Regulation of mitotic spindle orientation: an integrated view

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Abstract

Mitotic spindle orientation is essential for cell fate decisions, epithelial maintenance, and tissue morphogenesis. In most animal cell types, the dynein motor complex is anchored at the cell cortex and exerts pulling forces on astral microtubules to position the spindle. Early studies identified the evolutionarily conserved Gαi/LGN/NuMA complex as a key regulator that polarizes cortical force generators. In recent years, a combination of genetics, biochemistry, modeling, and live imaging has contributed to decipher the mechanisms of spindle orientation. Here, we highlight the dynamic nature of the assembly of this complex and discuss the molecular regulation of its localization. Remarkably, a number of LGN-independent mechanisms were described recently, whereas NuMA remains central in most pathways involved in recruiting force generators at the cell cortex. We also describe the emerging role of the actin cortex in spindle orientation and discuss how dynamic astral microtubule formation is involved. We further give an overview on instructive external signals that control spindle orientation in tissues. Finally, we discuss the influence of cell geometry and mechanical forces on spindle orientation.

See the Glossary for abbreviations used in this article.

Introduction

During division, cells face numerous challenges. First, to avoid genetic aberrations, the genetic material has to be correctly segregated into the daughter cells. This is achieved through the formation of a dedicated bipolar structure, the mitotic spindle, which involves profound remodeling of the microtubule network: Kinetochore microtubules (MTs) attach to chromosomal kinetochores, interpolar and central spindle MTs position the furrow upon cytokinesis, and astral microtubules anchor the furrow to the cell cortex [1,2]. Second, depending on the cell type and the developmental stage, a dividing cell may need to produce daughters of different size and/or fate. Here again, the spindle plays a critical role, since its orientation determines the axis of cell division and thereby decides about symmetry or asymmetry of the division. Correct spindle orientation is important for asymmetric segregation of polarized cell fate determinants in *Drosophila* neuroblasts thus allowing asymmetric cell divisions [3]. Similarly, during the first division of the *C. elegans* zygote, spindle displacement toward the posterior pole is crucial for the production of two daughter cells of asymmetric size and different fate [4,5]. Third, daughter cells resulting from a division must be correctly positioned in order to maintain tissue structure and/or contribute to tissue morphogenesis in metazoans. In epithelia, planar orientation of divisions is required for the maintenance of daughter cells in the plane of the tissue [6–8]. In addition, polarized orientation of cell divisions within the epithelium plane can contribute to tissue elongation [9,10]. Conversely, spindle orientation along the apico-basal axis is necessary for asymmetric cell division and epithelial stratification during skin development in the mouse embryo [11]. Altogether, spindle orientation and positioning are involved in fundamental developmental processes and in tissue homeostasis, and their deregulation has been correlated with different pathologies, including microcephaly and cancer [12,13]. This underscores the importance of understanding the mechanisms mediating these processes. The multiple roles of oriented cell divisions in animal development and pathologies have been reviewed elsewhere [14–19]. The focus of this review was to provide a comprehensive overview of the mechanisms and regulatory inputs of spindle orientation in metazoans. Of note, spindle positioning mechanisms are also extensively studied during asymmetric division of the budding yeast; however, this model shows important differences to higher eukaryotes and therefore will not be discussed here (see Box 1 for a brief overview).

The orientation of the mitotic spindle in animal cells can be influenced by geometric cues, internal cues, and external cues. Hertwig

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Glossary

Antxr2a	anthrax receptor 2a
APC	adenomatous polyposis coli
aPKC	atypical protein kinase C
APs	apical progenitors
Arp3	actin-related protein 3
AurA	Aurora A
CDK1	cyclin-dependent kinase 1
CYK4	Rho family GTPase-activating protein CYK4/MgcRacGAP
Dgrip75	*Drosophila* grip-motif-polypeptide 75
Dlg	Discs large
Dsh DEP domain	dishevelled/EGL10/pleckstrin domain
EB1	end binding family member 1
EB3	end binding family member 3
ECM	extracellular matrix
Ed	echinoid
ERM	Ezrin–radixin–moesin
EVL	enveloping cell layer
4.1G	band 4.1-like 2 protein/EPB41L2
4.1R	band 4.1 protein/EPB41
Fz–Dsh	frizzled/disheveled
GAP	GTPase-activating protein
GEF	guanine exchange factor
GOA1	guanine nucleotide-binding protein G (o) subunit alpha
GPA16	G protein alpha subunit
GPR	G protein regulator
GPR1/2	G protein regulator 1/2
HTT	huntingtin
ILK	integrin-linked kinase
Insc	inscuteable
Lgl	*lethal giant larvae*
LGN	leucine–glycine–asparagine
LIN5	spindle apparatus protein lin-5
MAP4	microtubule-associated protein 4
MDCK	Madin–Darby canine kidney
MISP	mitotic interactor and substrate of Plk1
MKLP1	mitotic kinesin-like protein 1
MT	microtubule
Mud	mushroom body defect
NB	neuroblast
NRK	normal rat kidney
NuMA	nuclear and mitotic apparatus
Par3	partitioning-defective 3
Pcnt	pericentrin
PCP	planar cell polarity
Pins	partner of inscuteable
PIP	phosphatidylinositol phosphate
PIP2	phosphatidylinositol 4,5-bisphosphate
PKC-ζ	protein kinase C-ζ
Plk1	polo-like kinase 1
Rab11	Ras-related protein Rab11, recycling endosome GTPase
Ran	Ras-related nuclear protein
SOP	sensory organ precursor
T2055	threonine 2055
TCJs	tricellular junctions
TPR	tetratricopeptide repeats
Tre1	trapped in endoderm 1
zDia2	diaphanous-related formin 2 (in zebrafish)
γ-TuRCs	γ-tubulin ring complexes

Box 1:   Spindle orientation in budding yeast

Spindle positioning is well characterized during the asymmetric division of the budding yeast *S. cerevisiae* [23]. In this model system, the spindle is positioned in relation to the bud neck to allow the correct segregation of chromosomes between mother and daughter cells. Spindle orientation in yeast also depends on the interaction of astral microtubules with cortically localized factors; however, cortical factors (e.g., Num1; reviewed in [24]) are not homologous to those found in higher eukaryotes. In addition, spindle positioning is achieved by two sequential and clearly distinct pathways [25]. In pre-anaphase, spindle orientation along the mother–bud axis is not linked to dynein-dependent forces, but instead depends on the displacement of astral microtubules along actin cables. This process depends on the interaction between the MT tip protein Bim1 (homologous to EB1) and the myosin Myo2 via the yeast-specific adaptor Kar 9 (homologous to APC) [25,26]. In anaphase, spindle displacement into the bud neck is mediated by pulling forces exerted by cortically anchored dynein. The switch between both pathways is linked to the removal of the dynein inhibitor She1 from astral microtubules in the metaphase–anaphase transition [27]. In anaphase, dynein (Dyn1) is delivered to the cortex, where it binds to the cortical factor Num1 through a mechanism of "off-loading" from astral microtubules [28]. Thus, pre-targeting of dynein to microtubule plus-ends is necessary for spindle positioning in yeast [25,28]. Dynein pre-targeting depends on Pac1 and Bik1 (LIS1 and CLIP-170 homologs, respectively) [25]. Interestingly, in mammalian interphase cells, dynein localizes to MT plus-ends in a CLIP170- and EB1-dependent manner [29]. However, whether the localization of dynein to MT plus-ends is important for its delivery to the cortex during vertebrate mitosis remains to be investigated. Notably, both pathways acting in spindle positioning in yeast do not rely on the polarization of cortical anchors as it is seen in higher eukaryotes. Instead, they rely on the asymmetric localization of Kar 9 and Dyn1 to the astral microtubule plus-ends emanating from the daughter spindle pole [26].

specific polarity cues. At the turn of the century, the realization that the orientation of the axis of division often correlates with fate choices in *Drosophila* and *C. elegans* models of asymmetric cell division prompted a series of studies that linked regulators of cell polarity with the molecular control of spindle positioning and orientation. In this context, a role of Gαi subunits of heterotrimeric G proteins and the adaptor molecule LGN (leucine–glycine–asparagine) in spindle orientation was initially identified in *Drosophila* embryonic neuroblasts [21,22]. Later work revealed the evolutionary conservation of this complex in numerous metazoans, and how it interacts with the NuMA (<u>n</u>uclear and <u>m</u>itotic <u>a</u>pparatus) adaptor to recruit the dynein motor complex to the cell cortex in symmetrically and asymmetrically dividing cells. Indeed, in most animal cell types oriented cell divisions involve the transmission of localized pulling forces located at the cell cortex to astral microtubules, resulting in the positioning of the mitotic spindle. As a consequence, the cell cortex, the specific mechanisms that recruit and localize force generators, and the astral microtubule network have emerged as the three essential levels of regulation for spindle orientation.

In this review, we will first briefly review the role of the so-called LGN complex and discuss recent literature that refines our understanding of the spatial and temporal regulation of the activity of this complex. We will also discuss recently described alternative mechanisms for the recruitment of force generators at the cell cortex. In the second part of the review, we will review the emerging roles of the actin cortex on spindle orientation. In the third part, we will

first proposed more than a century ago that cells orient their spindles along the long axis of the cell, arguing for a role of cellular geometry in controlling the plane of division [20]. While this rule applies to many situations, orientation of the spindle is also often set by

show how mechanisms that regulate astral microtubule nucleation, dynamics, and interaction with the cortex can influence the transmission of cortical forces to the spindle and control the orientation of cell divisions. Finally, we will consider cells in their tissue context and present the extracellular signaling pathways that have been recently demonstrated to modulate spindle orientation in different organisms and tissues. We will also review recent studies that address the influence of geometrical cues and external forces applied at the tissue scale on spindle orientation without the involvement of polarized signaling pathways.

Revisiting an old leader: the Gαi/LGN/NuMA/dynein complex

The historical complex

A number of genetic studies have shown that an evolutionarily conserved molecular complex composed of the heterotrimeric Gα protein Gαi, LGN, and NuMA (respectively Gαi, Pins, and Mud in *Drosophila*, and GOA1/GPA16, GPR1/2, and LIN5 in *C. elegans*, and hereafter called the LGN complex for simplicity; see Table 1 and Fig 1) has core functions in spindle orientation and positioning in different tissues both in invertebrate and vertebrate species [6,7,11,21,22,30–35] (reviewed in [19]). During mitosis, this complex is localized to a particular subcortical domain and directs the recruitment of the minus-end-directed microtubule motor dynein [36–38] (Fig 1A). The directed movement of cortically anchored dynein along astral microtubules generates pulling forces on the spindle poles leading to the orientation and/or positioning of the spindle. Therefore, the specific localization of the LGN complex determines the site of force concentration and the axis of spindle orientation. Consistently, the apical localization of Pins/Mud or LGN/NuMA directs spindle orientation along the apico-basal axis in *Drosophila* neuroblasts (Fig 1B) and mouse skin progenitors, respectively [11,22,39–42]. In the *C. elegans* zygote, enrichment of GPR1/2 at the posterior cortex is necessary for spindle positioning along the antero-posterior axis [32,34] (Fig 1B). Furthermore, the lateral localization of the LGN complex regulates planar spindle orientation of progenitors in chick and mouse neuroepithelium [6,7,33] as well as during epithelial morphogenesis of *Drosophila* and mammalian cells [8,43] (Fig 1B).

LGN Biochemical and structural analysis defined the different components of the LGN complex. LGN was first identified biochemically as an interaction protein that binds to Gαi subunits [44]. LGN is a modular protein composed of three main domains. Its N-terminal TPR domain contains 7 (6 or 8 depending on the authors) tetratricopeptide repeats, which mediate the interactions with multiple binding partners, including NuMA. Its central "linker" domain does not show any recognizable organization or binding motif, but is crucially required for its function (Fig 1). The C-terminal GPR (G protein regulator) domain contains four (3 in *Drosophila* Pins and 1 in *C. elegans* GPR1/2) GoLoco domains that mediate interaction with

Table 1. Genes mentioned in this review and names of their homologs in model organisms.

Vertebrates	*Drosophila*	*C. elegans*
Gαi 1, 2, 3	Gαi	GOA1/GPA16
LGN (GPSM2, mPins)	Pins (partner of inscuteable, Rapsynoid)	GPR1/2
NuMA	Mud	Lin-5
Insc (mInsc)	inscuteable	–
Par3	Bazooka (dPar3)	Par3
Afadin	Canoe	Ce-AF-6
RGS14, RGS12	loco	RGS-7
Ric8a	dRic8	Ric8 (synembrin)
Dlg1 (SAP97, hDLG)	Dlg	DLG-1
Dia2 (zDia2)	diaphanous	–
ERM (ezrin–radixin–moesin)	moesin	ERM-1
Dynactin-1 (DCTN1, p150)	p150-glued	DNC-1
GCP4	Dgrip75	–

Gαi/o subunits. LGN interacts with Gαi only when it is bound to GDP, and has a guanine dissociation inhibitory (GDI) activity [45]. The GTPase-activating protein (GAP) RGS14/Loco/RGS-7 (in vertebrates, *Drosophila* and *C. elegans*, respectively) and the guanine exchange factor (GEF) Ric8a control the interaction between LGN and Gαi, and therefore the stability of the complex, by modulating the GTPase activity of Gαi subunits and thereby the phosphorylation state of bound guanosine [46–53].

NuMA NuMA is a coiled-coil protein that can interact with LGN, dynein as well as with microtubules (Fig 1) [38,40,54–56]. NuMA is also present on the spindle, enriched near the poles, and regulates spindle formation and organization. In dividing cells, LGN and NuMA are usually observed as cortical crescents facing one or both spindle poles (Fig 1). It is well established that this specific cortical localization is instructive for spindle orientation in many systems.

Gαi Gαi subunits localize to the plasma membrane through myristylation, where they serve as an anchor to the complex. By default, Gαi subunits cover the whole cell inner surface and do not contribute to the polarization of LGN and NuMA crescents.

Additional factors, and in particular polarity proteins, must regulate the polarized cortical distribution of LGN and NuMA. Indeed, the LGN homolog Pins (partner of inscuteable) was initially identified in *Drosophila* in interaction screens with inscuteable [21,22], an apical protein known for its role in apico-basal spindle orientation in neuroblasts [3]. Apical localization of Insc, and consequently of Pins, requires the polarity protein Bazooka (*Drosophila* Par3) [22], as well as atypical protein kinase C (aPKC) [57,58]. Similarly, the

Figure 1. The LGN complex.
(A) The scheme shows the LGN domains and its interactions with Gαi membrane-anchored subunits, and with NuMA, as well as the interaction with cortical proteins (Dlg, Afadin) that regulate LGN cortical localization. (B) LGN complex localization in different systems, showing the polarity proteins regulating this specific localization when applicable. (i) *Drosophila* embryonic neuroblasts, (ii) *C. elegans* zygote, (iii) neural progenitors in the vertebrate neuroepithelium, and (iv) mammalian cell lines.
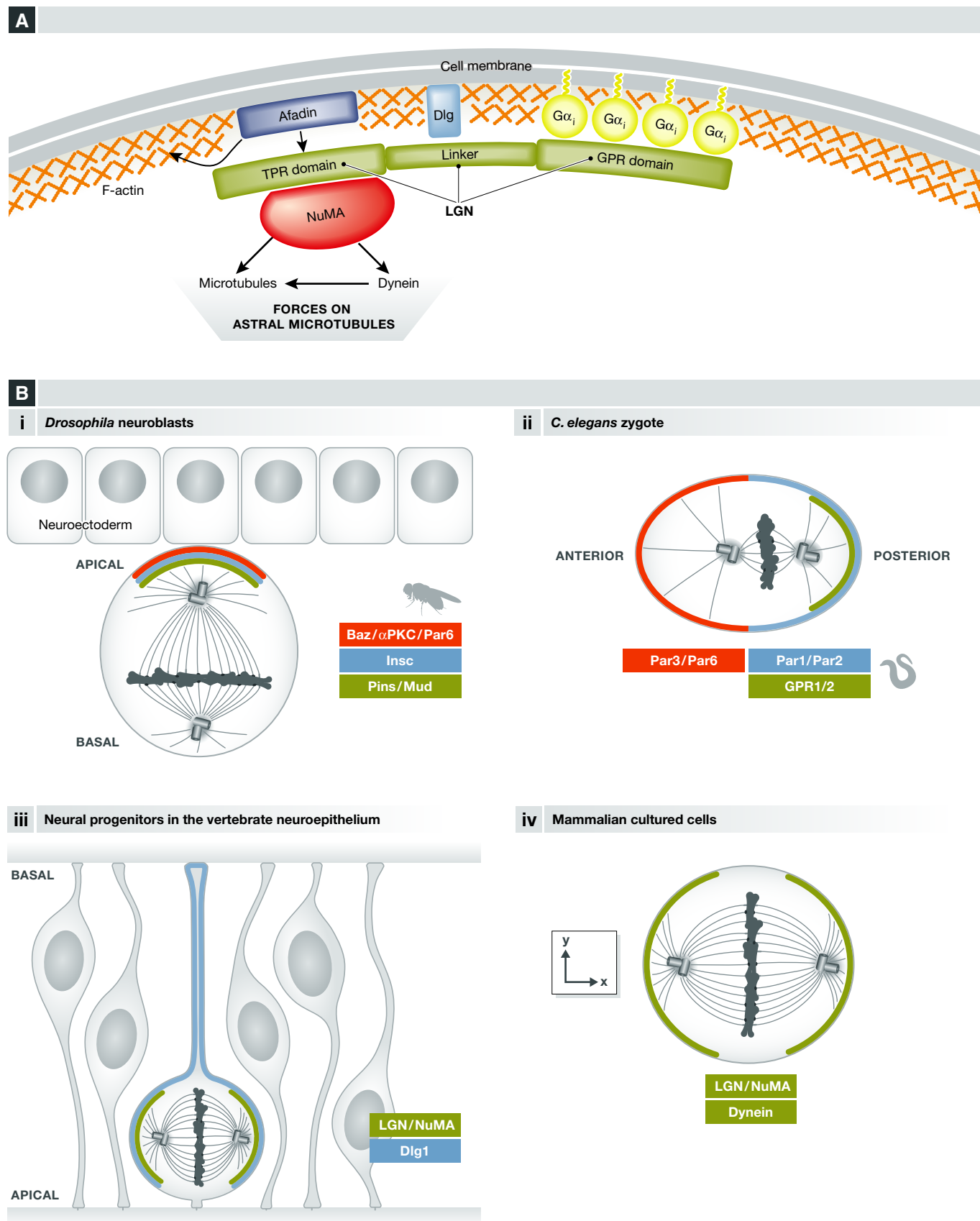
Figure 1.

Box 2:   Models for studying spindle orientation

The core components of spindle orientation have been discovered in invertebrate *in vivo* models of spindle orientation, which continue to be useful for dissecting new regulators and understanding the dynamics of this cellular process. In addition, an induced polarity assay has been developed in *Drosophila* S2 cells [60]. In this model (see the figure below), intracellular fusion to the transmembrane and extracellular domains of the echinoid (Ed) homophilic cell–cell adhesion protein is used to localize a protein of choice to the contacts between clustered cells, in this way generating a polarized distribution in each cell (i). Polarized localization of Pins by using this method results in spindle orientation in the direction of the Ed-Pins enrichment, constituting a model where the function of molecules in spindle orientation downstream of Pins can be evaluated. Alternatively, by fusing proteins or protein domains to echinoid, their ability to orient the spindle has been evaluated in different studies [60–63].
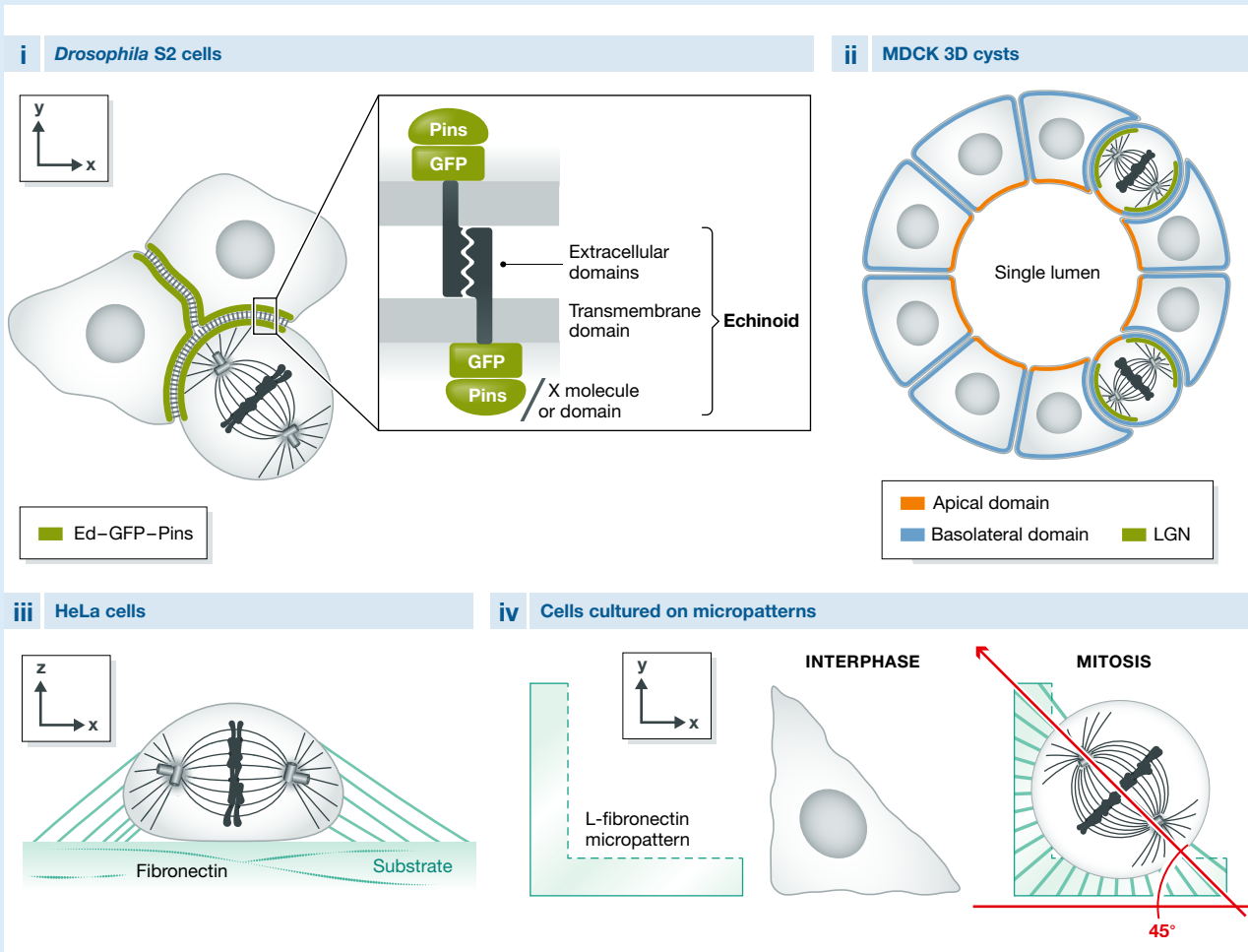
In vertebrate systems, in addition to the *in vivo* models of spindle orientation (e.g., mouse skin progenitors, mouse and chick neuroepithelial cells, fish epiblast cells), *in vitro* cultured cells are frequently used to study the molecular details and dynamics of this cellular process. The most frequently used *in vitro* models are:

- MDCK cysts (ii): a 3D model of epithelial morphogenesis. By culturing dog MDCK cells in Matrigel, cysts with a central lumen and defined polarity domains are generated. In this context, spindle orientation occurs in the plane of the epithelium and depends on LGN which localizes to the lateral cell cortex [8]. Defective spindle orientation commonly results in cysts with multiple lumina.

- HeLa cells cultured on a fibronectin substrate (iii): This human cell line has been shown to orient the mitotic spindle parallel to the substrate, which depends on astral microtubules [64].

- Cells cultured on micropatterns (iv; e.g., HeLa cells, MDCK cells, fibroblasts, MCF cells): In this model, single cells are cultured on micro surfaces of defined geometry, which dictates a specific shape and adhesion pattern to the cells. The adhesion pattern can induce a specific spindle orientation in the *xy*-plane. This orientation is dependent on the distribution of actin retraction fibers, as well as on astral microtubules [65–68].

It should be noted that knockdown of LGN or NuMA only shows weak spindle orientation phenotypes in these last two systems, suggesting that the involvement of the LGN complex is only marginal and that it acts in combination with, or as a complement to, additional pathways.

posterior cortical enrichment of GPR1/2 requires the Par2 and Par3 polarity proteins during the first division of the *C. elegans* embryo [32].

More recent work has revealed a surprising diversity in the mechanisms that control LGN localization at restricted cortical domains. While Pins/LGN localizes apically in *Drosophila* neuroblasts, it is found in a ring at the lateral cortex during planar spindle orientation in different epithelial contexts [7,8,43]. Remarkably, while aPKC is required for the apical recruitment of Pins in neuroblasts [57], it inhibits the apical localization of LGN and favors its lateral enrichment during cystogenesis in MDCK cells [8] (see Box 2). The mechanism mediating this inhibition involves the phosphorylation of LGN by apical aPKC, which increases locally LGN affinity to a 14-3-3 protein, competing with the interaction of LGN with Gαi at the apical domain [59], and favoring the planar orientation of the spindle in these cells.

New insights into the molecular regulation of LGN complex recruitment/stability at the cortex

Discs large In contrast to MDCK cysts, aPKC does not regulate the lateral localization of Pins/LGN, and the resulting planar orientation of divisions, in *Drosophila* follicular epithelia and chick embryonic neuroepithelium [7,43]. This function relies on the polarity protein Discs large (Dlg), known as a tumor suppressor in *Drosophila*. The role of Dlg in spindle orientation was identified in *Drosophila* larval sensory organ precursor (SOP) cells, where it regulates Pins localization to the anterior cell cortex [69]. Previous work in fly embryonic neuroblasts had identified a role for Dlg (and other tumor suppressors lgl and scribble) in the asymmetric division of these cells, but not in spindle orientation [70,71]. Later work identified that Dlg is part of a non-essential microtubule-based pathway driving cortical localization of LGN in neuroblasts, acting in parallel with the dominant inscuteable recruitment pathway [72] (reviewed in [19]). However, both the SOP and NB models relied on a polarized localization of Dlg, which differed from its classical "baso-lateral" localization, raising the question of its potential role in symmetrically dividing cells in canonical epithelia. Recent studies have shown that depleting Dlg/Dlg1 affects Pins/LGN cortical localization and results in defects in planar spindle orientation in *Drosophila* epithelia and in chick neuroepithelium [43,73]. Quite remarkably, Dlg/Dlg1 acts differently in each of these tissues. In *Drosophila* follicular epithelia, Pins becomes localized all around the cortex upon Dlg depletion, indicating that Dlg may act by restricting Pins localization to the lateral cortex [43]. In contrast, in the chick neuroepithelium, LGN is lost from the cortex when Dlg1 is depleted, suggesting that Dlg acts to recruit/stabilize LGN at the cortex in this context [73]. Similarly, DLG1 depletion in human HeLa cells reduces LGN/NuMA cortical localization and is associated with defects in micropattern-guided spindle orientation (see Box 2) [73]. Importantly, while Dlg/Dlg1 has been described as a polarity protein, acute depletion of Dlg/Dlg1 does not generate obvious defects in tissue polarity in follicular epithelia or in the neuroepithelium, indicating that this protein plays a specific role in spindle orientation independent of its function in cell polarity [43,73]. Direct interaction between Dlg/Dlg1 and Pins/LGN relies on the phosphorylation of a conserved serine residue in the linker domain of LGN [60,74–76] (Fig 1A). In *Drosophila*, Pins is phosphorylated by AurA

and regulates the interaction between Dlg and Pins. In contrast, AurA activity is not required for LGN cortical recruitment in HeLa cells [77], suggesting that AurA does not control LGN interaction with DLG1 in these cells.

Afadin The *Drosophila* scaffolding protein Canoe (Afadin in mammals) also regulates LGN complex formation and spindle orientation. This role was initially described in *Drosophila* neuroblasts, where Canoe localizes to the apical cortex and regulates apical–basal spindle orientation [78]. The molecular details have been dissected in the S2 cell induced polarity assay (Box 2) [62], where Canoe interacts with Pins and acts specifically in the spindle orientation pathway mediated by Pins^{TPR}/Mud [60]. In particular, Canoe is necessary for Mud recruitment to cortical Pins crescents through its interaction with the TPR domains [62,78]. Canoe interaction with Ran^{GTP} is necessary for Mud recruitment and spindle orientation, but the mechanisms by which Ran^{GTP} regulates these activities remain elusive [62]. Canoe's vertebrate homolog Afadin also plays a role in spindle orientation, albeit through a distinct mechanism: Afadin binds simultaneously cortical F-actin and the TPR region of LGN (Fig 1A). Afadin interaction with LGN is in competition with the NuMA/LGN interaction, although the affinity for Afadin is lower. However, upon Afadin depletion, LGN cortical recruitment is reduced, NuMA and dynein are not recruited, and the spindle is misoriented, both in adherent cells and in 3D cell cultures [79]. As NuMA is nuclear in interphase and only released upon nuclear envelope breakdown, one possible interpretation is that Afadin is necessary for the initial recruitment of LGN to the cortex and its interaction with Gαi subunits in early mitosis, before LGN interacts with and recruits NuMA at the cell cortex.

More generally the interplay between Gαi, Afadin, and Dlg1 in LGN cortical localization is not well understood. Gαi appears as an obligate membrane anchor, since LGN is completely absent from the cortex when the Gαi/LGN interaction is disrupted; in contrast cortical levels of LGN are only reduced in the absence of Afadin and Dlg1 [73,79]. Whether Afadin and Dlg1 are important for the initial recruitment of LGN by Gαi, or whether they are involved in maintaining LGN at the cortex, remains unclear. In addition, Dlg1 is involved in the polarization of the cortical localization of LGN at least in epithelia.

Huntingtin A series of recent investigations have focused on the role of huntingtin (HTT) in spindle orientation. This protein, mutated in Huntington's disease, regulates spindle orientation in mouse neural progenitors and basal mammary cells *in vivo*, as well as in *Drosophila* neuroblasts [80,81]. The mechanisms of action of HTT have been further evaluated in cultured cells, where it regulates spindle orientation with respect to the substrate [80]. HTT depletion leads to a decrease in the cortical levels of LGN, NuMA, and members of the dynactin/dynein complex. Contrary to the LGN interacting proteins Afadin and DLG1, HTT localizes to spindle poles during mitosis. Because HTT plays a role in anterograde vesicular transport in neurons, the proposed hypothesis is that HTT regulates the transport of LGN and dynein complex members via astral MTs from the spindle poles to the cortex. Accordingly, this transport depends on the plus-end-directed motor kinesin 1 [80].

Phosphorylation of Mud and NuMA Two recent reports identified the phosphorylation of Mud and NuMA as necessary for their cortical localization. In *Drosophila*, phosphorylation of a serine residue in the coiled-coil domain of Mud by the Hippo pathway kinase Warts induces a conformation change that uncovers the Pins binding domain and allows interaction with cortical Pins [82]. Warts can also phosphorylate human NuMA *in vitro*, but the relevance of this modification has not been tested in cells. However, phosphorylation of a distinct serine residue in another domain of NuMA by the mitotic kinase Aurora A (AurA) is also necessary for its cortical recruitment in human cells [77]. While the two mechanisms are different, it is remarkable that both Warts and AurA kinases localize to spindle poles in mitotic cells, suggesting that they act there to promote the release of phosphorylated Mud/NuMA from the spindle pole and thereby allow its interaction with cortical Pins/LGN. Accordingly, upon pharmacological inhibition or knockdown of AurA, NuMA is lost from the cortex and its concentration increases at the spindle poles [77].

Temporal and spatial regulation of LGN complex localization
In specific cell types, the centrosome maintains its position during all the cell cycle and the spindle forms directly with its correct orientation [83,84]. However, in many cases, the spindle forms in prometaphase with a random orientation, and the final axis of division observed at anaphase is set through spindle rotation during prometaphase and metaphase [7]. The switch from interphase to mitosis, and mitotic progression itself, are accompanied by the sequential activation of numerous signaling pathways and major changes in the organization of cellular structures. This section highlights a number of recent studies that describe the dynamics and molecular regulation of the subcellular recruitment of the LGN complex in relationship to mitotic progression (Fig 2).

Temporal regulation of the LGN complex formation in early mitosis In the last few years, different labs have studied the temporal and spatial aspects of LGN/dynein complex formation by mainly using mammalian cell lines. In HeLa cells, LGN protein levels increase during mitosis [30], which contributes to restrict LGN complex formation only in mitosis, but the molecular regulation of this increase in unknown. In addition, Du and Macara have also demonstrated that during interphase, LGN exists in a closed conformation and because it interacts poorly with Gαi in this state, it does not localize to the cortex. They proposed that interaction with NuMA is necessary to switch LGN to an open conformation that increases its ability to bind Gαi subunits. Because NuMA localizes to the nucleus during interphase, the formation of the Gαi/LGN/NuMA cortical complex would then be further restricted to mitosis in vertebrate cells [30] (Fig 2). However, whether NuMA is required for the cortical recruitment of LGN is unclear: Knockdown of the NuMA homolog lin-5 in *C. elegans* embryos results in the loss of cortical GPR1/2 (LGN) [34], whereas knockdown of NuMA in the chick neuroepithelium does not prevent LGN cortical localization [7]. Besides, upon AurA inhibition NuMA is lost from the cortex, but not LGN [77]. In contrast to vertebrate cells, *Drosophila* Mud is not nuclear and shows cortical localization during interphase in neuroblasts and the overlying neuroectoderm [22,40]. This may explain why Pins shows also cortical patterns in interphase in these cells. Similarly, LIN5 in *C. elegans* is not a nuclear protein.

Spatial regulation of LGN localization in early mitosis The subcellular localization of the LGN complex is very dynamic throughout mitosis (Fig 2). Using HeLa cells that stably express GFP–LGN cultured on fibronectin, Kiyomitsu and Cheeseman showed that LGN is initially recruited all around the cell cortex during prometaphase but its localization is later restricted to two cortical crescents facing the spindle poles during metaphase and anaphase [85]. Using different drugs that affect spindle organization and chromosome alignment, they observed that an abnormal proximity of chromosomes with the cortex inhibited cortical localization of LGN and NuMA, and concluded that chromosome-derived signals normally exclude LGN/NuMA from cortical sites in proximity to the chromosomal plate. Using a Ran^T24N dominant-negative mutant to disrupt the Ran^GTP chromosomal gradient, they went on to show that this gradient is responsible for cortical exclusion of LGN [85]. Therefore, the spatiotemporal restriction of LGN complex localization during mitosis is proposed to rely on a gradient of Ran^GTP that inhibits the formation of the complex in the vicinity of chromosomes. Once the metaphase plate is formed, LGN and NuMA are excluded from this location and appear enriched as two cortical crescents overlying each spindle pole (Fig 2). Therefore, in this model the localization of the complex is established once spindle orientation is set. This is in marked contrast to models in which the orientation is constrained by polarized molecular cues, such as the asymmetric division of fly neuroblasts. Interestingly, when HeLa cells are cultured on polarized micropatterns (see Box 2), the asymmetric distribution of retraction fibers imposes such a constraint, and both LGN and dynein complexes show a restricted cortical localization before the spindle is oriented along the correct axis [68,86].

In mitotic HeLa cells, the cortical distribution of dynactin/dynein complexes is dynamic during metaphase. Live imaging revealed redistribution of a polarized crescent that alternates between the cortical domains that face each spindle pole [85]. Remarkably, these oscillations are independent of the distribution of LGN and NuMA, which remain localized in two cortical crescents (Fig 2). They are followed by the asymmetric positioning of the spindle, whose poles are alternatively attracted to the dynein enriched cortical domains. Here, the kinase Plk1, localized at the spindle poles, negatively controls the cortical localization of dynein/dynactin. The proximity of a spindle pole to the cortex excludes dynein from this cortical site. Concomitantly, dynein/dynactin accumulates to the side of the cell facing the opposing (and more distant) spindle pole and generates pulling forces, which in turn will reposition the spindle [85]. Cortical targeting of dynein/dynactin during this oscillatory phase depends on astral microtubules [86].

A specific spatiotemporal regulation in anaphase More recently, different labs have described changes in the cortical recruitment of NuMA between metaphase and anaphase [87–89]. In contrast to LGN levels, cortical levels of NuMA increase from metaphase to anaphase. These changes are related to its phosphorylation state at T2055, which is regulated by the balance between the activities of the CDK1 kinase and the PP2CA phosphatase. During metaphase, phosphorylated NuMA is observed at spindle poles, while the cortical protein would correspond to non-phosphorylated NuMA. At anaphase onset, the decrease in CDK1 activity results in an increase in non-phosphorylated NuMA, which allows further enrichment of this protein at the cortex (Fig 2). Accordingly, altering NuMA
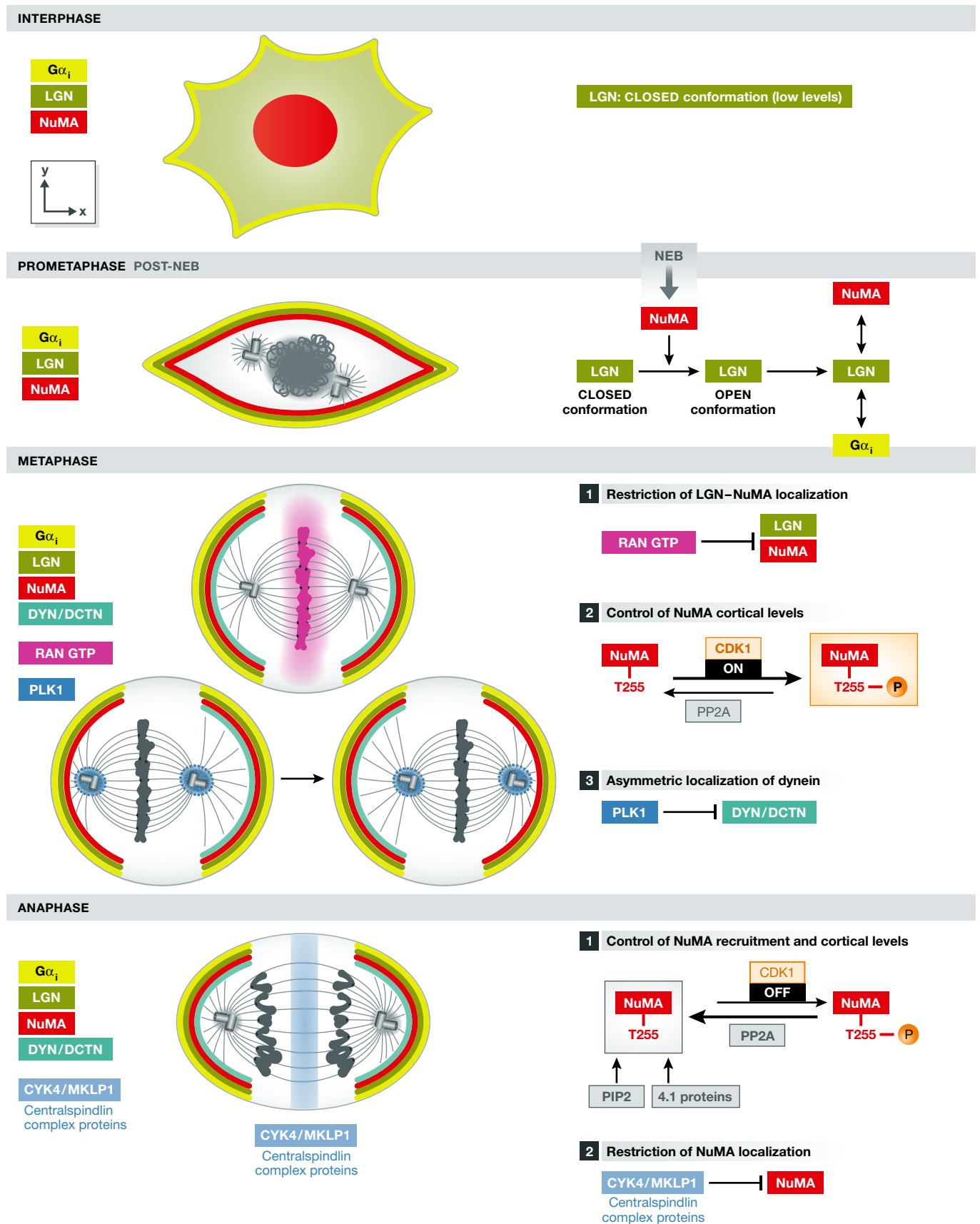
Figure 2.

Figure 2. Temporal–spatial regulation of LGN complex localization.
Left: Scheme of interphase and mitotic phases indicating the distribution of LGN, NuMA, and dynein, as well as the localization of specific molecules (Ran-GTP, PLK1, and centralspindlin proteins) that are involved in controlling this distribution in cultured HeLa cells. Note that NuMA and dynein cortical levels increase in anaphase. Right: Detail of the molecular mechanisms involved in the control of LGN/NuMA localization and of dynein by Ran-GTP/centralspindlin and PLK1, respectively. The control of NuMA cortical levels by CDK1 activity is also indicated.

phosphorylation states results in defects in spindle orientation with respect to the substrate [90].

In contrast to metaphase, an absence of LGN or Gαi in anaphase does not result in complete loss of NuMA from the cortex. Besides, LGN cortical levels do not increase in anaphase, indicating that additional molecules contribute to NuMA localization after the metaphase/anaphase transition. Kiyomitsu and Cheeseman showed that cortical 4.1G and 4.1R proteins interact directly with non-phosphorylated NuMA in anaphase, providing a potential mechanism for this increase [87]. However, whereas removal of both LGN and the 4.1 proteins completely deplete cortical NuMA in anaphase, depletion of the 4.1 proteins alone had no effect [87]. Moreover, Kotak *et al* [89] found that a GFP-tagged version of NuMA lacking the 4.1 protein interaction domain shows the same localization and levels during metaphase and anaphase than wild-type GFP-NuMA. As an alternative model, they proposed that an interaction between NuMA and the phosphoinositides PIP/PIP2 is involved in NuMA cortical recruitment in anaphase. Using different approaches to perturb PIP2 levels, they show changes in NuMA and dynein levels in anaphase. The authors found that this PIP2-mediated recruitment pathway is anaphase-specific. However, the dependence of the NuMA–PIP2 interaction on NuMA phosphorylation states remains to be elucidated. In the same study, Kotak and colleagues show that NuMA is excluded from the equatorial cortex by the centralspindlin proteins CYK4 and MKLP1 during anaphase [89], therefore maintaining the exclusion initiated in metaphase by the Ran-GTP signal. Increased cortical levels of NuMA in anaphase are important for spindle elongation and chromosome separation in human cultured cells [88]. However, whether this increase is also important for spindle orientation itself is not clear.

Taken together, these recent experiments in cultured symmetrically dividing human cells have revealed a complex regulation of the dynamics of LGN, NuMA, and dynein localization during mitosis by molecules located on chromosomes, centrosomes and at the cortex. Several questions remain. Firstly, are these pathways active and necessary to achieve oriented divisions *in vivo*? In the neuroepithelium, it is unlikely that the Ran-GTP mechanism is at play in metaphase. A continuous and homogenous ring of LGN/NuMA is observed at the lateral cortex, and the levels of LGN/NuMA are not lower in the vicinity of the metaphase plate, despite the very small cell size [7]. Secondly, how do these pathways integrate with the instructive signals, such as polarized inscuteable, that control spindle orientation in complex tissues? Further investigations in the field will shed light on these aspects.

In this section, we have shown how cell cycle-regulated changes in the activity of kinases and the assembly of central spindle complexes in anaphase result in a differential regulation of the cortical localization of NuMA. Remarkably, this reveals that NuMA can be recruited to the cortex independently of Gαi and LGN in anaphase. In the next section, we further discuss this notion by describing alternative spindle orientation complexes that

converge on NuMA and dynein cortical recruitment, independently of LGN.

Not a monopoly: Gαi/LGN-independent pathways in spindle orientation
The frizzled/disheveled (Fz/Dsh) PCP pathway regulates spindle orientation in different contexts, including zebrafish gastrulation and asymmetric division of the SOP pI cell (reviewed in [91]). pI cells in the fly notum divide along the antero-posterior axis, and the spindle is slightly tilted relative to the tissue surface. Although Gαi, Pins, and Dlg accumulate at the anterior cell cortex and are involved in the near planar orientation of the spindle, they are not necessary for its antero-posterior alignment [48]. This orientation is regulated by the Fz receptor and its cortical effector Dsh, which are located at the posterior cortex. Using the S2 cell induced polarity assay [60] (see Box 2), Segalen and colleagues identified Mud as the downstream effector of Dsh [63]. Accordingly, they showed that Mud recruitment by Dsh at the posterior apical cortex of the pI cell is necessary for spindle orientation along the antero-posterior axis (Fig 3A). Similarly, during zebrafish gastrulation, dishevelled and NuMA are necessary for spindle orientation along the animal–vegetal axis in epiblast cells [63]. This suggests that the dishevelled–NuMA pathway is conserved across different species.

Mechanistically, the Dsh DEP (dishevelled/EGL10/pleckstrin) domain mediates the recruitment of Mud and dynein. However, Johnston and colleagues found that the Dsh/NuMA pathway does not act alone and uncovered an accessory pathway in Dsh-mediated spindle orientation [61]. Using the induced polarity assay in S2 cells (Box 2), they showed that the DEP domain of Dsh on its own indeed recruits Mud, but surprisingly this was not sufficient to orient the spindle. Robust spindle orientation required the C-terminal domains of Dsh in addition to the DEP domain (DEP-CT) and an interaction of the Dsh-PDZL domain with Canoe. Indeed, RNAi experiments showed that Canoe is necessary for robust spindle orientation in this assay. However, contrary to its previously described role downstream of Pins [62], in this case Canoe does not work through the recruitment of Mud. Instead, it is required for the recruitment of RhoA and the formin diaphanous to the DEP-CT construct. Both RhoA and the actin nucleation activity of its effector diaphanous are necessary for spindle orientation. Consistently, actin accumulates in the cortical domain where DEP-CT is localized. The mechanisms that link cortical actin nucleation to spindle rotation, however, remain to be investigated. In support of their *in vitro* data, the authors demonstrated that diaphanous is indeed necessary for Dsh-mediated spindle orientation along the antero-posterior axis in *Drosophila* SOP cells [61] (Fig 2A).

While NuMA is a central component in several spindle orientation pathways (see "Spindle orientation in context: roles of cell geometry and mechanical forces" for an additional NuMA-dependent pathway), the molecular details of how it regulates spindle orientation remain to be clarified. Artificial targeting of dynein to the cell membrane independently of its interaction with endogenous
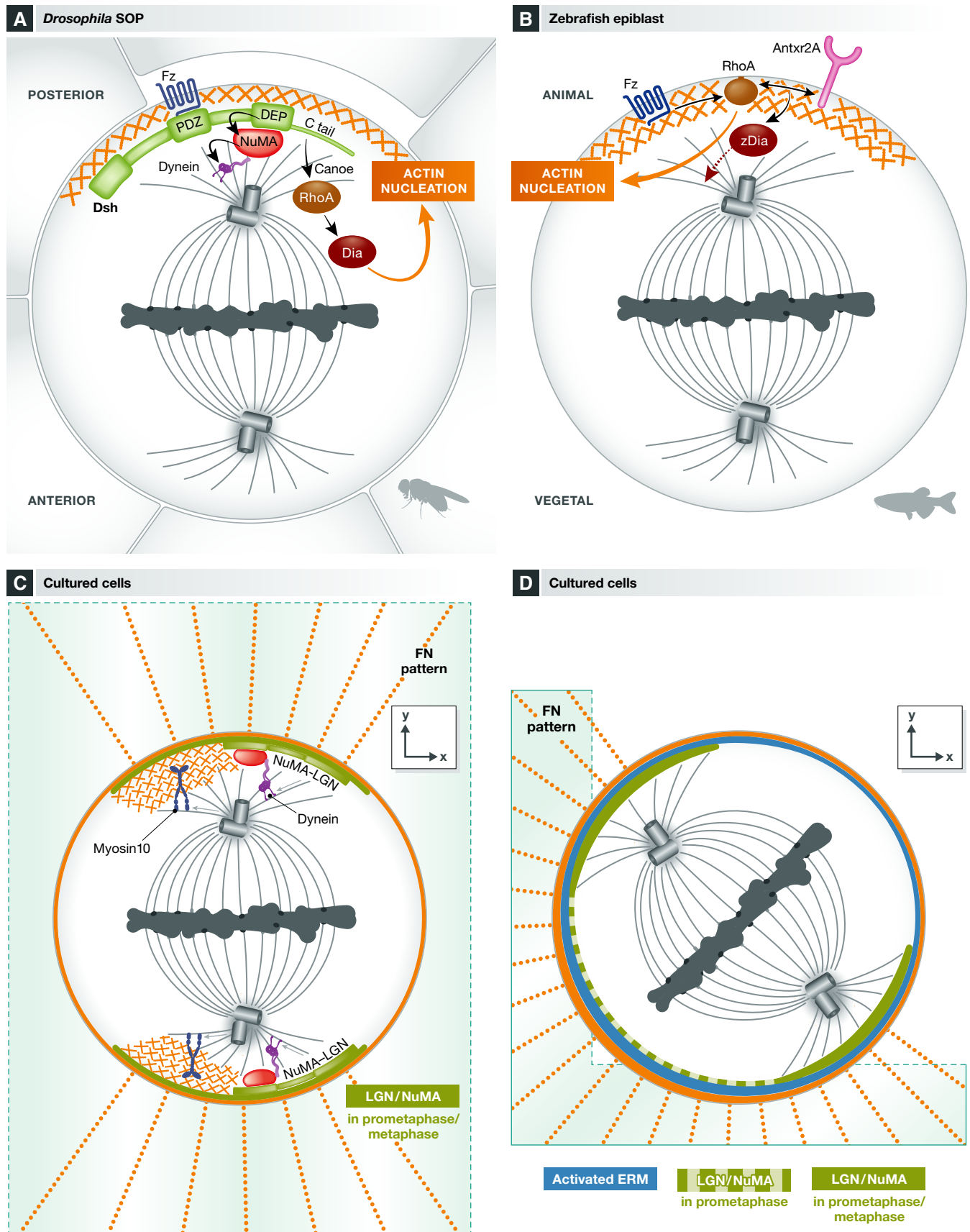
Figure 3.

Figure 3. The role of actin in spindle orientation.
(A) In *Drosophila* SOP, dishevelled localizes to the posterior cortex activating two parallel pathways required for spindle orientation: i) The recruitment of NuMA via the DEP domain allows dynein enrichment at this site, ii) a molecular cascade involving the tail domain of dishevelled, and the Canoe and RhoA molecules leads to the activation of the actin nucleator diaphanous at this cortical site. (B) RhoA and the anthrax receptor 2a (Antxr2a) orient the spindle along the animal–vegetal axis in zebrafish epiblast. Activation of Fzz promotes RhoA recruitment to the "animal cortex". In turn, RhoA induces actin nucleation leading to the formation of an actin cap, and together with the anthrax receptor activates the downstream effector zDia. (C, D) Involvement of different actin-related molecules in *xy* spindle orientation in single cells cultured on fibronectin micropatterns. In this context, the distribution of actin retraction fibers dictates the orientation of the spindle. (C) Polarized actin subcortical clouds make the link between the distribution of retraction fibers and spindle orientation. Myosin 10 mediates the link between actin and microtubules in this context. The classical LGN/dynein complexes are proposed to act in parallel to this pathway, leading to robust spindle orientation. (D) The ezrin–radixin–moesin proteins are enriched in the adhesive cortex in cells cultured in L patterns. These proteins control the initial distribution of LGN and NuMA, during prometaphase, which favor spindle rotation along the depicted axis.

NuMA induces excessive spindle rotation [38]. This suggests that dynein alone is sufficient to exert forces on astral microtubules and that NuMA may only be a passive anchor for the motor complex. However, artificially high levels of cortical dynein may cause excessive spindle rotations and bypass a regulation of dynein by NuMA that may occur at physiological expression levels. Alternatively, NuMA could itself contribute to force generation, either by regulating the motor activity of dynein, or through its ability to directly interact with microtubules [55]. It was proposed that NuMA localization to microtubule ends via its MT binding domain is necessary for spindle orientation [92]. Mouse keratinocytes depleted for the NuMA MT binding domain show spindle orientation defects without changes in the localization of the dynein complex. However, the exact mechanisms by which MT end-localized NuMA contributes to spindle orientation remain to be elucidated.

The emerging role of actin in spindle orientation

In the previous sections, we have used the words "cell cortex" and "cortical recruitment" in an improper (but very widely employed) manner while referring to the inner surface of the cell membrane. The cell cortex is actually defined as a cross-linked network of actin, myosin, and associated proteins located directly underneath the plasma membrane. In this section, we discuss the emerging role of this network in spindle orientation. The mechanics of the cell cortex are essential in the control of cell deformations that occur during cell division, and contribute to the transmission of forces. Furthermore, as already alluded to in the previous paragraph, several studies describe that specific polarization of the actin cortex controls spindle orientation. Finally, recent work shows that actin cross-linkers regulate the recruitment of the LGN complex.

Requirement of an intact actin cortex

When a cell enters mitosis, remodeling of its actin cytoskeleton leads to cell rounding and the establishment of a thinner, but stiffer actin/myosin cortex [93] (reviewed in [94]). Impairment of the actin cortex by latrunculin A/B or cytochalasin D treatment generates spindle orientation defects in cultured cells and *in vivo* in the mouse embryonic skin and in *Drosophila* wing disks [95–97]. In cultured cells and in the developing mouse skin, LGN cortical localization was perturbed by these treatments [68,95,97]. Hence, an intact cortex is required for the correct localization of the spindle orientation machinery and for the stabilization of force generators. How F-actin influences force generator localization at the cortex remains

elusive. Recent data showing that Afadin can bind simultaneously actin and LGN provide for the first time a direct mechanistic link between cortical actin and the force generator machinery [79]. Simultaneously, a sufficiently stiff actin cortex is likely important to prevent membrane deformations and to balance the forces exerted by force generators at the cell surface that pull the spindle, as suggested by experiments in the *C. elegans* zygote [98]. In addition, changes in cellular shape generated by disruption of the actin cortex may be involved in the observed phenotypes, as we will discuss in detail in "Spindle orientation in context: roles of cell geometry and mechanical forces". While the presence of an intact and stiff actin cortex can be seen as permissive for the correct localization of force generators and mitotic cell rounding, an active (or instructive) role of actin and actin-related molecules in guiding spindle orientation is becoming more apparent (as discussed below).

Anthrax receptor and actin polarization

In the context of zebrafish gastrulation, Castanon and colleagues described a novel molecular cascade controlling oriented divisions in epiblast cells [99]. Interestingly, the authors have observed the formation of an F-actin cap that co-localizes with an anthrax receptor (Antxr2a) cap during cell division. Depletion of Antxr2a causes spindle misorientation. By following spindle rotation and cap formation in a series of gene knockdown experiments, the authors dissected the cascade of events that leads to cap formation and spindle rotation. They propose that local activation of RhoA by Wnt leads to the cortical enrichment of actin in an oriented manner. Actin recruits Antxr2a to the actin cap where it contributes to the activation of a diaphanous-related formin, zDia2, which in turn allows spindle rotation in the direction of the cap (Fig 3B). However, it is not clear whether this pathway acts on dynein, which is also involved in spindle orientation in these cells [100]. It is also unknown whether Dsh and NuMA [63] act in parallel to or downstream of the Antxr2a orientation pathway.

Polarized subcortical actin clouds

In addition to cortical actin, Mitsushima and colleagues described the presence of a subcortical cluster of actin during mitosis in cultured cells. This actin cloud undergoes a rotational movement during metaphase and disappears into the contractile ring upon cytokinesis. The formation of this amorphous actin-rich structure depends on Arp3 [101]. Further studies support a role for the actin cloud in spindle orientation. When cells are cultured on micropatterned surfaces (Box 2), the adhesion pattern of the cell controls spindle orientation in the planar (*xy* axis) in an actin- and microtubule-dependent manner [65,67]. The polarized distribution of

retraction fibers during mitosis constitutes a memory of the adhesion pattern in interphase and influences the orientation of the spindle, as seen by laser ablation experiments [67]. Interestingly, the adhesion pattern and distribution of retraction fibers influenced the polarized distribution and movements of actin clouds, and dynamic analyses suggested that clouds influence the rotation of the mitotic spindle in an astral MT-dependent manner [67]. More recently, the function of these actin clouds in spindle orientation was formally demonstrated by inhibiting the Arp2/3 complex [102]. Kwon and colleagues further demonstrated that the unconventional microtubule binding myosin 10, an actin motor involved in spindle formation and integrity [103], regulates spindle orientation with respect to polarized actin clouds in cells cultured on micropatterns. This activity depends on its MT binding domain. Interestingly, myosin 10 localizes to retraction fibers and to dynamic actin clouds but it does not modify their dynamics or assembly. In contrast, depletion of myosin 10 specifically increases astral microtubule dynamics and decreases the interaction of these MT with the cortex, as demonstrated by dynamic analyses of EB3 in metaphase. This suggests that actin-localized myosin 10 regulates spindle orientation by modulating astral MT dynamics, constituting a link between actin and microtubules in the context of spindle orientation (Fig 3C). Of note, the action of myosin 10 differs from that of dynein, as myosin 10 depletion does not change the frequency of lateral transitions of microtubules in anaphase, in contrast to cells lacking cortical dynein caused by depletion of LGN using RNAi. In addition, depletion of myosin 10 and LGN together results in more dramatic defects on spindle orientation than depleting each protein alone, suggesting that the actin/myosin 10 and LGN/dynein pathways act in parallel to orient the spindle [102]. This reinforces the idea that in some cellular contexts, multiple pathways act to promote robust spindle orientation.

ERM proteins

The ezrin–radixin–moesin (ERM) proteins are a family of actin/membrane cross-linkers which control cortical rigidity and stability [104]. Depletion of moesin, the single member of the family in *Drosophila*, leads to massive cortical instability and blebbing in mitotic S2 cells. This results in exaggerated spindle oscillations and mispositioning [105,106]. Defects in spindle morphology (such as short spindle and asymmetric asters) make it difficult to properly evaluate spindle orientation in this model. In contrast, in the *Drosophila* larval wing disk, moesin RNAi does not induce massive blebbing during division, but affects cell rounding so that cells are more elongated along the apico-basal axis. This correlates with a loss of planar spindle orientation [96]. ERM proteins have been recently studied for their role in spindle orientation in vertebrate cells. In dividing human cells cultured on L-shaped micropatterns, activated ERM proteins are asymmetrically distributed, with an enrichment in the cortical domain facing the adhesive surface [66,68] (Fig 3D). Here, depletion of the three proteins as well as impairment of their activation through depletion of the SLK kinase (which was found to directly activate ERM proteins through phosphorylation) leads to spindle misorientation in the *xy* axis (see Box 2) [68]. This phenotype is associated with the loss of LGN and NuMA cortical localization and with reduced spindle rotation, suggesting that activated ERM proteins are necessary for cortical recruitment of LGN/NuMA or stability in this context. Importantly, in contrast to the effects observed upon depletion of moesin in *Drosophila* [105,106], depletion of ERM proteins does not generate obvious alterations in cell shape and spindle morphology in human cells, arguing for a specific role of these proteins in orienting the spindle by the control of LGN/NuMA localization [68]. ERM proteins probably act at the level of LGN, since no effect was observed on Gαi localization upon ERM inactivation or depletion. In addition, overactivation of ERM around the cortex leads to ectopic localization of LGN/NuMA and exaggerated spindle rotation, which also results in defects in spindle orientation. Remarkably, perturbing ERM activation in mouse apical neural progenitors *in vivo* impairs spindle orientation [68]. However, whether ERM proteins regulate LGN complex localization also in this context remains to be studied. Intriguingly, activated ERM can also bind microtubules and thus could also influence spindle orientation directly [107]. Detailed time-lapse microscopy indicated that spindles rotate in prometaphase in cells cultured on L-shaped micropatterns [66,68]. The finding that LGN and NuMA are first localized asymmetrically as a large crescent facing the adhesive matrix [68] likely explains the stereotyped spindle orientation in this system, as anticipated by previous theoretical modeling [65].

In this section we highlighted the role of actin and actin regulators in spindle orientation in different model systems. An important challenge is to understand the crosstalk between actin/dynein and NuMA/dynein pathways. Remarkably, actin-related pathways both seem to modulate or to act independently of the LGN/NuMA pathways. Indeed, ERM actin cross-linkers regulate the cortical localization of the LGN complex in cultured cells [68]. Whether this regulation goes through modulation of the actin cortex or if alternatively there is a direct molecular link between ERMs and LGN/NuMA, remains to be determined. In contrast, actin subcortical clouds and myosin 10 act in parallel to the LGN/dynein pathway to regulate spindle orientation in cells cultured on micropatterns [102]. Similarly, dishevelled controls spindle orientation in *Drosophila* S2 and SOP cells by activating two parallel cascades: a NuMA/dynein and a RhoA/diaphanous/actin pathway [61]. In these cases, it would be interesting to study if parallel pathways act simultaneously or not during spindle orientation. For instance, it could be imagined that one pathway determines the initial orientation of the spindle, having a more instructive role, while the other cascade maintains the orientation once it is set.

The molecular complexes that recruit force generators are located at the plasma membrane. Despite the size of these complexes, due to the thickness of the mitotic cortex (190 nm [93]), it is unlikely that force generators stick out beyond the cortex in the cytoplasm, and more probable that astral microtubules reach motor complexes close to the plasma membrane by growing through the actin meshwork. This also provides an additional layer of regulation for the cortical capture of microtubules, which will be addressed in the following section, dedicated to the regulation of astral microtubules. It will be interesting to explore whether the actin regulators described above influence this meshwork.

Modulation of spindle orientation through the specific regulation of astral microtubules

Except for positioning of meiotic spindles, which lack astral microtubules (reviewed in [108]), spindle orientation is thought to be

achieved by the interaction of astral microtubules with force generators at the cellular cortex (in the broader definition that includes the plasma membrane). Therefore, defects in spindle morphology and/or astral microtubules (MTs) can affect spindle orientation. Shorter spindles may indirectly affect the distance between astral microtubules and the cortex. Alternatively, abnormal astral microtubules may affect the correct transmission of forces necessary to orient the spindle. Indeed, many proteins affecting astral microtubules perturb spindle orientation. We will discuss how modulation of (i) astral MT nucleation/anchoring at the centrosome, (ii) astral MT dynamics and stability, (iii) astral MT cortical capture, (iv) astral MT behavior at the cortex, and (v) astral MT subpopulations impact on spindle orientation (Fig 4).

Astral microtubules nucleation

The role of the centrosomal protein pericentrin (Pcnt) in spindle orientation has been addressed by using cultured MEFs derived from Pcnt$^{-/-}$ knockout mice [109]. In these cells, both astral microtubule length and density (determined by measuring α-tubulin signal intensity) are decreased, and spindle orientation with respect to the substrate (see Box 2) is impaired. In addition, Pcnt was found necessary for spindle pole localization of a particular set of centrosomal proteins including ninein, centriolin, and Cep215. While the localization of these proteins at the centrosome is required for spindle orientation, it remains to be analyzed how each of them affects astral microtubules. However, the data obtained so far suggest that defective recruitment of centrosomal proteins by Pcnt depletion leads to defects in astral microtubules nucleation at the centrosome and thus induces spindle misorientation. Importantly, the cortical localization of NuMA and the dynactin subunit p150 glued are not affected in Pcnt$^{-/-}$ cells, suggesting that their transport and/or turnover are not affected by the observed defects on astral microtubules. This suggests that defects in centrosomal protein localization and/or astral microtubule density are responsible for the observed defects in spindle orientation. In agreement with the *in vitro* data, the authors found spindle orientation defects in neural progenitors and in heart septums of Pcnt$^{-/-}$ mice [109].

Related to MT nucleation activity, a novel role of Rab11 recycling endosomes (RE) in spindle orientation has been recently demonstrated in human cells [110]. These endosomes associate with the spindle and with spindle poles in a Rab11-dependent manner. Impairment of Rab11 function generates spindle misorientation with respect to the substrate. The authors proposed that disruption of astral microtubules is related to this phenotype, which could be explained by the fact that Rab11 RE transport microtubule nucleation components like γ-tubulin and GCP4. These effects may not be astral MT-specific as the overall spindle microtubule density is affected upon Rab11 depletion [110]. Assays of microtubule nucleation from spindle poles in Rab11 depleted vs. control cells demonstrated that Rab11 is indeed important for spindle pole MT nucleation. In conclusion, Rab11 endosomes would be important for the delivery of MT-nucleating components to the spindle poles, which would affect MT nucleation, spindle morphology and consequently, spindle orientation. However, it should be noted that Rab11 depletion also generates misaligned chromosomes. The proximity of misaligned chromosomes to the cortex could affect the cortical localization of LGN/NuMA in a Ran^{GTP}-mediated manner and thus indirectly affect spindle orientation, as described above

[85]. Alternatively, recent data show that artificially induced chromosomal misalignments result in kinetochore-derived Plk1 signaling, whose proximity to the cortex can locally inhibits LGN and NuMA recruitment [111], which may also explain the phenotype of Rab11 depletion. In any case, these results originally link membrane traffic with spindle orientation.

Astral microtubules dynamics and stability

Defects in astral microtubule stability also affect spindle orientation. Toyoshima and Nishida have first shown that depletion of the microtubule plus-end protein EB1, a regulator of microtubule stability, results in spindle misorientation with respect to the substrate in cultured cells (see Box 2), accompanied by a reduction in spindle length and of astral microtubules [112]. More recently, Bouissou and colleagues have shown in *Drosophila* S2 cells and human HeLa cells that γ-tubulin ring complexes (γ-TuRCs) localize to astral microtubules in addition to their well-known localization at centrosomes and spindle microtubules. Depletion of the γ-TuRCs component Dgrip75 in *Drosophila* impairs spindle orientation mediated by Ed-Pins$^{TPR + Linker}$ in the S2 induced polarity assay (see Box 2) and apico-basal spindle orientation in neuroblasts [113]. Similarly, depletion of GCP4, the human Dgrip75 ortholog, generates defects in spindle orientation with respect to the substrate in cultured human cells. Associated with these defects, spindles show longer astral microtubules in S2 cells. Interestingly, changes in astral microtubules do not result from defects in microtubule nucleation activity, a canonical function assigned to γ-TuRCs. In contrast, γ-TuRCs act by regulating astral microtubule dynamics. Indeed, depletion of Dgrip75 increases astral MT dynamics and the time that MTs spend in the growing state, possibly explaining the overall increase in MT length. Importantly, by suppressing MT dynamics using drug- and knockdown-based approaches, the authors were able to rescue spindle orientation defects in S2 cells [113]. This suggests that perturbed astral MT dynamics is directly responsible for the spindle orientation phenotypes observed.

While the effects on spindle orientation generated by the absence or shortening of astral MTs can easily be explained by the lack of interactions between the spindle and the force generators, the link between longer and more dynamic astral MTs and defective spindle orientation is less clear. One possibility is that longer astral microtubules establish abnormal interactions with the cortical sites facing the initial axis of spindle orientation, which in consequence could affect the rotation of the spindle to the cortical domains enriched in force generators [65]. Alternatively, the interaction of force generators with highly dynamic microtubules may be less effective. Consistently, exaggerated spindle oscillations are seen upon Dgrip75 depletion in S2 cells, which could indicate unstable MT–cortex interactions [113].

Astral MT cortical capture

While microtubule nucleation and dynamics regulate the number of microtubules reaching the cortex, these microtubules need to establish proper contacts with the cortex. The interaction between the cortex and astral MTs can be modified by molecules localized at the cortex. For instance, the actin-associated protein MISP localizes to the cellular cortex during mitosis and regulates spindle orientation with respect to the substrate in HeLa cells [114]. Depletion of MISP results in reduced astral microtubule intensity, which is not caused
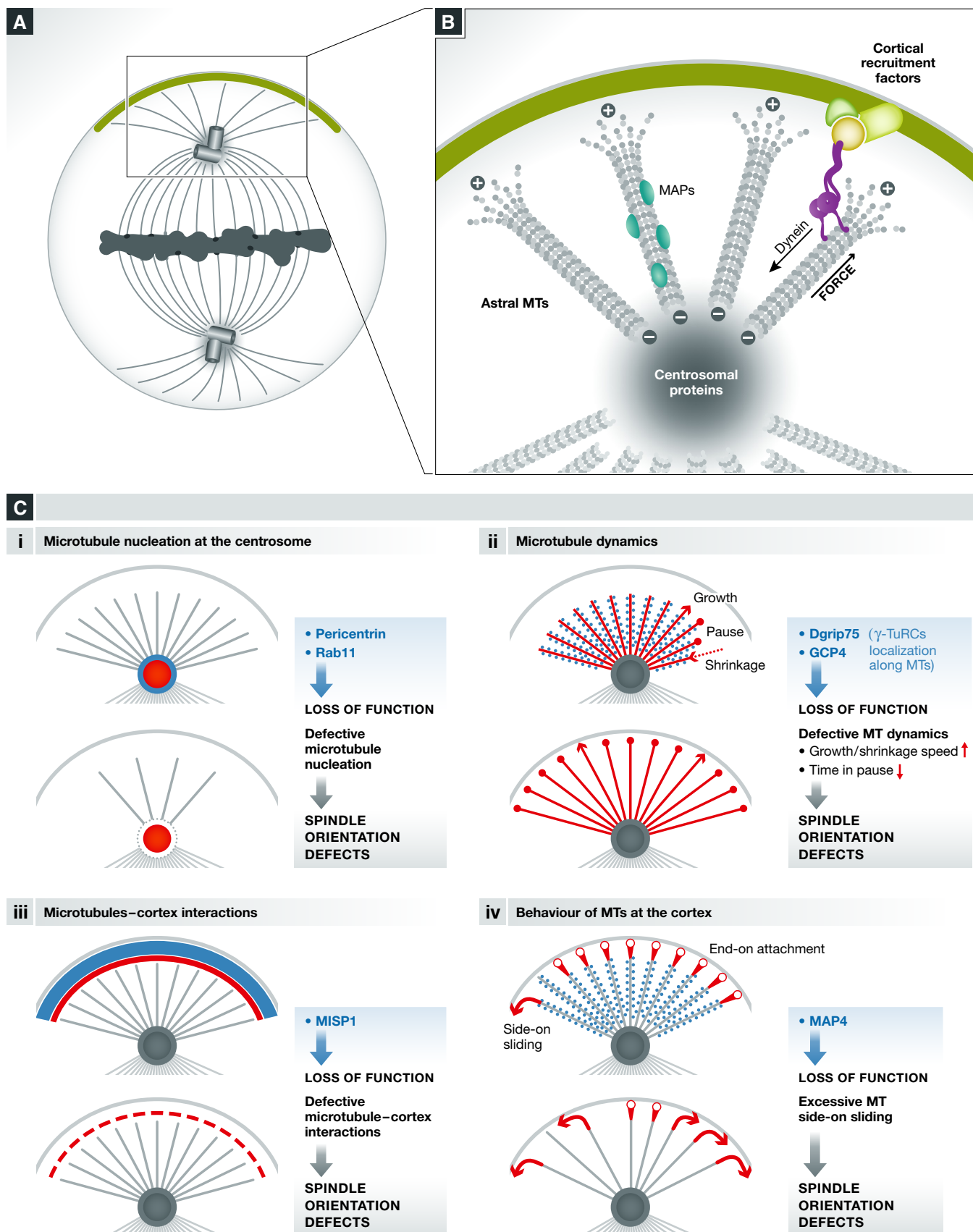
Figure 4.

◀

Figure 4. Modulation of spindle orientation through regulation of astral microtubules.
(A, B) Schema illustrating the centrosome and astral microtubules as well as generic proteins localized on these structures. Cortically recruited dynein is believed to walk on the minus-end direction of astral MT, generating the force that orients the spindle. (C) Regulation of different processes (i–iv) controls the density, length, and behavior of astral microtubules, and thus spindle orientation. The process and cellular structure concerned are indicated in red. Loss of function of specific proteins (in light blue) results in defects in the indicated processes and spindle misorientation. In (iii), MISP acts from the cellular cortex regulating cortex–MT interaction.

by defects in microtubule nucleation, as *in vitro* and *in vivo* polymerization assays showed. Because MISP does not localize to the spindle but to the cortex, the authors proposed that astral MT attachment to the cortex is impaired in the absence of MISP, resulting in destabilized astral MTs. However, it is noteworthy that MISP depletion generates fragmented centrosomes that are often located at the interior of the spindle, which could also contribute to disrupt astral MTs.

Behavior of astral microtubules at the cortex

Once microtubule plus-ends contact the cortex by end-on attachment, two different scenarios have been observed. After a few seconds of cortical dwell, they either undergo catastrophe and shrink or continue to grow along the cell cortex, a process known as side-on sliding. Samora and colleagues have shown that the microtubule-associated protein MAP4 regulates spindle orientation and positioning by modifying the behavior of astral MTs at the cellular cortex [115]. Dynamic analyses of EB3-Tomato during metaphase revealed that upon depletion of MAP4, side-on sliding of astral microtubules at the cortex is increased and leads to spindle pole displacement. Interestingly, these effects are lost upon the impairment of dynein activity, suggesting that MAP4 acts by moderating dynein-dependent forces that generate abnormal MT–cortex interactions.

Modulation of specific astral MT subpopulations

While most of the studies describing the role of astral MT in spindle orientation have been performed in cultured cells, progress has been made recently to understand their characteristics and *in vivo* function in apical progenitors (APs) of the mouse neocortex [116]. The authors defined two different astral MT subpopulations, which are differentially regulated between proliferating and neurogenic APs. In neurogenic APs, the numbers of apical and basal astral MTs (but not of central MTs), decrease with respect to proliferating APs, in correlation with an increase in the amplitude of spindle oscillation during metaphase. Therefore, the density of apical/basal astral MTs may regulate the stability of spindle orientation. Indeed, specific perturbation of this astral MT subpopulation impacts the amplitude of spindle oscillations observed in proliferating APs. Interestingly, this subpopulation of astral MTs is in part controlled by LGN enrichment in the basal cortex, which is higher in proliferating than in neurogenic progenitors. This suggests that cortical anchoring of apical/basal astral MTs by the LGN complex regulates their stability. While it could be imagined that a broader cortical distribution of the LGN complex would lead to a less stable spindle orientation, the authors propose that it acts in the opposite manner: Basal LGN would favor the stabilization of the spindle by anchoring apical/basal astral MTs. It can be hypothesized that forces exerted on apical/basal astral MTs are smaller than those exerted on central astral MTs. This would allow spindle orientation along the plane of the tissue, which will be further stabilized by the anchoring of astral

MTs to the apical/basal domains. Whether specific subpopulations of astral MTs exist in other cellular contexts and how they regulate spindle orientation remains to be investigated.

Finally, it should be pointed out that shortening of astral MTs might differentially impact spindle orientation depending on the spindle size relative to the cell size. In addition, reduced astral MT density can result in different outcomes depending on the available cues for spindle orientation that in turn determine the level of enrichment of force generators at the cortex.

Extracellular stimuli influencing spindle orientation

In a tissue, cells are exposed to a variety of environmental stimuli that can influence their axis of division by mobilizing and polarizing the internal machinery for spindle orientation discussed above. In this section, we will review the increasing diversity in signaling pathways involved in the upstream regulation of spindle orientation (Fig 4).

Tre1 GPCR signaling

The observation that spindle orientation was random in dissociated fly neuroblasts led to the proposal that the axis of division of these cells may be influenced by extrinsic cues from the underlying neuroectoderm [117]. More recently, Yoshiura and colleagues have shown that Tre1 GPCR expressed in neuroblasts is the receptor for polarizing signals from the neuroectoderm. Remarkably, signaling downstream of Tre1 recruits Pins to the cell membrane closest to the neuroectoderm, through unconventional (and *Drosophila*-specific) Pins binding to activated GTP-bound Gαo. Pins in turn recruits inscuteable and Par3, thereby aligning the apical–basal polarity axis of the neuroblast relative to the neuroectoderm. In addition, Pins controls the orientation of the spindle along the defined axis through Mud. In Tre1 mutants, the polarity axis of the cell (defined by Par3/Insc localization) and the orientation of the spindle remain coordinated with each other, but they are randomized relative to the neuroectoderm. Therefore, an unidentified signal from the neuroectoderm acts as a positional cue for the polarity axis through Tre1 signaling [118], and Pins plays a dual role in interpreting the external polarity cue and orienting the spindle.

Planar cell polarity (PCP) pathways

The role of planar cell polarity pathways in the modulation of spindle orientation has been extensively documented [19,91]. Both Wnt/Fz and Fat/Dachsous/Four-jointed (Fat/Ds/Fj) pathways modulate spindle orientation in both asymmetrically and symmetrically dividing cells.

One of the best-understood models regarding these signaling pathways is the EMS cell division in the *C. elegans* 4-cell embryo (Fig 5Ai). In this system, the EMS cell receives a secreted Wnt (MOM2) signal from the neighboring P2 cell, and this leads to the
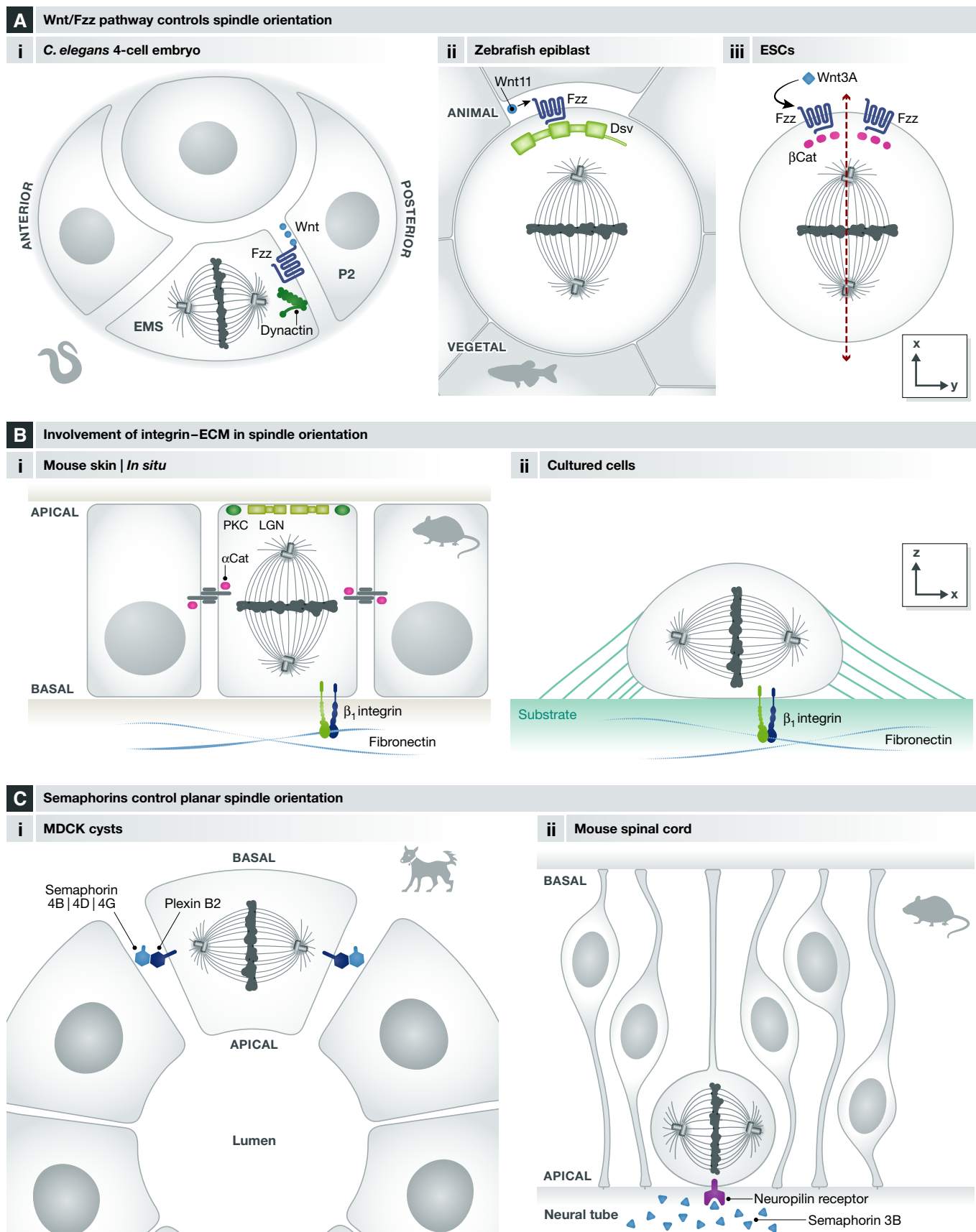
Figure 5.

◄

Figure 5. Extracellular stimuli influencing spindle orientation.
(A) The Wnt/Fz pathway controls spindle orientation in diverse contexts including (i) the 4-cell *C. elegans* embryo, (ii) zebrafish epiblast cells, and (iii) embryonic stem cells. (B) Involvement of integrins–ECM in spindle orientation: I) In mouse skin progenitors during stratification, β1 integrin is necessary for correct cell polarity (PKC), LGN apical localization and thus spindle orientation; II) in cultured cells, interaction of β1 integrin with extracellular matrix components is necessary for spindle orientation in parallel to the substrate. (C) Semaphorins control planar spindle orientation (i) in the context of epithelial morphogenesis in MDCK cysts and (ii) in neural progenitors of the mouse spinal cord.

activation of Fz (MOM5). As a consequence, signaling events trigger the accumulation of dynactin at the P2-EMS contact site, directing spindle rotation in the direction of this site. Similarly, during the asymmetric division of *Drosophila* SOP, the Fz/Dsh pathway dictates the orientation of the spindle along the anterior–posterior axis. This mechanism involves the localization of Fz and Dsh to the posterior cortex of the SOP cell, which allows the recruitment of NuMA, and possibly dynein [63]. In addition, PCP proteins are necessary for the correct localization of Dlg/Pins/Gαi to the anterior lateral cortex, which controls the orientation of the spindle relative to the apico-basal axis. The Fat/Ds/Fj pathway also controls spindle orientation in some invertebrate and vertebrate tissues. In the context of tissue elongation of *Drosophila* wing disks, the Fat/Ds/Fj pathway is necessary for spindle orientation along the proximal–distal axis [119]. Similarly, this pathway regulates the orientation of cell division in the context of tubule elongation during postnatal development of the murine kidney [120].

PCP pathways also modulate mitotic spindle orientation during symmetric division in the context of tissue morphogenesis. In particular, Wnt11 and Dsh regulate division orientation along the animal–vegetal axis in epiblast cells during zebrafish gastrulation [121] (Fig 5aii). In addition, the Wnt/Fz and Fat/Ds/Fj pathways regulate spindle orientation in kidney development in the mouse. Likewise, spindle orientation along the proximal–distal axis during limb bud growth in the mouse embryo is under the control of Wnt5A [122].

Finally, localized extracellular Wnt signals have recently been shown to orient the mitotic spindle in cultured embryonic stem cells [123]. In this system, asymmetric exposure of ES cells to Wnt3a beads promotes the asymmetric localization of Wnt components such as β-catenin, APC and frizzled. In this context, the spindle is oriented perpendicularly to the Wnt3A bead (Fig 5Aiii). This leads to the question how Wnt3A orients the spindle in ES cells. One possibility is the involvement of the Fz/Dsh/NuMA pathway [63], considering the asymmetric distribution of Fz-GFP observed in this context.

Integrins

The influence of signals from the extracellular matrix (ECM) on spindle orientation has first been demonstrated in the context of *in vivo* mouse skin stratification. Apical recruitment of LGN by inscuteable and Gαi controls the apico-basal spindle orientation of asymmetrically dividing progenitors during skin stratification [11,39,124]. In knockout mice for β1 integrin, a protein that is critical for basement membrane integrity, spindle orientation is randomized [11]. This phenotype correlates with the loss of PKC-ζ (a key determinant of the apical domain) from the apical cortex and the randomization of the position of LGN crescents. This suggests that the absence of β1 integrin signaling disrupts cell polarity and correct apical localization of the LGN complex, thus randomizing spindle orientation. Similarly, the loss of the cell–cell adhesion molecule

α-catenin impairs the apical localization of PKC-ζ and spindle orientation. However, LGN is no longer observed in the cell cortex. Because of the influence of adherens junctions on the actin cytoskeleton, the effects of α-catenin knockout on LGN complex formation could be due to defects in the actin cortex (see "The emerging role of actin in spindle orientation"). Therefore, both the basement membrane and cell–cell contacts are necessary for spindle orientation during skin stratification. In summary, β1 integrin and α-catenin may be seen as permissive rather than instructive cues for spindle orientation. By maintaining cell polarity, they allow LGN to be specifically localized to the apical cortex. In addition, α-catenin would be necessary for LGN recruitment or stability in the cell cortex (Fig 5Bi).

Similarly, Toyoshima and Nishida have shown that spindle orientation with respect to the cell–substrate adhesion plane is influenced by the extracellular matrix (ECM) in cultured cells [112] (Fig 5Bii). Cells cultured on fibronectin and collagen, which are integrin-binding ECM components, orient their spindles parallel to the substrate more tightly in comparison with cells cultured on poly-L-lysine, which does not bind to integrin. In addition, by using blocking antibodies and siRNA approaches, the authors showed that this adhesion-dependent orientation depends specifically on β1 integrin. Recent work by Morris and colleagues indicates that the direct interaction between integrin-linked kinase (ILK) and dynactin-2 (p50) links integrins to the dynein complex and controls the position of force generators [125]. It should be noted that in cells cultured on poly-L-lysine or in cells in which integrin, ILK, or p50 have been knocked down, spindle orientation remains strongly biased to angles lower than 40°, indicating that additional cues guide spindle orientation in this context (see "Spindle orientation in context: roles of cell geometry and mechanical forces").

Semaphorins

Semaphorin-mediated signaling has recently been proposed to regulate spindle orientation in different tissues. Xia and colleagues have demonstrated that cell–cell communication mediated by semaphorin–plexin signaling orients the mitotic spindle in kidney cells [126] (Fig 5Ci). In particular, plexin B2 is localized at the basolateral membrane in different epithelial tissues *in vivo* and in MDCK cells grown in 3D cultures. Depletion of plexin B2 generates defects in spindle orientation both in MDCK cysts and in renal tubules in the context of tissue repair [126]. Based on observations made in knockout mice, the authors suggested that the ligands semaphorin 4B, 4D, and 4G redundantly activate plexin B2. In addition, they demonstrated the involvement of Cdc42 in mediating plexin B2 effects on spindle orientation. Interestingly, upon plexin B2 depletion in MDCK cysts and regenerating renal tubules, spindle orientation is not randomized but appears to be biased toward the apico-basal axis. Whether this results from the re-localization of spindle orientation regulators like LGN or from the takeover of an alternative pathway remains to be investigated.

Similarly, Arbeille and colleagues have reported the effects of semaphorin 3B on the orientation of neural progenitor divisions in the mouse spinal cord [127] (Fig 5Cii). In semaphorin 3B knockout mice, the authors observed a small but significant change in the distribution of progenitor division angles at E10.5. Interestingly, these mice do not show defects in apico-basal polarity and F-actin organization, indicating that spindle orientation is not indirectly affected by changes in cell polarity. The authors showed that semaphorin 3B released from the ventral floor plate into the central canal binds to neuropilin receptors located at the apical surface of neural progenitors. At the molecular level, GSK3 and the microtubule stabilizing protein CRMP2 have been shown to be involved, but the pathway between signal reception at the apical surface and spindle positioning remains elusive. In particular whether the localization of LGN complex members is affected has not been determined, and whether semaphorin 3B signals during prometaphase/metaphase when the orientation of the spindle is set up is unknown.

Spindle orientation in context: roles of cell geometry and mechanical forces

Mitotic rounding is a common and remarkable feature of most dividing animal cells, whether in adherent cell culture or in intact tissues. Mitotic rounding implies reorganization of the actin cytoskeleton (reviewed in [128]), and cell ballooning is achieved through an increase in intracellular osmotic pressure [129]. The mitotic actin cortex is thinner, but stiffer than in interphase [93]. Mitotic rounding is viewed as a way to generate sufficient intracellular space to accommodate spindle formation and is indeed important for chromosome capture and bipolar spindle maintenance [130] (reviewed in [94]). Apart from non-adherent cells (such as one-cell zygotes), mitotic rounding in mitosis implies a profound remodeling of cells adhesion with their neighbors and/or the extracellular matrix.

Despite their rounding, mitotic cells are exposed to external forces generated by the contact with neighboring cells and with the substrate. These forces depend on the position of the cell within a tissue and on the changes in the tissue itself, especially during morphogenesis, and reflect a memory of cell shape and adhesion in interphase. In addition, rounding itself is often imperfect and cells retain a slightly elongated shape that corresponds to their shape in interphase and scales with tissue tension. In the following section, we will describe that both the memory of cell shape in interphase and a more direct sensing of cell shape in mitosis can influence spindle orientation.

Intrinsic cell geometry in mitosis impacts on spindle orientation

The empirical century-old "long axis" or "Hertwig rule", initially proposed by Oscar Hertwig in the late 19th century, posits that cells usually place their cleavage plane at the center of their mass and perpendicular to their longest axis [20]. Hertwig had explored this property through experimental deformation of single cell echinoderm embryos. These cells are normally perfectly spherical, and their first division is symmetric with no preferential orientation. However, by gently squeezing embryos between glass plates, Hertwig observed that the orientation of division could be controlled

by the deformation and aligned with the elongated axis. In line with Hertwig's observations, O'Connell and Wang used a similar approach to probe the relationship between cell shape and spindle orientation in cultured mammalian cells [131]. Using micromanipulation with glass pipettes, they forced shape deformations in dividing normal rat kidney (NRK) cells, which do not round up during mitosis and keep their interphase shape. In these cells, although the mitotic spindle can sometimes be observed orthogonal to the cell's longest axis in early metaphase, by anaphase it is aligned parallel to the longest axis. They observed that upon experimental deformation of mitotic cells, the spindle constantly reacted to cell shape changes and adapted by moving to the new cell center and realigning with the induced longest axis. They further showed that spindle movements occurred in an astral microtubule- and dynein-dependent manner.

Hertwig's rule was recently revisited in sea urchin embryos by Minc and colleagues, who used microfabricated 3-D molds to apply specific anisotropic shape deformations [132]. While the rule applied to most shapes, some specific shapes did not conform to its predictions. A model in which forces applied to spindle poles scaled with the length of individual astral microtubules predicted much better the orientations observed in all tested shapes. This model is difficult to reconcile with force generators combined at or near the cell cortex, which is the dominant model in other cell types, and it implies a role of force generators in the cytoplasm [132]. It should also be noted that single cell zygotes are usually very large and their spindle is comparatively small, with very long astral microtubules. In contrast, in many cell types, the size of the spindle scales with cell size [133,134]; mitotic rounding is indeed essential to allow sufficient space for the formation of the spindle, and artificial confinement results in chromosome missegregation [130].

Recently, Lazaro-Dieguez and colleagues studied the relationship between spindle orientation and cell shape in the context of imperfect rounding, making use of the natural variability of rounding in adherent MDCK and HeLa cells in mitosis [135]. In control conditions, adherent cells divide very precisely in the plane of the substrate, making it difficult to address the question. Disruption of force generation either by knockdown of the LGN pathway or pharmacological removal of astral microtubules disrupts this orientation, but a strong bias toward planar orientation remains. The authors attribute this bias to imperfect cell rounding: They compared the orientation of the spindle in cells treated with low doses of nocodazole (that primarily disrupt astral microtubules and abolish force generation) between perfectly round and more "flat" mitotic cells and found that the bias toward planar orientation was much more pronounced in flat cells (relative to the substrate), while orientation was close to random in cells with a more spherical shape. This indicates that cell shape can directly influence orientation independently of cortical force generators. The authors observed frequent deformation of the metaphase plate in these flat cells, suggesting that the effect on orientation may be a direct consequence of steric hindrance in cells where cytoplasmic volume and cell size are just sufficient to accommodate the size of the metaphase spindle.

This notion can be transposed to *in vivo* situations, where cell packing imposes constraints on cell shape both in interphase and during mitosis, and where rounding is unlikely to be perfect. In the mouse developing skin, where interphase cells are flat (e.g.,
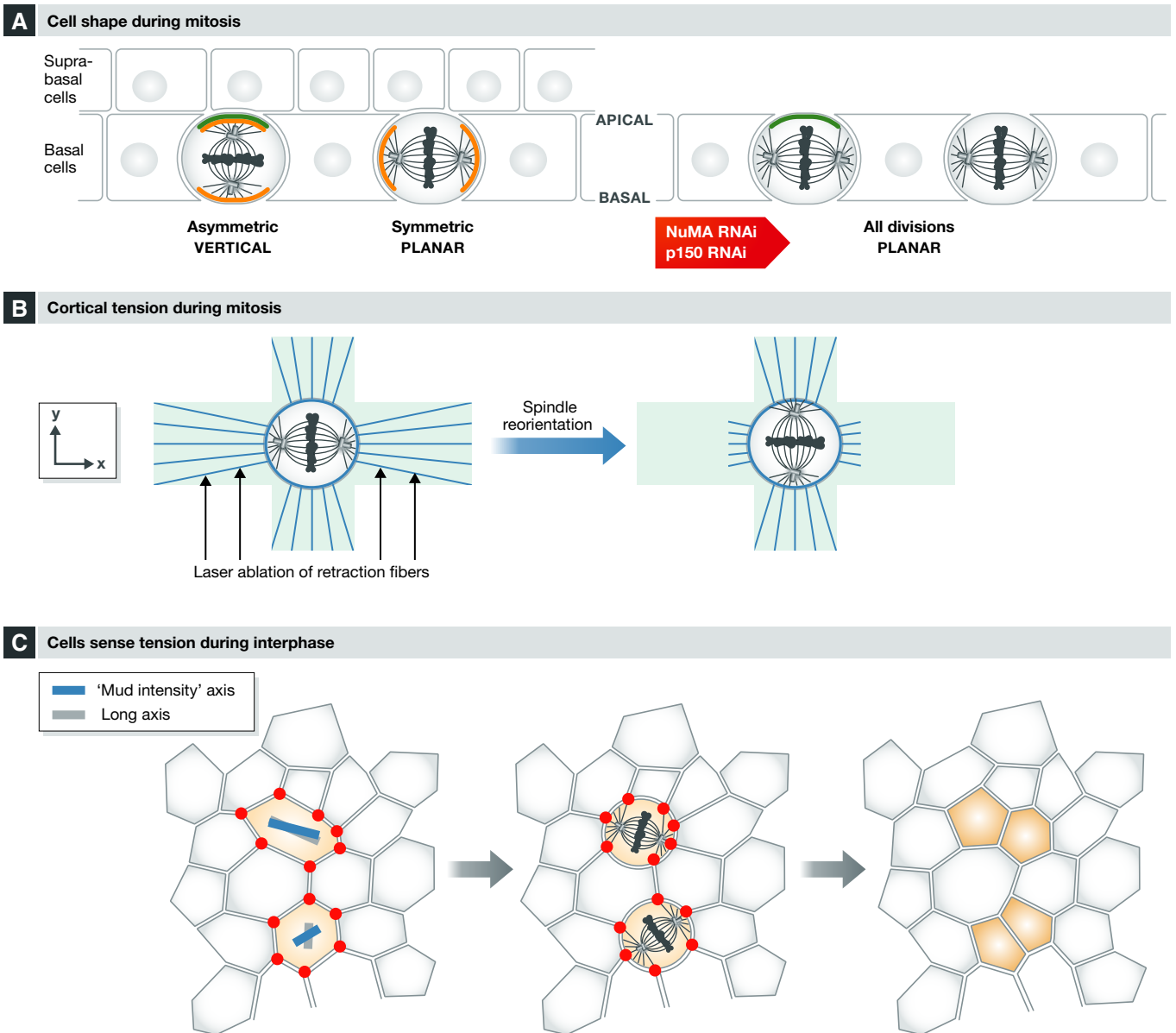
Figure 6. Role of cell geometry and external forces.

(A) Cells in the basal layer of the developing mouse epidermis adopt a binary orientation: Symmetric divisions occur in the plane of the epithelium, and asymmetric divisions divide along the apico-basal axis in an Insc/Gαi/LGN/NuMA/dynein manner, with one daughter cell delaminating into the suprabasal cell layer. Upon NuMA or p150 depletion, cortical force generators are not functioning and most divisions now take place in the plane of the epithelium, suggesting that the "default" planar orientation may be dictated by the flat cell shape in this tissue. Green lines: Insc and Gαi3 apical accumulation; orange lines: force generators (dynein). See Williams *et al* (2011) [39]. (B) In single cells cultured on fibronectin micropatterns (light blue), a field of maximal force is associated with polarized retraction fibers (blue lines). Cells cultured on "cross"-shaped patterns orient their spindle along the long arms of the cross, where maximal forces are observed. Laser ablation of retraction fibers on the long arms induces a 90° spindle rotation and alignment to face the "new" maximal forces. See Fink *et al* (2011) [67]. (C) In the fly notum epithelium, NuMA accumulates at tricellular junctions in the G2 phase. Left panel: A vector corresponding to the cells long axis (gray bar) or to the geometry of tricellular junctions (or Mud accumulation, red dots) can be drawn (blue bar). In elongated cells, both vectors are aligned (top light red cell), whereas they do not always align in cells with an isotropic shape (bottom light red cell). Middle panel: The "Mud accumulation" vector predicts the orientation of cell divisions more accurately than the long axis. Right panel: Position and shape of the daughter cells after division. See Bosveld *et al* (2016) [140].

with a relatively short apico-basal length), orientation of the spindle is biphasic: Symmetric divisions orient in the plane of the tissue and asymmetric divisions are perpendicular to this plane in an Insc/Gαi/LGN/NuMA-dependent manner [11,124]. Remarkably, disruption of force generators via knockdown of NuMA or p150 does not lead to random spindle orientation, but most divisions are planar, according to the main axis of cell elongation (Fig 6A). This suggests that a cell shape sensing mechanism independent of cortical force generators contributes to default planar orientation in this tissue [39].

Role of surrounding forces in spindle orientation

External forces influence spindle orientation in single cells
in vitro In cultured adherent cells, the distribution of retraction fibers in mitosis reflects the geometry of the adhesion of the cell to its substrate in the previous interphase. As mentioned above, the distribution of retraction fibers dictates, and therefore can indeed be used to predict the orientation of the mitotic spindle within the plane of the substrate [65–67]. Fink and colleagues demonstrated the function of retraction fibers by performing laser ablation of these cellular structures and by analyzing spindle movements (Fig 6B). Importantly, these authors observed changes in cell shape upon retraction fiber ablation, suggesting that retraction fibers exert forces on the cell. Indeed, this was confirmed upon measurement of the forces associated with retraction fibers by using optical tweezers. Remarkably, applying stretch forces to a cell without affecting its shape is sufficient for spindle rotation along the axis of the dominant force field [67]. Collectively, these experiments demonstrate that adhesion-related forces can control spindle orientation in single cells. In these experiments, reorientation was reduced in the presence of low doses of nocodazole that disrupt astral microtubules, indicating that force generators acting on the microtubule network work downstream of retraction fibers. The entire molecular cascade that links external forces from retraction fibers to the recruitment and activation of internal force generators is not completely understood and involves several pathways, as already detailed. On one hand, activation of the actin cloud/myosin 10 pathway provides a direct link with microtubule dynamics [102]; on the other hand, forces exerted on the cortex influence local ERM activation [66,68], which directly or indirectly promotes the asymmetric localization of LGN/NuMA and presumably of dynein to the cortex [68].

While retraction fibers have not been described in tissues, distinct structures may mediate the establishment of forces in an analogous manner through cell–matrix attachment or cell–cell interactions, as described below.

Influence of external forces on spindle orientation *in vivo* The influence of external forces on spindle orientation has also been addressed *in vivo* in developing tissues. During the spreading of the enveloping cell layer (EVL) of the zebrafish gastrula [136], cells orient their spindle along the animal–vegetal axis which coincides with the axis of maximal tension in this tissue. Importantly, artificial induction of local tension in the perpendicular direction induces spindle rotation and reorientation toward the axis of induced tension. Using a computational model, the authors found that the cell shape parameter in interphase can reliably be used to predict the visualized orientation of divisions in the EVL. Mechanistically, they showed that the molecular motor myosin II is involved in cell shape regulation and in mediating the connection between shape and spindle orientation [136].

Wyatt *et al* [137] used micromanipulation to apply stretch forces to a suspended monolayer of cultured MDCK cells. In response to stretch, cells elongate parallel to this homogeneous field force and divide along their longest axis. This restores cell shape isotropy in the stretched tissue; in short, divisions relieve tension. Similar results were obtained in the developing *Drosophila* wing disk, where cells divide according to local tension fields, therefore reducing the tension in the tissue [138,139]. During this morphogenetic process,

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Box 3:   In need of answers

1) How do different cortical molecules (like Gαi, Afadin, and Dlg1) interplay to regulate the recruitment and maintenance of LGN to the cellular cortex?
2) In cultured cells the dynamics of LGN, NuMA, and dynein localization during mitosis are regulated by molecules located on chromosomes, centrosomes and at the cortex. Are these pathways active and necessary to achieve oriented divisions *in vivo*? How do they integrate with the instructive signals, such as polarized inscuteable, that control spindle orientation in complex tissues?
3) How are LGN/dynein complex members transported to the cellular cortex in higher eukaryotes? Are they transported by astral MT as suggested by the dynein off-loading model in yeast?
4) How do actin and NuMA/dynein pathways interact during spindle orientation?
5) What other alternative force tethering systems exist?
6) How do geometric and molecular cues integrate in a complex tissue?
7) Can the recent models and molecular interpretations of the "long axis rule" be transferred in tissues with a different organization?
8) What is the actual contribution of spindle orientation to development and tissue homeostasis? How does spindle orientation contribute to cancer development and in what specific contexts?

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the tension fields themselves are generated by local variations in proliferation rates, showing an interesting feedback loop between proliferation, tissue tension, and oriented cell divisions.

How do cells sense "tension" and translate it into spindle orientation? When a tissue is under tension, cells tend to adopt an elongated shape that generally aligns with the axis of maximal tension. However, a minority of cells do not behave like this. Remarkably, Wyatt *et al* [137] found that the spindle aligns with the long axis even in the minority of cases when the long axis is not aligned with the stretch applied to the tissue, indicating that cell shape may be a better predictor than global tissue tension itself. However, a recent study by Bosveld and colleagues shows that while cell shape in interphase is a good indicator of spindle orientation when anisotropy is high, it does not predict orientation as efficiently in nearly isotropic cells. Under these conditions the topology of a cell's contacts with its neighbors during interphase is a better parameter [140]. In the epithelium of the fly pupal notum, the authors found that tricellular junctions (TCJs; the vertex where three neighboring cells are in contact) localize force generators in a Mud-dependent manner. Remarkably, Mud starts to accumulate at TCJs during G2 phase. When cells round up for mitosis, the position of cortical patches of Mud reflects the geometry of the cell contacts with its neighbors and dictates where greater forces will be generated. The authors show that a model using the position of the TCJs, and therefore of the Mud patches ("Mud intensity model"), to predict force generation faithfully recapitulates experimental data in this tissue. Remarkably, predictions in this particular tissue are more accurate than with a model that uses cell shape as one of its main parameters [132]. Bosveld and colleagues propose that in addition to their function as epithelial barrier structures, TCJs serve as polarity cues promoting geometry and mechanical sensing in epithelial tissues [140] (Fig 6C). Quite remarkably, this new orientation mechanism depends on Mud, dynein, and Dlg, but does not require Gαi or Pins, providing another example of a Pins-independent, but

Mud-dependent pathway. In contrast to *Drosophila* Mud, vertebrate NuMA is nuclear in interphase and has not been described at cellular junctions in epithelia; it is therefore unclear whether the mechanism described above reflects a generic property of TCJs. Future experiments in other model systems, either in tissues or in experimentally stretched cell layers (like those described by Wyatt *et al*), should explore whether TCJs carry a similar geometric information independently of NuMA.

In summary, it appears that mechanisms acting in interphase and during mitosis sense extrinsic tension and intrinsic geometry and contribute to translate these cell shape parameters into an oriented spindle. Despite the increase in osmotic pressure, mitotic rounding is probably never perfect in a tissue where cells are subjected to forces of adhesion and compaction. It is therefore difficult to completely uncouple the factors that depend on external forces from those related to intrinsic shape.

Conclusions

Over the last two decades, our knowledge of the molecular mechanisms that control the orientation of the mitotic spindle has significantly increased. Early findings in a limited number of models uncovered a common set of evolutionarily conserved basic players. However, recent work looking at different species has highlighted that regulatory mechanisms vary a lot, and a number of alternative pathways for force generation have been discovered. Remarkably, within a single organism, there are important tissue-specific differences concerning the modalities of spindle orientation and which pathway is used.

The actin cortex and astral microtubules are not just passive players that respectively anchor force generators and serve as a substrate for those forces: They are the core of complex regulatory pathways that tune force transmission to finely modulate spindle orientation, and many levels of regulation certainly remain to be uncovered.

Over the last years, advances in live molecular imaging have played a decisive role in this field, highlighting the importance of dynamic studies in the description and understanding of this highly choreographed process. Combinations of theoretical modeling with live observations, genetics, and physical manipulations have helped to refine Hertwig's remarkable observations, bringing forth a number of different interpretations and molecular explanations to his classical rule in different cell and tissue types.

The role of spindle orientation in normal and pathological development and homeostasis has been acknowledged, but is still poorly understood [14–19]. The research performed over the last few years, illustrates the remarkable diversity of strategies and mechanisms used in different systems. Hence, it is increasingly evident that we need to explore, in addition to further research into the details of known pathways in classical models of spindle orientation, the conservation and diversity in spindle orientation pathways in new cell and tissue types (Box 3).

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Conflict of interest

The authors declare that they have no conflict of interest.

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