is Required for Reliable Spindle Orientation

We have shown that a microtubule/Khc-73/Dlg pathway can polarize the neuroblast cortex in the absence of Ins/c/Par complex proteins. Here we test whether the microtubule/Khc-73/Dlg pathway has a function in the presence of Ins/c/Par protein function. We scored Khc-73 RNAi neuroblasts and dglaz/m22 null mutant neuroblasts—which have normal Ins/c/Par apical protein crescents—for proper spindle orientation relative to the center of the apical Ins/c/Par protein crescent. In wild-type embryonic or larval neuroblasts, the metaphase spindle is tightly aligned with the center of the Ins/c/Par protein crescent (Figure 6A). In contrast, Khc-73 RNAi-treated and dglaz/m22 larval mutants showed only 65–70% neuroblasts with tightly aligned mitotic spindles (Figures 6B and 6C). Thus, the microtubule/Khc-73/Dlg pathway is required for reliable linkage between the mitotic spindle and Ins/c/Par cortical proteins. Similarly, pins or Gaz zygotic mutant larvae had neuroblasts with only 50–55% spindles aligned with Ins/c/Par crescents (Figures 6D and 6E). We could not assay for a stronger phenotype in pins or Gaz maternal zygotic mutant embryos because they fail to form Ins/c/Par protein crescents (Yu et al., 2000, 2003). We conclude that Khc-73, Dlg, Pins, and Gaz are required for proper spindle orientation relative to cortical polarity (intrinsic spindle orientation). Thus, we have revealed evidence for bidirectional signaling between the mitotic spindle and the cell cortex: The astral microtubule/Khc-73 pathway can induce Pins/Gaz cortical polarity, and cortical Pins/Gaz/Dlg proteins regulate spindle orientation (Figure 7A).

DISCUSSION

Recent work has shown that microtubules can directly regulate cortical polarity in yeast (Behrens and Nurse, 2002)
during C. elegans meiosis (Cowan and Hyman, 2004; Wal- lenfang and Seydoux, 2000) and in migrating cells (Et- ienne-Manneville and Hall, 2001). An important question is the extent to which microtubules regulate cortical cell polarity in other contexts. Here we identify a microtubule/kinesin pathway for inducing cortical polarity in Drosophila neuroblasts. This pathway is sufficient to induce cortical polarization of the evolutionarily conserved Dlg, Pins, and Gai proteins and is necessary for reliable spindle orientation relative to apical Insc/Par cortical proteins.

Figure 4. Khc-73 Coimmunoprecipitates with Dlg and Is Required for Microtubule-Induced Pins/Gai/Dlg Crescent Formation

(A and B) Khc-73/Dlg coimmunoprecipitate from embryonic lysates. The maternal V32a-GAL4 line drives ubiquitous expression of UAS-HA/Khc-73FL or UAS-HA/Khc-73MBS during embryogenesis. Protein complexes from these lysates were immunoprecipitated with HA antibodies and detected by Western blot using either α-HA (A) or α-Dlg (B). As a control, α-HA fails to coimmunoprecipitate Dlg from a wild-type lysate (A and B).

(C) Dlg/Pins coimmunoprecipitate from embryonic CNS tissue. UAS-Dlg:eGFP was specifically expressed in the embryonic CNS using worniu-GAL4. Dlg:eGFP protein was immunoprecipitated with GFP antibodies, and the immunoprecipitant was probed for Pins by Western blot. β-galactosidase antibody was used as a negative control. IgG heavy chain, asterisk.

(D–H) Khc-73 is required to induce Pins and Dlg cortical polarity in the absence of Insc. Lateral view of stage 10 metaphase neuroblasts. Genotypes or RNAi treatments are listed above panels and markers listed within panels.

(D) insc Khc-73 double RNAi neuroblasts show loss of Pins localization while (E) Khc-73 single RNAi neuroblasts show normal Pins crescents.

(F) The same phenotype is seen in insc mutant neuroblasts expressing a Khc-73 hairpin dsRNA.

(G and H) Normal Pins and Dlg crescents in embryos overexpressing HA:KhcMBS requires Insc.

(I) Quantitation of Pins crescents formed in each genotype or RNAi treatment (inj, injected dsRNA).
The Microtubule/Khc-73/Dlg Pathway

A model for the microtubule/Khc-73/Dlg pathway, in the absence of the Insc/Par function, is shown in Figure 7D and summarized below.

1. At prophase, the microtubule/Khc-73/Dlg pathway is unable to polarize. Dlg/Gαi are uniform cortical, and Pins is predominantly cytoplasmic. It is not clear when Khc-73 is localized to microtubule plus ends because Taxol-treated neuroblasts metaphase arrest (Figure 5).

2. At metaphase, Khc-73 is localized at microtubule plus ends where it can contact cortical Dlg protein. We propose that Khc-73 first contacts Dlg at the cortex because we do not detect Dlg or Dlg:eGFP on astral microtubules or colocalized with Khc-73 at microtubule plus ends. Association of Khc-73 with microtubule plus ends may be mediated by its CAP-Gly domain, similar to the Clip-170 and APC plus-end binding proteins (reviewed in Galjart and Perez, 2003). The Khc-73/Dlg interaction could occur between the Khc-73 MBS stalk domain and the Dlg GK domain because the related GAKIN/hDlg domains directly interact (Asaba et al., 2003; Hanada et al., 2000). While we can immunoprecipitate Dlg with the Khc-73 MBS domain from embryonic lysate, we are unable to detect these direct protein–protein interactions between Khc-73 and Dlg in vitro, suggesting that this interaction may be highly regulated (Figure 4; data not shown).

3. Dlg clustering occurs over one spindle pole, although low levels persist around the cortex. An attractive model for Dlg clustering is that Khc-73/Dlg interaction blocks Dlg SH3-GK intramolecular interactions to favor Dlg intermolecular oligomerization (McGee and Bredt, 1999). In support of this model, we can express Dlg:eGFP specifically in neuroblasts and use an anti-GFP antibody to immunoprecipitate endogenous Dlg proteins (Figure S5). It is not clear why clustering occurs over just one spindle pole; perhaps the spindle poles are intrinsically different (Lambert and Nagy, 2002), or perhaps cortical heterogeneity (e.g., residual Par proteins) favors crescent formation over one spindle pole.

4. Pins/Gαi cortical clustering occurs. Pins/Gαi clustering requires Dlg and may be mediated by direct Dlg/Pins interactions (Bellaiche et al., 2001) and Pins/Gαi interactions (Schaefer et al., 2001). However, the Dlg/Pins interaction must be highly regulated or indirect as we have been able...
to coimmunoprecipitate Dlg/Pins from in vivo lysates but see no binding by in vitro assays (Figure 4 and data not shown).

(5) Khc-73/Dlg/Pins/Gαi signals to the mitotic spindle. Loss of any of these proteins results in spindle orientation and varying morphology defects, even in the presence of the Insc/Par pathway (Figure 6). It is not clear which protein most directly mediates the cortex to microtubule signal, but it is intriguing to note that the Pins ortholog LGN can bind the microtubule-associated protein NuMA and regulate spindle biology in mammals (Du and Macara, 2004). Gα subunits can bind tubulin to regulate microtubule dynamics (Roychowdhury et al., 1999), and finally Khc-73/Dlg microtubule binding could also directly regulate spindle behavior.
Two Pathways for Generating Cortical Polarity in Neuroblasts

Asymmetric localization of Pins/Gai proteins can be induced by two distinct pathways in embryonic neuroblasts: a well-characterized cortical pathway involving the Insc/Par proteins (reviewed in Betschinger and Knoblich, 2004) and a microtubule-dependent Khc-73/Dlg pathway (this work). Each pathway is regulated differently and has unique features that provide different temporal and spatial information for generating cortical polarity.

First, each pathway is initiated by a different mechanism and provides unique information for the timing of Pins/Gai polarization. The Insc/Par pathway is initiated at late interphase in response to an unknown extrinsic cue (Siegrist and Doe, 2006) and is required for the early prophase cortical polarization of Pins/Gai (Figure 7B). In contrast, the Khc-73/Dlg pathway is initiated later at prometaphase/metaphase by astral microtubules and is required for cortical polarization of Pins/Gai only in the absence of Insc/Par complex proteins (Figure 7C). Consistent with this timeline, asymmetric enrichment of Dlg normally occurs well after polarization of Insc/Par/Pins/Gai during the prometaphase/metaphase transition, and this temporal progression of Dlg cortical enrichment is not affected in insc mutants (Figure S6). The temporal polarization of Dlg coincides precisely with the onset of Pins/Gai cortical polarity at prometaphase/metaphase that occurs in the absence of the Insc/Par pathway.

Next, each pathway provides different spatial information for the cortical polarization of Pins/Gai. The Insc/Par pathway recruits Pins/Gai to the apical cortex of the neuroblast at a position just below the overlaying epithelium, thus coordinating neuroblast cortical polarity with the neuroblast environment. In the absence of this pathway (e.g., insc mutant neuroblasts), cortical polarity can be generated but is not linked to tissue polarity, resulting in mispositioning of neuroblast progeny. In contrast, the microtubule/Khc-73/Dlg pathway induces Pins/Gai crescent formation over one spindle pole, thus coordinating the neuroblast cortical polarity with the spindle axis. In the absence of this pathway (e.g., dlg mutant or Khc-73 RNAi neuroblasts), Insc/Baz can still recruit Pins/Gai to the apical cortex, yet the spindle is not always properly aligned with this cortical polarity. Together
these two pathways ensure the correct temporal and spatial positioning of apical complex proteins relative to extrinsic and intrinsic landmarks.

**Microtubule-Induced Cortical Polarity in Other Cell Types**

*Drosophila* sense organ precursors (SOPs) divide asymmetrically to generate an anterior pIIb cell and a posterior pIIa cell. During this division, Pins, Gαi, Dlg, and Numb form cortical crescents over the anterior spindle pole, and Baz localizes over the posterior spindle pole (reviewed in Betschinger and Knoblich, 2004). Cell division orientation is fixed along the anterior/posterior axis by planar polarity cues mediated by the seven pass transmembrane receptor Frizzled. However, Frizzled signaling is required only for the position of Dlg/Pins crescents, not for their formation (Bellaiche et al., 2001). When we remove both frizzled and microtubules together, we find about 10% of the mitotic SOPs lack Pins crescents (n = 50; Figure S7). This mild phenotype suggests that while astral microtubules may contribute to Dlg/Pins polarization in SOPs, there must be an additional mechanism involved. The best candidates for this third mechanism are the Par proteins because Par crescents still form in frizzled mutant SOPs at metaphase (Bellaiche et al., 2001).

There are many similarities between asymmetric division of fly neuroblasts and the *C. elegans* zygote (reviewed in Betschinger and Knoblich, 2004), but there are also striking differences. One of the most noteworthy differences is that *C. elegans* par mutants undergo symmetrically sized embryonic cell divisions, whereas in *Drosophila*, par or insc mutants maintain sibling cell size asymmetry. Our work provides an explanation for this discrepancy. We show that astral microtubules are capable of generating Pins/Gαi cortical polarity in the absence of localized Par proteins and that this microtubule-induced Pins/Gαi cortical polarity is fully functional for generating an asymmetric spindle, cell size, and unique daughter cell fates (data not shown and Kaltschmidt et al., 2000; Kraut et al., 1998). It is likely that *C. elegans* lacks this “microtubule-based pathway” for inducing GPR1/2 (Pins) and Gz cortical polarity, at least during the first embryonic cell division, because posterior cortical localization of GPR1/2 is absent in par mutants and the daughter cells are equal in size (Colombo et al., 2003; Gotta et al., 2003). Interestingly, we do observe an increase in symmetrically dividing neuroblasts in neuroblasts lacking both Insc/Par and microtubule pathways, compared to loss of single pathways alone (Figure S4, Table S1, and data not shown). It appears that either the Insc/Par or microtubule/Khc-73/Dlg pathway is sufficient to induce Pins/Gαi cortical polarity, which generates daughter cells of different sizes and fates.

The microtubule/kinesin-induced Dlg clustering pathway that we describe here may be evolutionarily conserved. In mammals, hDlg and the Khc-73 ortholog GAKIN are coexpressed in T cells and coimmunoprecipitate, and T cell activation leads to recruitment of hDlg to the immunological synapse (Hanada et al., 2000). Interestingly, GAKIN targets hDlg into ectopic cellular projections in MDCK cells, and this targeting depends on microtubules (Asaba et al., 2003). This has lead to the hypothesis that GAKIN may use a microtubule-based mechanism to target hDlg to the T cell immune synapse, similar to the microtubule/Khc-73 pathway described in this paper.

**EXPERIMENTAL PROCEDURES**

**Fly Stocks and Genetics**

Mutant phenotypes were analyzed using the following stocks: pins^{369D}, pins^{369F}, Gαi^{2}, Ins^{22}, Df(2L)TESBDC-3; apk^{v4083D}, dflg^{v52}, dflg^{v303}, dflg^{v230}, dflg^{v270}, dflg^{v330}, and dflg^{v330}. These alleles were used to generate double mutants. Mutations were recombined on either fzl-lacZ (for embryos), actin-GFP (for larvae), or Tb (for pupae) marked chromosomes to pick homzygous mutant animals. dflg^{v302} germline clones were generated using FL-P-DFS methods (Chou and Perrimon, 1996) and crossed to y/FM7c-fzl-lacZ males.

We used vls佳AL4-GAL4 for ubiquitous embryonic expression, womni-GAL4 (Albertson et al., 2004) for neuroblast-specific expression, and neurolgal-GAL4 for SOP expression. We used standard methods (Albertson and Doe, 2003) for generating transgenes. Mutant phenotypes were analyzed using the following stocks: Mutant females were separately recombined onto the standard chromosome for live imagening. Embryos and larvae were reared at 30°C for GAL4-induced gene expression.

**Khc-73 Subcloning and Constructs**

A full-length Khc-73 cDNA was made from joining the two partial yet overlapping ESTs LP11192 and GH09175, and it was subcloned in-frame following hemagglutinin (HA) sequence in *Drosophila* PAR-6, and guinea pig senseless (Nolo et al., 2000). In addition, anti-Baz (1:500; A. Wodarz), rabbit aPKC (1:500; Santa Cruz), rat Par-6 (1:200; Parnas et al., 2001), rabbit Dlg (1:100; Serotec), mouse Dlg 4F3E2 (Parnas et al., 2001), rabbit Pins (Yu et al., 2000), and rabbit Gαi (Schaefer et al., 2001), mouse Dlg F36E2 (Parnas et al., 2001), rabbit Ins (1:500; W. Chia), rabbit anti-Baz (1:500; A. Wodarz), rabbit aPKC (1:500; Santa Cruz), rat Par-6 (Rolls et al., 2003), and guinea pig Senseless (Nolo et al., 2000). In addition, anti-Par antibodies were generated against fly PAR-6. Fluorescent-conjugated secondary antibodies (Jackson) were used. Images were collected using a Biorad Radiance confocal using a 60 × 1.4NA objective. SOP images were collected on a Leica TCS SP2 confocal using a 63 × 1.4NA objective. Biorad LaserSharp, Metamorph, Velocity, Photoshop, and Illustrator software were used for data analysis, movies, or figures.

For drug studies, 0–1 hr embryos were aged for 4–6 hr and drug treated for 1 hr by standard methods (Albertson and Doe, 2003). Larval brains and pupal nota were fixed in Schneider’s medium with or without drugs for 2 hr or 45 min, respectively. Drug concentrations used: 0.5 µM Colcemid (Sigma-Aldrich) or 10 µM nocodazole (Sigma-Aldrich) for embryos and nota; and 10 µg/ml Colcemid or 5 µM Paclitaxel (Sigma-Aldrich) for larvae.

**Live Imaging**

DlgGFP localization in wild-type neuroblasts was imaged in embryos collected from a cross between UAS-DlgGFP and womni-GAL4 flies. DlgGFP localization in insc homozygous mutant neuroblasts was imaged in embryos collected from a cross between UAS-DlgGFP, insc^{v2} / CyO and womni-GAL4, insc^{v2} / CyO flies (only insc^{v2} homozygous embryos are GFP positive). Embryos were mounted in halocarbon oil on a Teflon membrane fixed within a stainless steel slide (Kehart et al., 2009).
et al., 1994). Three to five 1–1.5 μm Z steps were collected at 15 s intervals on a BioRad confocal as described above.

**RNAi Experiments**

For insc RNAi, T7 sites were created on both 5’ and 3’ ends of a SacI fragment (bp 1597–2249, from the start ATG) from an insc cDNA by PCR. For Khc-73 RNAi, a 562 bp fragment (bp 4185–4747 from the start ATG) was PCR-amplified from the EST GH09175 (BDGP) with T7 sites on both ends. dsRNA was synthesized using the MEGAscript kit (Ambion). dsRNA was injected for either Khc-73 alone or for both Khc-73 and insc at a final concentration of 1 mg/ml. The LEA-Khc-73 hairpin dsRNA construct was made using pWIZ (Lee and Carthew, 2003) with 621 nucleotides from exon 9.

**Biochemistry**

Five hundred microliters of 0–12 hr embryos were homogenized in an equal volume of Cytoskeletal Buffer (50 mM Hepes, pH 7.5, 150 mM KCL, 8% Glycerol, 1% Triton, 5 mM DTT, and Protease inhibitors). The lysate was centrifuged and the soluble fraction was precleared against Protein A or Protein G agarose (Roche); either α-APG (Torrey Pines) or α-HA (Sigma-Aldrich) antibodies were added for 2 hr at 4°C. Protein complexes were pulled down using either Protein A or G agarose. The aggregate was washed 4–6 times in Cytoskeletal Buffer, boiled in Sample Buffer, separated by SDS-PAGE gel electrophoresis, transferred to nitrocellulose, and probed with antibodies to Pins (1:12000), Dlg (1:500), GFP (1:1000), or HA (1:1000) using standard methods (Harlow and Lane, 1999). We used HRP-conjugated secondary antibodies (Jackson) and enhanced chemiluminescence.

**Supplemental Data**

Supplemental Data include seven figures, one table, and one movie and can be found with this article online at http://www.cell.com/cgi/content/full/123/7/1323/DC1/.

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**REFERENCES**


