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Lis1/dynactin regulates metaphase spindle orientation in *Drosophila* neuroblasts

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ABSTRACT

Mitotic spindle orientation in polarized cells determines whether they divide symmetrically or asymmetrically. Moreover, regulated spindle orientation may be important for embryonic development, stem cell biology, and tumor growth. *Drosophila* neuroblasts align their spindle along an apical/basal cortical polarity axis to self-renew an apical neuroblast and generate a basal differentiating cell. It is unknown whether spindle alignment requires both apical and basal cues, nor have molecular motors been identified that regulate spindle movement. Using live imaging of neuroblasts within intact larval brains, we detect independent movement of both apical and basal spindle poles, suggesting that forces act on both poles. We show that reducing astral microtubules decreases the frequency of spindle movement, but not its maximum velocity, suggesting that one or few microtubules can move the spindle. Mutants in the Lis1/dynactin complex strongly decrease maximum and average spindle velocity, consistent with this motor complex mediating spindle/cortex forces. Loss of either astral microtubules or Lis1/dynactin leads to spindle/cortical polarity alignment defects at metaphase, but these are rescued by telophase. We propose that an early Lis1/dynactin-dependent pathway and a late Lis1/dynactin-independent pathway regulate neuroblast spindle orientation.

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Introduction

Asymmetric cell division involving unequal partitioning of cell fate determinants is a widely used mechanism for generation of cellular diversity. Successful asymmetric cell division in animal cells requires the polarized localization of cell fate determinants and alignment of the mitotic spindle along this polarity axis; spindle orientation defects during asymmetric cell division have been implicated in tissue overgrowth and tumor formation (Caussinus and Hirth, 2007; Gonzalez, 2007; Knoblich, 2008; Morrison and Kimble, 2006). A central question is how cell polarity and spindle positioning are coordinated.

We are using *Drosophila* larval neuroblasts as a model system to investigate spindle-to-cortex interactions during asymmetric cell division. *Drosophila* neuroblasts repeatedly divide in a stem cell-like fashion, each division regenerating a larger apical neuroblast and producing a smaller basal ganglion mother cell (GMC) that typically forms two neurons. Mitotic neuroblasts are highly polarized, with distinct protein complexes localized to the apical and basal cell cortex. The apical cortical domain is formed around an evolutionary conserved protein complex containing Bazooka (Baz, the *Drosophila* Par-3 homolog), Cdc42, Par-6, and atypical Protein Kinase C (aPKC), and the associated Inscuteable, Partner-of-Inscuteable (Pins), Gαi, and Mushroom body defect (Mud) and Locomotion defects proteins. Basal proteins include the GMC cell fate determinants Miranda (Mira), Brain tumor (Brat), Prospero, and Numb (reviewed in Knoblich, 2008). The precise alignment of the mitotic spindle along the apical/basal polarity axis ensures segregation of these cell fate determinants into distinct daughter cells, which is correlated with the proper establishment of neuroblast/GMC cell fates (Caussinus and Hirth, 2007; Gonzalez, 2007). Despite the potential importance of spindle orientation in neuroblast/GMC cell fate specification, surprisingly little is known about the mechanism of spindle positioning in neuroblasts.

Studies in yeast and *Caenorhabditis elegans* (*C. elegans*) have provided strong evidence that cortex-spindle forces are generated at the astral microtubule–cortex interface by the action of cortically attached microtubule-based motors, proteins affecting microtubule length, and/or by proteins controlling the dynamics of astral microtubule–cortex interactions. Thus, spindle positioning requires dynamic attachment of astral microtubules to polarized cortical domains, and generation of forces exerted on these cortically-attached astral microtubules aligns the mitotic spindle (Cowan and Hyman, 2004; Pearson and Bloom, 2004). Consistent with this general model, reduction of either astral microtubule number or polarized cortical proteins can disrupt spindle positioning in *Drosophila* larval neuroblasts (Basto et al., 2006; Bowman et al., 2006; Giansanti et al., 2001; Izumi et al., 2006; Megraw et al., 2001; Nipper et al., 2007; Rebollo et al., 2007; Rusan and Peifer, 2007; Siegrist and Doe, 2005; Siller et al., 2006). Nevertheless, several important questions remain to be answered for *Drosophila* neuroblasts: Do both apical and basal spindle poles interact with the cortex during spindle orientation? What microtubule-binding proteins are required for productive spindle/

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cortex interactions? Here we address these questions using live imaging of larval *Drosophila* neuroblasts — including wild type, mutants that reduce astral microtubule number, and Lis1/dynactin mutants. We find that the apical and basal spindle poles show independent dynamic movement, suggesting that forces act on both spindle poles; that virtually eliminating all astral microtubules decreases the frequency of spindle movement, without altering the maximum velocity of spindle movement, suggesting that individual microtubules are sufficient to alter spindle orientation; and that the Lis1/dynactin complex is required for spindle movement and metaphase spindle orientation, indicating that this evolutionarily-conserved protein complex is responsible for spindle/cortex forces.

Results

Neuroblast spindle alignment occurs by prophase

We first determined when spindle orientation was established during the cell cycle, so we could focus our analysis of spindle/cortex interactions at the functionally relevant stage. Previous studies showed that larval neuroblasts establish spindle orientation at prophase relative to extrinsic cellular landmarks — i.e. GMCs in contact with the neuroblast basal cortex (Nipper et al., 2007; Rebollo et al., 2007; Rusan and Peifer, 2007; Siller et al., 2005), and relative to the intrinsic polarity marker Bazooka-YFP (Baz-YFP) at the neuroblast apical cortex (Siller et al., 2006). We used live imaging and fixed preparations to confirm that one centrosome is fixed at the apical cortex opposite the GMC contact site and adjacent to the apical Baz-YFP cortical crescent by prophase (Fig. 1; Movies 1 and 2; Table 1). In rare cases, centrosomes rotated during prophase, but their final position was established before the end of prophase (mean deviation from the final division axis 9.4±6.8°; n=14; Table 1), and spindles stayed aligned along this axis throughout mitosis. Together these data confirm that neuroblast spindle positioning relative to extrinsic and intrinsic cues is established during prophase.

Apical and basal spindle poles move independently

A full understanding of the mechanism of spindle orientation requires knowing which spindle pole interacts with the cortex – apical, basal, or both – and when these interactions occur. To address this question, we traced movements of apical and basal spindle poles throughout mitosis to determine their velocity, and most importantly whether they moved independently from each other. We find that during prometaphase and metaphase both spindle poles showed periods of vigorous movement, causing rocking of the spindle perpendicular to the spindle axis (Fig. 2A −7:07 to −1:27; Movies 3+4). These spindle oscillations did not affect the overall spindle orientation, which was constant from the end of prophase onwards (see above). We quantified peak velocity and frequency of high velocity movements (percent of time with a velocity ≥133 nm/s, see Experimental procedures). The peak velocity reached 320 nm/s, with apical and basal metaphase spindle poles spending an average of 24–30% moving at a velocity ≥133 nm/s (Fig. 2D+E). While rapid, this is likely an underestimate of spindle pole velocities, as we could not measure movement in the z-axis of the image stack. To determine if each spindle pole could move independently, we categorized spindle pole movement into the following categories: apical pole only, basal pole only, bipolar lateral, and bipolar rotational. We observed many examples of where only one spindle pole (apical or basal) showed movement (Fig. 2A). Interestingly, the basal spindle pole showed more vigorous movement than the apical spindle pole in third instar larval neuroblasts (Figs. 2A, D). This is unexpected because Gα promotes spindle rocking in *C. elegans* (Cowan and Hyman, 2004) but is localized over the less active apical spindle pole in neuroblasts (see Discussion). We conclude that the apical and basal spindle poles

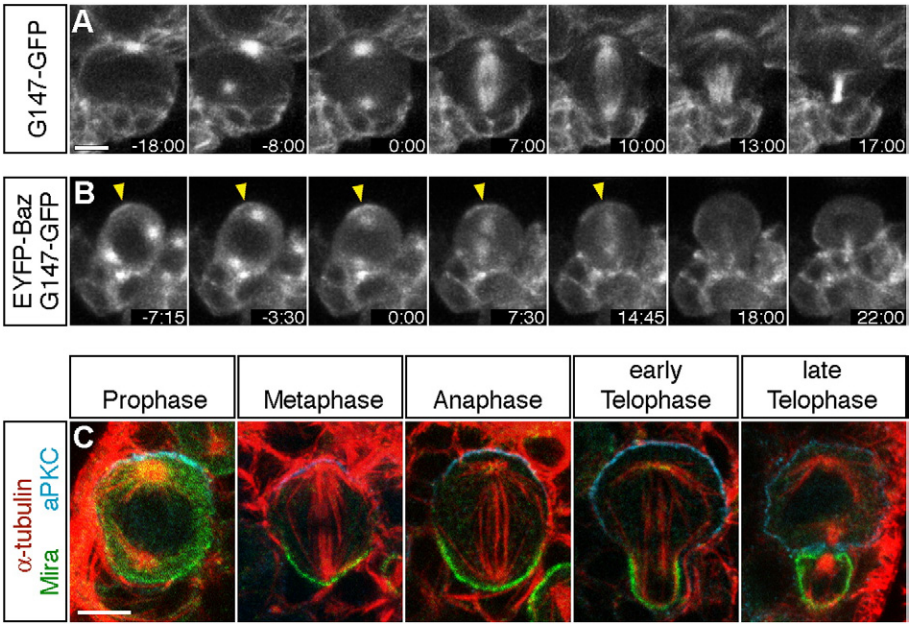

Fig. 1. Spindle position is determined during prophase in larval neuroblasts. (A–B) Temporal sequence of spindle alignment in wild type larval neuroblasts visualized in neuroblasts expressing the G147-GFP microtubule-associated protein (A) or co-expressing G147-GFP and EYFP-Baz (B). Time is given in min:sec relative to onset of prometaphase (0:00), which was evident by extension of microtubules into the cell center (A+B) and detection of EYFP-Baz in the cell center (B) after nuclear envelope breakdown (NEB). (A) Note that both spindle poles were positioned along the eventual division axis by the onset of prometaphase (0:00) and that spindle orientation was stabilized along this axis from this timepoint onward (Movie 1). (B) EYFP-Baz was concentrated in an apical cortex crescent (arrowheads) before centrosomes complete alignment along the apical/basal axis (−7:15). Separated centrosomes rotate during prophase to align along the apical/basal cell polarity axis (−3:30 to 0:00) (Movie 2). (C) In wild type larval neuroblasts, aPKC and Mira are localized to opposite cortical domains in late prophase, metaphase, anaphase, and telophase larval neuroblasts. Apical is up and basal is down. From late prophase until late telophase/cytokinesis the centrosome pair/mitotic spindle is tightly aligned along the cortical apical/basal polarity axis. Scale bars: 5 μm.

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Table 1
Time-lapse analysis of spindle orientation in larval neuroblasts

	Wild type[a] ($n=14$)	cnn^{hk21} ($n=10$)	*Lis1*[b] centrosome separation >160° ($n=4$)	*Lis1*[b] centrosome separation ≤160° ($n=6$)
Angle between centrosomes at end of prophase and division axis[c,d]				
Mean±S.D.	9.4±6.8°	24.7±17.8°	41.7±30.7°	53.5±38.5°
Max.	28°	56°	84°	88°
% of NBs with angle >15°	7.1%	60.0%	75.0%	66.7%
Angle between spindle axis at anaphase onset and division axis[d]				
Mean±S.D.	7.2±6.5°	20.1±16.4°	33.1±24.8°	12.7±9.5°
Max.	25°	47°	64°	26°
% of NBs with angle >15°	7.1%	60.0%	50.0%	33.3%

NB: neuroblasts.
[a] Genotypes: wild type (*G147-GFP*); cnn^{hk21} (cnn^{hk21}/cnn^{hk21} *G147-GFP*); *Lis1* ($Lis1^{k13209}/Lis1^{G10.14}$ *G147-GFP*).
[b] *Lis1* mutant neuroblasts were divided into two classes: (1) neuroblasts with complete centrosome separation (centrosome separation >160°); and (2) neuroblasts with centrosome separation defects (centrosome separation ≤160°).
[c] In cnn^{hk21} mutants the angle between spindle axis at the time of first successful bipolar focused spindle formation (during prometaphase/metaphase) and the eventual division axis.
[d] Division axis was defined as the axis through the center of the neuroblast and its GMC daughter at the time when central spindle microtubules had converged into a compact midbody (~5 min after anaphase onset in wild type and cnn^{hk21} mutant neuroblasts; ~10–15 min after anaphase onset in *Lis1* mutant neuroblasts due to slower completion of cytokinesis).

can move independently; this suggests that there are force-generating proteins associated with both spindle poles.

Reducing astral microtubule number decreases the frequency but not maximum velocity of spindle pole movement

Spindle movements could be due to a direct interaction between astral microtubules and the neuroblast cortex, or an indirect consequence of cytoplasmic flow or movement of neighboring cells. To determine if astral microtubules were required for spindle movements, we analyzed larval neuroblasts in *centrosomin* (cnn^{hk21}) mutants, which have specific defects in pericentriolar material assembly and lack most or all astral microtubules (Megraw et al., 1999; Vaizel-Ohayon and Schejter, 1999). We performed live imaging of spindle dynamics in cnn^{hk21} mutant neuroblasts (Fig. 2B; Movie 5). We find that cnn^{hk21} mutant neuroblasts have a slight delay in forming a bipolar spindle and have broader spindle poles (Fig. 2B −7:57 to −5:57; Movie 5), but this did not preclude analysis of spindle dynamics. We found that cnn^{hk21} mutant neuroblast have a significant reduction in the *frequency* of high velocity spindle pole movements (≥133 nm/s) for both apical and basal spindle poles (Figs. 2B, E). Interestingly, both apical and basal spindle poles in cnn^{hk21} neuroblasts occasionally exhibited high velocity spindle pole movements with peak velocities comparable to those seen in wild type neuroblasts (Fig. 2D). This suggests that there may be one or a few microtubules still contacting the cortex, which we confirmed in fixed preparations where we occasionally detected one microtubule (or microtubule bundle) contacting the cortex (Fig. 3B). We conclude that astral microtubules mediate spindle–cortex forces, and that just one or a few microtubules are capable of generating maximum velocity spindle movements.

Lis1 mutants abolish spindle pole movement

Astral microtubule-dependent spindle pole movements could be due to the activity of a microtubule/cortex force generation complex, or due to growth and collapse of individual microtubules (dynamic instability). To test whether a motor complex was required for spindle movement, we investigated the role of the dynactin complex and Lis1, which associate with the dynein motor and have been shown to regulate spindle movement in several cell types (Dujardin and Vallee, 2002; Pearson and Bloom, 2004). When not attached to the cell membrane, the complex translocates to microtubule minus-ends at the centrosome; when attached to the cortex it can pull astral microtubules towards the cortex. In *Drosophila*, dynein is required for germ cell spindle positioning (McGrail and Hays, 1997) and dynein/dynactin/Lis1 affect spindle assembly and progression through neuroblast mitosis (Siller et al., 2005; Wojcik et al., 2001), but no member of this protein complex has been tested for a role in regulating neuroblast spindle movement. Here we use mutations in Lis1 (see methods), a known co-factor of the dynein/dynactin complex (Vallee and Tsai, 2006), to determine whether Lis1/dynactin/dynein is required for neuroblast spindle movement. We assayed a *Lis1* transheterozygous mutant genotype (see methods) in which larval neuroblasts have undetectable Lis1 protein levels (Siller et al., 2005). Some of these mutant neuroblasts exhibit spindle assembly defects (Siller et al., 2005), so here we restrict our analysis to the neuroblasts that formed a normal bipolar spindle. *Lis1* mutant neuroblasts have apparently normal astral microtubules that contact the cortex, but the peak velocity of spindle pole movements rarely exceeded detection threshold (Fig. 2D, red line depicts detection threshold of spindle movement, see Experimental procedures), showing that Lis1 is required for spindle movement in neuroblasts. Interestingly, both apical and basal spindle poles were virtually static, with neither showing detectable rocking motion (Figs. 2C, 3C; Movie 6). We conclude that the Lis1 (and by association, the dynactin complex) is required for generating apical and basal spindle/cortex force in larval neuroblasts.

Lis1/dynactin and astral microtubules are required to align the mitotic spindle with cortical polarity during prophase/metaphase

We next determined whether astral microtubules or Lis1/dynactin were required to align the mitotic spindle with cortical polarity. We analyzed *cnn*, *Lis1*, and *dynactin* mutant neuroblasts from prometaphase/metaphase to telophase, as these stages always showed spindle alignment with cortical polarity in wild type neuroblasts (95.1% of all wild type metaphase neuroblasts showed ≤15° angle between spindle and cell polarity axis; Fig. 3A; Table 2). In contrast, cnn^{hk21} mutant metaphase neuroblasts showed spindle orientation defects at metaphase (Fig. 3B; Table 2), consistent with previous studies (Megraw et al., 2001). Similarly, *Lis1* and *dynactin (Glued; Gl)* mutant neuroblasts showed spindle positioning defects at metaphase without apparent defects in cortical polarity as assayed by normal apical Baz/aPKC and basal Mira localization (Figs. 3C, D; Table 2). Although some *Lis1* and *Gl* mutants had neuroblasts with spindle morphology defects (Siller et al., 2005), the observed spindle orientation defects were present even in mutant neuroblasts with normal bipolar spindle morphology,

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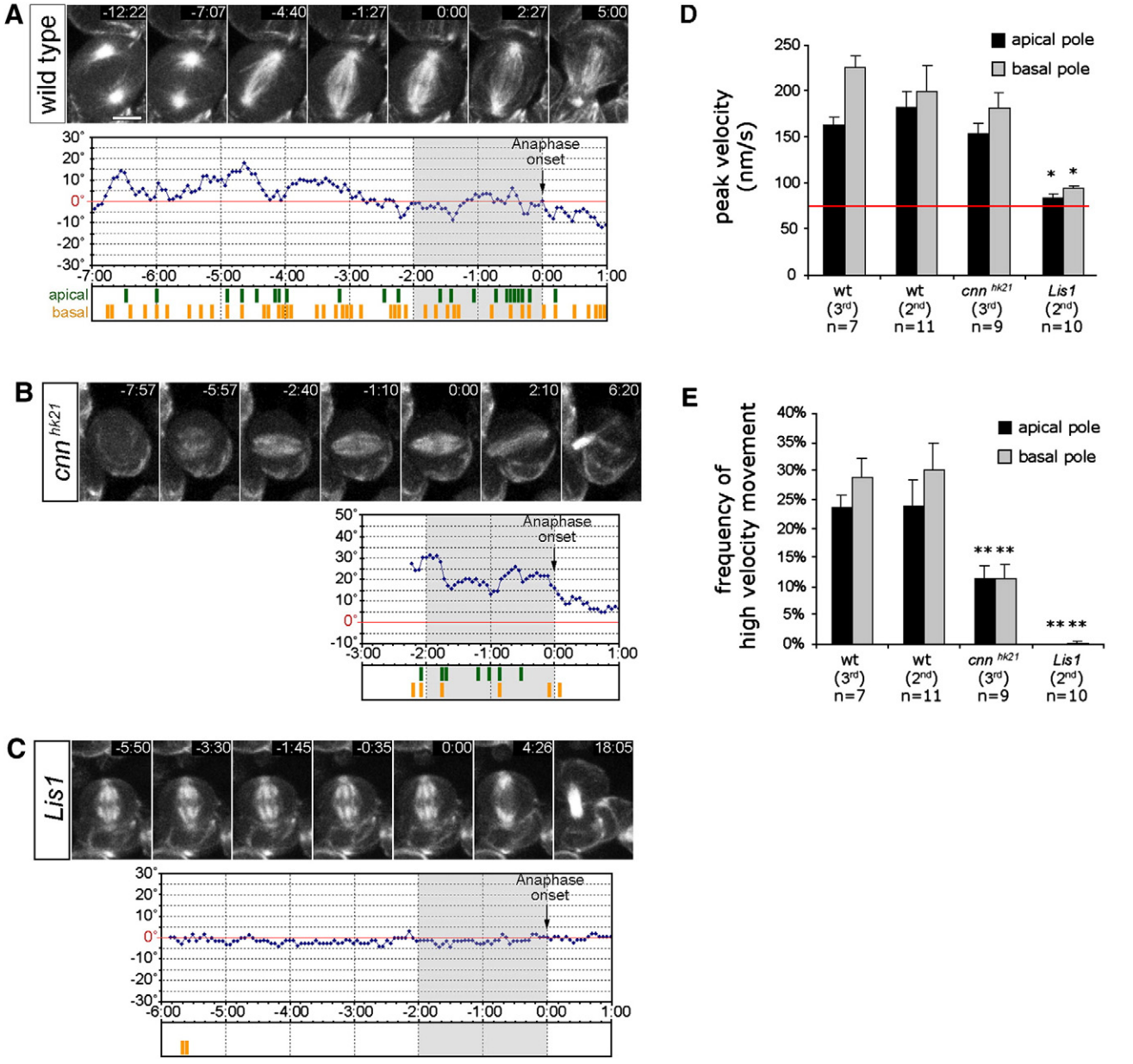

Fig. 2. Lis1 and Cnn regulate apical and basal spindle pole movements. (A–C) Spindle pole movement in larval metaphase neuroblasts expressing the G147-GFP microtubule-associated protein. Time is given in min:sec relatively to the onset of anaphase (0:00). Diagrams depict the angular deviation of the spindle axis at a given time point (blue line) from the final division axis (red line = 0°, determined 5:00 min after anaphase onset). Grey shaded regions indicate the 2 min period of late prometaphase/metaphase prior to anaphase onset (0:00) used for the analysis of spindle pole movements quantified in panels D and E. High velocity movement (≥133 nm/s or ≥2 pixels/frame) of the apical spindle pole (green) and basal spindle pole (yellow) are depicted below each diagram. (A) In 2nd instar wild type neuroblasts (48h ALH), both apical and basal spindle poles move extensively, causing oscillatory movements of the mitotic spindle around the final division axis. (Movie 3+4). No significant difference was observed between 2nd instar (48h ALH) and 3rd instar (96 h ALH) larval neuroblasts (see panels D and E). (B) In 3rd instar *cnn*^{hk21} mutant neuroblasts (96 h ALH), apical and basal spindle pole movements with velocities ≥133 nm/s were less frequent than in wild type counterparts. Measurements were started at the time a bipolar spindle with focused spindle poles had formed (Movie 5, see panels D and E). (C) In 2nd instar *Lis1* mutant neuroblasts (48h ALH), spindle pole movements were undetectable or significantly reduced (Movie 6; see panels D and E). (D+E) Quantification of 'peak velocity', and 'frequency of high velocity movements' (≥133 nm/s) of apical and basal spindle poles in 2nd instar wild type (48 h ALH; $n=11$), 3rd instar wild type (96 h ALH; $n=7$), 3rd instar *cnn*^{hk21} (96 h ALH; $n=9$), and 2nd instar *Lis1* (48 h ALH; $n=10$) mutant metaphase neuroblasts. For fair comparison, only the 2 min period prior to anaphase onset (−2:00 to 0:00, indicated by grey shaded diagram areas in panels A–C) was analyzed for each genotype. Depicted are means ± S.E.M. The 'frequency of high velocity movement' corresponds to the frequency of time periods during which spindle pole velocity was ≥133 nm/s. This velocity corresponds to spindle pole movement by ≥2 pixels between two consecutive frames; movement by 1 pixel between consecutive frames corresponds to 63–67 nm (red line) and cannot be distinguished from noise. Differences in 'peak velocity' and 'frequency of high velocity movements' between genotypes were analyzed by Student's *t*-tests. Significant differences are indicated (*, $p<0.005$; **, $p<0.001$). Scale bar: 5.

tightly focused spindle poles, and well-developed microtubule asters (Figs. 3C, D). We confirmed these results by time lapse analysis of spindle orientation in wild type, *cnn*^{hk21}, and *Lis1* mutant neuroblasts. Wild type neuroblasts maintain spindle orientation from the end of prophase to telophase (Figs. 1A, 2A, 4A; Movie 7; Table 2). In contrast, *cnn*^{hk21} mutant neuroblasts showed either no spindle movement or a slow drifting spindle that was unrelated to the cortical polarity axis (Figs. 4B, D; Movie 8; Table 1) — consistent with reduced astral microtubule–cortex interactions described above. We conducted a similar analysis in *Lis1* mutant neuroblasts, scoring only neuroblasts that formed a clear bipolar spindle with well-focused spindle poles. We found that in *Lis1* mutant neuroblasts, centrosome position at the end of prophase deviated from the eventual division axis by 42 ± 31° (Fig. 4C; Movie 9; Table 1); that is, spindle rotation occurred during

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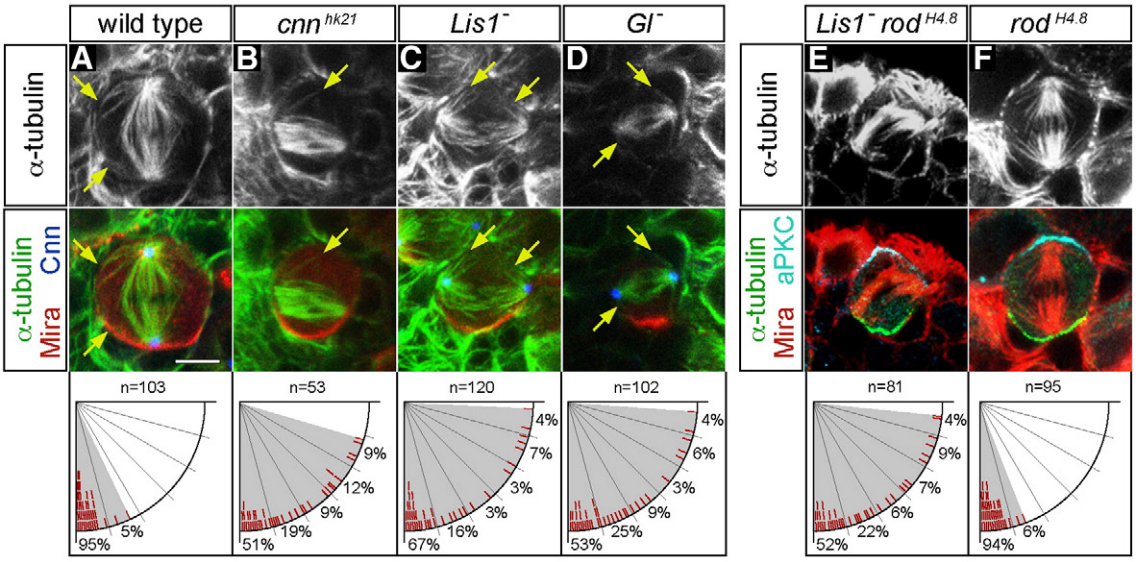

Fig. 3. Defective spindle orientation in *cnn*, *Lis1*, and *dynactin* (*Gl*) mutant neuroblasts. (A–D) wild type (A), cnn^{hk21} (B), *Lis1* (C) and *Gl* (D) mutant neuroblasts were triple labelled for α-tubulin (top row), Cnn, and Mira (shown as merged images in bottom row). (A) Wild type metaphase neuroblasts showed spindles containing robust apical and basal asters nucleated by centrosomes (marked with Cnn). Note the proximity of astral MTs to the cortex (arrows) and the tight alignment of the spindle along the cell polarity axis (defined by basal Mira localization). (B) cnn^{hk21} mutant neuroblasts lacked detectable Cnn protein, formed bipolar spindles with very few astral MTs, and often failed to align the spindle with the cell polarity axis. (C, D) *Lis1* ($Lis1^{G10.14}/Lis1^{k13209}$) and *Gl* ($Gl^{1-3}/Df(3L)fz\text{-}GF3b$) mutant neuroblasts showed spindle alignment defects despite formation of bipolar spindles and astral microtubules (arrows) nucleated by centrosomes (marked with Cnn). The deficiency *Df(3L)fz-GF3b* removes the entire *Gl* locus. (E, F) *Lis1* $rod^{H4.8}$ double and $rod^{H4.8}$ single mutant neuroblasts were triple labelled for α-tubulin (top row), aPKC, and Mira (shown as merged images in bottom row). (E) *Lis1* $rod^{H4.8}$ double mutant metaphase neuroblasts showed spindle alignment defects as observed in *Lis1* single mutants. (F) Metaphase spindle orientation is normal in $rod^{H4.8}$ single mutant neuroblasts. Scale bar: 5 μm.

metaphase–telophase, which was never observed in wild type neuroblasts. We conclude that the Lis1/dynactin complex and astral microtubules are required to align the mitotic spindle with the cortical polarity axis in prophase neuroblasts.

"Telophase rescue" of spindle/cortical polarity alignment in Lis1/dynactin mutants is not due to a delay in cell cycle progression

Lis1 and *dynactin* mutants had defective spindle alignment relative to cortical polarity at metaphase (see above), but surprisingly showed normal spindle/cortical polarity alignment by telophase (Fig. 5; Table 2). One possible mechanism is that a delay in anaphase onset, characteristic of *Lis1* and *dynactin* mutants (Siller et al., 2005), allows enough time for proper spindle/cortical polarity alignment. To address this issue, we tested whether spindle positioning defects still occurred in *Lis1* $rod^{H4.8}$ double mutants, where cell cycle delay is suppressed (Siller et al., 2005). Rod (Rough deal) is a checkpoint protein required to maintain metaphase arrest until kinetochore microtubules have attached to kinetochores, which is delayed in *Lis1* mutants (Siller et al., 2005). We found that *Lis1* $rod^{H4.8}$ double mutant metaphase neuroblasts showed metaphase spindle orientation defects as well as "telophase rescue" of spindle/cortical polarity alignment (compare Figs. 3 and 5; Table 2). Thus, the defects in spindle/cortical polarity alignment observed in Lis1/dynactin mutants are not due to a delay in cell cycle progression.

Table 2
Spindle orientation relative to cell polarity axis in larval neuroblasts

	Wild type[a]	cnn^{hk21}	*Lis1*	*Lis1 rod*	*rod*	Gl^{1-3}	Gl^{1-3}/Df
Metaphase[b]	*n=103*	n=53	n=120	n=81	n=95	n=101	n=102
0–15°	95.1%	50.9%	66.7%	54.3%	93.6%	62.4%	55.9%
16–30°	4.9%	20.8%	15.8%	19.8%	6.4%	26.7%	23.5%
31–45°	0.0%	11.3%	3.3%	7.4%	0.0%	6.9%	7.8%
46–60°	0.0%	7.6%	3.3%	6.2%	0.0%	2.0%	3.9%
61–75°	0.0%	9.4%	6.7%	8.6%	0.0%	1.0%	4.9%
76–90°	0.0%	0.0%	4.2%	3.7%	0.0%	1.0%	3.9%
Telophase	n=95	n=86	n=45	n=79	n=81	n=19	na
Mira asymmetrically segregated	100.0%	100.0%	100.0%	97.5%	100.0%	100.0%	na
Mira symmetrically segregated	0.0%	0%	0.0%	2.5%	0.0%	0.0%	na

na: not assayed.
[a] Genotypes: wild type (*Oregon R*); *cnn* (cnn^{hk21}/cnn^{hk21}); *Lis1* ($Lis1^{k13209}/Lis1^{G10.14}$); *Lis1 rod* ($Lis1^{k13209}/Lis1^{G10.14}rod^{H4.8}/rod^{H4.8}$); *rod* ($rod^{H4.8}/rod^{H4.8}$); Gl^{1-3} (Gl^{1-3}/Gl^{1-3}); Gl^{1-3}/Df ($Gl^{1-3}/Df(3R)fz\text{-}Gf3b$).
[b] Deviation of spindle axis from center of Mira crescent in metaphase neuroblasts (compare to Fig. 5).

Discussion

Here we show that spindle/cortical polarity alignment is established at prophase in *Drosophila* larval neuroblasts, and that both apical and basal spindle poles move independently, as if spindle/cortex forces are applied to both poles. We show that reducing astral microtubule number reduces the frequency of spindle pole movements, but that maximum spindle pole velocity is unaffected, suggesting that maximum velocity may occur when only one or a few microtubules are simultaneously contacting the cortex. We show that the Lis1/dynactin complex is required for spindle pole movement; reducing Lis1/dynactin complex activity reduces the maximum and average spindle velocity, even though astral microtubules still contact the cortex. This suggests that Lis1/dynactin is required to translate microtubule–cortex contact into spindle movement. Finally, we show that Lis1/dynactin is required for spindle orientation at metaphase but not at telophase. These findings are summarized in Fig. 6.

Apical and basal spindle poles move independently

We observed Lis1-dependent dynamic microtubule–cortex interactions at both apical and basal spindle poles, as well as asynchronous movements of apical and basal spindle poles. What are the candidate apical or basal cortical proteins that might regulate spindle pole movement? Insight into the role of cortical proteins in regulating spindle movement has been made in both *C. elegans* and mammals,

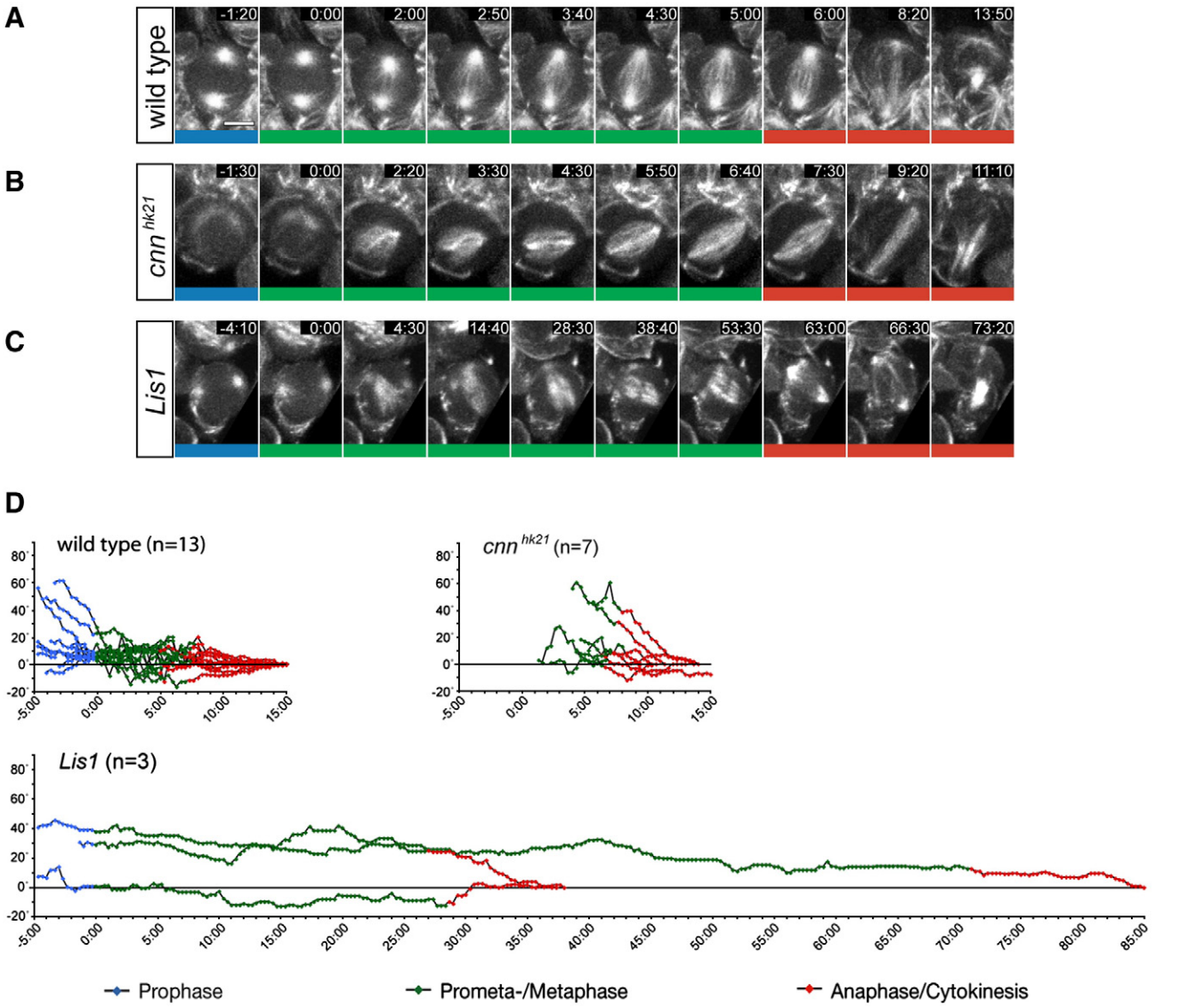

Fig. 4. Live imaging reveals aberrant spindle movement during metaphase/anaphase in *Lis1* and *cnn* mutant neuroblasts. (A–C) Centrosome positioning and spindle orientation in larval neuroblasts expressing the G147-GFP microtubule-associated protein. Time is given in min:sec relative to onset of prometaphase (0:00). (A) In wild type neuroblasts, centrosomes were already aligned with the final division axis by NEB (0:00) and spindle orientation remained fixed from prometaphase to telophase (0:00–13:50; Movie 7). (B) In *cnn*^{hk21} mutant neuroblasts, bipolar spindle formation was delayed (0:00–4:30). Eventually bipolar spindles formed, yet spindles could rotate during metaphase, anaphase, and telophase (4:30–11:10; Movie 8). (C) In *Lis1* mutant neuroblasts, centrosome position was abnormal at the onset of prometaphase (0:00) leading to formation of metaphase spindles with aberrant orientation (e.g. 38:40). Misoriented spindles could rotate during metaphase (14:40–38:20) and anaphase (63:00–66:30; Movie 9). Note the prolonged prometaphase/metaphase interval due to delayed bipolar spindle assembly and defects in mitotic checkpoint silencing. (D) Representation of centrosome/spindle position in live larval neuroblasts. Depicted is the angular position of the centrosome pair/spindle over time relative to the centrosome pair position at the end of mitosis. Scale bar: 5 μm.

and can be used to model spindle dynamics in *Drosophila*. Apical proteins in neuroblasts known to regulate spindle force in *C. elegans* include Gαi, Pins and Mud. During the first division of the *C. elegans* zygote, enrichment of the Gα-binding and activating Pins-related GPR1/2 proteins at the posterior cortex leads to increased Gα activity, resulting in higher cortex-spindle force generation, spindle pole rocking, and posterior spindle displacement (Colombo et al., 2003; Couwenbergs et al., 2007; Gotta et al., 2003; Grill et al., 2003; Nguyen-Ngoc et al., 2007; Srinivasan et al., 2003). This suggests that Gαi/Pins/Mud may promote movement of the apical spindle pole in *Drosophila* neuroblasts, which is supported by our recent finding that reducing Gαi can decrease spindle rocking (Siller et al., 2006).

In *C. elegans*, Lin-5 mediates the physical interaction of Lis1/dynein/dynactin with the cortical Gα and the Pins-related GPR1/2 proteins (Couwenbergs et al., 2007; Nguyen-Ngoc et al., 2007), and reduction of dynein or Lis1 function also reduces spindle pole rocking and posterior spindle displacement (Couwenbergs et al., 2007; Nguyen-Ngoc et al., 2007; Schmidt et al., 2005). Furthermore, in mammalian tissue culture cells Gαi overexpression can induce robust spindle rocking that requires LGN (a Pins/GPR-related protein) and NuMA (a Mud/Lin-5-related protein that binds dynein/dynactin (Du and Macara, 2004; Merdes et al., 1996). An attractive model is that Gαi/LGN activates NuMA, which interacts with dynein/dynactin/Lis1-loaded astral microtubules. In *Drosophila* neuroblasts, Gαi, Pins (LGN-related) and Mud (NuMA-related Pins-binding protein) are all enriched at the apical cortex and required for proper metaphase spindle orientation (Bowman et al., 2006; Izumi et al., 2006; Nipper et al., 2007; Parmentier et al., 2000; Schaefer et al., 2001, 2000; Siller et al., 2006; Yu et al., 2000). Thus, it is tempting to propose that apical Gαi/Pins/Mud interacts with dynein/dynactin/Lis1-loaded astral microtubules to center the apical spindle pole with the apical cortical domain. Identifying a physical link between Mud and dynein/dynactin/Lis1, and determining its functional importance in spindle orientation, would be a good test of this model.

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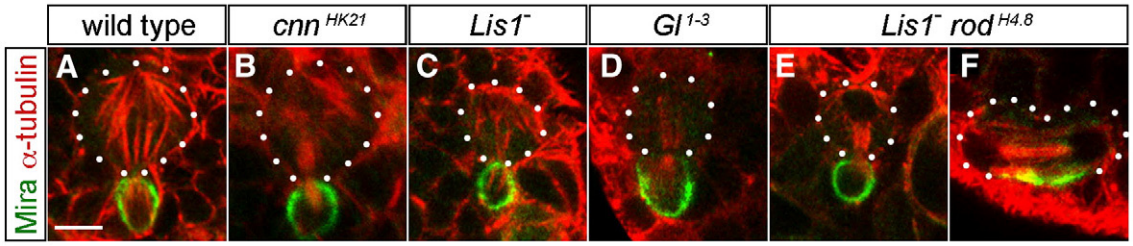

G

	wild type (n=95)	cnn HK21 (n=86)	Lis1- (n=45)	Gl 1-3 (n=19)	Lis1- rod H4.8 (n=79)	rod H4.8 (n=81)
	100%	100%	100%	100%	97%	100%
	0%	0%	0%	0%	3%	0%

Fig. 5. *cnn*, *Lis1*, and *dynactin* (*Gl*) mutant metaphase neuroblasts show defective spindle alignment in metaphase but not telophase neuroblasts. (A–F) Spindle orientation relative to cortical polarity axis in telophase. (A) In wild type telophase neuroblasts the mitotic spindle is aligned such that Mira gets exclusively segregated into the smaller GMC. (B–F) Telophase spindle alignment was normal in the vast majority of *cnn* (B), *Lis1* (C), Gl^{1-3} (D), *Lis1* $rod^{H4.8}$ (E), and $rod^{H4.8}$ (data not shown) mutant neuroblasts. Only in *Lis1* $rod^{H4.8}$ mutants we observed a few telophase neuroblasts with oblique spindle orientation causing equal partitioning of Mira into both daughter cells (F). (G) Quantification of spindle alignment in telophase neuroblasts for the indicated genotypes; see Table 2 for complete quantification. Scale bar: 5 μm.

Surprisingly, we find that third instar larval neuroblasts have more vigorous basal spindle pole rocking than apical spindle pole rocking, revealing a Gαi-independent spindle force generation mechanism at the basal cortex. Basal cortical proteins include Armadillo, DE-cadherin, β-catenin, APC2, and Mud (Akong et al., 2002; Bowman et al., 2006; Izumi et al., 2006; McCartney et al., 1999; Siller et al., 2006). Components of the APC2/DE-cadherin/α-catenin/β-catenin complex physically interact with the dynein complex in mammalian cells, and are required for spindle positioning in the *Drosophila* pre-cellular embryo, epithelial cells, and germline stem cells (Akong et al., 2002; Bowman et al., 2006; Izumi et al., 2006; Ligon et al., 2001; Lu et al., 2001; McCartney et al., 1999, 2001, 2006; Yamashita et al., 2003). Previous studies indicated no spindle positioning defects in neuroblasts after reduction of APC2 function (Akong et al., 2002), however these findings do not rule out a role for APC2 in spindle orientation because it may function redundantly with an apical cue, such as the Gαi/Pins/Mud pathway (Bowman et al., 2006; Izumi et al., 2006; Nipper et al., 2007; Siller et al., 2006).

Lis1/dynactin regulation of spindle positioning forces

We have demonstrated that both apical and basal spindle pole movements are greatly diminished in *Lis1* mutant larval neuroblasts (even in those with well-formed bipolar spindles and asters), providing first evidence that Lis1/dynactin is a critical component in the regulation of both apical and basal cortex-spindle forces. How does

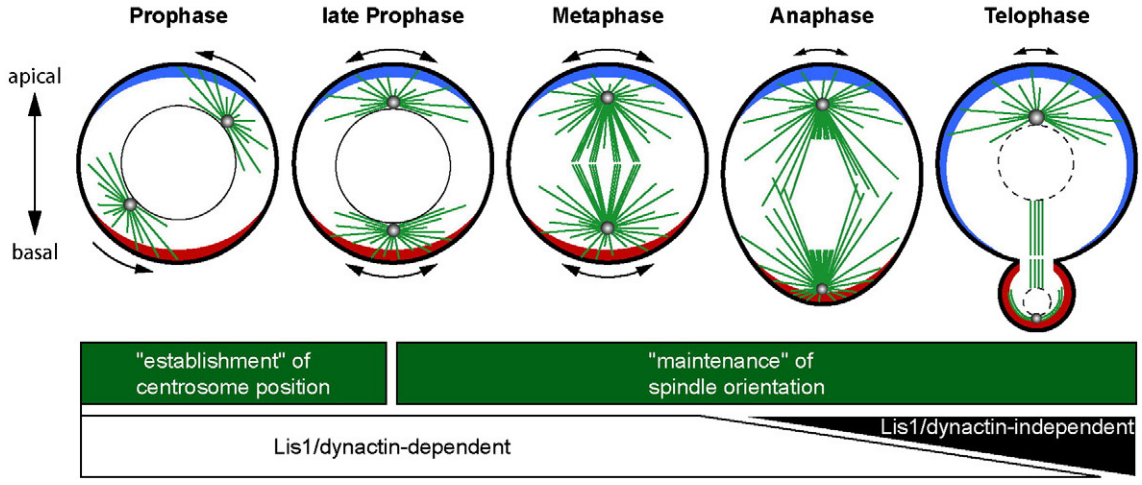

Fig. 6. Summary of spindle positioning mechanism in larval neuroblasts. In wild type neuroblasts, apical/basal positioning of centrosomes is established during prophase and maintained from prometaphase onward. Apical/basal centrosome/spindle positioning during the period from prophase to metaphase depends on Lis1/dynactin function, presumably due to their role in regulation of cortex-spindle forces. The short-lived imbalance of these forces is manifest in the rocking movements of both apical and basal spindle poles (indicated by double headed arrows outside the neuroblasts). Spindle rocking ceases during anaphase and telophase – only the apical pole shows sporadic lateral movements – possibly as a consequence of reduced or more balanced force generation/transduction. Spindle positioning in anaphase and telophase neuroblasts is largely Lis1/dynactin-independent, suggesting the existence of a novel yet unidentified spindle positioning pathway that can re-establish correct spindle alignment before the end of telophase in the absence of Lis1/dynactin (see Discussion for details).

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Lis1 regulate cortex-spindle forces? One possibility is that translocation of cortically associated motor proteins towards microtubule(−) ends results in movement of the microtubule towards the cortex. Consistent with this hypothesis, Lis1 colocalizes with and binds the microtubule minus-end motor dynein/dynactin complex (Dujardin and Vallee, 2002; Vallee et al., 2001; Xiang, 2003). Specifically, the budding yeast Lis1 homologue (Pac1) targets dynein to astral microtubule plus-ends (Lee et al., 2003; Sheeman et al., 2003) where it promotes movement of astral microtubules towards the cortex, resulting in translocation of the spindle apparatus through the bud neck (Adames and Cooper, 2000). By analogy, Lis1 may regulate spindle pole movement in neuroblasts by promoting dynein-dependent movement of astral microtubules towards the cortex. Alternatively, Lis1 may modulate the polymerization/depolymerization cycle (dynamic instability) of cortically-attached astral microtubules or the duration of astral microtubule–cortex interactions. In support of this latter hypothesis, loss of Lis1 or dynein function in *Aspergillus nidulans* or budding yeast results in reduced microtubule catastrophe and/or decreased shrinkage rates, thereby promoting assembly of overly long microtubules (Carvalho et al., 2003). Currently, we are not able to visualize astral microtubule plus-ends with sufficient spatial and temporal resolution to distinguish between these models for Lis1 function.

Both models for Lis1 function described above would require association of Lis1 protein with astral microtubules and/or the neuroblast cortex. Indeed, Lis1/dynactin complex proteins have been detected on astral microtubule plus-ends or at the cortex in mammalian, nematode, and yeast cells (Wynshaw-Boris, 2007). We previously analyzed the localization of HA-tagged Lis1, GFP-tagged Lis1, endogenous Lis1, and endogenous dynactin protein distribution using various fixation and live imaging methods in embryonic and larval neuroblasts, but were unable to detect enrichment of Lis1/dynactin at the cortex or at astral microtubule plus-ends (Siller et al., 2005). The most likely explanation is that Lis1/dynactin at these sites is masked by the high level of cytoplasmic protein present in neuroblasts.

Two spindle orientation pathways

The Lis1/dynactin complex is required for reliable spindle orientation with the apical/basal polarity axis in metaphase neuroblasts. These spindle orientation defects may be due in part to failure in anchoring one centrosome at the apical cortex during interphase, as recently reported for wild type neuroblasts (Rebollo et al., 2007; Rusan and Peifer, 2007); we have observed mis-positioned interphase centrosomes in *Lis1* mutants, but have not analyzed this phenotype in detail. We were surprised to find that spindle orientation was essentially normal at telophase in *Lis1* and *dynactin* (*Gl*) mutant neuroblasts, despite severe defects at metaphase. This indicates that there are two pathways for regulating spindle orientation: an early Lis1/dynactin-dependent pathway (prophase/metaphase), and a late Lis1/dynactin-independent pathway (anaphase/telophase). There are several models consistent with these findings: (1) *Lis1* and *dynactin* mutants have a delay in anaphase onset (Siller et al., 2005) which allows sufficient time for "telophase rescue" to occur. (2) A spindle orientation checkpoint – analogous to the yeast spindle orientation checkpoint (Lew and Burke, 2003) – may delay cytokinesis until proper spindle orientation has occurred. These first two hypotheses are disproven by our finding that *Lis1 rod* double mutants have normal metaphase progression but still show metaphase defects and "telophase rescue" of spindle orientation. (3) The cleavage furrow may be positioned by cortical polarity cues, such that cell elongation at early anaphase may mechanically re-orient the spindle along the long axis of the neuroblast. This model is unlikely because it is commonly accepted that the position of the cleavage furrow is determined by the position of the mitotic spindle and not by cortical cues (Burgess and Chang, 2005). (4) Additional microtubule–cortex regulators unrelated to Lis1/dynactin promote telophase spindle orientation.

The fourth model is the most likely, except that microtubule–cortex regulators unrelated to Lis1/dynactin have not yet been identified in *Drosophila* neuroblasts. Help may come from analysis of budding yeast spindle orientation pathways, where Lis1/dynactin-dependent and -independent pathways have been identified (Huisman and Segal, 2005). Several components of the yeast Lis1/dynactin-independent pathway are evolutionarily conserved, including the microtubule plus-end binding protein Bim1p, called EB1 in *Drosophila*. It is tempting to speculate that these proteins may regulate the Lis1/dynactin-independent pathway in *Drosophila* neuroblasts.

Experimental procedures

Fly genetics

The *Oregon R* strain was used as wild type control in all experiments. To analyze the *Lis1* mutant phenotype we used $Lis1^{G10.14}/Lis1^{k13209}$ transheterozygotes (Liu et al., 1999). Other strains used include: cnn^{hk21}/CyO (Megraw et al., 1999); $Gl^{1-3}/TM6B$ (Harte and Kankel, 1982; Siller et al., 2005); *Df(3R)fz-GF3b/TM6B* (Bloomington stock center); $rod^{H4.8}/TM6B$ (Karess and Glover, 1989); the gene trap line P{PTT-GA}Jupiter^{G00147}, expressing a GFP-tagged microtubule-associated protein CG17238 (Morin et al., 2001). EYFP-Baz was expressed in larval neuroblasts by crossing *pUAST-3xEYFP-Baz* transgenic flies (Siller et al., 2006) to a *worniu-Gal4* driver line (Albertson and Doe, 2003). Mutant alleles were rebalanced over *Cyo actin-GFP* or *TM3 actin-GFP Ser*. Newly hatched mutant larvae (identified based on absence of GFP expression in the gut) were aged to appropriate stage at 25 °C.

Time-lapse analysis of larval neuroblast divisions

Larval brains of wild type, cnn^{Hk21}, or *Lis1* mutants were dissected and neuroblasts were imaged using a BioRad Radiance 2000 laser scanning confocal microscope equipped with a 60× 1.4NA oil immersion objective as previously described (Siller et al., 2005). Time-lapse sequences were processed using MetaMorph (Universal Imaging Corp) and ImageJ and were converted into movies using QuickTime Pro.

Analysis of spindle pole movements in live neuroblasts

Stacks of 3 focal planes, 1.5 μm apart, were imaged in 3.3 to 3.5 s intervals with a resolution of 0.222 μm/pixel (Fig. 2, Movies 3–5). The 'frequency of high velocity movement' of spindle poles corresponds to the frequency of time periods with a velocity ≥133 nm/s (corresponding to spindle pole movement by ≥2 pixels between two consecutive frames). Thus, movement by 1 pixel between consecutive frames corresponds to 63–67 nm, which corresponds to the "detection threshold" (red line in Fig. 2D).

Antibodies and immunofluorescent staining

Wild type (48 h ALH), cnn^{hk21} (96–120 h ALH), $Lis1^{G10.14}/Lis1^{k13209}$ (48 h ALH), $Lis1^{G10.14}/Lis1^{k13209}$ $rod^{H4.8}$ (48 h ALH), $rod^{H4.8}$ (48 h ALH), Gl^{1-3} (96–120 h ALH), and $Gl^{1-3}/Df(3R)fz\text{-}GF3b$ (96–120 h ALH) larval brains were dissected, fixed and immunofluorescently labelled as previously described (Siller et al., 2005). Primary antibodies were: rat anti-Miranda (Irion et al., 2004); rabbit anti-nPKCλ (C20, Santa Cruz Biotechnology Inc; 1:1000); rabbit anti-Baz (gift from Andreas Wodarz; 1:500); mouse anti α-tubulin (DM1A, Sigma; 1:2000); rabbit anti-Cnn (Heuer et al., 1995). Fluorescently conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories and Molecular Probes. Confocal images were acquired on a BioRad Radiance 2000 or Leica TCS SP2 laser scanning confocal microscope equipped with a 60× or 63× 1.4 NA oil immersion oil respectively. Final figures were arranged using ImageJ and Adobe Photoshop.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.03.018.

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