nature cell biology

The NuMA-related Mud protein binds Pins and regulates spindle orientation in *Drosophila* neuroblasts

Karsten H. Siller[1], Clemens Cabernard[1] and Chris Q. Doe[1,2]

Asymmetric cell division generates cell diversity during development[1,2] and regulates stem-cell self-renewal in *Drosophila* and mammals[3,4]. In *Drosophila*, neuroblasts align their spindle with a cortical Partner of Inscuteable (Pins)–Gαi crescent to divide asymmetrically, but the link between cortical polarity and the mitotic spindle is poorly understood. Here, we show that Pins directly binds, and coimmunoprecipitates with, the NuMA-related Mushroom body defect (Mud) protein. Pins recruits Mud to the neuroblast apical cortex, and Mud is also strongly localized to centrosome/spindle poles, in a similar way to NuMA. In *mud* mutants, cortical polarity is normal, but the metaphase spindle frequently fails to align with the cortical polarity axis. When spindle orientation is orthogonal to cell polarity, symmetric division occurs. We propose that Mud is a functional orthologue of mammalian NuMA and *Caenorhabditis elegans* Lin-5, and that Mud coordinates spindle orientation with cortical polarity to promote asymmetric cell division.

Drosophila neuroblasts are a model system for studying asymmetric cell division. Neuroblasts are polarized along their apical–basal axis: apical cortical proteins include the Par protein complex (atypical protein kinase C (aPKC), Par6 and Bazooka (Baz)/Par3), Inscuteable (Insc), Partner of Inscuteable (Pins) and Gαi; basal cortical proteins include Miranda (Mira) and Prospero[1]. Neuroblasts reliably undergo asymmetric cell division by aligning their mitotic spindle along the apical–basal cell polarity axis, resulting in the formation of a larger self-renewing apical neuroblast and a smaller, basal ganglion mother cell (GMC) that typically differentiates into a pair of neurons.

Pins and Gαi are key regulators of neuroblast asymmetric cell division: they control cortical polarity, spindle orientation, spindle asymmetry and the establishment of sibling cell size differences[1]. How these proteins execute each of these functions has been difficult to establish. A central function of Pins–Gαi is to orient the mitotic spindle. This is supported by three lines of evidence. First, partial reduction of Pins–Gαi levels results in defects in aligning the mitotic spindle with the residual Par–Insc cortical crescents[5]. Second, neuroblasts with delocalized Par–Insc proteins still show tight alignment of the mitotic spindle with cortical Pins–Gαi crescents[5]. Third, vertebrate and *Caenorhabditis elegans* Pins homologues (LGN and GPR1/2, respectively) can bind microtubule-associated proteins[6–9]. All Pins-related proteins contain at least one carboxy-terminal GoLoco domain that interacts with Gα proteins: whereas the amino-terminal domain of Pins binds Insc, LGN binds NuMA and GPR1/2 binds Lin-5 (refs 6,7,9–12). Vertebrate NuMA and *C. elegans* Lin-5 are coiled-coil proteins that directly or indirectly associate with spindle microtubules, and Lin-5 (together with GPR–Gα proteins) is required for spindle positioning and sibling cell size differences in the early *C. elegans* embryo[8,9,13]. Insc has been proposed to have a NuMA-like function in *Drosophila*[14], but Insc and NuMA have unrelated protein-domain organization and Insc has not been shown to bind tubulin or to localize to spindle microtubules. Thus, it remains unclear whether *Drosophila* has a functional NuMA/Lin-5 orthologue that links Pins to the mitotic spindle during asymmetric cell division, or how Pins–Gαi interact with spindle microtubules to regulate spindle orientation.

Pins and Gαi — and the associated Insc, Loco and Dlg proteins — are all required for proper cortical polarity and spindle orientation[5,12,15,16], and thus it is difficult to determine whether they regulate spindle orientation directly or indirectly. We have been investigating Pins-binding proteins that regulate spindle orientation without altering cortical polarity, and thus are more likely to provide a direct link between Pins and the mitotic spindle. We previously showed that the kinesin Khc-73 regulates spindle orientation without affecting cortical polarity[5]. Here, we characterize the Mushroom body defective (Mud) protein[17] — which has been identified as a 'low confidence' Pins interactor in a genome-wide yeast two-hybrid screen[18] — for its ability to bind Pins and to regulate spindle orientation in *Drosophila* neuroblasts. *mud* mutants are viable but are female-sterile, with structural defects in the mushroom body region of the brain[17,19,20]. The Mud protein is predicted to have three isoforms, all sharing a central coiled-coil domain and one or two C-terminal predicted transmembrane domains[17] (Fig. 1a). In addition, the longest Mud isoform contains an evolutionarily conserved short sequence motif that is also found in human NuMA[20]. We extended this analysis and found a conserved ~30 amino-acid sequence motif that is shared by vertebrate NuMA, *C. elegans* Lin-5 and insect Mud proteins, which we term the NLM homology domain (Fig. 1b). Blast analysis of vertebrate genomes

[1]Institute of Molecular Biology, Institute of Neuroscience, Howard Hughes Medical Institute, University of Oregon, Eugene, OR 97403, USA.
[2]Correspondence should be addressed to C.Q.D. (e-mail: cdoe@uoregon.edu)

Received 20 March 2006; accepted 10 April 2006; published online 30 April 2006; DOI: 10.1038/ncb1412

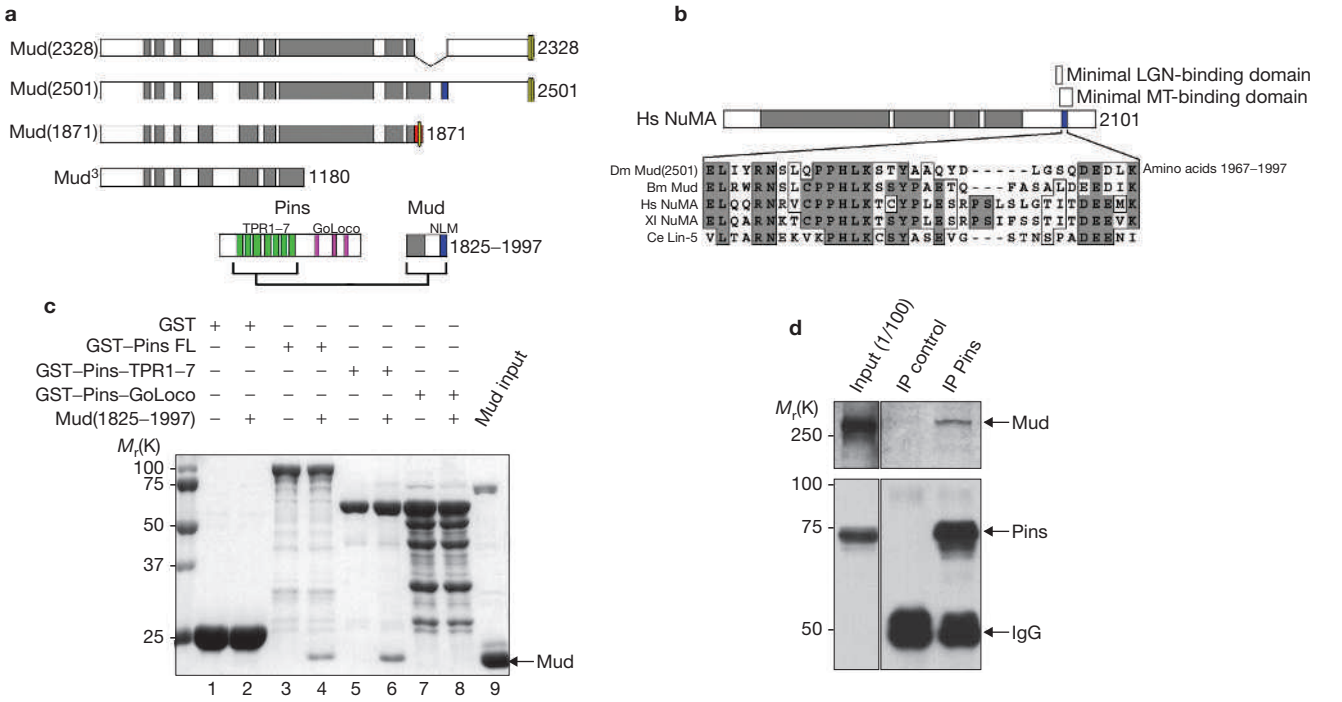

Figure 1 *Drosophila* Mud protein domains and interaction with Pins. (**a**) Schematic of the predicted Mud isoforms[17]. Grey: coiled-coil domains; blue: NLM motif (amino acids 1967–1997); red: isoform Mud(1871)-specific domain; yellow: putative transmembrane domains. Also shown are the truncated Mud protein encoded by the *mud*^{3} allele[20] and the Mud(2501)-specific domain (amino acids 1825–1997) used for the *in vitro* binding assay (panel **c**, below); this domain interacts with the Pins TPR1-7 (green) but not the GoLoco (purple) domains. (**b**) Schematic representation of human NuMA[28] and the proposed minimal microtubule- and LGN-binding domains[7,10]. Note that the NLM motif (blue) is embedded in the proposed microtubule-binding domain. Below, amino-acid sequence alignment of the NLM motif. Dm: *Drosophila melanogaster*; Bm: *Bombyx mori*; Hs: *Homo sapiens*; Xl: *Xenopus laevis*; Ce: *Caenorhabditis elegans*. (**c**) Glutathione *S*-transferase (GST) pulldown experiment. The Mud 1825–1997 fragment from the Mud(2501) protein contains the NLM domain and is pelleted with GST-bound full-length Pins (amino acids 1–658; lanes 3, 4) and GST-bound Pins amino-terminal TPR domain (amino acids 42–398; lanes 5, 6), but not with the GST-bound Pins carboxy-terminal GoLoco domain (amino acids 372–658; lanes 7, 8) or GST alone (lanes 1, 2). Lane 9 was loaded with 50% of the Mud-PB input. (**d**) Mud and Pins immunoprecipitated (IP) from wild-type embryonic lysates with anti-Pins antibody but not with non-specific control antibodies. A total of 1/100 of the embryonic lysate used as input was loaded with the immunoprecipitates on the same gel (non-adjacent lanes) and blotted on the same membrane for comparison. IgG, immunoglobulin G.

with the insect NLM motif exclusively recovers NuMA-related proteins. Thus, Mud is an excellent candidate for a *Drosophila* NuMA orthologue: it has a similar domain structure and is reported to interact with Pins.

We confirmed the Mud–Pins interaction[18] by showing that a short C-terminal portion of Mud containing the NLM domain and 142 amino acids of the amino-terminal sequence (amino acids 1825–1997) directly interacted with Pins *in vitro* (Fig. 1c). Further analysis revealed that Mud could bind the amino-terminal Pins tetratricopeptide (TPR)1–7 domain, but not the C-terminal GoLoco domain (Fig. 1c). Although we found that Insc could bind TPR1–4, we could not observe Mud binding to any region of Pins that was smaller than TPR1–7 (data not shown), indicating that all seven TPRs are required for proper presentation of the Mud-binding epitope. Consistent with the Mud–Pins direct interaction, Mud and Pins could be coimmunoprecipitated from embryonic lysates (Fig. 1d). The Mud–Pins interaction is likely to be evolutionarily conserved, as homologous domains in NuMA and Mud may mediate their interaction with LGN and Pins, respectively[7] (Fig. 1b). We conclude that the Mud C terminus can bind the Pins TPRs, and both proteins are part of a common protein complex *in vivo*.

In embryonic neuroblasts, we found that Mud and Pins were both enriched at the cortex over the apical centrosome/spindle pole from late interphase and up to the end of metaphase (Fig. 2a, b, d, e; Fig. 3a). By late anaphase–telophase, Mud showed bipolar apical and basal cortical crescents over both spindle poles (Fig. 2c); this can be seen most clearly in neuroblasts that are cultured *in vitro*, where there are fewer surrounding cells (Fig. 2f). In addition, Mud showed strong spindle-pole/centrosome localization and weaker spindle and astral microtubule localization in all neuroblasts (Fig. 2b–f). In larval neuroblasts, Mud was always present at the apical cortex with Pins ($n > 20$): it either formed cortical crescents over both spindle poles (Fig. 3e) or was uniformly cortical (data not shown). In *mud*^{4} null mutants[17,20], larval neuroblasts had virtually no detectable Mud protein, confirming the antibody specificity (Fig. 3g). We conclude that Mud and Pins form apical cortical crescents during late interphase and prophase (this is the time when spindle orientation is established in larval neuroblasts, see below), and that Mud is also detected at the basal cortex later in mitosis, as well as on spindle poles and microtubules. The minor differences in Mud localization between embryonic and larval neuroblasts may be due to differences in fixation/visualization or in cell types. Thus, *Drosophila* Mud, *C. elegans* Lin-5 and mammalian NuMA all share a common localization profile of cell cortex, spindle poles and spindle microtubules[9,13,14].

We next tested whether Mud and Pins relied on each other for apical cortical localization. In *mud* mutant larval neuroblasts, we observed normal apical–basal localization of Pins–Gαi and all other tested cortical polarity proteins (Figs 3, 4, and see Supplementary Information, Fig. S1). By contrast, *pins* or *Gαi* maternal-zygotic null-mutant

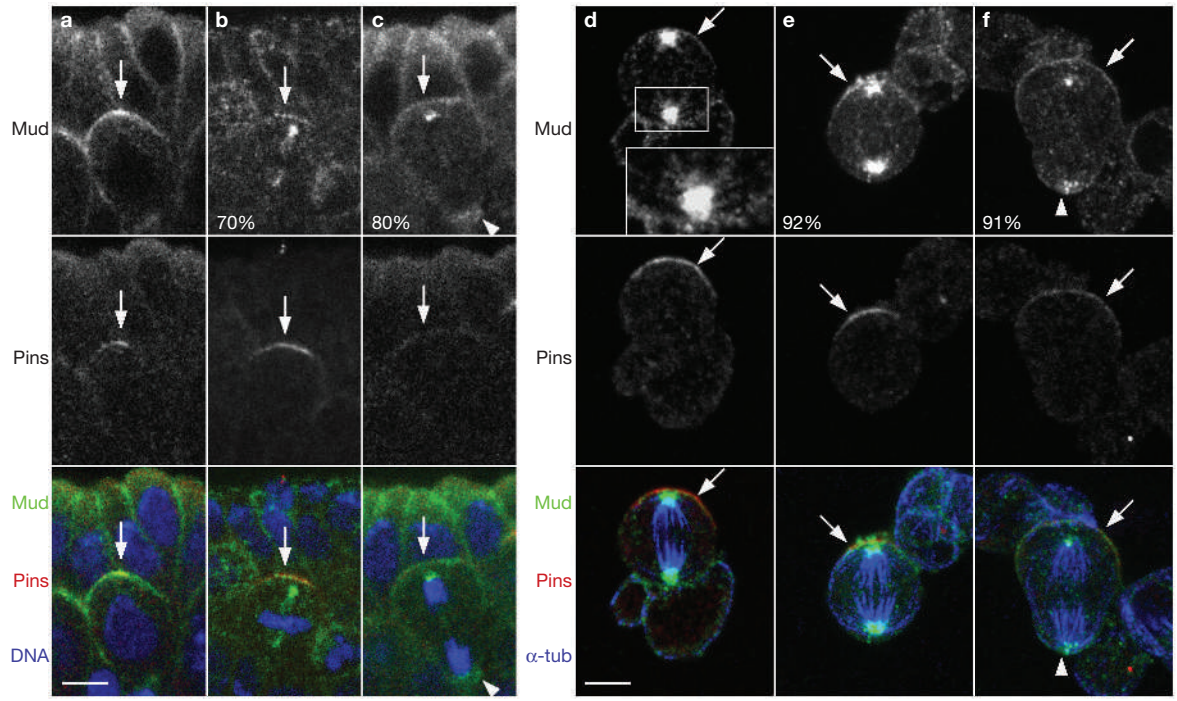

Figure 2 Mud is localized to the cortex, spindle poles and spindle microtubules. (**a**–**c**) Wild-type embryonic neuroblasts *in vivo* were triple-labelled for Mud (top row), Pins (middle row) and DNA (merged panels in bottom row). (**a**) At late interphase, Mud and Pins were both enriched at the apical cortex. (**b**) At metaphase, Mud and Pins were both enriched at the apical cortex (70%; $n = 43$); in the remaining neuroblasts, Mud was additionally detected at the lateral or basal cortex. (**c**) At anaphase/telophase, Mud was detected in apical and basal cortical crescents (80%; $n = 10$), with the remaining neuroblasts showing uniform cortical Mud. At all stages of mitosis, Mud was strongly associated with spindle poles/centrosomes. Note the lateral cortical association of Mud in the overlying ectoderm. (**d**–**f**) Wild-type embryonic neuroblasts cultured *in vitro* triple-labelled for Mud (top row), Pins (middle row) and α-tub (merged panels in bottom row). At pro-metaphase/metaphase (**d**, **e**), Mud and Pins were both enriched at the apical cortex (Mud, 92%, $n = 12$), or Mud had an additional basal crescent (8%, $n = 12$). At anaphase (**f**) or telophase (not shown), Mud showed bipolar cortical localization (91%, $n = 11$). At all stages of mitosis, Mud was strongly associated with spindle poles/centrosomes, including microtubules radiating from the spindle pole (**d**, inset). Arrows, Mud or Pins at the apical cortex; arrowheads, Mud at the basal cortex. Apical, up (as defined by cortical polarity markers, in this and all subsequent figures). Scale bars, 5 μm.

neuroblasts always lacked apical enrichment of Mud: it was either cytoplasmic or cytoplasmic with residual uniform cortical localization, although centrosome/spindle-pole localization was unaffected (Fig. 3b–d; quantified in figure legend). In addition, the C-terminal truncated Mud protein that was encoded by the *mud*[3] allele failed to localize to the cortex or spindle poles in larval neuroblasts (Fig. 3g). We conclude that Pins recruits Mud to the neuroblast apical cortex, probably via interaction with the Mud C-terminal domain.

We next determined the function of Mud in spindle orientation. Because Mud is maternally provided and required during meiosis[20], we analysed spindle orientation in larval neuroblasts. Wild-type larval neuroblasts invariably aligned their metaphase spindle within 15° of the centre of the Pins apical crescent (96% aligned, $n = 41$; Fig. 4a–c, g) or the Mira basal crescent (data not shown). By contrast, *mud* mutant neuroblasts showed significant defects in metaphase spindle alignment with the apical Pins crescent (only 40–43% aligned for *mud*[2] or *mud*[4]; Fig. 4d–g). We also observed formation of bent spindles in 29–40% of all *mud* mutant neuroblasts (described and quantified in Supplementary Information, Fig. S2), but these are not correlated with spindle-orientation defects (Fig. 4g) and arise after spindle orientation is fixed (see below). We conclude that Mud is required for metaphase spindle orientation. Despite severe defects in metaphase spindle orientation, we found that the mitotic spindle and cortical polarity markers were nearly always re-aligned by telophase in *mud* mutant neuroblasts (96–98% aligned; Fig. 4i, k). In the rare neuroblasts in which 'telophase rescue' of spindle-cortex alignment failed to occur, and the spindle axis remained nearly perpendicular to the cell polarity axis, we found that the neuroblast division was invariably symmetric with regards to cortical polarity and sibling cell size (Fig. 4j, k). Thus, Mud specifically regulates spindle orientation, but spindle orientation defects can affect the asymmetry of cell division. We conclude that: Mud is required to align the mitotic spindle with Pins cortical polarity at metaphase; a Mud-independent mechanism can rescue spindle–cortex alignment at telophase; and proper spindle–cortex alignment is necessary to promote asymmetric cell division of larval neuroblasts (Fig. 4k).

We next used time-lapse imaging of larval neuroblasts to address two important questions: when do the spindle orientation defects arise in *mud* mutants, and how are the spindle orientation defects 'rescued' at telophase? We imaged mitotic larval neuroblasts in whole brain explants[21] expressing a spindle marker labelled with green fluorescent protein (transgenic line G147)[22] and/or an enhanced yellow fluorescent protein (EYFP)–Baz apical cortical marker. In wild-type neuroblasts, we found that the two fully separated centrosomes were always aligned along the future apical–basal axis by the end of prophase (mean deviation, 9.6° ± 6.7°; $n = 22$; Fig. 5a). Thus, in contrast to embryonic neuroblasts in which spindle rotation is reported to occur at metaphase[23], larval neuroblasts fix spindle-pole/centrosome alignment at prophase and maintain spindle orientation up to the end of telophase.

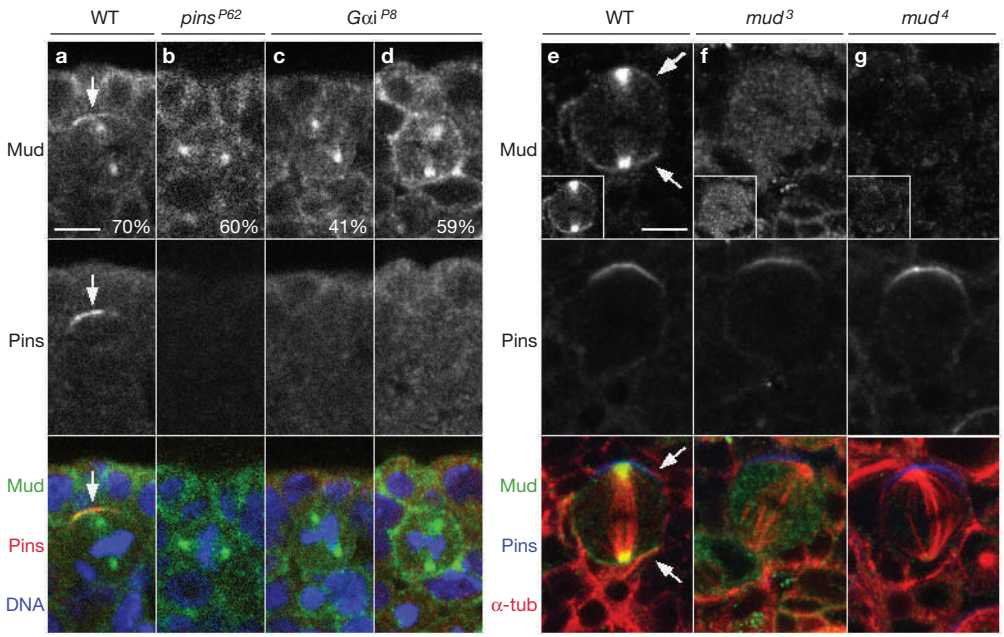

Figure 3 Pins recruits Mud to the neuroblast apical cortex. (**a**–**d**) Wild-type, *pins* and *Gαi* mutant embryos triple-labelled for Mud, Pins and DNA. (**a**) In wild-type, Mud and Pins are both enriched at the apical cortex. (**b**) In maternal-zygotic *pins*^{P62}-null mutants, Mud was cytoplasmic (60%) or uniformly cortical (40%, not shown) and was never enriched at the apical cortex ($n = 16$). (**c**, **d**) In maternal-zygotic *Gαi*^{P8}-null mutants, where Pins is delocalized[29], Mud was cytoplasmic (41%, **c**) or uniformly cortical (59%, **d**) and was never enriched at the apical cortex ($n = 34$). Centrosome/spindle-pole association was unaffected in all neuroblasts in both mutants. Arrows, cortical Mud crescents. (**e**–**g**) Wild-type, *mud*^{3} and *mud*^{4} mutant larval neuroblasts triple-labelled with Mud, Pins and α-tubulin (α-tub) and imaged using identical settings. (**e**) Wild-type. Mud localization is bipolar cortical and on centrosomes/spindle poles ($n > 20$). (**f**) *mud*^{3} mutant. This Mud protein lacks the NLM and putative transmembrane domains (Fig. 1a) and fails to localize to the cortex or spindle poles ($n > 15$). (**g**) *mud*^{4} null mutant. Only weak Mud staining on centrosomes/spindle poles was detectable. Similar results were seen with *mud*^{2} mutants. Insets show the same neuroblasts imaged at higher gain to confirm lack of Mud^{3} protein localization. Scale bars, 5 μm.

Analysis of the EYFP–Baz apical cortical marker revealed that cortical polarity was always established prior to fixation of centrosome position and accurately predicted the final axis of spindle orientation (Fig. 5b; $n = 8$). This is consistent with the tight alignment of centrosomes and cortical polarity axes that were observed from the end of prophase to telophase in fixed preparations (Fig. 4). We conclude that wild-type neuroblasts establish cortical polarity by prophase, establish centrosome position by the end of prophase and maintain tight spindle–cortex alignment during telophase.

In *mud* mutant neuroblasts, we found that spindle orientation was also established at prophase, with little or no spindle movement through telophase (mean angular movement, 13.7° ± 9.1°, $n = 20$, Fig. 5c). However, we found significant defects in the alignment of the mitotic spindle with the EYFP–Baz cortical crescent, including neuroblasts in which the spindle and cortical polarity axes were nearly perpendicular (Fig. 5d, e; $n = 4$). When we examined *mud* mutant neuroblasts during anaphase/telophase, we never observed movement of the mitotic spindle to bring it into alignment with the EYFP–Baz cortical polarity axis (Fig. 5d, e; $n = 4$), despite data from fixed preparations showing that the majority of metaphase spindle orientation defects are corrected by telophase (Fig. 4i, k). Finally, we observed that *mud* mutants could divide asymmetrically (Fig. 5d; $n = 2$) or symmetrically (Fig. 5e; $n = 2$). Symmetric divisions occurred only when the spindle was nearly orthogonal with the cortical polarity axis and we infer that these neuroblasts correspond to the equally dividing neuroblasts that were seen in fixed preparations (Fig. 4j). We draw three conclusions from our live imaging experiments. First, the *mud* spindle-orientation defects are due to a failure in centrosome/spindle-pole positioning at prophase, prior to the formation of the metaphase spindle. This further supports our conclusion that metaphase spindle morphology defects are not the source of the spindle-orientation defects. Second, *mud* mutants do not rotate their spindle towards the cortical polarity axis at anaphase–telophase, indicating that the observed 'telophase rescue' of spindle-cortical polarity occurs by modification of cortical protein distribution to match the spindle axis. Third, Mud does not directly promote asymmetric cell division, but it does regulate spindle orientation relative to cortical polarity, and only when the spindle is orthogonal to the cortical polarity axis does the cell division become symmetric. Thus, spindle orientation dictates whether the cell division is symmetric or asymmetric (Fig. 4l).

Here, we have shown that Mud has the properties of a functional orthologue of the vertebrate NuMA and *C. elegans* Lin-5 proteins. All three proteins contain coiled-coil regions and an adjacent NLM domain (found only in NuMA-related proteins), and all three proteins directly interact with similar Gα-binding proteins (Pins, LGN, GPR1/2). In addition, all three proteins are localized to the cell cortex, spindle poles and spindle microtubules, and at least Mud and Lin-5 have some role in spindle orientation and generating unequal daughter cell size[8,9,13]. However, there are differences. NuMA and Lin-5 cortical association depends on LGN and GPR1/2, respectively[8,9,14], whereas Mud can localize to the cortex (albeit uniformly) in the absence of cortical Gαi and Pins. Pins-independent Mud cortical localization may be mediated by the Mud C-terminal putative transmembrane domains, which are absent in NuMA and Lin-5 proteins. Conversely, NuMA and Lin-5 facilitate cortical localization of LGN and GPR1/2, respectively[8,9,14], whereas Mud is not required for Pins localization. Finally, it is unknown how

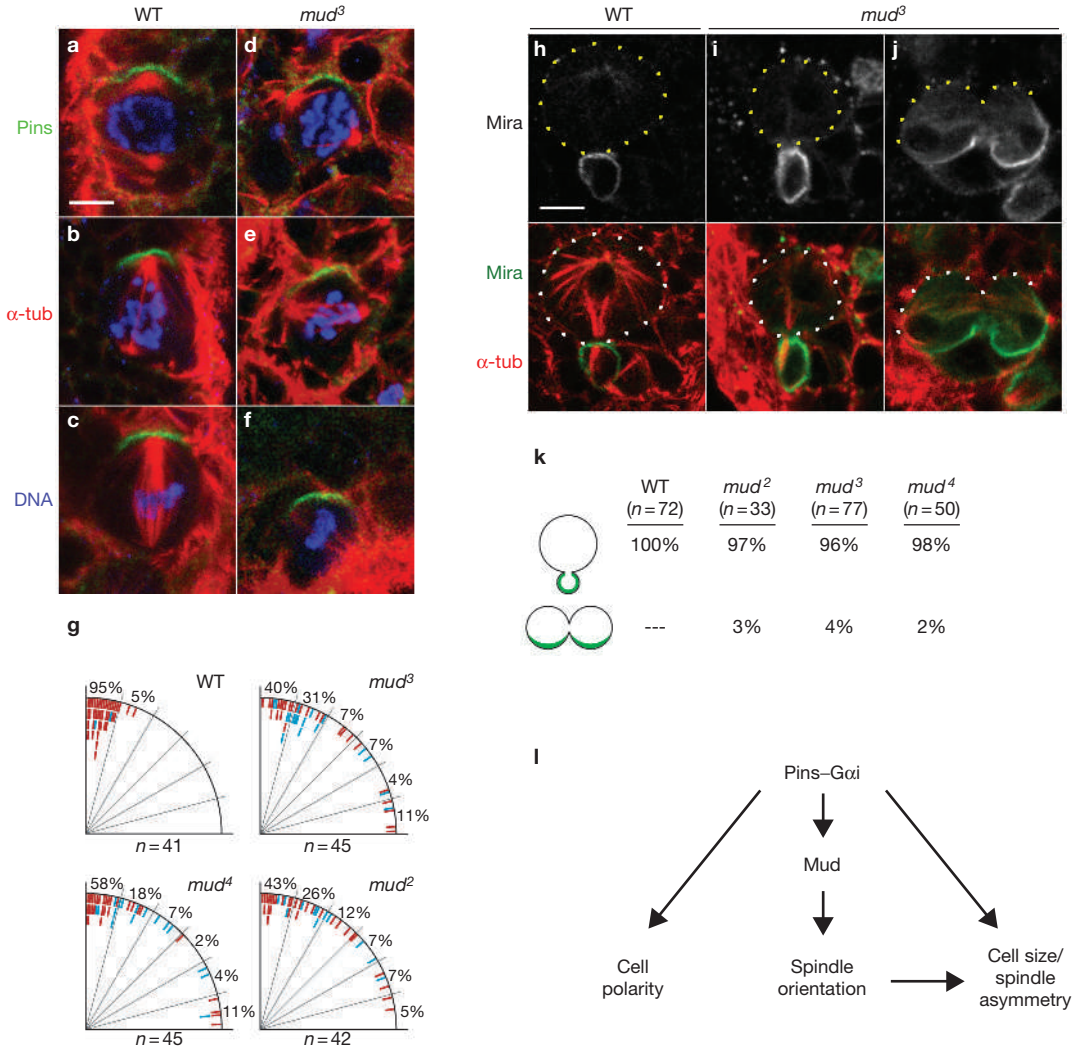

Figure 4 Mud is required for spindle orientation in metaphase neuroblasts. (**a**–**g**) Wild-type and *mud* mutant larval metaphase neuroblasts triple-labelled with Pins, α-tubulin (α-tub) and DNA. (**a**–**c**) In wild-type, the mitotic spindle was tightly aligned with the centre of the apical cortical Pins crescent. (**d**–**f**) In *mud*^{3} mutants, the spindle frequently failed to align with the apical Pins crescent. (**a**, **d**) Prophase; (**b**, **e**) pro-metaphase; (**c**, **f**) metaphase. (**g**) Quantification of metaphase spindle alignment for wild-type, *mud*^{2}, *mud*^{3} and *mud*^{4} mutants (red bars, straight spindles; blue bars, bent spindles) to the centre of the Pins crescent (vertical line). Note lack of correlation between spindle orientation and spindle morphology. (**h**–**k**) Wild-type and *mud*^{3} mutant larval telophase neuroblasts double-labelled with Mira and α-tubulin. (**h**) In the wild type, the spindle was aligned such that the cortical Mira protein was specifically segregated into the smaller cell, forming the ganglion mother cell ($n > 70$). (**i**) Most *mud* neuroblasts showed normal spindle orientation by telophase (96–98%). (**j**) A small number of *mud* mutant neuroblasts showed misaligned spindles leading to the symmetric partitioning of Mira protein in both daughter cells; in these cases, both daughter cells and spindle asters were equally sized (2–4%). (**k**) Quantification of telophase spindle alignment and sibling cell size labelling as in (**g**). Scale bars, 5 μm. (**l**) Pins–Gαi regulate cell polarity, spindle orientation and spindle/cell size asymmetry. By contrast, Mud specifically mediates the Pins–Gαi spindle orientation function. When spindle orientation is perpendicular to the cortical polarity axis, however, asymmetric cell division fails to occur and two equal-sized daughter cells are formed (thin arrow).

Mud interacts with the mitotic spindle. NuMA directly binds tubulin through a domain containing the NLM motif (Fig. 1b)[7,10], raising the possibility that the Mud NLM domain mediates microtubule association. Alternatively, Mud may associate with the spindle via dynein/dynactin, as has been shown for NuMA[24].

Pins and Gαi regulate cortical polarity, spindle orientation, spindle asymmetry and the establishment of sibling cell size differences[1]. Previously, all *Drosophila* mutants in cortical polarity proteins either severely disrupted cortical polarity, thereby precluding analysis of cortical-spindle alignment, or had no effect on spindle orientation[1]. Reduction in Mud or Khc-73 levels affects spindle orientation without altering cortical polarity[5]; each has a partially penetrant phenotype, so they may function redundantly. *mud* mutants affect only spindle orientation without directly regulating any other known Pins–Gαi-dependent functions, such as regulation of cortical polarity or sibling cell size (Fig. 4g). Only when the spindle is aligned orthogonally to the Pins–Gαi crescent are there defects in sibling cell size, presumably due to the equalized activity of Pins–Gαi in both siblings. Whether each of the many essential Pins–Gαi functions has a unique effector protein, similar to the role of Mud in regulation of intrinsic spindle orientation, will be an interesting question for the future. □

Note added in proof: an accompanying manuscript by Izumi, Y. et al. *(*Nature Cell Biol. ***8,*** *doi: 10.1038/ncb1409; 2006) is also published in this issue.*

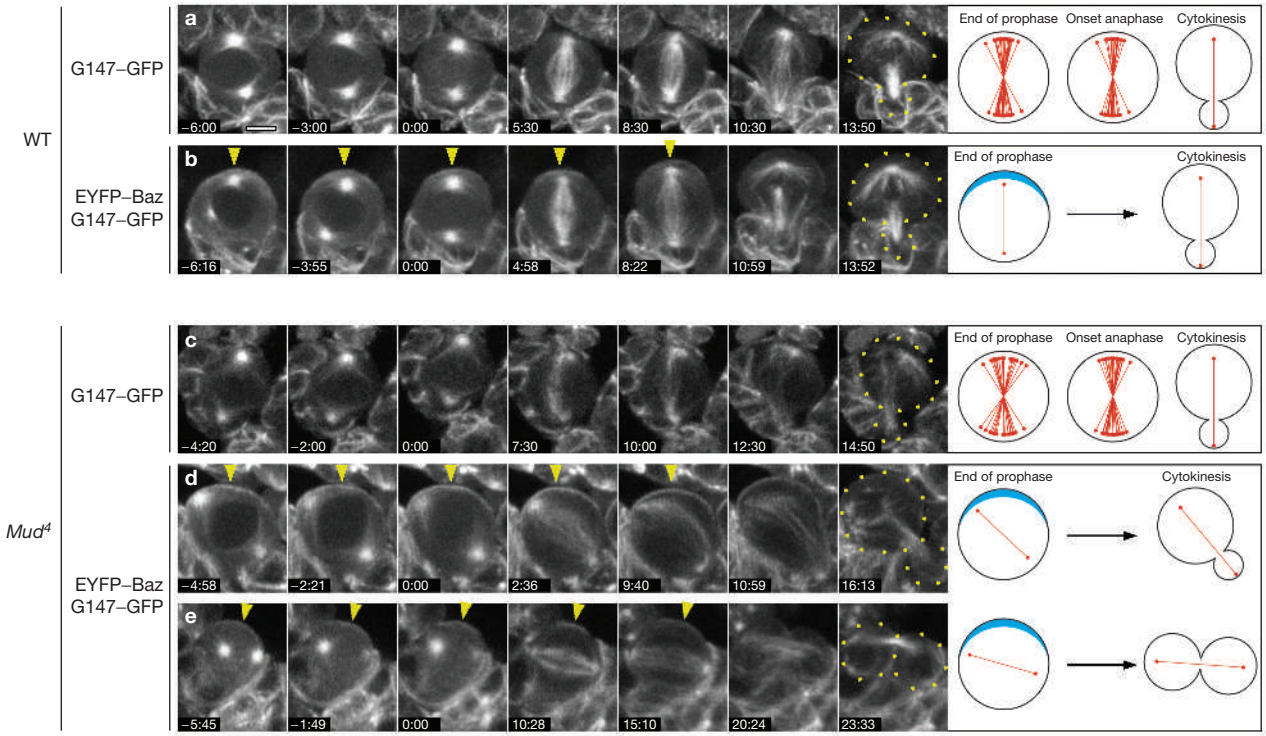

Figure 5 Mud aligns the centrosome/spindle poles with cortical polarity. Larval neuroblasts expressing microtubule-associated protein G147–GFP[22] (green fluorescent protein; **a**, **c**) or G147–GFP and enhanced yellow fluorescent protein (EYFP)–Baz apical cortical protein (**b**, **d**, **e**). Time is given in min: s relative to onset of pro-metaphase (0:00). Cartoons depict positioning of centrosome pairs (solid circles connected by solid lines) from the end of prophase and up to cytokinesis. (**a**, **c**) Centrosome alignment relative to final division axis. (**b**, **d**, **e**) Centrosome alignment relative to the EYFP–Baz apical cortical crescent (blue). (**a**) In wild-type neuroblasts, centrosomes were aligned with the final division axis from the end of prophase and up to telophase (0:00–13:50; Movie 1, $n = 22$). (**b**) In wild-type neuroblasts, centrosomes were aligned with both EYFP–Baz and the final division axis from the end of prophase and up to telophase (0:00–13:52; Movie 2). Average deviation of spindle angle from centre of EYFP–Baz crescent was 8.2° ± 5.6° at the end of prophase and 3.1° ± 1.9° at anaphase onset ($n = 8$). (**c**) In *mud*^{4} mutant neuroblasts, centrosomes were aligned with the final division axis from the end of prophase and up to telophase (0:00–14:50; Movie 3, $n = 20$). The fixation of spindle orientation at prophase is fully penetrant, and thus is not due to centrosome detachment from the nuclear envelope, which was observed in a minority of neuroblasts (data not shown). (**d**–**e**) In *mud*^{4} mutant neuroblasts, the centrosome/spindle poles did not show reliable alignment with the EYFP–Baz apical cortical crescent, and did not move towards the crescent during anaphase/telophase (**d**, 0:00–16:13, Movie 4; **e**, 0:00–23:33, Movie 5). The average deviation of spindle angle from the centre of the EYFP–Baz crescent was 58.9° ± 10.5° at the end of prophase and 61.2° ± 16.6° at anaphase onset ($n = 4$). The average duration of pro-metaphase/metaphase was similar in wild-type neuroblasts (7 min 0 s ± 1 min 30 s) and *mud*^{4} mutant neuroblasts (7 min 18 s ± 1 min 46 s). Scale bar, 5 μm.

METHODS

***In vitro* binding assay.** PGEX-4T1 vectors containing cDNA encoding full-length Pins (glutathione *S*-transferase (GST)–Pins-FL; amino acids 1–658), Pins amino acids 42–398 (containing the tetratricopeptide motifs; GST–Pins-TPR) or Pins amino acids 372–658 (containing the GoLoco motifs; GST–Pins–GoLoco) downstream and in-frame of the GST coding sequence were kindly provided by Rick Nipper and Ken Prehoda (University of Oregon, Eugene, OR). A DNA fragment encoding Mud amino acids 1825–1997 was PCR-amplified from expressed sequence tag LD31911 and subcloned downstream and in-frame of a 6 × histidine encoding sequence in the bacterial pBH expression vector[25]. GST, GST–Pins-FL, GST–Pins-TPR and 6 × His–Mud(1825–1997) were expressed in the *Escherichia coli* strain BL21(DE3). 6 × His–Mud(1825–1997) was purified on Ni-NTA resin. For the GST pulldown experiments, the GST fusion proteins were adsorbed on glutathione agarose, washed three times with binding buffer (10 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM DTT, 0.1% Triton-X-100), incubated with ~10 μg purified 6 × His–Mud(1825–1197) for 15 min, and washed four times in binding buffer. Bound proteins were eluted in sample buffer and analysed by SDS–PAGE and Coomassie staining.

Immunoprecipitation experiments. Embryos (0–16 h) were homogenized at 4 °C in five volumes of IP buffer (50 mM HEPES, pH 7.2, 150 mM KCl, 0.9 M glycerol, 0.5 mM DTT, 0.1% Triton X-100, with protease inhibitor mix; Sigma, St Louis, MO). Following centrifugation for 15 min, the supernatant was collected and pre-cleared against washed protein G-Agarose beads (Roche, Basel, Switzerland). Cleared lysates were incubated with equal amounts of polyclonal rat anti-Pins or control rat anti-HB9 antibodies overnight at 4 °C. After addition of protein G-Agarose beads and 2 h incubation at 4 °C for 2 h, beads were washed four times in IP buffer. Pellets were eluted into 40 μl sample buffer and samples were analysed by SDS–PAGE, followed by western blot analysis, using anti-Pins or anti-Mud antibodies.

Fly strains. Oregon R was used as a wild-type control. The Baz coding sequence was PCR-amplified and subcloned into the pUAST vector downstream of, and in-frame with, three repeats encoding EYFP. Transgenic flies were generated by standard methods. EYFP–Baz was expressed in larval neuroblasts by crossing *pUAST–3xEYFP–Baz* transgenic flies (line #3A.3) to a *worniu–Gal4* driver line (line #2)[5]. All other fly strains have previously been described[17,20,22].

Antibodies and immunofluorescent staining. Whole-mount embryos, *in vitro* cultured embryonic neuroblasts and larval brains were fixed and stained as described previously[21,26]. Wild-type and *mud* mutant larvae were aged at 25 °C at 96–120 h after larval hatching. Primary antibodies: rabbit anti-Mud (against amino acids 375–549; 1:2000)[20]; rat anti-Miranda (1:1000); rat anti-Pins (1:500)[5]; rabbit anti-Gαi (raised against peptides, amino acids 327–355; 1:500); rabbit anti-PKCζ (C20; 1:1000; Santa Cruz Biotechnology Inc, Santa Cruz, CA); guinea pig anti-Baz (raised against amino acids 1–297; 1:1000); mouse anti α-tubulin (DM1A; 1:2000; Sigma); rabbit anti-Phospho-Histone H3 (1:1000; Upstate, West Grove, PA); rabbit anti-Cnn (1:2000)[27]. Secondary antibodies were from Jackson

ImmunoResearch Laboratories (Charlottesville, VA) and Molecular Probes (Eugene, OR). Confocal images were acquired on a Leica TCS SP2 microscope equipped with a 63 × 1.4 NA oil-immersion objective. Final figures were arranged using ImageJ, Adobe Photoshop and Adobe Illustrator.

Identification of larval neuroblasts and their apical–basal polarity. We identified central brain neuroblasts at 96–120 h after larval hatching based on their superficial position in the brain, large size (8–13 μm) and expression of Miranda, aPKC or Pins. We defined neuroblast apical–basal polarity based on cortical markers: aPKC, Baz, Par6, Pins, Gαi or Insc defined the apical cortex, and Miranda defined the basal cortex.

Spindle morphology and orientation. Spindle morphology was scored in late pro-metaphase/metaphase larval neuroblasts and categorized into two classes by assaying whether kinetochore fibres were assembled in parallel bundles on either side ('straight spindles') or only on one side of a straight line through both spindle poles ('bent spindles'). Spindle orientation in larval brain neuroblasts of a fixed specimen was analysed at late pro-metaphase/metaphase relative to the position of the cortical Pins crescent. At telophase/cytokinesis, spindle orientation was assayed relative to the positioning of Mira crescents.

Time-lapse analysis of larval neuroblasts. Brains of wild-type or *mud*^{4} mutants at 96–120 h after larval hatching expressing the microtubule-associated protein CG17238 (line G147)[22] alone or in combination with EYFP–Baz (expression controlled by neuroblast-specific *worniu–gal4* driver) were dissected and neuroblasts were imaged using a BioRad Radiance 2000 laser scanning confocal microscope equipped with a × 60 1.4 NA oil-immersion objective[21]. Time-lapse sequences were processed using ImageJ and were converted into movies using QuickTime (Apple).

Note: Supplementary Information is available on the Nature Cell Biology website.

ACKNOWLEDGEMENTS

We would especially like to thank H. Nash for providing Mud antibody and mutants ahead of publication, and for sharing unpublished data. We also thank F. Matsuzaki for communicating results prior to publication; R. Nipper, K. Prehoda, T. Kaufman, T. Megraw, F. Yu, X. Morin, W. Chia and A. Wodarz for generously sharing reagents; S. Siegrist for providing *in vitro* cultures of embryonic neuroblasts; S. Schneider for his help with sequence alignments; K. Hirono and K. Robinson for excellent technical assistance; and S. Siegrist, B. Bowerman, K. Prehoda and S. Schneider for stimulating discussions. This work was supported by an American Heart Association pre-doctoral fellowship (K.H.S.) and by the Howard Hughes Medical Institute (C.Q.D).

COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

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