Spindle orientation during asymmetric cell division

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Development of a multicellular organism from a fertilized egg depends on a precise balance between symmetric cell divisions to expand the pool of similar cells, and asymmetric cell divisions to create cell-type diversity. Spindle orientation can influence the generation of symmetric or asymmetric cell fates depending on how it is coupled to cell-intrinsic polarity cues, or how it is positioned relative to cell-extrinsic cues such as niche-derived signals. In this review, we describe the mechanism of spindle orientation in budding yeast, *Drosophila melanogaster*, *Caenorhabditis elegans* and mammalian neural progenitors, with the goal of highlighting conserved mechanisms and indicating open questions for the future.

The mitotic spindle consists of two spindle poles that nucleate microtubules from their minus-ends, and three classes of microtubules: kinetochore microtubules that attach to chromosomes, interpolar microtubules that form an antiparallel array between the spindle poles and astral microtubules that radiate out from the spindle poles and probe the cytoplasm and cell cortex with their plus-ends. Interactions of astral microtubules with the cell cortex and cytoplasmic anchor sites are thought to be the main source of information for spindle alignment[1], although cell shape can constrain the orientation of the linear mitotic spindle[2]. Spindle microtubules dynamically grow and shrink through the addition and removal of tubulin dimers, respectively, a property referred to as 'dynamic instability'. Microtubule dynamic instability allows probing for microtubule anchor sites, and can be coupled to spindle positioning force generation. Spindle positioning typically involves pulling-forces exerted on astral microtubules, which can be generated by; (1) plus-end depolymerization of astral microtubules that remain attached to the cell cortex, (2) cortically-attached microtubule minus-end directed motor activity or (3) translocation of microtubule plus-ends by attachment to actin-based motors. In these cases, the precise regulation of microtubule length is essential for productive spindle orientation, thus regulation of microtubule dynamic instability is critical for correct spindle positioning.

Budding yeast

Spindle orientation is best understood in the budding yeast *Saccharomyces cerevisiae*, and many of the relevant yeast proteins are evolutionarily conserved. Thus, investigators curious about spindle orientation mechanisms in other cell types would be wise to pay careful attention to yeast.

The cell polarity axis of budding yeast is used to direct polarized growth of the daughter cell (bud), as well as to align the mitotic spindle along this axis to ensure proper DNA segregation to both mother and daughter cells. Establishment of cell polarity requires the localized cortical activation of Cdc42, a Rho GTPase family member, which marks the incipient bud site. Activated Cdc42 organizes polarized actin cables extending from the bud site into the mother cell: subsequently a collar of septin proteins accumulates around the bud site and marks the bud neck[3]. Genetic analyses show that spindle positioning is controlled by two partially redundant pathways: an 'early' pathway that aligns the mitotic spindle along the bud axis of the mother cell before anaphase, and a 'late' pathway that translocates the aligned spindle through the bud neck during anaphase (Fig. 1).

The early pathway for spindle orientation. The first step in spindle orientation is the polarized transport of astral microtubule plus-ends along the actin cables into the bud, thereby positioning one spindle pole body (SPB) — the fungal centrosome — at the bud neck and leaving the other SPB at the base of the mother cell. Elegant genetic, biochemical and imaging studies have led to the following model for early spindle orientation. Adenomatous polyposis coli (APC)-related Kar9 is recruited to the daughter SPB by EB1-related Bim1. Kar9–Bim1 translocate to the microtubule plus-ends where Kar9 binds Myo2, a class V myosin, resulting in polarized transport of the SPB and its microtubules along actin cables to the bud neck (Fig. 1a). In support of this model, mutants lacking Kar9, Bim1 or Myo2 have spindle alignment defects, as also caused by actin cable disruption[4]. Expression of a chimaeric Myo2–Bim1 fusion protein suppresses spindle alignment defects in *kar9* mutants, indicating that Kar9 functions as a linker between the microtubule-associated Bim1p and the actin-associated Myo2 (ref. 5). Furthermore, live imaging revealed that microtubule plus-ends move in sweeping motions along actin cables towards the bud neck and into the bud without microtubule shortening, suggesting that Myo2 provides the force for spindle orientation at this stage, rather than cortical microtubule capture and depolymerization[5,6].

After actin-dependent spindle alignment, astral microtubules do shorten while attached to the cortex at the bud tip and bud neck, resulting in the final, precise positioning of the pre-anaphase spindle[6–8]. Cortical microtubule attachment requires Bud6, an actin- and

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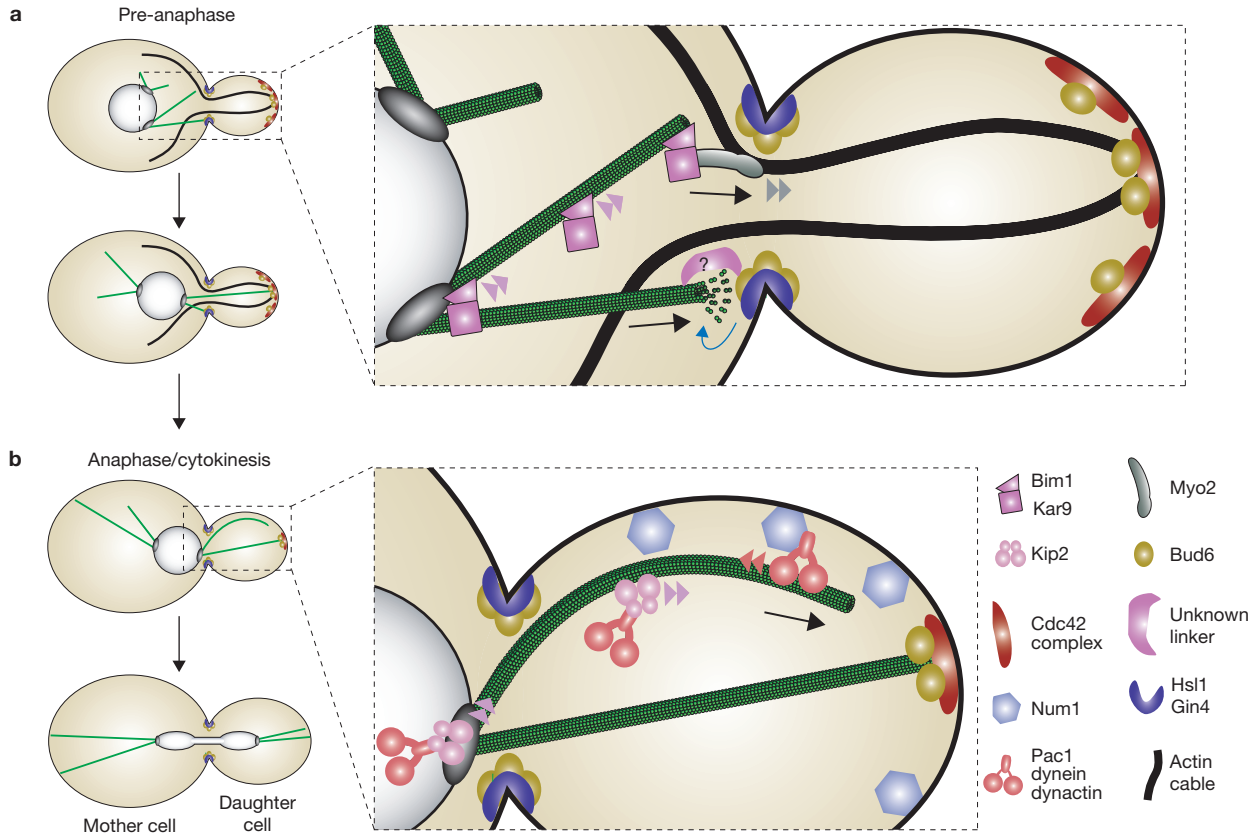

Figure 1 Spindle orientation and positioning in budding yeast. (**a**) The early pathway (pre-anaphase). Bim1–Kar9 are recruited to the spindle pole body (SPB) translocate to microtubule plus-ends (pink arrowheads) and associate with the myosin motor Myo2. Myo2 motor activity (grey arrowheads) pulls attached microtubules into the bud resulting in positioning of the SPB at the bud neck. Bim1–Kar9 movement to microtubule plus-ends may require the Kip2 kinesin motor. The bud neck kinases Hsl1and Gin4 promote microtubule shortening (blue arrow), which facilitates spindle alignment. (**b**) The late pathway (anaphase). The Kip2 kinesin transports the presumably inactive Pac1–dynein–dynactin complex from the SPB to microtubule plus-ends, and then the dynein complex is 'off-loaded' to the cortex, where it is activated by Num1. Cortical Num1–dynein–dynactin pulls the daughter centrosome to the centre of the bud cortex. Bold black arrows indicate the direction of the net spindle positioning force.

formin-binding protein localized to both the bud tip and neck[9]. How Bud6 captures microtubules is unknown, but premature Bud6 localization to the bud neck increases microtubule capturing events at this site, indicating an instructive role[7,9]. Microtubule depolymerization at the bud neck is promoted by the septin-associated kinases Hsl1 and Gin4 (refs 7, 8), but it is unknown whether these proteins directly affect tubulin or exert their function on microtubule-associated proteins. Interestingly, Hsl1 and Gin4 are related to the MARK/Par-1 kinase, which can phosphorylate and inactivate the microtubule-stabilizing Tau protein[10], raising the possibility that Hsl1 and Gin4 may destabilize microtubules by a similar mechanism. Despite these advances, several important questions remain. How are microtubules anchored to Bud6? How is astral microtubule-shortening regulated? Nevertheless, the existing data on the early pathway highlight the importance of actin-and microtubule -based molecular motors, microtubule–cortex interactions and regulated microtubule dynamics — mechanisms that are all used in higher eukaryotes as well.

The late pathway for spindle orientation. During anaphase, the SPB closest to the bud is translocated through the bud neck into the future daughter cell to establish its final position along the cell polarity axis. This process requires the microtubule minus-end directed dynein–dynactin motor complex (Box 1, refs 11–13) and involves microtubule plus-end-directed transport of an inactive dynein–dynactin complex to the cortex, followed by activation of the cortically anchored complex (Fig. 1b). Proteins required to get the dynein–dynactin complex to the cortex include the associated proteins Bik1, Pac1 and Ndl1 (related to mammalian CLIP-170, Lis1 and Ndl, respectively). Bik1 recruits dynein–dynactin to the SPB and transports the complex to microtubule plus-ends through binding to the kinesin Kip2 (ref. 4). In the absence of Pac1, dynein fails to accumulate at microtubule plus-ends[14,15], thus Pac1 may facilitate dynein–Kip2 binding, or may inhibit the minus-end directed motor activity of dynein.

Once dynein-loaded astral microtubules reach the cortex, dynein is activated by the membrane-bound pleckstrin-homology domain protein Num1 (refs 14–17). Activated dynein then pulls the SPB to the cortex using its microtubule minus-end directed motor activity[6,17]. Loss of dynein, dynactin or Num1 results in failure of the spindle to enter the neck, and the generation of a binucleate mother cell and an anucleate daughter cell[6,11,12,18]. In addition, Num1 mutants lack microtubule-sliding along the bud cortex and show increased dynein at microtubule plus-ends, consistent with a role for Num1 in cortical dynein activation[14–17]. Dynein may also be activated by the bud-cortex-localized Bud14 and associated Glc7 phosphatase: both Bud14 and Glc7 mutants show defects in spindle positioning, and Bud14 overexpression results in excessive

BOX 1 The dynein–dynactin complex: an evolutionarily conserved spindle force generator

Dynein is a motor protein complex that uses ATP hydrolysis to translocate towards microtubule minus-ends. Dynein associates with the multiprotein dynactin complex which increases dynein processivity and tethers dynein to its cargo proteins[20]. The dynactin complex includes the rod-shaped p150 dynactin protein, which directly binds dynein subunits and associates with microtubule plus-ends through its CAP-Gly domain; dynein complex structure and function is conserved from fungi to mammals. Other regulatory proteins include Lis1 (mutated in lissencephaly), a WD40 domain protein that directly binds dynein and dynactin subunits, and Nde (formerly mNudE) and Ndl (NUDEL), two coiled-coil proteins that bind Lis1. The dynein–dynactin complex regulates many processes, including organelle positioning, centrosome separation and spindle orientation. In animal cells, dynein, dynactin and Lis1 are all required for positioning the mitotic spindle in response to cortical polarity cues. Coupling of cell polarity and dynein function can be mediated through the coiled-coil protein NuMA (homologous to *C. elegans* LIN-5 and *Drosophila* Mud), which mediates a physical link between dynein–dynactin–Lis1 and G-protein regulators LGN–AGS-3 (GPR-1/2 in *C. elegans* and Pins in *Drosophila*). These G-protein regulators show polarized localization during asymmetric division in response to the activity of the Par protein complex in *C. elegans* blastomeres, *Drosophila* neuroblasts and mammalian cortical progenitor cells *Drosophila* (see Box 3).

BOX 2 The Par complex: a conserved regulator of cortical polarity

The establishment of cell polarity in many animal cells requires the partitioning defective (Par) complex, first discovered in pioneering genetic screens done by Ken Kemphues and collaborators in *C. elegans*. The Par complex contains three proteins: Par-3, a PDZ domain scaffolding protein (Bazooka in *Drosophila*); Par-6, a CRIB and PDZ domain protein and atypical protein kinase C (aPKC; PKC-3 in *C. elegans*). Par-3 is required for the polarized cortical localization of Par-6–aPKC; Par-6 regulates the kinase activity of aPKC (Par-6 alone inhibits aPKC, but Par-6 bound to Cdc42 or Rac1 monomeric GTPases activates aPKC) and aPKC is the effector of the Par complex that regulates cortical polarity by phosphorylating and driving target proteins off the cortex. The Par complex localizes to the apical cortex in invertebrate and vertebrate epithelial cells (including epidermal and neural progenitors), to the anterior cortex in the *C. elegans* zygote and to the apical cortex in *Drosophila* neuroblasts. At present it is believed that the Par complex is required for establishing cortical polarity in all metazoan cell types that undergo regulated spindle orientation, and in each case defects in Par complex localization lead to aberrant spindle orientation. Par-dependent spindle positioning is mediated through polarized localization of the G-protein binding proteins LGN–AGS3 (mammals), GPR-1/2 (*C. elegans*) and Pins (*Drosophila*), and the associated NuMA (LIN-5, Mud) proteins. In mammalian and *Drosophila* cells, the ankyrin protein Insc provides a physical link between Par-3–Baz and LGN–AGS3–Pins, thereby coupling cortical cell polarity and spindle position (see Box 3).

microtubule-sliding and aberrant translocation of the entire spindle into the bud[19]. This phenotype is suppressed in dynein mutants, suggesting that the primary cause for aberrant spindle translocation is dynein hyperactivity[19].

Spindle pole asymmetry. Proper spindle positioning requires SPB asymmetry, which ensures that only one SPB is pulled towards the bud[1]. This is accomplished by asymmetric localization of Kar9 and dynein specifically to the daughter SPB[21–23]. Kar9 localization to the daughter SPB requires the SPB-associated proteins Bim1, Bik1 and Stu2 (related to the microtubule-binding protein XMAP215), although none of these proteins are themselves restricted to the daughter SPB[4,24,25]. Kar9 localization to the daughter SPB also requires the early cyclins Clb4 and Clb5 and the associated Cdc28 kinase activity to prevent it from binding to the mother SPB[23,26]; this may be due to a daughter SPB-specific protein that binds phosphorylated Kar9[24,25].

In contrast, dynein localization to the daughter SPB occurs after asymmetric localization of Kar9, is Kar9-independent and requires the late cyclins Clb1/Clb2 and Cdc28 (ref. 21). Interestingly, the bud-neck-localized kinases Hsl1 and Gin4 are also required, suggesting that a signal is conveyed from astral microtubules contacting the bud neck back to the SPB to promote dynein asymmetry[21]. The identity of this putative signal and the substrates of the Cdc28, Hsl1 and Gin4 kinases are currently unknown.

C. elegans embryonic blastomeres

The *C. elegans* early embryo is unique in permitting powerful genetic analysis, as well as having a large cell size that facilitates mechanical experiments such as spindle severing and offers superb optical properties for time-lapse imaging. The genetic attributes have led to the discovery of evolutionarily-conserved cell polarity proteins (Box 2); whereas the large cell size and experimental accessibility have made this the premier system for understanding spindle force-generating mechanisms[27,28].

The fertilized *C. elegans* zygote is elongated along the anterior-posterior axis, with the two juxtaposed pronuclei and associated centrosomes (nucleus centrosome complex, NCC) positioned in the posterior half of the zygote, the centrosomes aligned perpendicular to the anterior-posterior axis. Before the first mitosis, the NCC moves to the cell centre in a posterior to anterior direction (centration; Fig. 2a) and rotates 90° to align the centrosome pair along the anterior-posterior axis (spindle orientation; Fig. 2b). During early anaphase the mitotic spindle moves towards the posterior pole (spindle positioning; Fig. 2c), resulting in asymmetric cell division[27].

Cortical polarity determines spindle orientation and position. The evolutionarily conserved Par complex (Par-3–Par-6–aPKC; Box 2) is localized to the anterior cortex of the zygote; whereas the posterior cortex is occupied by the PAR-1 kinase and the PAR-2 RING-finger protein[27] (Fig. 2a). In *par-2* mutants, spindle orientation (NCC rotation) is absent leading to the assembly of a transversely oriented spindle, which 'passively' aligns along the anterior-posterior axis when it elongates during anaphase, owing to the elliptical shape of the zygote[29,30]. In contrast, *par-3* mutants show normal NCC rotation in elliptically shaped zygotes, but lack NCC rotation in spherical blastomeres induced by egg-shell removal[29,30]. In addition, loss of cortical Par polarity disrupts anaphase spindle positioning, leading to the formation of equally sized anterior and posterior daughter cells[27]. Thus, both Par cortical polarity and cell shape regulate spindle orientation and position.

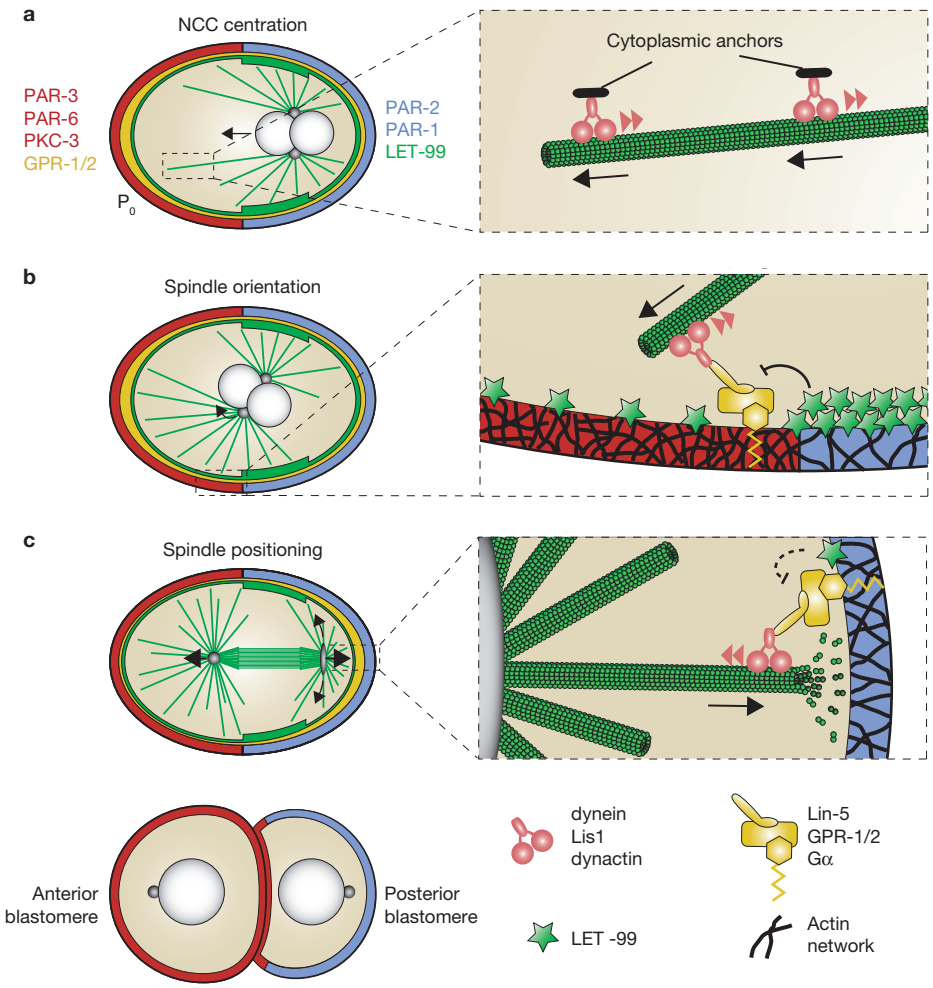

Figure 2 Spindle orientation and positioning in the *C. elegans* zygote. (**a**) The nucleus/centrosomal complex (NCC) moves anteriorly to the cell centre (centration) primarily owing to activity of dynein–dynactin complex anchored to an unknown substrate in the cytoplasm (activation of cortical dynein by the GPR-1/2 complex may also contribute, **b**). (**b**) NCC rotation (spindle orientation) aligns the centrosomes along the anterior-posterior axis due to the combined activity of cortical Par polarity proteins, the cortical GPR-1/2 complex and associated cortical dynein–dynactin complex. (**c**) At anaphase, GPR-1/2 is enriched at the posterior cortex, where it activates cortical dynein resulting in posterior spindle displacement and generation of a larger anterior and smaller posterior blastomere. LET-99 is enriched in a lateral cortical belt and restricts dynein activation in this domain by restricting cortical GPR-1/2 localization. Light red arrowheads indicate the direction of dynein motion; bold black arrows indicate the direction of the net spindle positioning force. Anterior is to the left and posterior to the right, in all panels.

Centrosome and spindle positioning forces. We will first briefly discuss NCC centration, and then focus on NCC rotation and spindle positioning mechanisms. NCC centration requires the dynein–dynactin complex but not cortical Par polarity[31]. This suggests that the force driving centration is provided by cytoplasm-anchored dynein–dynactin complex: the posteriorly located NCC generates longer microtubules in the anterior direction, which consequently have more associated dynein–dynactin complex motors, thereby pulling the NCC anteriorly until all forces are balanced at centration[32,33] (Fig. 2a). However, recent work shows that cortical polarity has a supporting role in centration. GPR-1/2 and LIN-5, components of the receptor-independent heterotrimeric G-protein pathway (Box 3), are transiently enriched at the anterior cortex at the time of centration, and are required for its timely occurence[34]. LIN-5 is known to interact with members of the dynein–dynactin complex[35,36], which could provide an anterior-directed pulling force. In addition, disruption of microtubule interactions with the cortical acto-myosin network slows centration[37]. It seems likely that cytoplasmic dynein is sufficient for centration, with cortical dynein and the acto-myosin network providing an additional anterior-directed force.

Data from spindle severing experiments, genetics and theoretical modelling show that both spindle orientation and positioning are driven by the attachment of astral microtubule plus-ends to cortically-anchored dynein–dynactin complex, resulting in pulling forces moving the centrosomes and spindle towards the cortex[28,31,35,36,38–41] (Fig. 2b–c). In contrast to budding yeast, microtubules initiate end-on contact with the cortex in *C. elegans*[42,43], although lateral microtubule-cortex interactions have also been proposed[44]. End-on engagement of cortical dynein must be coordinated with microtubule plus-end depolymerization to avoid counteracting force due to microtubule plus-ends pushing against the cortex, and indeed time-lapse imaging shows that microtubules maintain end-on contact with the cortex only transiently (≤ 1 sec) before they undergo catastrophe and depolymerize[43]. NCC rotation is due to differential pulling forces on each centrosome; however, both centrosomes seem to have similar initial positions relative to anterior-posterior cortical polarity cues

and, in contrast to yeast, no molecular or morphological centrosome asymmetry is apparent. Therefore it is likely that an initial stochastic difference in force on the two microtubule asters is amplified through a positive feedback loop that results in anterior-posterior alignment of the NCC, although molecular components of such a feed-back loop are unknown. In contrast, during anaphase spindle-positioning anterior and posterior microtubule asters are probing largely distinct cortical compartments, and the posterior spindle movement is due to larger net pulling forces acting on the posterior spindle pole as a result of this cortical polarity[28,35,36,39,40]. Dynein–dynactin complex and Lis1 are detected uniformly in the cytoplasm, at the cell cortex and on microtubules, throughout the cell cycle[31,38], raising the question of how dynein pulling forces are temporally and spatially regulated to confer posterior-directed spindle positioning. A series of papers convincingly demonstrate that heterotrimeric G-proteins have a central role in this process.

Heterotrimeric G-proteins control dynein-dependent spindle positioning. The Gα(GAO-1 or GPA-16)–Gβ–Gγ heterotrimeric complex is inactive, but on dissociation both Gα and Gβ–Gγ are activated (Box 3). All three subunits show uniform cortical localization during the first zygotic division, and each is required for proper centrosome migration, spindle orientation and spindle positioning[45–47]. Reduction in Gα levels or activity results in spindle-positioning defects[39,46–48], similar to those caused by depletion of dynein–dynactin[35,36]. In contrast, Gβ inhibition increases free Gα levels resulting in the opposite phenotype of excessive centrosome movements[48–50]. Thus Gα is the protein required for spindle positioning, whereas the Gβ–Gγ dimer attenuates Gα function by sequestering Gα into a non-functional complex. This raises the questions of how cortical Par proteins regulate the receptor-independent heterotrimeric G-protein pathway, and how free Gα activates dynein–dynactin complex.

The answer to both questions involves the TPR/GoLoco domain protein GPR-1/2 (Pins in flies; LGN/AGS-3 in mammals). Par polarity cues result in the enrichment of GPR-1/2 at the anterior cortex during prophase when NCC centration and rotation occurs, and at the posterior cortex during anaphase spindle positioning[34,48,50,51] (Fig. 2). Binding of GPR-1/2 to Gα activates both proteins: GPR-1/2 activates Gα by displacing Gβ–Gγ [48,51,52], and Gα activates GPR-1/2 by preventing TPR/GoLoco intramolecular interactions[53,54], thus making the TPR domain available for intermolecular interactions. The 'opened' GPR-1/2 uses its TPR domain to bind LIN-5 (Mud in flies; NuMA in mammals)[52], and LIN-5 and GPR-1/2 can associate with the dynein activator Lis1, and dynein itself, to exert a spindle pulling force[35,36] (Box 3). This model is supported by *in vivo* and *in vitro* protein interactions, protein localization epistasis, genetic interactions and the observation that reducing levels of Gα, GPR-1/2 or LIN-5 gives the same spindle positioning defects as loss of dynein–dynactin function[31,35,36,38,46,47,52,55].

Recent work has helped define the mechanism that translates Par-polarity into cortical GPR-1/2 asymmetry, which is critical for polarized cortical dynein activation and directionality of cortex-spindle force production. LET-99, a DEP domain G-protein regulator, has a central role this process by inhibiting cortical association of GPR-1/2. It is localized in a lateral cortical belt in response to Par polarity, which promotes the exclusion of GPR-1/2 from this domain[30,34,50] (Fig. 2a). The mechanism leading to cortical LET-99 enrichment and LET-99-dependent GPR-1/2 exclusion remains to be further investigated. Other questions also remain unanwered. For example how do LIN-5–Lis1 interactions activate the dynein–dynactin complex? Is this function conserved in flies and mammals? Is this the only pathway required for spindle positioning?

BOX 3 Receptor-independent heterotrimeric G-protein pathway

Heterotrimeric G-protein complexes consist of α, β, and γ subunits tethered to the plasma membrane by lipid modifications on Gα and Gγ subunits. Canonical receptor-dependent heterotrimeric G-protein signalling is activated by ligand-binding to a seven-pass transmembrane receptor, which promotes dissociation of active Gα-GTP from Gβ–Gγ. In contrast, receptor-independent heterotrimeric G-protein activity utilizes a GEF (Ric-8) to stimulate production of Gα-GTP, followed by a GAP (RGS-7 in *C. elegans*) generating Gα-GDP which is likely to be the active form in this pathway. Gα-GDP binds a tetratricopeptide (TPR)–GoLoco (Gα-binding) domain protein — for example, Pins (*Drosophila*), GPR-1/2 (*C. elegans*) or LGN–AGS3 (mammals) — and activates this protein by disrupting intramolecular TPR–GoLoco interactions. The 'open' TPR–GoLoco protein then binds a coiled-coil NuMA-related protein (LIN-5 in *C. elegans*, Mud in *Drosophila* and NuMA in mammals[56–58]). In this way, Gα-GDP triggers the formation of a tripartite protein complex (for example, Gα–Pins–Mud) that is required for spindle orientation. In *Drosophila* and mammals, the Insc protein can link the Pins–LGN–AGS3 to the Par complex. Whether the Pins–LGN–AGS3 TPRs can bind NuMA–Mud and Insc concurrently is unknown. The spindle positioning function of this receptor-independent G-protein pathway is, at least in part, mediated through dynein–dynactin–Lis1, which has been shown to physically associate with mammalian NuMA and *C. elegans* LIN-5. This interaction is likely to be conserved in *Drosophila*, although it has not been tested.

Drosophila neuroblasts

Neuroblasts are the stem cell-like progenitors of the *Drosophila* central nervous system. Embryonic neuroblasts delaminate as single cells from an apical/basal polarized neuroectoderm and divide asymmetrically perpendicular to the plane of the neuroectoderm to 'bud off' a series of small ganglion mother cells (GMCs). Larval neuroblasts derive from embryonic neuroblasts but contact a glial cell rather than the neuroectoderm[59]. Neuroblast asymmetric division can be subdivided into three steps: (1) apical/basal cortical polarity is established during late interphase/early prophase; (2) spindle orientation along the cell polarity axis is established by prometaphase and (3) spindle position is shifted towards the basal cortex during anaphase. This results in a molecularly and physically asymmetric cell division (Fig. 3).

Cortical polarity determines spindle orientation and position. Neuroblasts show no detectable cortical polarity during most of interphase. Apical cortical polarity is first seen at late interphase/early prophase for the Par-complex proteins Bazooka (Baz/Par-3)–Par-6–aPKC (Box 1) and the associated Inscuteable (Insc), Pins and Gαi proteins[60] (Box 3). Basal proteins such as Miranda, Prospero and Numb are subsequently localized at prometaphase. In embryonic neuroblasts, loss of Baz or Insc leads to randomization of the spindle orientation relative to the overlying neuroectoderm, whereas loss of basal proteins has no effect on spindle orientation[61–64]. In larval neuroblasts, reduction of Pins or Gαi uncouples

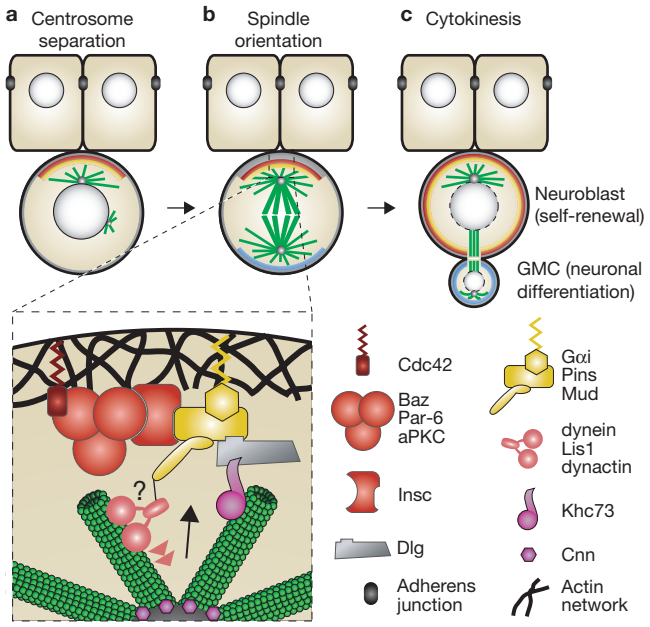

Figure 3 Spindle orientation and positioning in the *Drosophila* neuroblast. (**a**) Late interphase/prophase. Par-proteins (Baz, Par-6 and aPKC) and Cdc42 (associated through Par-6) are enriched at the apical cortex. One centrosome is anchored at the apical cortex by Gα–Pins–Mud (possibly through dynein–dynactin, although this remains to be tested) and by Pins–Dlg–Khc73. The second centrosome nucleates few microtubules and migrates basally. (**b**) Prometaphase/metaphase. Tight coupling of the spindle to the apical/basal polarity axis requires the motor proteins Khc73 and dynein; Khc73 binds Dlg and may facilitate cortical microtubule anchoring, whereas spindle positioning forces on microtubules are probably due to dynein complex activity. The Insc protein directly binds Baz and Pins, thereby coupling Par polarity with Mud- and Dlg–Khc73-dependent spindle positioning pathways. (**c**) Anaphase. The mitotic spindle becomes asymmetric leading to unequal sized daughter cells. Light red arrowheads indicate direction of dynein motion; bold black arrows indicate the direction of the net spindle positioning force.

spindle alignment from the Baz cortical polarity axis[54,62], whereas loss of Par-6, aPKC or basal cortical proteins have no effect on spindle orientation[65]. Furthermore, loss of Baz and Pins together leads to failure to generate spindle pole asymmetry, absence of basal displacement of the spindle and the production of two equally sized daughter cells[66,67]. From these observations, it seems that Baz, Insc, Pins and Gαi are the key polarity proteins regulating spindle orientation and spindle positioning, and that they may function through more than one pathway.

Spindle orientation pathways. Spindle orientation has been characterized in both embryonic and larval neuroblasts. In both, centrosomes remain associated with the apical cortex during interphase[68–70], despite the lack of any known cortical polarity cues. At prophase, one centriole pair moves basally to establish the bipolar mitotic spindle; although occasionally both centrosomes move 90 ° from the apical cortex during centrosome separation, one spindle pole always rapidly resumes contact with the apical cortex[58,68,69,71]. The mitotic spindle undergoes gentle rocking movements during metaphase, showing that microtubules are constantly probing the neuroblast cortex and exerting pulling forces, but the spindle never strays far from the apical/basal polarity axis[58,68,69,71]. These studies suggest that spindle orientation is fixed by the end of prophase, and remains stable despite the rocking movements.

Two pathways are known to regulate neuroblast spindle orientation: the Gα–Pins–Mud pathway and the Pins–Dlg–Khc73 pathway. Gαi, Pins and Mud (homologues of *C. elegans* Gα, GPR-1/2 and LIN-5, respectively) are members of an evolutionarily-conserved receptor-independent G-protein pathway (Box 3); Pins binds Gαi through its GoLoco domains[72–75] and Mud through its TPR domain[56–58] to form a tripartite protein complex. This protein complex is linked to the apical Par complex by the adapter protein Insc, which binds Baz and the TPR domain of Pins[64,72,74,75] . Recent data show that the PDZ-domain protein Canoe also associates with Pins *in vivo*[76]. Reducing the level of Gαi, Pins, Mud or Canoe prevents spindle alignment to the apical Par complex[54,56–58,72–76]. Mammalian and *C. elegans* orthologues of Mud (NuMA, LIN-5) associate with components of the dynein–dynactin complex[35,36,77], and the dynein–dynactin complex plus Lis1 are required for dynamic spindle rocking and spindle orientation in *Drosophila* larval neuroblasts[71]. Thus, it is likely that the Gαi–Pins–Mud pathway works by recruiting the dynein–dynactin complex to the apical cortex, which exerts a pulling force to recruit and maintain one centrosome at the apical pole, thereby aligning the mitotic spindle along the apical/basal polarity axis. However, dynein–dynactin complex proteins have not been detected at the apical cortex of neuroblasts[71,78], and an interaction between Mud and the dynein–dynactin complex remains to be documented.

A second spindle orientation pathway involves Pins, the tumor suppressor Discs large (Dlg; a PSD95 family member containing PDZ, SH3 and GK domains) and the microtubule plus-end-directed kinesin heavy chain 73 (Khc73; a kif13A-related protein). This pathway was discovered owing to the ability of astral microtubules and Khc73 to induce the formation of Dlg–Pins–Gαi crescents in embryonic neuroblasts lacking a functional Par complex[62]. The study showed that Khc73 localizes to microtubule plus-ends (after taxol stabilization), Khc73 binds Dlg *in vitro* and *in vivo*, similar to interactions between their mammalian orthologues[79] and Dlg co-immunoprecipitates with Pins[62]. Thus, Khc73^{+} astral microtubules can induce Pins–Dlg cortical polarity. Interestingly, reducing Dlg or Khc73 levels leads to partial spindle-orientation defects without affecting apical Pins–Gαi cortical polarity, suggesting a 'reverse' signal flow with cortical Pins–Dlg directing spindle orientation through Khc73 (ref. 62). It is unknown whether the partial phenotype is due to residual Dlg or Khc73 protein remaining in the mutant or RNAi background, or whether it is because of redundancy with the Gαi–Pins–Mud pathway.

Spindle pole asymmetry. *Drosophila* larval neuroblasts show pronounced mitotic spindle pole asymmetry at anaphase: the apical spindle pole contains more pericentrosomal Centrosomin (Cnn), nucleates longer astral microtubules and the spindle is shifted towards the basal cortex[68,69]. Although spindle pole asymmetry is necessary for spindle orientation in yeast, it is not essential for spindle orientation in neuroblasts. Mutants in *cnn*, *sas-4, asl* or *spd-2* have morphologically identical spindle poles, yet most of these neuroblasts undergo normal asymmetric cell division[80–83].

Spindle orientation and cell fate. Mutants in genes such as *aurora-A*, *mud* and *polo* have both spindle orientation defects and increased neuroblast numbers[56,84–86], suggesting that precise spindle alignment is required for normal neuroblast/GMC fate. However, *aurora-A* and *polo* mutants have additional cell polarity defects, which complicates interpretation. In contrast, *mud* mutants show defects in spindle orientation and a moderate increase in neuroblast number yet have normal cortical

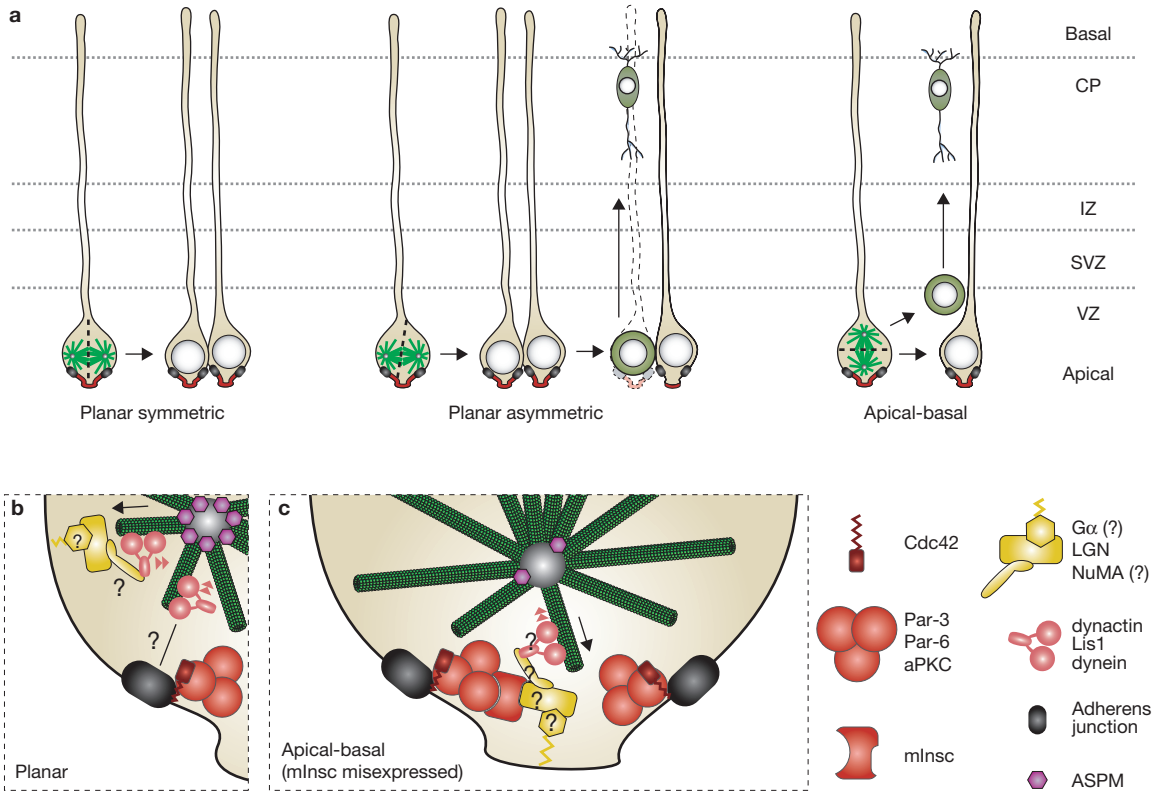

Figure 4 Spindle orientation and positioning in the mammalian neuroepithelium. (**a**) The mammalian cortex is a pseudostratified epithelium with morphologically distinct layers, including cortical plate (CP), intermediate zone (IZ), subventricular zone (SVZ) and ventricular zone (VZ). Neural epithelial cells or radial glia progenitors in the VZ (tan cells) can undergo planar and molecularly symmetric divisions (left); planar but molecularly asymmetric divisions (centre) or apical/basal molecularly asymmetric divisions (right). Molecularly asymmetric divisions generating a basal progenitor/neuron (green) and a neuroepithelial progenitor (tan) are shown, but the fate of sibling cells after each type of neuroepithelial progenitor division is now controversial (see text). (**b**) Planar divisions require basolateral LGN protein and Lis1 (dynein) function; it is likely but not proven that dynein activation involves Gα signalling and is linked to LGN through NuMA. In addition, dynein controls planar apical spindle positioning through direct binding to adherens junction components. The centrosome-associated ASPM protein is required for planar spindle orientation, but the mechanism is unknown. (**c**) Misexpressed mInsc colocalizes with the apical Cdc42–Par complex and increases the frequency of apical-basal divisions. mInsc can associate with LGN and the related AGS3 protein, thus it is possible that apical/basal spindle reorientation involves relocalization of Gα–LGN(AGS-3)–NuMA to the apical cortex and subsequent dynein engagement of apically positioned astral microtubules. Support for this model awaits further experimental testing. Light red arrowheads indicate direction of dynein motion; bold black arrows indicate the direction of the net spindle positioning force.

polarity[56–58], leading to the model that divisions with a transverse spindle lead to the formation of two neuroblasts through symmetric division. This is an attractive model, but it has not been rigorously tested. Time-lapse imaging studies of neuroblasts undergoing transverse divisions are needed to determine the cell fate of their progeny, and whether cell fate is correlated with inheritance of the apical or basal cortical domain.

Mammalian neuroepithelia

It has been proposed that spindle orientation regulates the determination of cell fate, the timing of neurogenesis and the evolution of brain size in mammals, but recent results suggest that spindle orientation might in fact have little or no effect on these processes. Here we review the evidence for regulated spindle orientation in the cerebral cortex and retina, highlight mechanistic similarities with other model systems and discuss the relationship between spindle orientation and sibling cell-fate specification.

Cortical cell polarity. The mammalian cerebral cortex and retina both contain multipotent neuroepithelial progenitors with pronounced apical/basal polarity (Fig. 4a). Their very small apical cortical domain or 'apical endfoot' contains Cdc42–Par-3–aPKC–Par-6, and the transmembrane protein Prominin (CD133)[87]. Their extensive basolateral domain or 'basal process' contains LGN protein (a mammalian GPR-1/2 and Pins orthologue; Box 3)[88,89], and the two domains are separated by adherens junctions containing E-cadherin, α-catenin and β-catenin[87].

Planar spindle orientation. When the mitotic spindle is aligned perpendicular to the neuroepithelial progenitor apical/basal axis — that is, in the plane of the neuroepithelium — it is termed planar spindle orientation. True planar spindle orientation ('planar symmetric', Fig. 4a, left) results in a cleavage furrow that bisects the apical membrane domain to generate two molecularly identical neuroepithelial cells. However, some apparently planar divisions in fact partition the apical domain to just one cell, resulting in a 'planar symmetric' cell division. Only very recent studies using molecular markers have distinguished these two forms of planar cell divisions[88–90]. Planar spindle orientation requires the basolateral LGN protein, a member of the LGN/Pins family (Box 3). Inactivation of LGN function randomizes spindle orientation during the early proliferative phase of neuroepithelial progenitor divisions in

mouse[88] and chick[89]. How does LGN induce planar spindle orientation? Apical/basal polarity markers and adherens junction markers are normal after LGN knockdown[89], so LGN does not act by disrupting cell polarity. LGN binds NuMA, which associates with the dynein–dynactin complex[77,91], suggesting that LGN recruits NuMA–dynein–dynactin to the basolateral domain (although this has not yet been shown). In support of this model, LGN recruits NuMA to the cortex overlying the spindle poles in epithelial Madin-Darby canine kidney (MDCK) cells[53], and reducing the level of dynein regulators Lis1/Nde1 disrupts planar spindle orientation in mouse cortical neuroepithelial progenitors[92–94]. How is the planar spindle located apically, and not randomly, within the extensive LGN^+ basolateral domain? The subapical adherens junctions may provide an additional spindle orientation cue, as the adherens junction component β-catenin binds dynein[95]. Taken together, these data support a model in which subapical adherens junctions and basolateral LGN/NuMA utilize the evolutionarily-conserved spindle orientation dynein–dynactin complex to promote planar spindle orientation.

Proper spindle orientation requires both a cortical capture site and dynamic microtubules that probe the cortex, and indeed mutations in centrosomal and microtubule -binding proteins are known to affect planar spindle orientation. Mice lacking the centrosomal protein ASPM (abnormal spindle-like microcephaly associated protein) or the microtubule -binding protein DCLK (Doublecortin-like kinase) have defects in planar spindle orientation and small brain size[96–98]. Consistent with these findings, mutations in the human *ASPM* gene, and five other genes, are linked to autosomal recessive primary microcephaly[99]. These genes may encode new components of the planar spindle positioning pathway, and two (*CDK5Rap2* and *CenpJ*) have *Drosophila* orthologues (*Cnn* and *Sas-4*) required for spindle orientation in neuroblasts[80,83].

Apical/basal spindle orientation. When the neuroepithelial progenitor mitotic spindle is aligned parallel to the apical/basal axis it is termed 'apical/basal spindle orientation' (Fig. 4a, right). True apical/basal spindle orientation seems to be relatively rare during all phases of cortical neurogenesis[88], although it may be more common in the retina[100,101] (and in epidermal progenitors[102]). In the retina, mouse Insc (mInsc) is apically localized in progenitors undergoing apical/basal divisions, and a reduction in mInsc levels results in persistent planar spindle orientation that expands the progenitor pool[103]. How does mInsc anchor one spindle pole at the apical cortex? The mechanism is likely to be highly conserved from flies to mammals. mInsc binds LGN–AGS3[102,103], which has the potential to recruit the LGN–AGS3 planar spindle orientation pathway to the apical cortex, thereby reorienting the mitotic spindle. In the mouse cortex, depletion of AGS3 is reported to switch apical/basal to planar spindle orientation[104]. LGN–AGS3-associated NuMA may associate with microtubule -bound dynein[77,91], to pull one spindle pole to the apical cortex. However, it remains to be seen whether NuMA or dynein–dynactin complex proteins are localized to the apical cortex in cells undergoing apical/basal spindle orientation.

Spindle orientation and cell fate. It has been proposed that spindle orientation regulates cell fate in the cortex and retina, with planar divisions generating two identical cell fates (neuroepithelial progenitor/neuroepithelial progenitor or neuron/neuron, depending on whether the division occurs early or late in neurogenesis), and apical/basal spindle orientation generating two different cell types (for example, neuroepithelial progenitor/neuron)[101,105,106]. Testing this model has become possible with advances in live imaging methods, allowing neuroepithelial progenitor apical and basolateral domains to be followed from cell division to sibling cell fate specification. Initial experiments reported that inheritance of the apical cortical domain was a good predictor of neuroepithelial progenitor fate in the mouse cortex[90,104], although more recent work shows that only cells that inherit both the apical and basal domain acquire neuroepithelial progenitor fate[88]. Other labs report that spindle orientation has no effect on progenitor fate, but merely regulates the position of the cells within the neuroepithelium; that is, cells lacking the apical domain and/or adherens junctions move away from the ventricular zone but retain neuroepithelial progenitor fate[89]. Consistent with this finding, reducing RhoA function increases apical/basal neuroepithelial progenitor divisions, but the basal daughter cell maintains neuroepithelial progenitor fate[107].

In the retina, evidence for a causal relationship between spindle orientation and cell fate is more convincing. In the retinal neuroepithelium, Numb protein is localized apically (whether to the apical domain or the adherens junctions is unknown). Planar divisions give rise to two $Numb^+$ photoreceptor neurons, whereas apical/basal divisions typically generate two molecularly distinct siblings ($Numb^+$/$Numb^-$) that assume different cell fates[101,108]. Reduction of mInsc reduces the frequency of apical/basal divisions and increases the frequency of symmetric proliferative divisions[103]. It is thought that Numb promotes neuronal differentiation, because overexpression of Numb in progenitor cells lead to formation of photoreceptor neurons at the expense of Muller glia[101]. Numb may act by inhibiting Notch, as in *Drosophila*, because overexpression of Notch results in the opposite phenotype of excess Muller glia at the expense of photoreceptors[109].

CONCLUSIONS

Great progress has been made over the past few years in revealing conserved mechanisms of spindle orientation from yeast to mammals, yet much remains to be learned. The next few years should reveal more about the biochemical mechanisms used to assemble and activate the protein complexes regulating spindle orientation. More difficult will be defining the role of spindle orientation in specifying cell fate. Future studies will have to identify cell fate determinants, observe their distribution relative to spindle orientation and track the subsequent sibling cell fates. This is experimentally challenging, but advances in imaging technology have made these experiments possible in worms, flies and even the mammalian brain.

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