

Mechanisms underlying spindle assembly and robustness

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Abstract

The microtubule-based spindle orchestrates chromosome segregation during cell division. Following more than a century of study, many components and pathways contributing to spindle assembly have been described, but how the spindle robustly assembles remains incompletely understood. This process involves the self-organization of a large number of molecular parts – up to hundreds of thousands in vertebrate cells – whose local interactions give rise to a cellular-scale structure with emergent architecture, mechanics and function. In this Review, we discuss key concepts in our understanding of spindle assembly, focusing on recent advances and the new approaches that enabled them. We describe the pathways that generate the microtubule framework of the spindle by driving microtubule nucleation in a spatially controlled fashion and present recent insights regarding the organization of individual microtubules into structural modules. Finally, we discuss the emergent properties of the spindle that enable robust chromosome segregation.

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Introduction

The spindle, first described in the 1880s¹ by Walther Flemming, is a bipolar, microtubule-based structure that positions chromosomes at its centre and segregates them into two daughter cells. Errors in spindle assembly and function can lead to aneuploidy, cancer and birth defects^{2,3}. The process of spindle assembly, which occurs between prophase and metaphase of cell division (Fig. 1a), serves as an excellent system to study both fundamental concepts of cellular self-organization and mechanisms whose dysregulation leads to disease.

During spindle assembly, the most basic building block of microtubules, tubulin, self-organizes from nanometre-scale dimers to micrometre-scale structural modules such as microtubule-based kinetochore fibres (k-fibres) (Fig. 1b). Although the identities of nearly all spindle parts (for example, microtubule nucleation factors, motors, crosslinkers) are known in many species, how these parts come together to give rise to the spindle's robust architecture, mechanics and function remains far from clear. Indeed, both conceptually and technically, there is currently a gap between bottom-up approaches (reconstituting systems from their parts) and top-down approaches (breaking systems apart) to study spindle assembly.

We organize this Review into three parts, from the smaller to the larger spatial scales of spindle assembly (Fig. 1b). We first describe how the microtubule framework is built across space and time for efficient spindle assembly. Second, we introduce how motor proteins (such as dynein) and non-motor proteins (such as crosslinkers) organize this microtubule framework into key structural modules, including k-fibres, bridging fibres and astral microtubules, each of which are essential to spindle function. Third, we describe how these structural modules give rise to the metaphase spindle and discuss the emergent properties of the spindle, including spindle dynamics, mechanics and size, which ultimately enable accurate chromosome segregation. Although studying spindle assembly across diverse species will continue to reveal rich insights into the fundamental mechanisms of the cell division machinery, we focus our discussion on animal spindles. We highlight recent studies that have expanded classic concepts and describe how novel developments in molecular, physical and imaging approaches are improving our understanding of animal spindle assembly (Box 1).

Spindle microtubule nucleation

At the onset of cell division, interphase microtubule structures are disassembled into their basic $\alpha\beta$ -tubulin subunits, supplying the cell with material to build the spindle framework. Efficient spindle assembly requires rapid formation of thousands of microtubules. However, spontaneous microtubule nucleation in cells is inefficient. The dividing cell uses several microtubule nucleation pathways to overcome this nucleation barrier at defined locations within the cell such as centrosomes, kinetochores and other microtubules. Together with proteins that organize microtubules and regulate their stability, these nucleation pathways give rise to the bipolar structure of the spindle, in which most microtubule plus ends (characterized by exposed β -tubulin subunits and usually fast growth) are oriented towards chromosomes and most microtubule minus ends (with exposed α -tubulin and slow growth) point towards spindle poles. Individual microtubules are dynamic, nucleating from their minus ends and constantly growing and shrinking, which occurs largely from their plus ends. The lifetimes of individual spindle microtubules (seconds to minutes) are much shorter than that of the spindle, which persists up to an hour to support chromosome segregation. Thus, microtubules must not only be nucleated to build the spindle but also to maintain it and allow it to remodel

itself as cell division progresses. Here, we provide an overview of how the barrier to nucleation is overcome, how nucleation pathways regulate microtubule formation in space and time, and how these pathways cooperate to build the spindle.

Molecular basis of nucleation

Microtubules are cylindrical polymers containing 13 filaments composed of $\alpha\beta$ -tubulin heterodimers, arranged in a head-to-tail configuration. For microtubules to form, individual tubulin dimers must interact laterally to create the cylindrical microtubule shape. This process requires the formation of a tubulin oligomer consisting of approximately eight tubulin dimers⁴, which is energetically unfavourable and makes spontaneous microtubule nucleation inefficient⁵ (Fig. 2a). In higher eukaryotes, efficient nucleation is promoted by a universal microtubule nucleation template, the γ -tubulin ring complex (γ -TuRC). This ~2.2-MDa cone-shaped complex is formed by γ -tubulin complex proteins 2–6 (GCP2–6), mitotic spindle organizing proteins 1 and 2 (MZO1 and MZO2), and actin^{6–9}. Together, these proteins provide a scaffold to position 13 γ -tubulin monomers into a left-handed helix with 13-fold symmetry, roughly mimicking microtubule geometry^{6–9}. This γ -TuRC geometry provides a microtubule minus end template upon which $\alpha\beta$ -tubulin dimers assemble, facilitating the formation of critical lateral interactions between individual dimers necessary to overcome the kinetic nucleation barrier¹⁰. Recent studies revealed that γ -TuRC-facilitated nucleation requires the assembly of approximately four to seven tubulin dimers to nucleate a microtubule^{9,10}, as opposed to the eight-dimer assembly needed for spontaneous nucleation¹⁰. Additionally, γ -TuRC requires the chTOG protein (also known as CKAP5, or Stu2 and XMAP215 in yeast and *Xenopus*, respectively), which recruits tubulin dimers to the template for assembly. Together, chTOG and γ -TuRC synergistically act to nucleate microtubules and form the universal microtubule nucleation module^{9–14} (Fig. 2a). Although mechanisms for γ -tubulin-independent nucleation have been reported in cells^{15,16} and studied in vitro^{17,18}, bipolar spindle assembly requires γ -tubulin^{15,19} and relies mainly on γ -TuRC-dependent pathways, which are discussed in the next section.

Key to building a spindle is the spatial and temporal regulation of γ -TuRC nucleation activity. After nucleation, microtubule dynamics are largely modulated by proteins that tune growth and shrinkage, and regulate catastrophe and rescue rates²⁰. It has been proposed that interactors of γ -TuRC, specific to each nucleation pathway, may serve as activators of its nucleation activity^{21–23} and we describe these in the following sections. How these activators function mechanistically is an open question. Proposed modes of activation include an induced conformational 'ring closure' of γ -TuRC to better mimic microtubule geometry, as observed in vitro with yeast γ -TuRC²⁴. This conformational change may be achieved simply as a result of the lateral $\alpha\beta$ -tubulin interactions formed between dimers upon assembly on γ -TuRC, which may promote a more microtubule-like configuration of the γ -TuRC structure¹⁰. Consequently, increased tubulin concentration alone would increase tubulin binding to γ -TuRC and bias γ -TuRC towards a conformationally activated state¹⁰. Recently, γ -TuRC regulation by local tubulin enrichment, driven by the formation of biomolecular condensates, was proposed to occur at centrosomes and on the microtubule lattice^{25–27}. This enrichment may not only overcome the nucleation barrier^{9,10} but also provide the necessary raw material for spindle assembly exactly where it is needed.

γ -TuRC is targeted to defined locations to construct the spindle in a regulated manner. We next discuss these microtubule nucleation

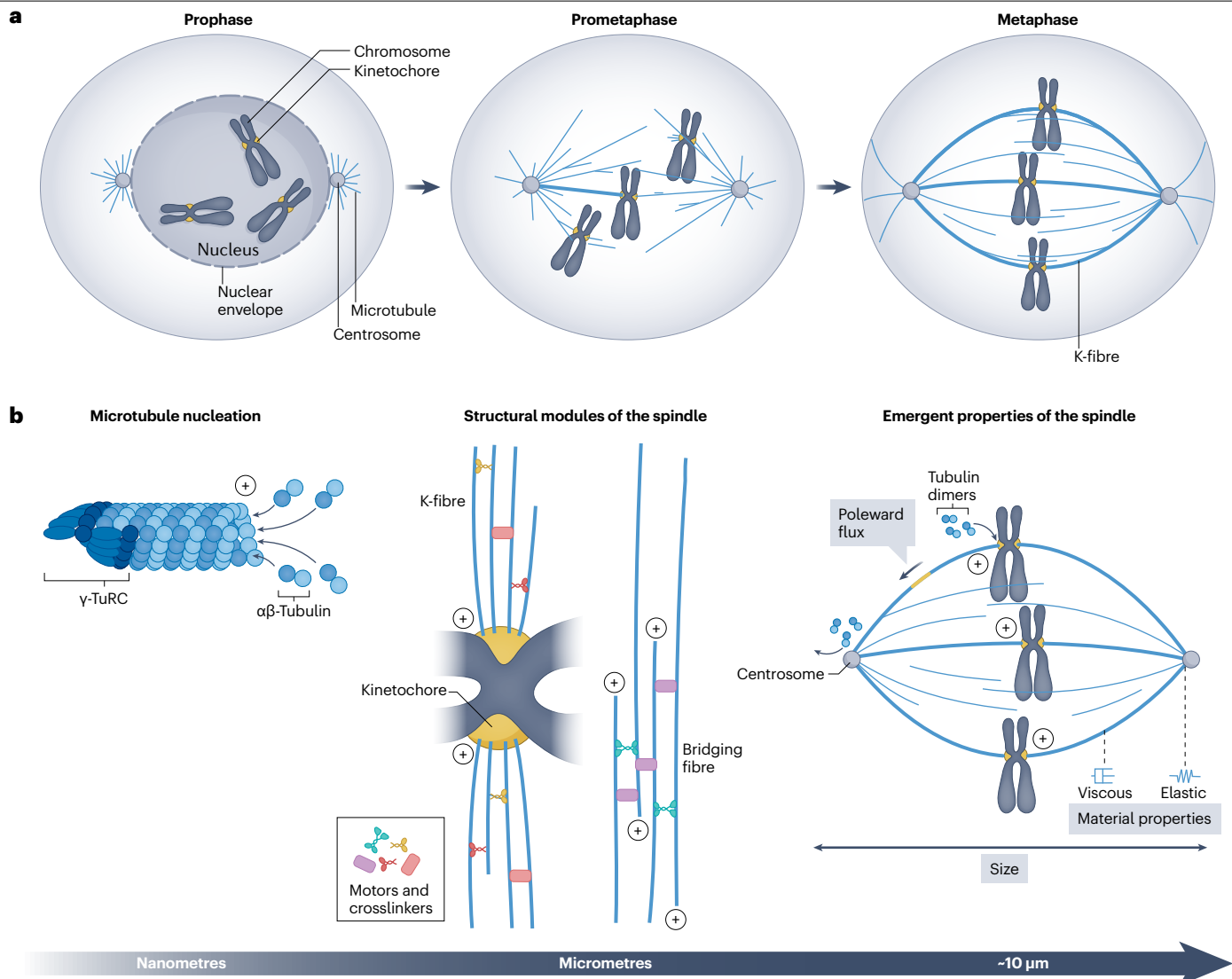


Fig. 1 | Overview of spindle assembly, from molecular parts to cellular-scale properties. **a**, Stages of spindle assembly. During prophase, centrosomes separate to opposite sides of the nucleus and chromosomes become condensed, followed by nuclear envelope breakdown at the end of prophase. In prometaphase, microtubules are nucleated from several sources (see also Fig. 2), and kinetochores begin to capture microtubules. Microtubules that interact with chromosome arms aid in chromosome congression to the middle of the cell. In metaphase, chromosomes align to form the metaphase plate, and bundles of microtubules

attached to each kinetochores (termed k-fibres) mature. **b**, Spatial scales of spindle assembly. Spindle assembly requires formation of microtubules from $\alpha\beta$ -tubulin dimers. This process relies on nucleation pathways that target the γ -tubulin ring complex (γ -TuRC) to promote microtubule nucleation in specified locations. Individual spindle microtubules assemble into structural modules through the action of crosslinkers and motor proteins. The spindle is characterized by emergent properties – that is, characteristics of the ensemble that are not exhibited by its component parts – such as its material properties and poleward microtubule flux.

pathways, which are characterized by their means of specifically localizing and activating γ -TuRC at centrosomes and around chromosomes (Fig. 2b–e).

Centrosome-mediated nucleation

In centrosome-containing spindles, such as in animal somatic cells, centrosomes are located at the spindle poles. In preparation for cell division, centrosomes mature by expanding their surrounding pericentriolar material (PCM) through protein accumulation, which is largely controlled by PLK1 and Aurora A kinase activity^{28,29}.

Various PCM proteins then perform the key task of recruiting and anchoring γ -TuRC to centrosomes and recruiting microtubule nucleation effectors^{30–32} (Fig. 2b). The PCM protein CEP192 activates a cascade of protein phosphorylation through Aurora A and PLK1 kinases. While many details remain elusive, this cascade drives γ -TuRC and chTOG localization to centrosomes^{28,33} and activation of microtubule nucleation³⁴, possibly through phosphorylation of the γ -TuRC interactor NEDD1 (ref. 35). CEP192 and NEDD1 localization is also facilitated by the PCM component pericentrin²⁹, which can also directly anchor γ -TuRC³⁶. Therefore, indirect effects caused by the interdependence

Box 1

Emerging technical approaches to study the spindle

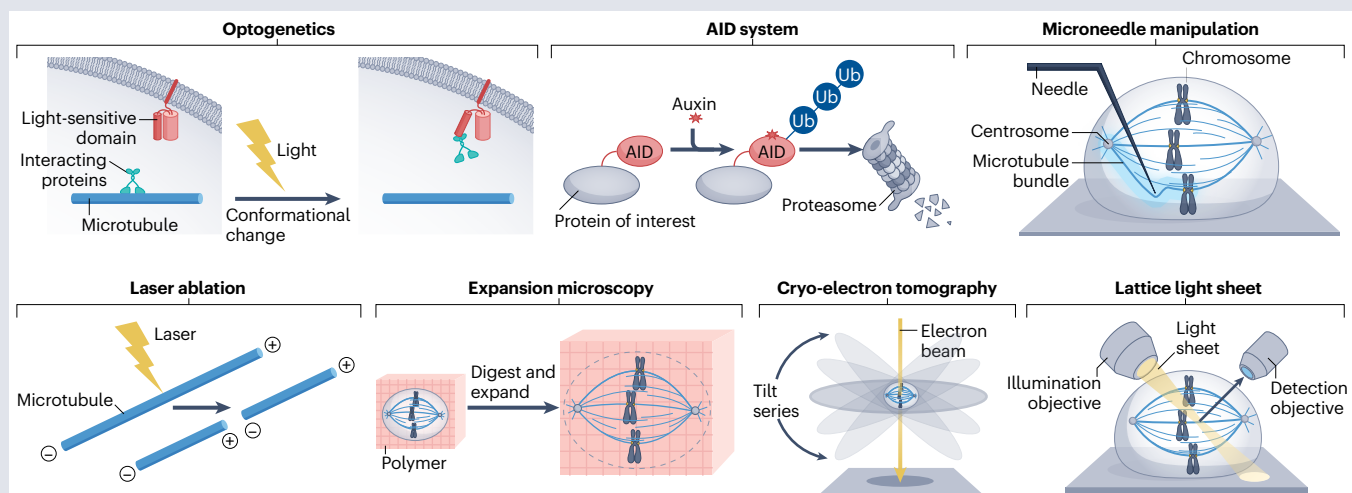
The process of spindle assembly is highly dynamic in space and time. Several emerging approaches, capable of manipulating and imaging the spindle with high spatiotemporal control and resolution, have complemented classical approaches to improve our understanding of spindle assembly.

Molecular approaches aim to define the mechanisms underlying spindle function. Molecular perturbations with improved spatiotemporal control include optogenetics and the auxin-inducible degron (AID) system. Optogenetic approaches entail tagging proteins of interest with engineered domains whose conformations are sensitive to specific wavelengths of light. This allows their localization or activity to be finely controlled via the spatial pattern, timing and/or intensity of illumination. This strategy has been employed to study the roles of the spindle proteins PRC1 (ref. ¹⁵¹) and EB1 (ref. ¹⁶⁵), among others. The AID system takes advantage of a protein degradation system found in plants to deplete proteins with temporal but not spatial precision. This system can be applied to non-plant cells by genetically modifying proteins, such as NuMA, HURP and components of the RanGTP pathway^{15,176}, to contain a small degron tag, which targets tagged proteins for rapid ubiquitylation (Ub) and degradation following induction with the plant hormone auxin. Both optogenetics and AID acutely affect protein behaviour, allowing for protein function at discrete stages of cell division to be studied. Acute perturbations can also reveal phenotypes previously obscured by cellular adaptation after long-term protein depletion. However, optogenetics and AID may not be possible for all proteins, including those with very slow turnover or those for which adding a tag disrupts protein function.

Physical approaches aim to measure and manipulate mechanical forces in the spindle. The spatiotemporal control that some physical approaches provide has been especially helpful in uncovering the specialized mechanics of the spindle in space and time, and probing their molecular basis. Microneedle manipulation, first

developed half a century ago¹²⁶ and recently adapted to mammalian cells^{212,217}, exerts controllable forces of varying magnitude, direction and duration on specific spindle substructures such as kinetochore fibres. Microneedle manipulation in *Xenopus* egg extract spindles, where no surrounding membrane or cortex is present, can also provide quantitative measures of spindle material properties such as viscoelasticity^{238,239}. Laser ablation acutely and locally severs spindle microtubules, allowing for the relaxation of spindle forces and breaks in spindle architecture. These approaches, while low throughput, provide insights to the material properties and robust structural maintenance of the spindle, and their more recent combination with molecular approaches is beginning to reveal their molecular basis.

Innovations in imaging approaches have improved the spatial and temporal resolution at which we can now observe spindle assembly. Expansion microscopy is a fixed-sample technique in which a sample is covalently bound to a swellable polymer, homogenized via enzymatic digestion, and linearly expanded to ~4.5-fold its original size. This expansion decreases crowdedness within the cell and allows higher spatial resolution of protein localization within the dense spindle architecture, observable through immunofluorescence and standard confocal microscopy²⁷¹. Cryo-electron tomography utilizes thin frozen samples and acquisition of a series of data points at various (tilted) angles to provide three-dimensional reconstructions of spindle microtubules and structures, such as kinetochore fibres^{117,118}, at angstrom resolution. Lattice light sheet microscopy scans samples using thin sheets of light, allowing high-resolution volumes of whole cells to be acquired with minimal phototoxicity at sub-second intervals^{30,272–274}. With lattice light sheet microscopy, resolving the dynamics of all spindle microtubules is now achievable^{272,275}. However, the slow speed associated with processing and analysing the large data sets generated from these experiments currently limit this technique.



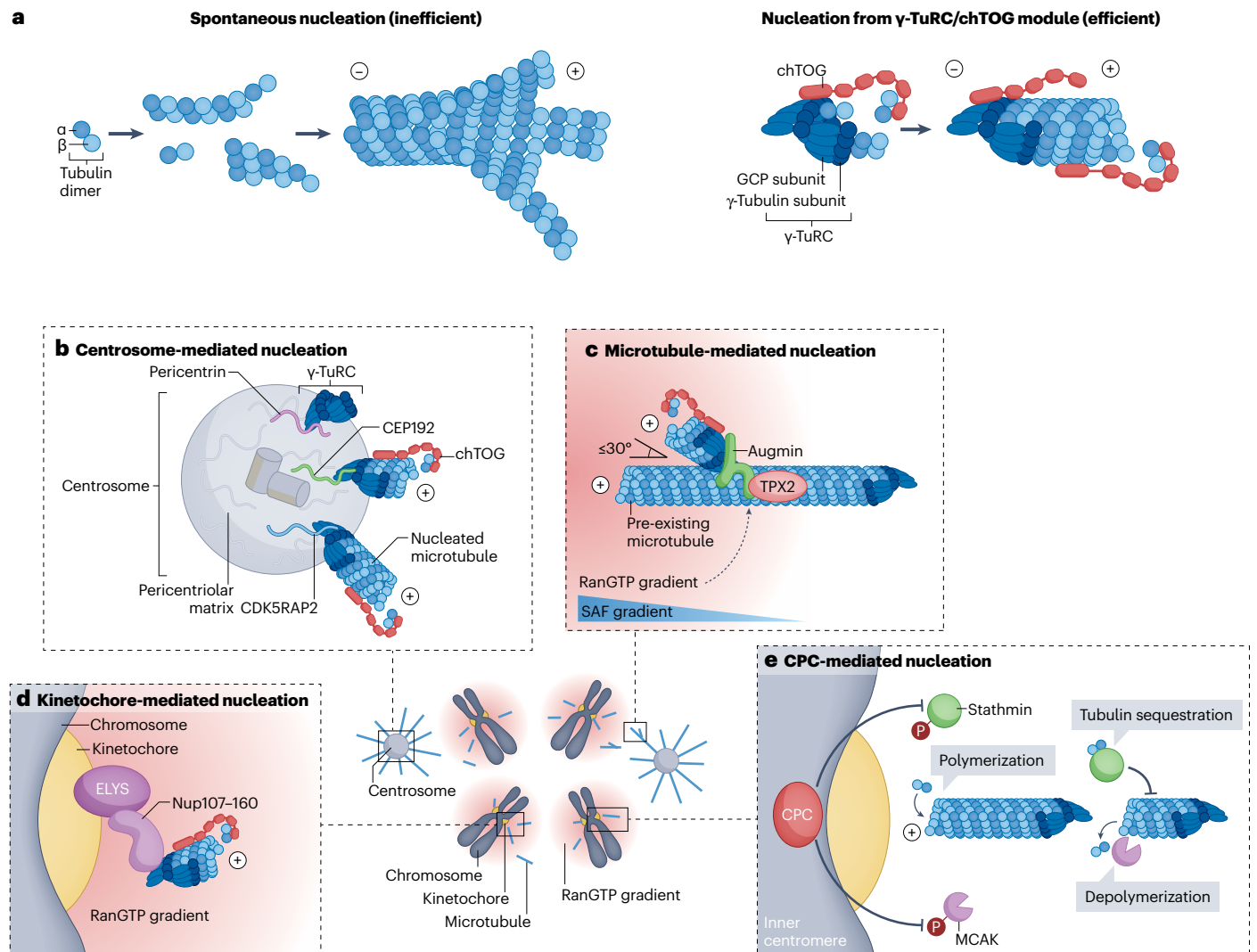


Fig. 2 | Microtubule nucleation during spindle assembly. **a**, Spontaneous microtubule nucleation from $\alpha\beta$ -tubulin dimers is energetically unfavourable and therefore an inefficient process. Nucleation is facilitated by the universal microtubule nucleation module that comprises the chTOG protein and the γ -tubulin ring complex (γ -TuRC), which mainly consists of γ -tubulin and γ -tubulin complex proteins 2–6 (GCP2–6). γ -TuRC provides a microtubule minus end template for nucleation whereas chTOG recruits $\alpha\beta$ -tubulin dimers to speed up microtubule assembly. Microtubule nucleation is localized to specific spindle sites, such as centrosomes (**b**), existing microtubules (**c**), kinetochores (**d**) and centromeres via the chromosomal passenger complex (CPC) (**e**). Chromosomes play a key role in activating the small GTPase Ran to form RanGTP, which forms a gradient around chromosomes following nuclear envelope breakdown. This gradient spatially activates nucleation on existing microtubules (**c**) by creating a corresponding gradient of active spindle assembly factors (SAFs). Additionally, RanGTP activates nucleation at kinetochores (**d**). **b**, The pericentriolar matrix components pericentrin, centrosomal protein 192 (CEP192) and, possibly,

CDK5 regulatory subunit-associated protein 2 (CDK5RAP2) localize γ -TuRC to the centrosome. **c**, In microtubule-mediated (branching) nucleation, the γ -TuRC-interacting complex augmin binds and localizes γ -TuRC and chTOG to the microtubule lattice. In *Xenopus laevis* egg extract, this mechanism also requires the RanGTP-activated SAF TPX2, which facilitates recruitment of the remaining factors to microtubules. This pathway results in microtubules of conserved polarity relative to the pre-existing microtubule, which are nucleated at a shallow angle ($<30^\circ$) and often parallel to the mother microtubule. **d**, γ -TuRC is localized to kinetochores by the nucleoporin (Nup) complex Nup107–160 and Nup-interactor ELYS and activated in a RanGTP-dependent manner. **e**, Formation and localization of the chromosome passenger complex (CPC) to the inner centromere activates the CPC component Aurora B kinase. Aurora B phosphorylates and inactivates the microtubule destabilizing protein MCAK and the tubulin-sequestering protein stathmin to facilitate microtubule nucleation and polymerization towards kinetochores, independently of RanGTP.

of PCM protein accumulation make it difficult to disentangle the individual role of PCM proteins in γ -TuRC recruitment and can complicate functional studies. Additionally, the PCM component CDK5 regulatory subunit-associated protein 2 (CDK5RAP2) can activate γ -TuRC

nucleation activity via its γ -TuRC nucleating activator (γ -TuNA) motif (also known as the CM1 motif)^{21,23,37}, although conflicting reports exist on whether CDK5RAP2 can also directly localize γ -TuRC to centrosomes^{32,38}.

In *Caenorhabditis elegans*, the CDK5RAP2 homologue SPD5 forms a protein-rich compartment, potentially through condensation, that is enhanced by PLK1 and the CEP192 homologue SPD2. This SPD5 compartment recruits the chTOG homologue ZYG9 and other regulatory factors (such as the *C. elegans* homologue of the Ran-regulated protein TPX2, discussed below), and enriches centrosomal tubulin concentrations tenfold^{25,26}. These findings present a possible model of how tubulin and nucleation factors become enriched at the centrosome in the absence of a membrane, and thus may turn the centrosome into a potent microtubule organizing centre capable of facilitating spindle assembly in centrosome-containing cells.

Chromosome-mediated nucleation

Spindles in most plant cells, *Xenopus* egg extract and many other animal oocytes form in the absence of centrosomes^{39–42}. Even in many centrosome-containing cells, centrosomes are dispensable for spindle assembly^{43–45}. The ability of spindles to assemble without centrosomes is largely due to several other microtubule nucleation pathways that originate from chromosomes, many of which are regulated by the Ran pathway.

Ran-driven spindle assembly. Ran is a small GTPase that is converted to its active RanGTP form by the chromatin-associated factor RCC1 (ref. ⁴⁶). In interphase, RanGTP is concentrated in the nucleus and functions to release nuclear cargo proteins from the transport complex importin- α/β ⁴⁷, whose binding inhibits the function of many nuclear cargo proteins^{48–51}. Many of these proteins regulate microtubule nucleation, organization and dynamics during cell division, and are termed spindle assembly factors (SAFs)^{48–51}. After nuclear envelope breakdown, RanGTP diffuses into the surrounding cytoplasm, where GTP is hydrolysed to form inactive RanGDP, creating a chromatin-centred gradient of RanGTP (Fig. 2). This allows the release of SAFs from importin- α/β in a corresponding gradient^{52,53}. This active SAF gradient facilitates spindle assembly in many systems⁵³, although the mechanistic contributions of many individual SAFs to spindle assembly remain undetermined. Inhibition of the RanGTP pathway leads to severe spindle assembly defects in *Xenopus* egg extract and mild defects in mammalian cells^{50,53}, which may indicate that chromosome-driven and centrosome-driven systems differentially utilize the Ran pathway during spindle assembly. Below, we describe the possible role of RanGTP in microtubule-mediated and kinetochore-mediated nucleation.

Microtubule-mediated nucleation. Assembly of the spindle requires the formation of dense and uniformly polar microtubule arrays. The use of existing microtubules to form new microtubules with conserved microtubule polarity provides an elegant means of accelerating this process and has been observed in plants, *Drosophila*, *Xenopus* egg extract and human cells^{54–60}. This process, also termed branching microtubule nucleation, nucleates microtubules at shallow angles along the surface of pre-existing microtubules, resulting in exponential microtubule self-amplification⁵⁸. In vitro reconstitution of this process has been achieved using *Xenopus*, *Drosophila* and human proteins. In all cases, the minimal branching components consist of γ -TuRC and the octameric protein complex augmin^{61–63}. The augmin complex is capable of both microtubule binding and γ -TuRC binding, allowing it to localize γ -TuRC to microtubules^{64,65} (Fig. 2c). Depletion of this universal branching factor leads to defects in k-fibre formation in *Drosophila* S2 and mammalian cells, and greatly reduces spindle microtubule mass in all spindles studied to date^{54,56,60,66–68}. Thus, the functions of this pathway

are conserved despite species-specific differences in its contribution to spindle assembly.

Additional factors are required in some species to drive efficient branching nucleation. In experiments based on *Xenopus* egg extract and in vitro assays using *Xenopus* proteins⁶³ (but not in *Drosophila* S2 cells⁶⁹), the branching pathway was found to require the SAF TPX2, which itself is regulated by the Ran pathway⁵⁸. Although this process does not appear to be necessary for branching activity using human proteins in vitro⁶², the contributions of TPX2 and the Ran pathway in branching microtubule nucleation in human cells require further study.

In *Xenopus* egg extract, a branch site is initiated by the localization of RanGTP-released TPX2 to the microtubule lattice^{58,70}. In vitro, this occurs by TPX2 condensates forming regularly spaced droplets on microtubules. These droplets confine the space along microtubules in which branching and nucleation factors must search to find each other, thus increasing the speed of branching complex formation in comparison to a uniform TPX2 coating^{27,71}. In addition, TPX2 co-condenses with tubulin in vitro and thus may also supply the branch site with building material for more efficient nucleation²⁷. Apart from mediating branching, TPX2 may stabilize growing microtubules, as seen in vitro^{72,73}. However, further study is required to determine if any of these mechanisms are relevant to branching microtubule nucleation in vivo.

Besides TPX2, the microtubule-binding protein EML3 may facilitate binding of augmin to the microtubule lattice⁷⁴. Additionally, augmin binding to γ -TuRC may be further supported by interaction with NEDD1 (ref. ⁵⁶). Altogether, this nucleation pathway promotes spindle self-organization by increasing the number of spindle microtubules while preserving their orientation^{68,75}, which may enable rapid kinetochore capture.

Kinetochore-mediated nucleation. How spindle microtubules find kinetochores within large eukaryotic cells during the limited timescale of mitosis has been a major question of study. In principle, a solution is for kinetochores to nucleate their own microtubules. Initially proposed to be a safeguard for timely kinetochore attachment to the spindle in case other pathways fail^{43,76,77}, recent studies suggest that microtubule nucleation from kinetochores begins at early prometaphase and is critical for timely kinetochore biorientation^{77–79}. In HeLa cells and *Xenopus* egg extract, the nucleoporin (Nup) proteins Nup107–160 can localize γ -TuRC to unattached kinetochores via the Nup-interactor ELYS^{80–82}. In *Xenopus* egg extract, microtubules nucleate from this complex in a RanGTP-dependent manner⁸¹ (Fig. 2d). Whereas the Nup107–160 complex is required for bipolar spindle assembly in *Xenopus* egg extract⁸⁰, it is not necessary in HeLa cells⁸³. Therefore, the relevance of this mechanism may be species dependent. Interestingly, the formation of kinetochore-associated microtubules requires TPX2 in mammalian cells⁸⁴. This suggests that branching microtubule nucleation or TPX2-mediated microtubule stabilization at kinetochores may contribute to the formation of these kinetochore-associated microtubules.

Although microtubule nucleation from kinetochores would, in principle, result in incorrectly oriented microtubules with minus ends oriented towards kinetochores, electron microscopy reveals that these microtubules have the correct orientation with plus ends pointing to kinetochores⁸⁵. The proper orientation could possibly be achieved if the kinetochore rapidly released newly nucleated microtubules, interacted with microtubule walls via the motor CENP-E and the depolymerase MCAK (also known as KIF2C), and subsequently recaptured plus ends^{78,86}. However, a recent study visualizing the minus-end-localized protein ASPM during kinetochore-associated

microtubule regrowth in *Drosophila* S2 cells argues against polarity inversion of kinetochore-mediated microtubules and favours a model in which kinetochores bind short microtubules nucleated in close proximity to the kinetochore by other mechanisms such as chromosome-mediated nucleation pathways⁸⁷. Thus, visualization methods with better spatial and temporal resolution are required to establish exactly how microtubules form at kinetochores and how prevalent this mechanism is in comparison to other mechanisms active near chromosomes such as nucleation mediated by the chromosomal passenger complex (CPC).

CPC-mediated nucleation. In the absence of a RanGTP gradient, *Xenopus* egg extract spindles still form around sperm chromatin, whereas no microtubules form around chromatin beads, which lack both kinetochores and centromeres⁸⁸. This implies the existence of another spatially regulated pathway at either kinetochores or centromeres that acts complementary to and independent of RanGTP-dependent pathways. This pathway, which is able to compensate for loss of RanGTP-dependent microtubule formation, is mediated by the CPC⁸⁸. In mammalian cells, this mechanism is required for kinetochore-associated microtubule formation⁸⁴, reflecting a conserved role in generating spindle microtubules. The CPC consists of four proteins localized at the inner centromere: an inner centromere protein (INCENP) scaffold, Aurora B kinase, and the non-enzymatic subunits Borealin and Survivin (also known as BIRC5)⁸⁹. Aurora B activity, promoted by CPC formation, phosphorylates and inactivates microtubule-destabilizing proteins such as MCAK and stathmin (Fig. 2e). This effectively promotes microtubule nucleation near kinetochores to facilitate kinetochore capture^{90–92}. Passive diffusion of these phosphorylated Aurora B substrates, such as stathmin, away from the centromere creates a gradient that stabilizes spindle microtubules globally^{93,94}, including microtubules nucleated by other pathways. Interestingly, the CPC appears to form a biomolecular condensate that can concentrate tubulin and directly nucleate microtubules *in vitro*⁹⁵. *In vivo*, the ability of the CPC to form a condensate was proposed to be correlated to its localization to the centromere⁹⁵. However, the formation of these condensates and their functional significance require validation⁹⁶.

Regulation and synergy of nucleation pathways

How are these microtubule nucleation pathways coordinated in space and time to give rise to a unified spindle? Centrosomes and chromosomes both begin to nucleate microtubules within a tightly controlled time window, between 9 and 13 minutes following nuclear envelope breakdown in *Drosophila* spermatocytes⁹⁷. In larger spindles (such as those in human cells and *Xenopus* egg extract), branching microtubule nucleation generates the majority of spindle microtubules^{55,56,67}. In human cells, the dominance of the nucleation pathways undergoes a temporal shift, whereby the rate of centrosomal microtubule nucleation is initially higher (directly following nuclear envelope breakdown) than branching nucleation, which becomes the dominant pathway later⁶⁰. A clear hierarchy of microtubule nucleation also exists in acentrosomal spindles where acentrosomal poles need to be established before acting as a nucleation centre; thus, microtubule nucleation from poles is delayed compared to chromosomal microtubule formation^{67,98}. However, exactly when and where individual chromosome-mediated pathways are activated remains to be defined.

The presence of multiple nucleation pathways may safeguard spindle assembly by providing redundancy. For example, lack of centrosomal nucleation can be compensated for by Ran-mediated

microtubule formation and vice versa^{43,59}. Similarly, branching microtubule nucleation can be partially compensated for by the centrosome-mediated pathway in *Drosophila*⁵⁴, and the CPC-mediated and Ran-mediated microtubule nucleation pathways can compensate for each other^{88,99}. Despite these redundancies, nucleation pathways largely act in parallel and may work synergistically to speed up spindle assembly^{53,59}. Interestingly, initial microtubule formation during meiosis I occurs at the nuclear envelope during breakdown, suggesting an additional Ran-independent and centriole-independent mechanism that contributes microtubules during spindle assembly^{100,101}.

While microtubule nucleation pathways are regulated in space and time, microtubule nucleators and their activation factors are also transported throughout the spindle. For example, TPX2, although released in a RanGTP-dependent manner surrounding chromatin, is actively and passively transported to poles by motor proteins and poleward flux, respectively¹⁰², and thus may distribute branching microtubule nucleation further away from chromosomes. Similarly, γ -TuRC itself is, after binding the spindle, also transported to poles by motor proteins^{103,104}, where it can continue nucleating microtubules. Thus, microtubule nucleation gives rise to spindle architecture and transport of nucleating factors reinforces this architecture by guiding where microtubule-based nucleation takes place.

Structural modules of the spindle

The complex task of chromosome segregation requires that, once nucleated, spindle microtubules are organized into structural modules that fulfil distinct roles such as generating force at kinetochores (k-fibres), providing structural stability (bridging fibres and other non-kinetochore microtubules) and positioning the spindle (astral microtubules) (Fig. 3). Microtubules must also be assembled into a bipolar structure with two focused poles to guide chromosome segregation into two daughter cells.

This modular organization is mediated by a plethora of motor and non-motor microtubule-associated proteins (MAPs), which bundle, slide and focus microtubules. Mitotic motors include cytoplasmic dynein and members of several kinesin families (see table 1 in ref. ¹⁰⁵). Many of these motors bind to microtubules through both a motor domain and a second microtubule-binding domain, allowing them to bridge two microtubules and thereby crosslink and slide them in an ATP-dependent manner. Non-motor MAPs include passive microtubule crosslinkers, which consume no energy but function in an ATP-independent fashion to regulate bundling, elasticity and friction within the spindle¹⁰⁶. Microtubule polymerases and depolymerases also localize to specific structural modules, fine-tuning the dynamics of these modules as they arise during spindle assembly.

Some structural modules have been reconstituted *in vitro*, including microtubule asters that resemble spindle poles^{107–110} and anaphase midzone-like antiparallel microtubule bundles^{111–113}. However, other modules, such as k-fibres and bridging fibres, have not yet been reconstituted outside the cell, limiting our understanding of how they form. In the following sub-sections, we discuss recent work, both *in vitro* and *in cells*, that has improved our understanding of the ultrastructure, regulation and function of the structural modules that together comprise the spindle and of their variation between species.

K-fibres

Microtubules with their plus ends embedded in kinetochores and their minus ends at or pointing towards spindle poles are known as kinetochore microtubules (KMTs). KMTs attached to the same kinetochore

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interact with each other and with non-KMTs in a parallel fashion to form k-fibres¹¹⁴. K-fibres generate pulling forces to move chromosomes, and their attachment to kinetochores satisfies the spindle assembly checkpoint¹¹⁵, allowing anaphase entry. Much of what we know about k-fibres comes from mammalian cells, where k-fibres are clearly identifiable by light and electron microscopy. The number of KMTs in a k-fibre varies between kinetochores within a cell and between species. For example, k-fibres vary from 4–40 KMTs in human RPE1 and HeLa cells^{116–118} to 25–60 KMTs in Indian muntjac skin fibroblasts¹¹⁹. KMTs are tightly bundled near kinetochores and gradually splay out to interact

with other KMTs and non-KMTs towards poles^{117,118}. Individual KMTs contact spindle poles either directly or indirectly through interactions with other pole-bound microtubules^{114,117,118}.

How plus end polymerization and depolymerization of KMTs are coordinated within a k-fibre bundle to move chromosomes remains an open question. The coordinated regulation of k-fibre plus end and minus end dynamics is also poorly understood, and factors that accumulate on KMTs in a length-dependent manner may have important roles in this process. For example, the processive motor KIF18A accumulates more strongly at the plus ends of longer KMTs due to the

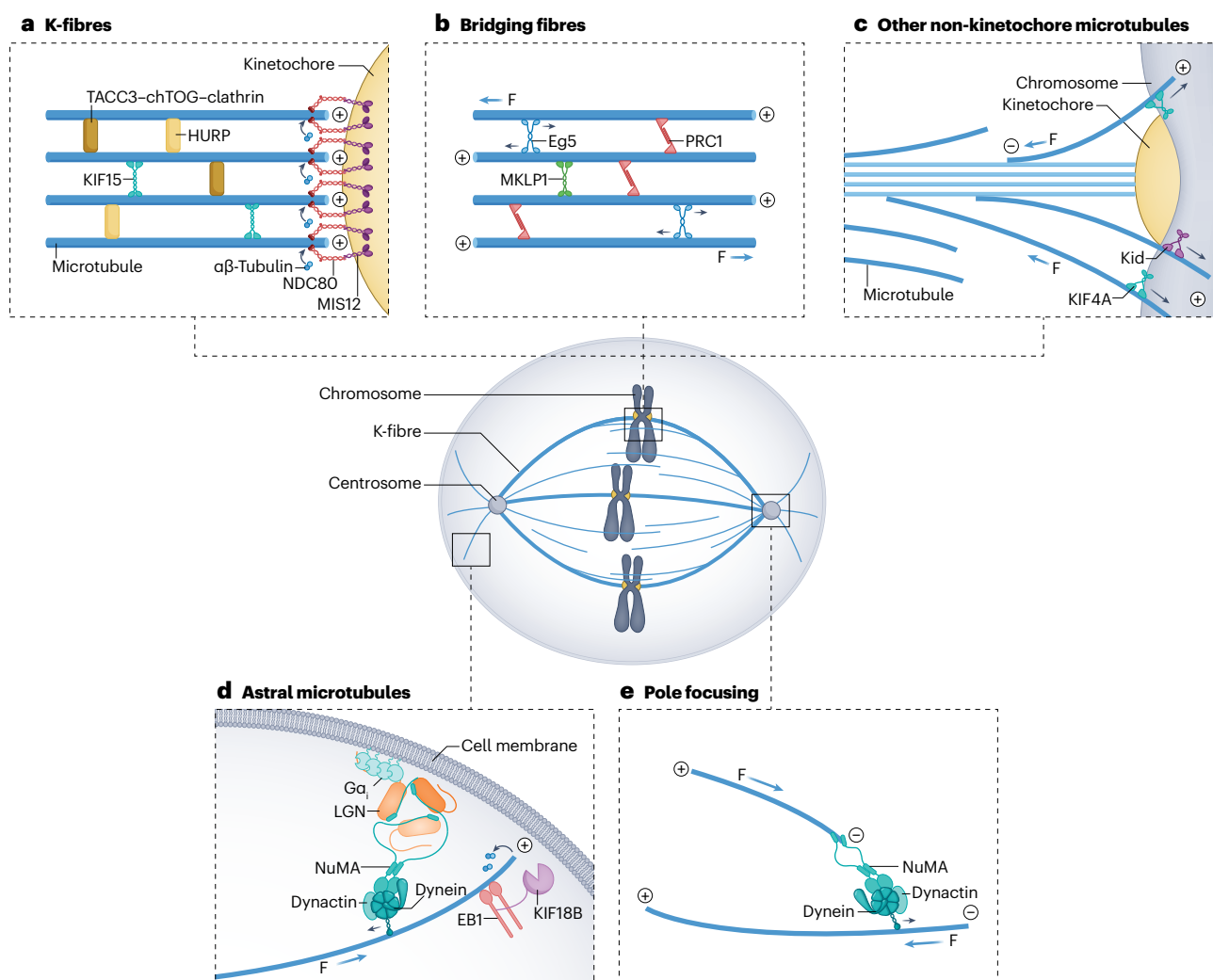
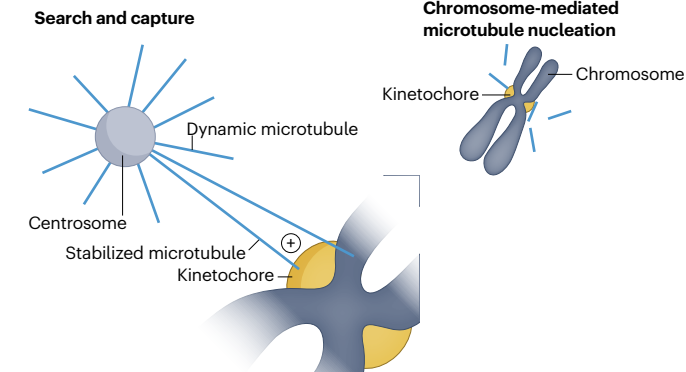


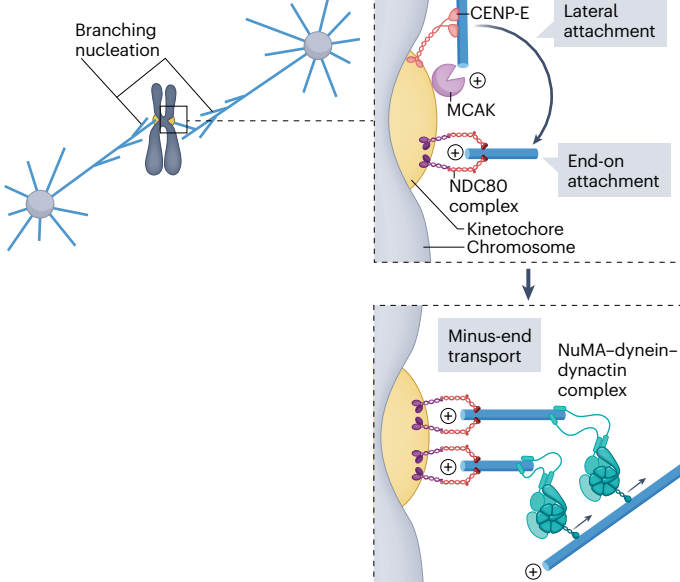
Fig. 3 | Structural modules in the spindle. Microtubule-associated proteins, including motors, crosslinkers and regulators of microtubule dynamics, organize microtubules into distinct structural modules. **a**, Kinetochore fibres (k-fibres) are bundles of parallel microtubules with their plus ends bound to the outer kinetochore protein NDC80. K-fibres are crosslinked by microtubule-associated proteins, including the kinesin KIF15, the transforming acidic coiled-coil-containing protein 3 (TACC3)–chTOG–clathrin complex, and HURP. **b**, Bridging fibres are bundles of antiparallel microtubules that maintain tension on k-fibres and align chromosomes. PRC1 and the kinesin-5 Eg5 bind and crosslink antiparallel microtubules, and PRC1 recruits the plus-end-directed motor MKLP1 to bridging fibres. **c**, Other non-kinetochore microtubules play

important mechanical roles in stabilizing the spindle, anchoring k-fibres, and mediating polar ejection forces via the chromokinesins Kid (KIF22) and KIF4A. **d**, Astral microtubules extend from spindle poles towards the cell cortex, where they interact with the G_{α_i} –LGN–NuMA complex to position the spindle. Their lengths and numbers are regulated by the depolymerases MCAK and KIF18B, that are recruited to plus ends by plus-end-tracking protein EB1. **e**, Pole focusing is mediated by motors such as dynein that, together with its regulators (NuMA, dynactin), dwells at minus ends and carries these minus end cargos towards neighbouring minus ends. Black arrows indicate directions of motor stepping; blue arrows labelled ‘F’ indicate direction of force on microtubules.

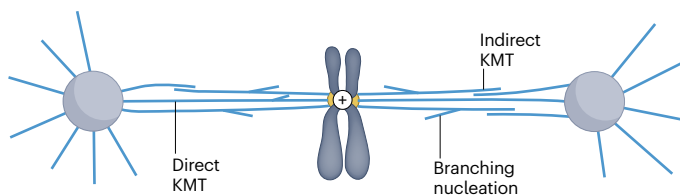
a K-fibre formation



b Nascent k-fibre



c Mature k-fibre



higher number of motors that can bind (the 'antenna model' of length regulation)^{120,121}. Upon arriving at plus ends, KIF18A suppresses microtubule dynamics^{122,123}, serving as a feedback mechanism to maintain KMT length and promote chromosome alignment. In contrast, the microtubule stabilizer HURP (also known as DAP5) accumulates near the plus ends of k-fibres in a manner inversely proportional to their length through a mechanism that remains unclear¹²⁴.

KMTs are thought to be the longest-lived microtubules in the spindle, with a half-life of ~7 min (10–20% of mitosis) in mammalian PtK1 cells¹²⁵. Their stability stems from the tension they are under following plus end capture by the kinetochore-localized NDC80 complex¹²⁶, and can be regulated by the phosphorylation state of NDC80 (ref. 127). The long lifetime of KMTs has both mechanical and biochemical

Fig. 4 | K-fibre formation mechanisms. **a**, Kinetochore fibre (k-fibre) formation can occur through two pathways, search-and-capture and chromosome-mediated assembly. In search-and-capture, dynamic centrosome-nucleated microtubules 'search' the surrounding cytoplasm for kinetochores as they grow and become stabilized upon kinetochore attachment. In chromosome-mediated assembly, microtubules nucleate via chromosome-mediated and kinetochore-mediated pathways described in Fig. 2. **b**, A nascent k-fibre typically begins to form when a kinetochore binds to the lateral surface of a microtubule. This laterally bound microtubule undergoes reorientation driven by the plus-end-directed kinesin CENP-E and the microtubule depolymerase MCAK, resulting in stable attachment of microtubule plus ends to the outer kinetochore NDC80 complex. The minus ends of these kinetochore-bound microtubules (KMTs) become bound by the minus-end-binding motor complex NuMA–dynein–dynactin. This minus-end-directed motor complex transports KMTs towards spindle poles (arrows). These nascent k-fibres additionally undergo branching microtubule nucleation, which serves to quickly amplify KMT numbers. **c**, The mature k-fibre forms both direct and indirect connections with the spindle poles, whereby KMTs of a single k-fibre that are bundled together near the kinetochore gradually splay out towards their minus ends to interact with other microtubules. Ongoing microtubule nucleation, mainly through branching nucleation, maintains the density of the k-fibre.

implications. Mechanically, k-fibres can propagate and respond to forces on longer timescales than other microtubule populations in the spindle. Biochemically, KMTs can recruit distinct, slower-binding factors compared to other microtubules¹²³. For accurate chromosome segregation, KMTs must be stable enough to preserve dynamic attachments between chromosomes and the spindle, and yet dynamic enough to allow for error correction when attachments are incorrect¹²⁸. Many microtubule crosslinking proteins, such as the TACC3–chTOG–clathrin complex¹¹⁶, HURP^{129,130} and KIF15 (ref. 131), specifically localize to k-fibres (Fig. 3a), possibly due to a preference for binding bundled parallel microtubules. These proteins are thought to reinforce the bundling of KMTs and maintain k-fibre integrity¹³² and, upon physical manipulation with a microneedle (Box 1), k-fibres appear to behave as a single entity¹³³.

How k-fibres form varies between species and remains an area of active research. In centrosome-containing cells, the search-and-capture model for k-fibre formation posits that dynamic centrosome-originated pioneer microtubules search the cytoplasm until they eventually capture kinetochores and become stabilized¹³⁴ (Fig. 4a). This process could indeed be recapitulated via computational modelling¹³⁵ considering: (1) RanGTP-dependent microtubule stabilization¹³⁶; (2) pre-alignment of kinetochores towards poles through initial lateral microtubule–kinetochore interactions¹³⁷; and (3) the expansion of the outer kinetochore during early mitosis^{138,139}, which increases the surface area of kinetochores and their probability of capture^{135,139}.

In addition to this search-and-capture mechanism, and in acentrosomal systems, k-fibres can originate through chromosome-mediated microtubule nucleation (Fig. 4a), followed by efficient lateral binding of these microtubules by kinetochores^{78,137,140,141} and subsequent conversion of kinetochore-bound microtubules to end-on attachments^{78,86} (Fig. 4b). The microtubule-binding protein NuMA can then bind the minus ends of KMTs, recruiting dynein and dynactin¹⁴². This NuMA–dynein–dynactin complex walks these minus ends as cargo towards the minus ends of nearby microtubules and thereby towards spindle poles^{76–78,84,143,144} (Fig. 4b). Upon formation of an initial KMT, amplification mechanisms ensure the establishment of k-fibres rather than relying on capture of additional KMTs (Fig. 4b). Branching microtubule

nucleation biases microtubule amplification of the k-fibre towards kinetochores^{60,68}. In vitro, TPX2 accumulates on more stable, long-lived microtubule lattice regions⁷⁰, which could provide a mechanism to target branching microtubule nucleation to k-fibres. Altogether, the cooperation of multiple mechanisms ensures the robust and efficient formation of mature k-fibres (Fig. 4c). After formation, k-fibres are stabilized in part by bridging fibres, another structural module discussed in the next sub-section.

Bridging fibres

Many non-KMTs that cross the metaphase plate are crosslinked in antiparallel bundles known as bridging fibres^{118,145,146} (Fig. 3b), a structural module with important mechanical functions in the mammalian spindle. Near chromosomes, these bundles form one-to-one associations with pairs of sister k-fibres, that is, k-fibres that form on each of two sister chromatids¹⁴⁷. Electron tomography has revealed that like k-fibres, bridging fibres splay apart closer to poles and their microtubules intercalate with multiple k-fibres¹¹⁸. Their minus ends form junctions with KMTs along the length of the k-fibre, although the identities of the factors mediating these junctions remain unknown. Some of these junctions are established as close as 2 µm from kinetochores in RPE1 cells¹¹⁸, suggesting that k-fibre anchorage within the spindle microtubule network differs outside this region. The number of microtubules within bridging fibres varies between cell types (6 ± 1 in PtK1 versus 14 ± 2 in HeLa cells, ~25% and ~82% of the average number of microtubules in k-fibres, respectively)¹⁴⁶. The higher number of bridging fibre microtubules in HeLa cells may reflect a more important mechanical role for bridging fibres in more curved spindles.

The crosslinker PRC1, which is specifically enriched on antiparallel microtubules^{111,148–150}, has a central role in maintaining and organizing bridging fibres^{146,147,151}. Optogenetic removal of PRC1 results in a ~2.5-fold reduction of bridging fibre microtubule density and reduces the levels of other molecular regulators localized to bridging fibres, including the plus-end-directed motors KIF4A and MKLP1 (also known as KIF23)¹⁵¹. Reduced levels of these factors after PRC1 removal have been proposed to result in increased antiparallel overlap, reduced outward sliding of the bridging fibre and disrupted chromosome alignment¹⁵¹.

Bridging fibres play an important mechanical role, acting to support k-fibre tension and curvature, which are required to maintain correct kinetochore–microtubule attachments and chromosome alignment. Optogenetic removal of PRC1 causes spindles to become more diamond shaped¹⁵¹. Laser severing of k-fibres in HeLa cells close to the kinetochore (and thus disconnecting the remaining k-fibre stub from the bridging fibre) has been shown to result in greater relaxation between sister kinetochores, further corroborating the role of bridging fibres in maintaining tension between kinetochores¹⁴⁶. Bridging fibres may also accelerate chromosome biorientation by providing antiparallel microtubules in the middle of the spindle upon which dynein can transport nascent KMTs towards each pole⁷⁹. Together, ultrastructural, optogenetic and biophysical approaches (Box 1) have revealed that the bridging fibre is a key structural module that aids in the assembly and mechanical stabilization of k-fibres.

Other non-KMTs

Much of the microtubule density of spindles is composed of non-KMTs that are not bundled into bridging fibres¹⁴⁵. This microtubule population plays important roles in stabilizing spindle structure. The proportion of non-KMTs in the spindle ranges from ~50% of total microtubules in PtK1 cells¹⁵² to ~95% in *Xenopus laevis* extract spindles¹⁵³

and ~86% in HeLa cells¹¹⁷. These non-KMTs are shorter on average than KMTs^{117,154,155} and arise mainly via branching microtubule nucleation^{55,60}. Non-KMTs undergo rapid turnover (polymerizing and depolymerizing within seconds)¹⁵⁶, which may allow spindles to quickly remodel as chromosomes move during mitosis.

During spindle assembly, non-KMTs aid in chromosome alignment via interactions with the chromokinesins Kid (also known as KIF22) and KIF4A. These plus-end-directed motors bind chromatin and generate polar ejection forces by walking along non-KMTs, thereby pushing chromosomes away from poles and towards the spindle equator^{157–159} (Fig. 3c). Non-KMTs are densest near poles owing to their continuous poleward transport¹⁰⁴, generating higher polar ejection forces there and thus contributing to a self-organized chromosome-centring mechanism^{160–162}.

Non-KMTs also interact with k-fibres and bridging fibres^{114,117,118}, mechanically linking the various structural components of the spindle. As discussed below, these interactions are important to many emergent properties of the spindle such as k-fibre load bearing, poleward flux and elasticity. In sum, the dense network of non-KMTs helps align chromosomes and structurally integrate the spindle, making it robust against mechanical force.

Astral microtubules

Astral microtubules are dynamic, non-bundled microtubules that are nucleated at the centrosome and extend towards the cell cortex, with important roles in positioning the spindle (Fig. 3d). Their numbers and length are largely controlled by EB1 (also known as MAPRE1), a MAP that binds growing microtubule plus ends. EB1 recruits the depolymerases KIF18B and MCAK to mediate continuous astral microtubule pruning^{163,164}, required to maintain spindle length as shown by optogenetic disruption of EB1 (ref. 165). Astral microtubules are molecularly distinguished by relatively low levels of tubulin detyrosination, whereas microtubules pointing towards the metaphase plate are detyrosinated. Tyrosinated tubulin inhibits the motility of the kinetochore motor CENP-E, biasing chromosome congression towards the metaphase plate and preventing chromosome accumulation near the cell cortex¹⁶⁶.

The role of astral microtubules in positioning the spindle has been summarized in recent reviews^{167,168}. Correctly positioning the spindle is important in both symmetric cell divisions, where astral microtubules enable the spindle to respond to cell shape cues^{169–171}, and in asymmetric divisions, where daughter cells take on different fates. Throughout mitosis, astral microtubules are linked to the cell cortex via the evolutionarily conserved ternary complex of Gα_i–LGN–NuMA^{172–174}. This complex recruits dynein and dynactin to generate pulling forces that translate the spindle. Optogenetics (Box 1) has been valuable in studying spindle positioning, revealing that cortical recruitment of NuMA but not dynein itself is sufficient to orient the spindle in *C. elegans* and human HCT116 cells^{175,176}. Although astral microtubules are not required to assemble a bipolar spindle, they fulfil a critical function in linking the spindle to cues from its tissue environment.

Spindle poles

Focused spindle poles incorporate microtubules belonging to several of the classes described above (KMTs, astral microtubules and other non-KMTs), and we here consider them as an independent architectural module that serves as a regulatory hub for microtubule nucleation and dynamics. Although spindle poles contain centrosomes in most cells, the formation of focused poles in acentrosomal oocytes^{39–41} and somatic cells after centrosome ablation^{43,45,177–180} has established that

centrosomes are not necessary for the formation of focused spindle poles. Instead, pole focusing is driven by minus-end-directed motors that are able to dwell at microtubule minus ends and transport these minus end cargos along neighbouring microtubules – the same mechanism that forms microtubule asters in vitro¹⁰⁹.

Dynein and its cofactors dynactin and NuMA are key players in pole focusing (Fig. 3e), and their inhibition leads to unfocused barrel-shaped or turbulent spindles^{39,107,108,181–184}. NuMA localizes to minus ends, where it recruits dynactin and dynein to walk along neighbouring microtubules, facilitating the end-dwelling behaviour¹⁴². The large and multifunctional NuMA likely contributes to pole focusing in many ways: in addition to localizing dynein, it is a putative activating adaptor of dynein motility^{176,185} and may have auxiliary roles in organizing microtubules via passive crosslinking¹⁸⁶ and/or phase separating at spindle poles^{187,188}. The kinesin-14 family of motors, including *Xenopus* XCTK2, *Drosophila* Ncd and human HSET (also known as KIFC1), also contributes to pole focusing, although the exact mechanisms by which these motors organize spindle poles remain unclear. The relative importance of dynein and kinesin-14 varies between species, with Ncd playing a dominant role in *Drosophila*¹⁸², whereas HSET depletion has only mild phenotypes in human mitotic spindles^{189,190}.

Functionally, poles and the motors that focus them are important in concentrating minus end regulatory factors like γ -tubulin. As γ -tubulin accumulates during pole maturation, it enhances microtubule nucleation and stabilizes the focused pole, even in acentrosomal spindles that initially lack nucleation at poles^{67,104}. Pole focusing also functions to maintain a steady-state spindle shape, thereby ensuring that growing and shrinking microtubules are confined to the spindle region without interacting with organelles or generating cytoplasmic flows¹⁸³. Interestingly, many plant spindles lack focused poles, suggesting that other geometric cues like the plant cell preprophase band (a ring of microtubules below the plasma membrane that marks the future division plane) can facilitate bipolar divisions while circumventing the need for pole focusing⁴².

Spindle bipolarity

The assembly of a spindle with two poles is critical to segregate chromosomes into two daughter cells. Monopolar, aster-shaped spindles are often the result of failed spindle assembly, and multipolar spindles are associated with genomic instability and cancer¹⁹¹. The bipolar homotetrameric kinesin-5 motors (Eg5 (also known as KIF11) in humans) play a central role in establishing spindle bipolarity in both acentrosomal and centrosome-containing spindles^{192,193} due to their ability to crosslink and slide antiparallel microtubules apart^{194,195}.

In prophase, spindle bipolarity is templated by the sliding apart of duplicated centrosomes to opposite sides of the nucleus. This movement is driven by the pulling forces of dynein attached to the nuclear envelope¹⁹⁶ and by Eg5-mediated antiparallel sliding of centrosomal microtubules. After nuclear envelope breakdown, microtubules from opposite centrosomes form additional antiparallel overlaps on which Eg5 can localize. Nascent k-fibres also promote bipolarity, potentially due to an outward pushing force on centrosomes from k-fibre polymerization at kinetochores^{197,198}. In metaphase, spindles in many mammalian cell types remain bipolar even if Eg5 is inhibited due to the partially redundant activity of the kinesin-12 KIF15 (refs. 199–202), which allows maintenance though not initial generation of spindle bipolarity.

Branching microtubule nucleation also contributes to spindle bipolarity. Because new microtubules are formed at shallow branch angles from pre-existing microtubules⁵⁸, this pathway both maintains

microtubule polarity and generates closely juxtaposed arrays to which motors and crosslinkers can rapidly bind. Indeed, augmin depletion leads to multipolar spindles in both acentrosomal¹⁶⁷ and centrosome-containing⁵⁵ spindles. Thus, the cooperation of multiple motor-based and nucleation pathways between prophase and metaphase promotes the robust assembly of a bipolar spindle, setting the stage for chromosome biorientation and equal segregation into two daughter cells.

Emergent properties of the spindle

The spindle exhibits many cellular-scale emergent properties that are qualitatively different from those of its individual components and enable the spindle to segregate chromosomes accurately. These include dynamic features such as microtubule flux and continuous self-repair, mechanical features such as spatially varying viscoelasticity, and architectural features such as helical twist and the scaling of spindle size with cell size. While the full complexity of the emergent properties of the spindle cannot yet be reconstituted in vitro, recent studies have begun to elucidate the mechanistic basis for some of these features.

Robustness

The spindle is subject to mechanical and biochemical noise such as variations in gene expression and forces from neighbouring cells. However, even a single segregation error can give rise to disease²⁰³. Several failsafe mechanisms have been identified that allow the spindle to maintain its architecture and accurate function in the face of such variations.

Redundancy. Redundancy exists in many spindle processes, including microtubule nucleation and motor activity. As discussed above, the spindle robustly generates the microtubule mass required for bipolar assembly. The Ran pathway can compensate for the absence of centrosomes^{43,59} and vice versa⁵³. Several motors with partially redundant function also cooperate to promote correct spindle architecture (Fig. 5a). KIF15 maintains bipolarity when Eg5 is inhibited in metaphase^{199,200} and, when overexpressed, can fully compensate for loss of Eg5 in bipolar spindle assembly^{198,199}. Further, kinesin-14 motors focus poles in a manner partially redundant with dynein, and cells can tolerate loss of the human kinesin-14 HSET during mitosis^{189,190} unless dynein is simultaneously inhibited^{204,205}.

Motor redundancy has important implications for the therapeutic targeting of cell division such as in anticancer therapy. For example, KIF15 is essential for cells to acquire resistance to Eg5 inhibitors²⁰⁶. An interesting case of redundancy involves the opposing motors Eg5 and dynein. Inhibiting either motor alone leads to monopolar or unfocused spindles, respectively, but inhibiting both together results in bipolar spindles of normal size as the redundant motors discussed above can compensate for their functions^{205,207–209}. However, these Eg5-inhibited and dynein-inhibited spindles are more mechanically fragile and more error-prone in chromosome segregation²⁰⁵, potentially as a consequence of locally altered microtubule organization^{205,210}. This indicates that Eg5 and dynein make the spindle more robust to perturbations although their combined inhibition does not disrupt overall spindle architecture.

Local k-fibre remodelling to preserve global architecture. Specialized mechanisms reinforce and repair key spindle connections at both the plus and minus ends of k-fibres. The NuMA–dynein–dynactin complex acts at minus ends to anchor k-fibres in the spindle. Not only does this complex transport KMT minus ends poleward during k-fibre

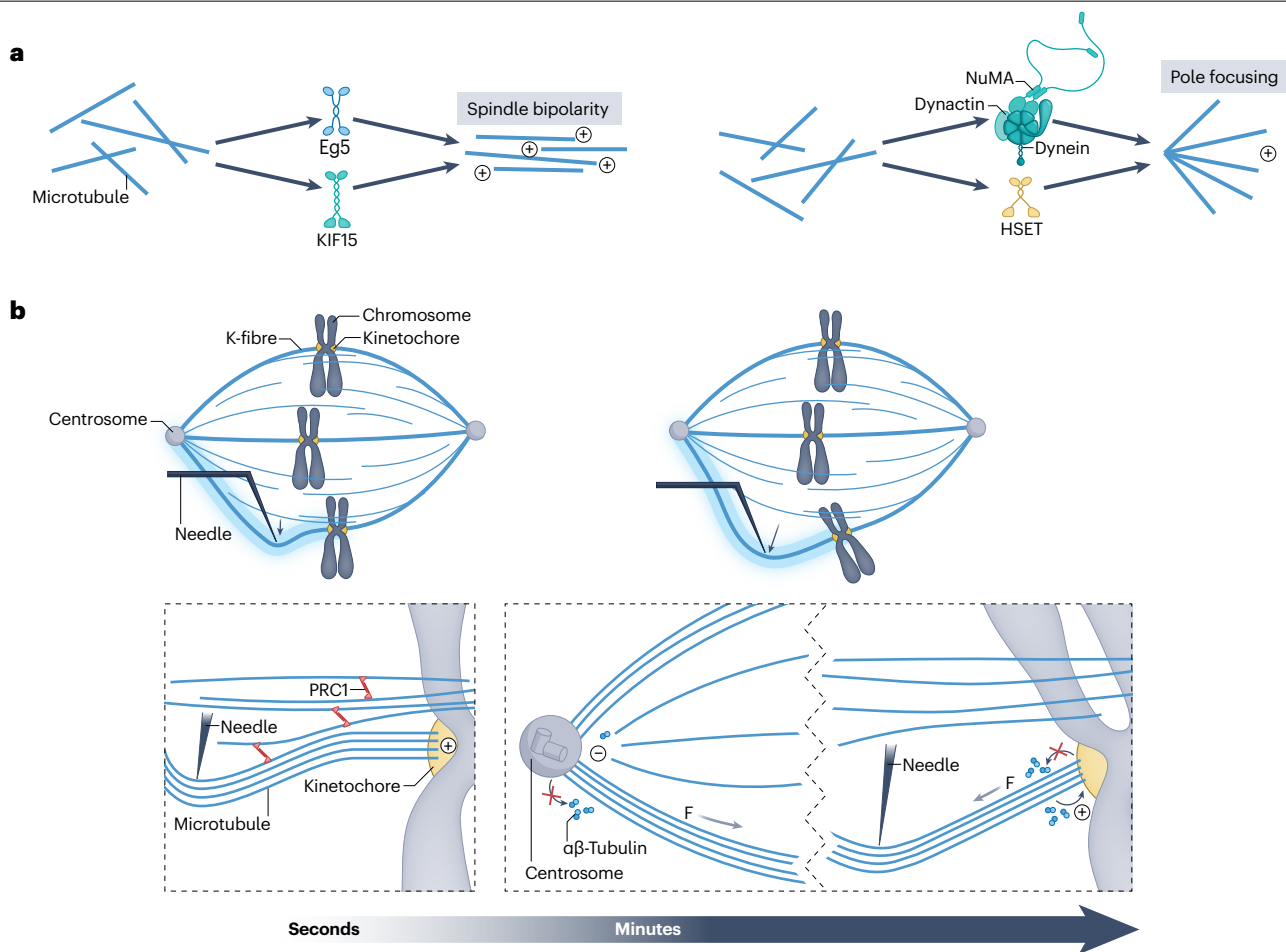


Fig. 5 | Mechanisms underlying spindle robustness: redundancy, anchorage and dynamics. **a**, Partial redundancy between motor proteins makes spindle assembly robust to perturbations and variations in motor activity. KIF15 can compensate for Eg5 inhibition to maintain spindle bipolarity. Similarly, NuMA–dynein–dynactin complexes have dominant roles in pole focusing in human spindles but the pole-focusing activity of HSET can compensate for reduced dynein activity. **b**, Plus-end-specific and minus-end-specific mechanisms reinforce key connections between the kinetochores (k-fibre), pole, and kinetochores and these can be experimentally dissected

by microneedle manipulation. On short (<20 s) timescales, PRC1-mediated crosslinking maintains k-fibre orientation, preventing k-fibres from pivoting around kinetochores. On longer (minutes) timescales, this reinforcement near kinetochores relaxes, allowing k-fibres to pivot around kinetochores. Plus ends undergo persistent polymerization while depolymerization at minus ends ceases, allowing the k-fibre to lengthen in response to external manipulation. These responses allow the k-fibre to locally dissipate force (F), remodelling at the individual k-fibre level to preserve global spindle architecture.

formation, as discussed above, but it is also sufficient to re-establish k-fibre connections to the poles after acute perturbations such as laser severing^{143,144} (Box 1). Recent studies have also identified mechanisms that anchor the first few microns near k-fibre plus ends within the spindle microtubule network, protecting kinetochores–microtubule attachments from acute perturbation^{77,146,211,212}. PRC1 has been shown to provide short-lived reinforcement in this region^{146,212}, presumably by crosslinking KMTs with nearby antiparallel non-KMTs, thus preventing microtubule pivoting near kinetochores in response to external force supplied by a microneedle²¹² (Fig. 5b). NuMA has also been shown to support k-fibre anchorage near the plus end²¹¹, an effect that may reflect crosslinking between KMTs and the minus ends of short microtubules in the centre of the spindle. On the longer (approximately minutes) timescale, these reinforcements are relaxed²¹², allowing

spindle remodelling and chromosome movement (Fig. 5b). Together, these mechanisms ensure that k-fibres remain robustly attached at kinetochores and anchored at poles.

Tension regulates microtubule dynamics at both plus and minus ends, enhancing spindle robustness to mechanical perturbation. Tension on microtubules decreases the catastrophe rate and increases the rescue frequency of microtubules at purified yeast kinetochores, stabilizing kinetochores–microtubule attachments under force^{213,214}. In microneedle manipulation experiments in newt epithelial cells²¹⁵, grasshopper spermatocytes²¹⁶ and, more recently, mammalian PtK2 cells²¹⁷, individual k-fibres elongated under applied force, demonstrating that similar principles apply to bundled k-fibres composed of many microtubules. At minus ends, depolymerization is inhibited when tension is applied to k-fibres either globally via cell compression²¹⁸

Glossary

Barrelling-type instability

A compression-induced deformation that is symmetric about the axis of compression such as a compression that transforms a cylinder into a barrel shape.

Biomolecular condensates

Micro-scale compartments that lack surrounding membranes and are formed through weak multivalent interactions, concentrating proteins and nucleic acids.

Biorientation

The state of a chromosome when both sister kinetochores are attached to microtubules linked to opposite spindle poles.

Catastrophe

A parameter of microtubule dynamic instability that describes the stochastic switching of a microtubule into a rapidly shrinking, depolymerizing state.

Chromokinesins

Microtubule plus-end-directed motors that bind to chromosome arms via specific DNA-binding motifs.

CLASPs

TOG domain-containing proteins that recognize microtubule plus ends, where they suppress catastrophe and promote rescue.

Elasticity

Ability of a material, by storing energy, to return to its original shape after being deformed. The stiffness (elastic modulus) determines the magnitude of a deformation.

Emergent properties

Properties of an ensemble, produced by interactions between smaller components, that the smaller components in isolation do not exhibit.

Metaphase plate

The plane formed by chromosomes after alignment at metaphase, located at the spindle equator.

NDC80 complex

The core outer kinetochore protein complex that mediates direct binding to microtubules. The NDC80 complex consists of four subunits (Hec1/Ndc80, Nuf2, Spc24 and Spc25) and is present in multiple copies per kinetochore.

Nematic

The liquid crystal phase in which filaments of mixed polarity are roughly aligned in parallel.

Nucleoporin

(Nup). Broadly conserved family of proteins that comprise the nuclear pore complex, facilitating molecular transport between the cytoplasm and nucleus.

Pericentriolar material

(PCM). The dense, structured protein matrix, composed mainly of scaffold proteins and microtubule nucleation factors, that accumulates around centrioles to create the centrosome.

Polar ejection forces

Forces present during chromosome congression that push chromosome arms away from spindle poles.

Self-organization

A stable order arising from local interactions between energy-consuming parts.

Spindle assembly checkpoint

Signalling mechanism that monitors the attachment of kinetochores to spindle

microtubules and inhibits progression of the cell cycle from metaphase to anaphase until sufficient attachments are made.

Stathmin

A protein that regulates microtubule dynamics by sequestering tubulin dimers, which destabilizes microtubules and inhibits their growth.

Rescue

A parameter of microtubule dynamic instability that describes the stochastic switching of a microtubule into a growing, polymerizing state.

Rheology

The study of the deformation and flow of materials with both solid and fluid characteristics.

Viscosity

Internal friction resisting deformation of a material, stemming from rearrangement of its components. The viscous drag coefficient determines the timescale of a deformation.

or individually via a microneedle²¹⁷ (Fig. 5b). The molecular mechanisms underlying these responses remain unknown, and they present an exciting opportunity to identify how the spindle physically and biochemically remodels under force. Collectively, these mechanisms allow the spindle to accommodate local deformations while limiting force propagation in space and time, suggesting a strategy to preserve global architecture and connectivity.

Microtubule flux

Spindle microtubules continuously treadmill towards poles, undergoing net polymerization at plus ends and depolymerization at minus ends in a phenomenon known as poleward flux²¹⁹. Individual motor and non-motor MAPs with microtubule transport, polymerization or depolymerization activities must be spatially coordinated to allow spindle microtubules to maintain a steady-state length distribution while undergoing poleward flux. This is a complex process that has only recently been reconstituted for single microtubules, but not yet for bundles, *in vitro*²²⁰.

Eg5 was the first driver of flux identified in *Xenopus* egg extract²²¹, and Eg5-mediated outward sliding of antiparallel microtubules is also important for flux in *Drosophila*²²². However, Eg5 has a minor role in mammalian cells as flux is only reduced by around 25% in its

absence^{223,224}. Stronger effects on mammalian and *Drosophila* poleward flux were found by inhibiting the minus end destabilizing activity of the kinesin KIF2A (refs. 225–227) or kinetochore-localized CLASPs^{227–230}. A recent study in human U2OS spindles proposed a model where instead of being the primary drivers of flux, KIF2A and CLASPs regulate plus and minus end dynamics in response to a driving force generated by multiple mitotic kinesins acting in concert²²⁴. According to this model, flux is driven by Eg5 and KIF15 acting in coordination with CENP-E in prometaphase and KIF4A in metaphase^{157,224}. These antiparallel sliding and chromokinesin motors exert poleward forces on non-KMTs, which are only partially transmitted to k-fibres through crosslinkers such as NuMA and HSET^{224,227}, explaining why non-KMTs flux faster than k-fibres^{104,224,231}. Reliance on multiple motors would thus explain the difficulty in finding a principal driver of flux in mammalian cells.

Various functions have been proposed for flux, including maintaining and/or equalizing tension at kinetochores^{227,231}, correcting erroneous kinetochore–microtubule attachments^{225,227}, and regulating spindle length²²⁴. However, these functions remain controversial despite the high conservation of flux across all metazoan cell types studied to date. Multiple studies using complementary approaches have now shown that, in conditions where flux is reduced but spindle length is unchanged, chromosome segregation errors frequently occur

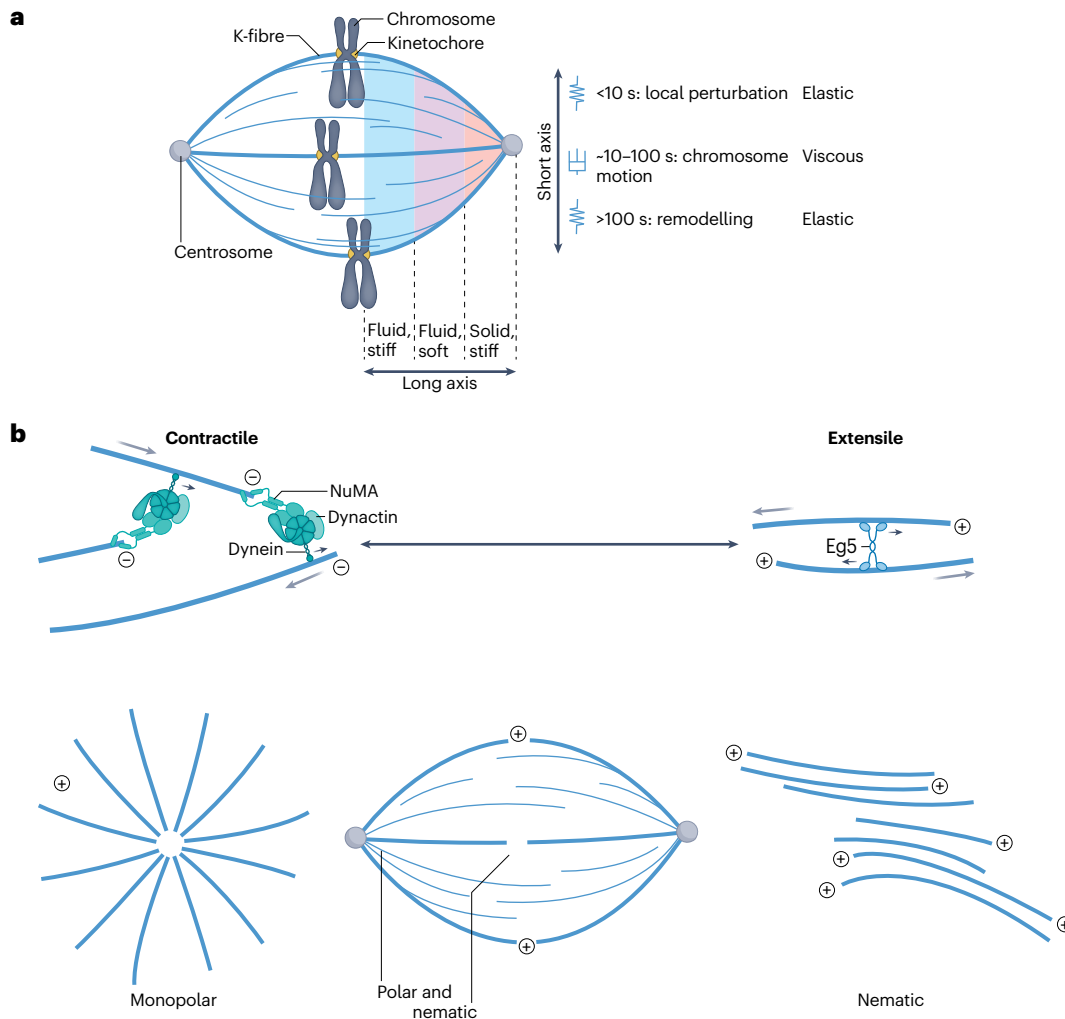


Fig. 6 | Spindle material properties. **a**, The responses of the spindle to force are spatially and temporally heterogeneous. Along the short axis, *Xenopus* extract spindles behave elastically on short (<10 s) and long (>100 s) timescales. On intermediate timescales, corresponding to the time required for chromosome movement, the short axis of the spindle exhibits more viscous behaviour, dissipating energy as it deforms. The long axis of the spindle is also mechanically heterogeneous: it is stiffest near the poles and kinetochores but less stiff in the middle. The pole region exhibits solid-like behaviour, recovering its shape after

deformation, whereas the middle and equator regions are more fluid-like.

b, Dynein and its cofactors NuMA and dynactin generate contractile stress in vitro and in the spindle, clustering minus ends together. Eg5 mediates extensile stress, giving rise to nematic motifs of aligned, mixed-polarity microtubules. These opposing motor activities must be balanced to give rise to a bipolar spindle: an excess of contractile activity leads to monopolar spindles, and an excess of extensile sliding produces turbulent microtubule networks.

in anaphase^{205,225,227}. These studies support the view that poleward flux is an emergent phenomenon resulting from the coordinated activities of several motor and non-motor MAPs that is required to maintain accurate and dynamic kinetochore–microtubule attachments.

Material properties

Although the biophysical properties of microtubules and many individual MAPs are well understood in vitro, far less is known about the material properties of the spindle – its deformation, remodelling and structural failure under force. The spindle must respond to forces generated both internally (by motors and dynamic microtubules) and externally (by cortical motors and by neighbouring cells). Mechanical forces regulate many aspects of cell division, including chromosome

movement²³², spindle positioning²³³ and error correction at kinetochores^{126,234,235}, and understanding each of these processes requires knowledge of the emergent mechanics of the spindle.

Several elegant studies using concepts of rheology have begun measuring the material properties of the spindle as a continuum, such as elasticity and viscosity, that exists in the fully assembled spindle but not in its component parts. These studies shed light on the heterogeneity of the spindle as a material as well as the mechanical principles that make it well suited to the timescales and force regimes of chromosome segregation. Experiments using microneedles, force-sensing cantilevers and cell confinement have demonstrated that invertebrate, mammalian and *Xenopus* egg extract spindles are stiffer in their long axes compared to their short axes^{218,236,237}. This property may be functionally

important for chromosome movement: for example, it may permit efficient longitudinal movements during chromosome congression, oscillation and segregation, while allowing chromosomes to deform their surroundings laterally. The dynamic and complex microstructure of the spindle also mediates distinct responses at different timescales of force application. In *Xenopus* egg extract spindles, the short axis deforms elastically on short (<10 s) or long (>100 s) timescales but is more viscous on intermediate timescales²³⁸. On the long axis, stiffness is highest at the pole and equator but the spindle is softer between them²³⁹ (Fig. 6a). These behaviours allow maximal structural plasticity on the timescale at which chromosomes move through the spindle, with minimal deformability near microtubule connections to chromosomes and poles. Microneedle manipulation of mammalian PtK2 cells has revealed that similar principles of spatially heterogeneous mechanics apply to spindles with prominent k-fibres^{212,217}. Finally, recent studies of molecular diffusivity in mammalian spindles²⁴⁰ and mass density in *Xenopus* egg extract spindles²⁴¹ have explored how particles move between closely packed spindle microtubules, which might be expected to impede diffusion. Surprisingly, diffusion and density do not differ between the spindle and the surrounding cytoplasm; instead, dynamic microtubules appear to fluidize the spindle region²⁴⁰, which may be important in enhancing the diffusion of large molecular complexes, such as the CPC or γ -TuRC, throughout the spindle. Together, these rheological studies illustrate mechanical features of the spindle across both axes and at different timescales that are optimized for its role in moving chromosomes.

The molecular components of the spindle constantly dissipate energy in the form of GTP (microtubule dynamics) and ATP (motor proteins such as dynein), meaning that, although the spindle reaches a steady-state structure at metaphase, it does not reach thermodynamic equilibrium. Accordingly, the full complexity of the spindle cannot be fully captured by the rheological models described above. Additional

insight into spindle material properties has been gained from active matter theories that model non-equilibrium systems. The microtubule architecture and dynamics of *Xenopus* egg extract spindles have been described by active liquid crystal theory, modelling the spindle as a droplet of microtubules that is shaped by microtubule turnover, mutual alignment, surface tension and motor-driven active stresses²⁴². In this model, focused poles arise from a barrelling-type instability resulting from dynein activity²⁴³. Active matter theory has also been applied to the human spindle, where the cell membrane provides boundary conditions absent in the *Xenopus* egg extract system. In human cells, unchecked extensile sliding by Eg5 results in the bending and buckling of microtubules against the cell cortex, leading to chaotic, turbulent microtubule networks. This effect is opposed by dynein-mediated contractile stress that allows the spindle to maintain a steady-state shape^{183,244} (Fig. 6b).

Despite these quantitative physical measurements and theoretical models, many open questions remain regarding the molecular mechanisms that give rise to these material properties. Furthermore, many of the functional implications ascribed to these properties, such as their impact on chromosome motion, remain speculative and require more direct testing.

Spindle twist

The regulation and function of spindle twist is a new area of research sparked by a recent report that metaphase human U2OS and HeLa spindles are, on average, slightly left-handed²⁴⁵. Twist varies between cell types, with HeLa and U2OS spindles showing a stronger left-handed twist than RPE1 spindles^{205,245,246}, and between mitotic stages, where twist peaks in early anaphase²⁴⁶. Interestingly, spindles of the amoeba *Naegleria* are, on average, right-handed²⁴⁷, suggesting that the left-handed bias may not be a highly conserved feature of the spindle.

Many mitotic motors have an off-axis (that is, they do not move exactly parallel to the microtubule) component in their stepping

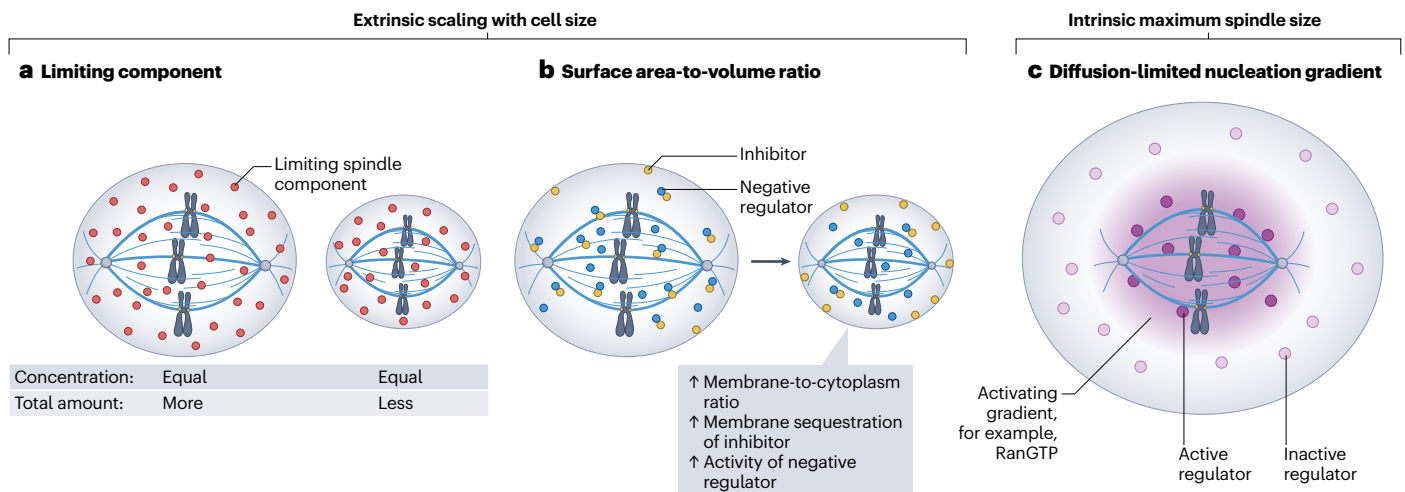


Fig. 7 | Spindle scaling mechanisms. The scale of the spindle is set by extrinsic and intrinsic mechanisms. **a**, Extrinsic mechanisms, whereby spindle size scales with cell size, include the limiting components model. This model posits that the size of the spindle is limited by the amount of spindle-building components available. Given that the concentration of the limiting component remains equal irrespective of cell size, a larger cell would contain more of this component and be able to build a larger spindle than a smaller cell. **b**, Extrinsic scaling can also result from changes in the cellular surface area-to-volume ratio. For example,

this occurs if an inhibitor of a negative regulator of spindle size localizes to the cell membrane. As a result, in smaller cells with larger membrane-to-cytoplasm ratios, the inhibitor becomes sequestered at the membrane and enables the negative regulator to scale spindle size down. **c**, Intrinsic mechanisms for setting maximum spindle length have also been proposed. In very large cells such as early embryonic cells, spindle size can be determined by the scale of a gradient of microtubule nucleation activity such as a RanGTP gradient emanating from the chromosomes.

behaviour *in vitro*, resulting in helical motion around the microtubule track. Plus-end-directed motors studied thus far show a left-handed bias^{248–250} and minus-end-directed motors have a right-handed bias^{251–253}, but how the spindle resists these additive torques to maintain its relatively untwisted global architecture is not known. The individual inhibition of several motors and MAPs, including Eg5, KIF18A and augmin, revealed that each contributes, albeit to a small degree, to left-handed twist^{245,246}. Deletion of NuMA results in strong left-handed twist²⁰⁵, suggesting that NuMA–dynein motor activity or crosslinking may be important in counteracting left-handed twist.

Although the function, if any, of spindle twist remains unknown, a recent study proposed that twist allows the spindle to bear mechanical load along the pole–pole axis (for example, forces originating from neighbouring cells)²⁴⁶. Further research is required to determine whether maintaining spindle twist within a specific range is important for spindle function.

Spindle size and scaling

Spindle size scales roughly linearly with cell size in all species studied to date^{254,255}, which allows the spindle to properly segregate chromosomes between daughter cells despite variation in cell size between developmental stages, tissues and species. Multiple models, involving both spindle-extrinsic and spindle-intrinsic mechanisms, have been proposed to explain spindle size scaling in different contexts. The limiting component model posits that there is a limiting component, or components, required for spindle assembly, such as the *Xenopus* protein XMAP215 (ref. ²⁵⁶), and spindles are larger in larger cells owing to greater amounts of this limiting component, assuming equal concentration between cells²⁵⁷ (Fig. 7a). Evidence for this mechanism was provided by encapsulating *Xenopus* egg extract in microfluidic droplets of defined sizes, where the total amount of cytoplasm could be carefully controlled while keeping cytoplasm composition unchanged^{258,259}. Cellular surface area-to-volume ratio can also tune spindle size: as cells shrink and the surface area-to-volume ratio increases at later stages of *X. laevis* embryo development, the microtubule destabilizing activity of KIF2A is enhanced as more of its inhibitor becomes sequestered to the membrane²⁶⁰ (Fig. 7b). Similarly, studies in *Xenopus* and zebrafish suggest that membrane sequestration of a microtubule nucleation inhibitor may regulate spindle scaling²⁶¹. Additionally, differing expression levels or activities of the MAPs KIF2A (ref. ²⁶²), the microtubule severing protein katanin²⁶³ and TPX2 (refs. ^{264,265}) have been shown to regulate spindle size in a species-specific manner.

At very large cell diameters ($\geq 140 \mu\text{m}$), spindle size reaches a plateau^{254,255,258,261,266}, revealing a second emergent phenomenon by which the spindle sets an intrinsic maximum length. This maximum is set, in part, by the spatial profile of microtubule nucleation activity, which peaks near chromosomes in large oocytes and large *Xenopus* egg extract spindles and is limited by diffusion at increasing distances from chromatin^{210,261,267} (Fig. 7c). Microtubule polymerization rates²⁶⁸, transport^{210,269} and lifetime²⁶⁹ also contribute to setting the intrinsic size scale of the spindle. Whether these models can account for the sizes of spindles with diverse architectures, such as mammalian spindles with prominent k-fibres, remains to be determined.

Future perspectives

Recent advances from *in vitro* reconstitution, *Xenopus* egg extract spindles and mammalian cells have provided a wealth of new insights into spindle assembly, allowing us to better link the structural and biochemical properties of individual molecules to the microtubule

modules they build and the emergent properties they give rise to. Despite this progress, many open questions remain, and several new approaches promise to further unravel the mechanisms by which microtubules are nucleated and organized to build the spindle. Advances in imaging techniques, both the tracking of individual molecules as well as imaging whole spindle volumes at high spatiotemporal resolution, will enable us to identify individual events of interest within the dense spindle network and follow the dynamics of specific spindle substructures throughout their entire lifetimes. Improved approaches to apply spatiotemporal perturbations, such as optogenetics, auxin-inducible degrons and microneedle manipulation, now allow us to acutely modulate forces and molecular interactions in a specific region or mitotic phase (Box 1). Finally, new three-dimensional culture models provide exciting opportunities to understand how the tissue context can influence spindle assembly²⁷⁰ and how the spindle is rewired in disease. These approaches will help close the gap between *in vitro* and *in vivo* studies, reconstituting the spindle from the bottom up and dissecting its emergent properties from the top down.

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Author contributions

V.A.V. and L.N. created the first draft of the text and figures. All authors contributed equally to discussion, revision and editing of the manuscript.

Competing interests

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