Microtubule-Induced Pins/G\(\alpha\)i Cortical Polarity in Drosophila Neuroblasts

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DOI 10.1016/j.cell.2005.09.043

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/G\(\alpha\)i 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/G\(\alpha\)i cortical polarity through Dlg/Khc-73 interactions. The microtubule/Khc-73/Dlg pathway acts in parallel to the well-characterized Insuteable/Par pathway, but each provides unique spatial and temporal information: The Insuteable/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 de-laminate 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 Insuteable (Insc), Partner of Insuteable (Pins), and G\(\alpha\)i 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 Discs large (Dlg), Scribble (Scrib), and Lethal giant lar-vae (Lgl) (Albertson and Doe, 2003).

Recent work has highlighted the central role played by the two apical cortical proteins, Pins and G\(\alpha\)i, 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). G\(\alpha\)i can form a heterotrimer with G\(\beta\)/G\(\gamma\) proteins, while Pins has three G\(\alpha\)i binding GoLoco domains as well as seven tetratricopeptide repeats (TPRs) that directly bind Insc. In all animals studied to date, Pins is proposed to activate both G\(\alpha\)i and G\(\beta\)/G\(\gamma\) in a receptor-independent fashion by binding G\(\alpha\)i and disrupting the inactive G\(\alpha\)i/G\(\beta\)/G\(\gamma\) heterotrimer (reviewed in Willard et al., 2004).

Despite the importance of Pins/G\(\alpha\)i cortical polarity in regulating Drosophila asymmetric cell division, it remains unclear how Pins/G\(\alpha\)i cortical localization is regulated. In neuroblasts, Insc is initially reported as essential for Pins/G\(\alpha\)i 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/Gai (Bellaiche et al., 2001; Schaefer et al., 2001).

Here we describe a novel astral microtubule-dependent pathway for inducing Pins/Gai 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 pathway are partially redundant for inducing Pins/Gai 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/Gai proteins, presumably via Insc-Pins direct interactions (reviewed in Betschinger and Knoblich, 2004). We term this the cortical “Insc/Par pathway” of Pins/Gai localization to distinguish it from the Insc-independent “microtubule-based pathway” of Pins/Gai localization that is the focus of this paper.

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

We confirmed that insc22 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) (Petronek and Knoblich, 2001; Wodarz et al., 1999, 2000), but interestingly we found that Pins, Gai, 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/Gai/Dlg crescents form in insc mutants, the timing and position of crescent formation differed from wild-type. First, in wild-type neuroblasts Pins/Gai/Dlg crescents always formed at the apical surface adjacent to the underlying ectoderm, whereas in insc mutant neuroblasts Pins/Gai/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/Gai/Dlg crescents formed by early prophase (94%, n = 50; data not shown), whereas in insc mutants Pins/Gai/Dlg 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/Gai/Dlg cortical crescents.

A clue to the identity of the Insc/Par-independent pathway was the observation that Pins/Gai/Dlg crescents were always colocalized 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/Gai/Dlg cortical crescents. We found that Colcemid treatment of insc mutant neuroblasts resulted in a nearly complete loss of Pins/Gai/Dlg crescents: Pins is mostly cytoplasmic and Gai/Dlg are uniform cortical (Figures 1F and 1O; data not shown). In contrast, Colcemid treatment of wild-type neuroblasts had no effect on Pins/Gai/Dlg crescent formation (Figures 1G and 1O; data not shown), likely due to the association of Pins/Gai/Dlg with the apical Insc/Par complex. In fact, the Insc/Par pathway of Pins/Gai/Dlg localization requires only Ins and Baz proteins, because aPKC mutants that lack aPKC/Par-6 protein localization but retain Baz/Insc localization still formed Pins/Gai/Dlg crescents in the absence of microtubules (Figures 1O, S1B, and S1C).

We conclude that spindle microtubules have the ability to induce Pins/Gai/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/Gai/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 (Megrav et al., 2001). Despite loss of astral microtubules, wild-type nocodazole-treated neuroblasts progressed through mitosis and showed normal colocalization of Pins/Gai/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/Gai/Dlg crescent formation (Figures 1L and 1O, and Supplemental Figures 2C and 2D), suggesting that astral microtubules were required to induce Pins/Gai/Dlg crescent formation. In addition, we abolished astral microtubules genetically using the fuzzy7 mutation. Fuzzy encodes an essential component of the anaphase-promoting complex, and fuzzy 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). Fuzzy single mutant neuroblasts formed normal Pins/Gai/Dlg crescents colocalized with Insc/Par, but insc fuzzy double mutant neuroblasts showed a loss of Pins/Gai/Dlg crescents: Pins was delocalized from the cortex, and Gai/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/Gai/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/Gai/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 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, CRIP1, APC, or KIF1B (Brenman et al., 1998; Hanada et al., 2000; Matushime et al., 1996; Mok et al., 2002; Passafaro et al., 1999).

![Figure 1. Astral Microtubules Induce Pins/Gαi/Dlg Cortical Polarity in Neuroblasts](image)

(A–N) Lateral view of stage 10 metaphase neuroblasts. Genotypes and/or drug treatments are listed above panels. (F and G) Colcemid (COL) and (K and L) 20 μM nocodazole (NOC). Markers are listed within each panel, and double-labeled neuroblasts are shown in the bottom row of (A–G) and (K–N); the mitotic marker phospho-histone H3, PH3. Crescents are indicated by arrowheads, and neuroblasts without crescents are in brackets in this and all subsequent figures.

(O) Quantitation of the Pins crescents formed in each genotype with or without drug; number of neuroblasts scored indicated within each bar. Apical is up and scale bar is 10 μm in this and subsequent figures unless noted.

![Cell 123, 1323–1335, December 29, 2005](image)
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 \textit{dlg}m52 insc double mutant embryos showed severe defects in Pins/Gai cortical polarity: Pins was mostly cytoplasmic (80%, \( n = 58 \)), and Gai 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/Gai cortical polarity. In contrast, when the Insc/Par pathway is functional, Pins/Gai 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/Gai 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 (Ikeshima-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 \textit{dlg}m52 germine clone embryos also failed to form basal Miranda crescents at metaphase yet exhibited “telophase rescue” (Figure S4B). We found that \textit{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/Gai 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/Gai crescents in \textit{dlg} insc mutants that selectively affect the SH3 domain (\textit{dlg}m30) or GK domain (\textit{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 (\textit{dlg}m30 insc and \textit{dlg}1P20 insc) showed uniform cortical mutant Dlg, mostly cytoplasmic Pins, and uniform cortical Gai (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/Gai cortical polarity.

Figure 2. Dlg Is Required for Microtubule-Induced Pins/Gai 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/Gai crescents formed in each genotype: solid black/gray bars, normal to weaker cortical crescents; white bars, no cortical asymmetry (Gai uniform cortical, Pins mostly cytoplasmic); number of neuroblasts scored indicated within each bar.
The Dlg GK Domain Is Required to Maintain Pins/Ga\textit{i}/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 insc} neuroblasts that form weak Pins/Ga\textit{i} 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/Ga\textit{i}/Dlg cortical crescent. As expected, the mitotic spindle was tightly aligned with the Pins/Ga\textit{i}/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/Ga\textit{i}/Dlg crescent in the \textit{dlg}^{\textit{sh3}} \textit{insc} double mutant, indicating that the low levels of maternal Dlg were insufficient for spindle orientation (Figure 3C). Interestingly, the \textit{dlg}^{\textit{30}} \textit{insc} neuroblasts that had a Dlg SH3 domain mutation maintained excellent spindle alignment with the Pins/Ga\textit{i}/Dlg crescent (Figure 3D), whereas the \textit{dlg}^{\textit{1P20}} \textit{insc} mutant neuroblasts that had a premature Dlg GK domain truncation failed to maintain mitotic spindle alignment with the Pins/Ga\textit{i}/Dlg crescent (Figure 3E). Thus, the ability of the mitotic spindle to tightly align with the Pins/Ga\textit{i}/Dlg crescent requires the Dlg GK domain.
Khc-73 Coimmunoprecipitates with Dlg and Is Required for Microtubule-Induced Pins/Gai/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 (Bremman 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 do-

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/Gai (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 Insc/Par complex proteins. Here we test whether the microtubule/Khc-73/Dlg pathway has a function in the presence of Insc/Par protein function. We scored Khc-73 RNAi neuroblasts and dag1411 null mutant neuroblasts—which have normal Insc/Par apical protein crescents—for proper spindle alignment relative to the center of the apical Insc/Par protein crescent. In wild-type embryonic or larval neuroblasts, the metaphase spindle is tightly aligned with the center of the Insc/Par protein crescent (Figure 6A). In contrast, Khc-73 RNAi-treated and dag1411 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 Insc/Par cortical proteins. Similarly, pins or Gai zygotic mutant larvae had neuroblasts with only 50%–55% spindles aligned with Insc/Par crescents (Figures 6D and 6E). We could not assay for a stronger phenotype in pins or Gai maternal zygotic mutant embryos because they fail to form Insc/Par protein crescents (Yu et al., 2000, 2003). We conclude that Khc-73, Dlg, Pins, and Gai 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/Gai cortical polarity, and cortical Pins/Gai/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; Walfenfang and Seydoux, 2000) and in migrating cells (Etienne-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 Gαi proteins and is necessary for reliable spindle orientation relative to apical Insc/Par cortical proteins.
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/Gaq 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/Gaq cortical clustering occurs. Pins/Gaq clustering requires Dlg and may be mediated by direct Dlg/Pins interactions (Bellaiche et al., 2001) and Pins/Gaq 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/Gai 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.

Figure 6. Khc-73, Dlg, Pins, and Gαi Are Required for Tight Coupling of the Apical Spindle Pole with Cortical Insc/Par Proteins
Metaphase neuroblasts scored for apical spindle pole alignment with the aPKC cortical crescent. Markers and genotypes indicated. First row, neuroblasts triple-labeled for aPKC (marked with arrowhead), γ-tubulin (marked with yellow arrows), and DNA. Dashed arc indicates distance between the two. Second row, neuroblasts labeled with aPKC (crescents marked with arrowhead) and the DNA marker PH3. Third row, same neuroblast with α-tubulin staining to show position of the mitotic spindle; spindle poles labeled with yellow arrows. Fourth row, same neuroblasts triple-labeled to show position of the mitotic spindle relative to cortical polarity. Bottom row, quantitation of spindle orientation defects. Each red line indicates the angle (represented by the dashed lines in the fourth row panels) between the spindle axis and the center of the Pins/Gai crescent. Only α-tubulin-labeled neuroblasts with a clear apical spindle pole were scored; in some neuroblasts, spindle morphology defects precluded scoring. Neuroblasts are shown at the stage where the phenotype is most penetrant (this varies because there are different amounts of residual maternal protein present in each genotype) or at the stage where we have the strongest Gal4 driver.
(A, C, and E) Third larval instar.
(B) Embryonic stage 10.
(D) Second larval instar. Scale bar in top left panel = 5 μm, and scale bar in panel below = 10 μm.
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 asymmetri-
cally to generate an anterior pIIb cell and a posterior pIl cell. During this division, Pins, Gαi, Dlg, and Numb form cor-
tical crescents over the anterior spindle pole, and Baz local-
izes 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. How-
ever, 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 to-
gether, 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 po-
larization 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 Bet-
schinger and Knoblich, 2004), but there are also striking dif-
fences. One of the most noteworthy differences is that
*C. elegans* par mutants undergo symmetrically sized embry-
onic cell divisions, whereas in *Drosophila*, par or insc mu-
tants maintain sibling cell size asymmetry. Our work provides
an explanation for this discrepancy. We show that astral mi-
crotubules are capable of generating Pins/Gαi cortical polar-
ity in the absence of localized Par proteins and that this
microtubule-induced Pins/Gαi cortical polarity is fully func-
tional for generating an asymmetric spindle, cell size, and
unique daughter cell fates (data not shown and Kaltschmidt
et al., 2000; Kraut et al., 1996). 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 embry-
onic 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). Inter-
estingly, we do observe an increase in symmetrically dividing
neuroblasts in neuroblasts lacking both insc/Par and micro-
tubule pathways, compared to loss of single pathways alone
(Figure S4, Table S1, and data not shown). It appears that ei-
ther the insc/Par or microtubule/Khc-73/Dlg pathway is suf-
ficient 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 coex-
pressed in T cells and communoprecipitate, and T cell activ-
ation leads to recruitment of hDlg to the immunological syn-
apse (Hanada et al., 2000). Interestingly, GAKIN targets hDlg
into ectopic cellular projections in MDCK cells, and this tar-
geting depends on microtubules (Asaba et al., 2003). This
has lead to the hypothesis that GAKIN may use a microtu-
bule-based mechanism to target hDlg to the T cell immune
synapse, similar to the microtubule/Khc-73 pathway de-
scribed in this paper.

**EXPERIMENTAL PROCEDURES**

**Fly Stocks and Genetics**

Mutant phenotypes were analyzed using the following stocks: *pin(1)nces*, *pak(1)*, Gαi8, *inscC 22*, Df(2)[ST8898C-3], *apk(1)nes3*; *dlg(3)g3* and *dlg(3)g30*, *dpy-16*, *tubG4a*, *Df(3)LD121*, and *iso(1)21*. These alleles were used to generate double mutants. Mutations were rebalanced over either *ftz-lacZ* (for embryos), *actin-GFP* (for larvae), or *Tb* (for pupae) marked chromosomes to pick homozygous mutant animals. *dlg(3)g32* germline clones were generated using FLP-DHS methods (Chou and Per-
rimon, 1996) and crossed to y/fM7C-fltZ-lacZ males.

We used v32a-GAL4 for ubiquitous embryonic expression, *worniu-
GAL4* (Albertson et al., 2004) for neuralblast-specific expression, and
*neurolg-GAL4* (Bellaiche et al., 2004) for SOP expression. *Fozz* (Koh
et al., 1996), UAS-DA/HKc73, FlUAS-HA/Khc-73 MBS, and UAS-Khc-73
haipn dsRNA lines. The UAS-DlgGFP and worniu-GAL4 transgenes
were separately recombined onto the *inscC* chromosome for live imag-
aging. Embryos and larvae were raised at 30°C for GAL4-induced gene ex-
pression.

**Khc-73 Subcloning and Constructs**

A full-length Khc-73 cDNA was made from joining the two partial yet overlap-
ing ESTs LP11192 and GH09175, and it was subcloned in-frame fol-
lowing hemagglutinin (HA) sequence in

**Antibody Staining and Drug Treatment**

Embryos, larval brains, and pupal nota were fixed and processed by stan-
dard methods (Albertson and Doe, 2003; Bellaiche et al., 2001). Primary
antibodies used for these studies include rabbit phospho-Histone H3
(1:10,000; Upstate), mouse α-tubulin (1:2000; Sigma-Aldrich), rat α-tu-
bulin (1:1000; Serotec), mouse γ-tubulin (1:2000; Sigma-Aldrich), rabbit Pins
(Yu et al., 2000), rat Pins (1:500; F. Yu), rabbit Gαi (Schaef er et al., 2001),
mouse Dlg 4F3E2 (Parnas et al., 2001), rabbit Ins (1:500; W. Chia), rabbit
anti-Baz (1:500; A. Wodarz), rabbit αPKC (1:500; Santa Cruz), rat Par-6
(Rolls et al., 2003), and guinea pig Senseless (Nolo et al., 2003). In addi-
tion, rat antibodies were generated against fls-tagged full-length Pins.
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, Meta-
morph, 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 placed in Schneider’s medium with or without
drug for 2 hr or 45 min, respectively. We used UAS-DlgGFP (Koh
et al., 2004) and visualized crescent over the anterior spindle pole, and Baz local-
izes 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. How-
ever, 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 to-
gether, 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 po-
larization 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 Bet-
schinger and Knoblich, 2004), but there are also striking dif-
fences. One of the most noteworthy differences is that
*C. elegans* par mutants undergo symmetrically sized embry-
onic cell divisions, whereas in *Drosophila*, par or insc mu-
tants maintain sibling cell size asymmetry. Our work provides
an explanation for this discrepancy. We show that astral mi-
crotubules are capable of generating Pins/Gαi cortical polar-
ity in the absence of localized Par proteins and that this
microtubule-induced Pins/Gαi cortical polarity is fully func-
tional for generating an asymmetric spindle, cell size, and
unique daughter cell fates (data not shown and Kaltschmidt
et al., 2000; Kraut et al., 1996). 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 embry-
onic 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). Inter-
estingly, we do observe an increase in symmetrically dividing
neuroblasts in neuroblasts lacking both insc/Par and micro-
tubule pathways, compared to loss of single pathways alone
(Figure S4, Table S1, and data not shown). It appears that ei-
ther the insc/Par or microtubule/Khc-73/Dlg pathway is suf-
ficient 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 coex-
pressed in T cells and communoprecipitate, and T cell activ-
ation leads to recruitment of hDlg to the immunological syn-
apse (Hanada et al., 2000). Interestingly, GAKIN targets hDlg
into ectopic cellular projections in MDCK cells, and this tar-
geting depends on microtubules (Asaba et al., 2003). This
has lead to the hypothesis that GAKIN may use a microtu-
bule-based mechanism to target hDlg to the T cell immune
synapse, similar to the microtubule/Khc-73 pathway de-
scribed in this paper.
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 662 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 LEAS-Khc-73 harpin 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., 0.5 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-γ-FG (Torry 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 agarose was washed 4–6 times in Cytoskeletal Buffer, boiled in Sample Buffer, separated by SDS-PAGE gel electrophoresis, transferred to microcellulose, and probed with antibodies to Pins (1:2000), 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/.

ACKNOWLEDGMENTS
We thank P. Adler, H. Beiken, K. Bhat, V. Budnik, P. Bryant, W. Chia, X. Yang, F. Matsuzaki, D. St. Johnston, J. Roste, M. Simon, A. Wodarz, J.E. Gomes, Bloomington Stock center, the Berkley Drosophila Genome Project, and especially Jürgen Knoblich for providing fly, DNA, or antibody reagents. We thank Amy Sheehan, Amanda Marusich, Victoria Robinson, Kristen Robinson, and especially Keiko Hirono for help in cloning, antibody production/stainings, and RNAi injections; and B. Hampolz and Ken Prehoda for protein biochemistry assistance. Karsten Siller, Stephan Schneider, Morgan Goulding, and Bruce Bowerman provided helpful comments on the manuscript. This work was funded by an NIH training grant (S.E.S.) and HHMI (C.Q.D.).

Received: June 5, 2005
Revised: August 26, 2005
Accepted: September 22, 2005
Published: December 28, 2005

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