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Microtubule nucleation for spindle assembly: one molecule at a time

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Abstract

The cell orchestrates the dance of chromosome segregation with remarkable speed and fidelity. The mitotic spindle is built from scratch after interphase through microtubule (MT) nucleation, which is dependent on the γ-tubulin ring complex (γ-TuRC), the universal MT template. Although several MT nucleation pathways build the spindle framework, the question of when and how γ-TuRC is targeted to these nucleation sites in the spindle and subsequently activated remains an active area of investigation. Recent advances facilitated the discovery of new MT nucleation effectors and their mechanisms of action. In this review, we illuminate each spindle assembly pathway and subsequently consider how the pathways are merged to build a spindle.

Microtubule (MT) organizing centers in the spindle

The mitotic spindle has the crucial job of partitioning genetic material into each daughter cell during cell division. The spindle is a self-organizing structure, driven by local biochemical interactions between MTs and their associated proteins, including motor proteins, which are responsible for bundling and sorting MT arrays. The spindle is highly dynamic due to the inherent properties of MTs and its many regulatory factors. Prior to mitosis, the MT cytoskeleton is completely disassembled and then, by metaphase, is rebuilt to form the mitotic spindle.

MTs emanate from specific sites called **microtubule organizing centers (MTOCs)** (see Glossary), which serve as the origin of a MT nucleation pathway. Originally, the centrosome was considered the main MTOC. With the discovery of dynamic instability [1], whereby MTs undergo cycles of polymerization and disassembly (i.e., catastrophe), came the first proposed mechanism of spindle assembly [2]. Specifically, a 'search and capture' model was postulated, where MTs nucleated from the centrosome undergo dynamic instability throughout the cytoplasm ('search') until they find the **kinetochore (KT)**, where they are captured and stabilized. An apparent example of 'search and capture' was first observed in live newt lung cells [3], but later studies indicated that a model based solely on stochastic

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MT growth and shrinkage is not enough to recapitulate the stereotypic timescales of spindle assembly. Similarly, it was realized that there must be a spatial bias to direct MTs towards the KT [4]. Furthermore, spindles in cells were discovered that entirely lacked centrosomes (as in oocytes). These mysteries are now being resolved with the ongoing discoveries of many additional acentrosomal MT nucleation pathways.

In this review, we discuss the most recent work that describes when and where MTs are made to assemble the spindle. Recent reviews have addressed spindle assembly mechanisms and the emergent properties of the metaphase spindle [5,6], while others have solely focused on the assembly and structure of γ-tubulin complexes [7,8], the universal MT template. Our review seeks to bridge these two topics, where we discuss the specific MT nucleation pathways, including the molecular players involved in each one and how γ-tubulin is recruited to different MTOCs in the spindle.

We first discuss the structure and mechanism of MT nucleation by the **γ-tubulin ring complex (γ-TuRC)**, the universal MT template. Once assembled, γ-TuRC is targeted and activated at MTOCs throughout the spindle to nucleate MTs. A newly emerging feature of some of these nucleation sites is that these are established by **liquid–liquid phase separation (LLPS),** which provides an additional means to regulate spindle assembly. Finally, we discuss how these pathways cooperate and scale with respect to one another to build a robust spindle. Excitingly, as a result of these discoveries, the field is beginning to approach a minimal parts list and a blueprint for spindle assembly.

MT nucleation forms the basis for all spindle MTS

MTs are composed of α/β-tubulin heterodimers arranged head-to-tail with a structurally distinct plus end (β-tubulin exposed) and minus end (α-tubulin exposed). Despite the discovery of tubulin roughly 50 years ago, the exact way of nucleating a MT remains an active area of investigation.

γ-Tubulin, another member of the tubulin family, was initially identified in a genetic screen of *Aspergillus nidulans* [9] and it was later shown to be essential for spindle assembly [10]. γ-Tubulin forms a complex with several accessory proteins to form a ring-shaped structure competent to build MTs. In yeast, γ-tubulin self-assembles along with **spindle pole body (SPB)** component 97 (Spc97) and Spc98 to form the **γ-tubulin small complex (γ-TuSC**; Figure 1A, left). Spc97 and Spc98 bind two copies of yeast γ-tubulin to form a V-shaped tetramer, where Spc97/98 interact laterally through their respective N-terminal regions and the two copies of γ-tubulin are held apart [11,12]. Seven γ-TuSC tetramers are assembled at the SPB and form a left-handed spiral ring with γ-tubulin molecules exposed at the top resembling the ring of a MT (referred to here as yeast γ-TuRC). Recent near-atomic resolution cryogenic electron microscopy (cryo-EM) structures revealed that yeast γ-TuRC contains an average of 6.5 γ-TuSC molecules per turn, resulting in 13 γ-tubulin molecules per γ-TuRC, reminiscent of a 13 protofilament MT [13].

In vertebrates, γ-tubulin complex proteins (GCPs) 2 and 3 (analogs of Spc97/98) interact with two copies of γ-tubulin to form vertebrate γ-TuSC. Five copies of γ-TuSC comprise

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the symmetric core of vertebrate γ-TuRC. Asymmetry is introduced into the ring with the addition of GCPs 4–6, which are present in higher organisms [14–19]. Mozart1 (MZT1) and Mozart2 (MZT2), Nucleoside diphosphate kinase 7 (NME7), and Neural precursor cell-expressed developmentally down-regulated protein 1 (NEDD1) were also identified as components of vertebrate γ-TuRC, though it was not known which proteins were an essential part of the complex, their relative stoichiometry in the complex, nor their positioning [15,20–23] (Figure 1A, left).

In recent years, several high-resolution structures of γ-TuRC have been solved that successfully addressed open questions related to its assembly and organization [24–29]. γ-TuRC in vertebrates contains 14 γ-tubulin/GCP spokes, which are ordered $(GCP2–3)_4$, GCP4, GCP5, GCP4, GCP6, $(GCP2–3)_1$ (Figure 1A, right). Given that MTs are typically 13 protofilaments, this result is intriguing and introduces a substantial mismatch between the γ-TuRC template and the geometry of the MT it nucleates. Activation of γ-TuRC is discussed in the following section.

Also contained within the structure is a stabilizing scaffold within the lumenal space at the bottom of the cone and it consists of the N terminus of GCP6 and GCP3, along with two copies of MZT1 and, surprisingly, one copy of globular actin (Figure 1A). However, from these structures, it was still unclear which proteins were required for assembly of the native complex and which were additional factors pulled down during purification. Using recombinant expression systems and reconstitution, it was revealed that γ-tubulin, GCP2–6, MZT1/2, and actin are sufficient to form γ-TuRC [30,31]. Additionally, one study showed the formation of the native complex requires coexpression with RuvB-like protein 1 (RUVBL1) and RUVBL2, AAA+ ATPases that act as chaperones and facilitate assembly of γ-TuRC [30]. With this leap in understanding γ-TuRC composition and structure, the next key question is how MT nucleation is turned on at the right location and time to build the spindle.

γ-TuRC stimulation and regulation by additional factors

Despite these advances, a key lingering question remains: how is γ-TuRC activated? Purified γ-TuRC has comparatively low nucleation activity *in vitro*, not matching its potent *in vivo* activity. Moreover, γ-TuRC is ubiquitous throughout the cytosol and yet ectopic MT nucleation is rare. This suggests additional factors are required for its activity and regulation *in vivo*. A major advance came from the discovery that XMAP215 (ch-TOG in humans), a well-characterized MT polymerase, is in fact a γ-TuRC co-nucleation factor necessary to nucleate MTs [32–34].

XMAP215 was initially thought to solely stimulate MT polymerization by binding and releasing tubulin dimers at the growing plus end. However, recent studies showed that XMAP215 directly interacts with γ-TuRC through its C-terminal domain. At the same time, N-terminal tumor overexpressed gene (TOG) domains bind incoming α/β tubulin heterodimers, thereby enhancing nucleation by concentrating soluble tubulin at the γ-tubulin ring interface [33]. The XMAP215 ortholog in budding yeast, Stu2, also promotes assembly of γ-TuSC for cytoplasmic MT nucleation by yeast γ-TuRC [34,35]. These findings hence demonstrate that XMAP215 family members together with γ-tubulin complexes form the

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conserved MT nucleation module in the cell (Figure 1C). Nevertheless, the question remains how this γ-TuRC:XMAP215 nucleation module is spatiotemporally regulated to build the spindle.

Of specific interest in the field has been the question of how a 30-nm wide γ-TuRC assumes a conformation that matches the 25-nm diameter of an MT (Figure 1B), which is hypothesized to be one way to activate γ-TuRC. *in vitro* experiments in yeast γ-TuRC showed that activation could be triggered through an artificial ring closure forced by the introduction of engineered disulfide linkages, resulting in a fivefold increase in nucleation rate [13]. At the same time, the hunt was on for activation factors that could similarly induce ring closure in vertebrate γ-TuRC. A simple model postulated that tubulin binding alone might facilitate ring closure, as derived by *in vitro* single molecule experiments combined with Monte Carlo simulations [36]. While the initial addition of α/β tubulin to the lattice is the rate limiting step for nucleation, approximately four α/β tubulin subunits form the critical nucleus and represent the γ-TuRC transition state (Figure 1B). This can be further aided by the tubulin recruitment activity of XMAP215, but does not address how MT nucleation is spatiotemporally regulated.

It is assumed that each MT nucleation pathway contains localization factors that recruit γ-TuRC to its site of action as well as specific activators for each pathway that tune the kinetics and lower the activation barrier of MT formation (Figure 2). Therefore, when, where, and how γ-TuRC is recruited and activated remains a key to understanding spindle assembly. In the remainder of this review, we will discuss how γ-TuRC is targeted to centrosomal and acentrosomal sites during mitosis.

Generating MTs at spindle poles

MTs are generated at spindle poles in cells containing a centrosome as well as those without. One way to specify the spindle poles as sites of MT nucleation is through the recruitment of γ-tubulin and accessory proteins canonically found at the centrosome. A high local concentration of soluble tubulin will also promote MT nucleation, which can be achieved either by specific targeting of nucleation factors like chTOG/XMAP215 or through phase separation, a mechanism used by the cell to locally enrich protein concentrations at specific sites.

MT nucleation at the centrosome

Centrosomes were initially described by Van Beneden and Boveri in the late 1800s and connected to cell division soon after [37]. The centrosome core consists of two **centrioles**, a mother and a daughter, composed of triplet MT structures that form a circular ninefold symmetric ‘cartwheel’ structure. Surrounding the centrioles is a dense cloud of proteins known as the **pericentriolar material (PCM)**. Upon mitotic entry, the PCM expands in a process regulated by at least two kinases downstream of the master regulator, CDK1: Polo-like kinase 1 (PLK1) and Aurora A kinase (Figure 3A) [38–40]. This expansion leads to a fivefold increase in the number of nucleated MTs. The PCM scaffold mediates recruitment of γ-TuRC to the centrosome and anchors MT minus ends. Specifically, PCM proteins such as pericentrin, AKAP450, CEP192, and CDK5RAP2 have been implicated in

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the recruitment of γ-TuRC to centrosomes [28,41–44]. The recruitment may be mediated by γ-TuRC subunits such as NEDD1 and MZT1/2, which act as binding partners with centrosomal proteins (Figure 3A) [45].

Of particular interest is CDK5RAP2, which has been studied in the context of recruitment, anchoring, and activation of γ-TuRC at the centrosome [41,46,47]. CDK5RAP2 interacts with γ-TuRC via its centrosomin-1 (CM1)-motif, also known as the γ-TuNA domain [41,46]. Besides targeting γ-TuRC to the centrosome, the γ-TuNA domain directly activates γ-TuRC [48]. Its activation *in vivo* is so potent that the CM1 domain must be autoinhibited to prevent ectopic nucleation activity [49]. Previously, γ-TuNA-mediated γ-TuRC activation could not be detected *in vitro*, which may be due to experimental conditions, including size and position of exogenous tags used relative to the conserved γ-TuNA sequence [25,36,46,48]. Hence, it is not surprising that binding of γ-TuNA with an activity-inhibiting tag location did not change the diameter of γ-TuRC.

In a structure of yeast γ-TuRC, CM1 mediates γ-TuRC assembly and binds at the Spc98/97 interface, where six molecules of CM1 bind to a single yeast γ-TuRC [50]. The CM1 binding site is conserved in metazoan γ-TuRC, but only one CM1 binding site is known, where one molecule of γ-TuNA binds at the GCP2–6 interface [26]. However, in the structure of human γ-TuRC, the symmetry is still mismatched compared with ideal MT geometry, even when γ-TuNA is bound [25], compared with a more ideal geometry for yeast γ-TuRC in the closed state. This may illustrate species-specific differences and additional regulatory factors in metazoans.

A breakthrough in the field demonstrated that key components of the PCM can undergo LLPS and this may play an important role in centrosome assembly and function [51]. In *Caenorhabditis elegans*, the essential elements of PCM assembly are SPD-2/CEP192, PLK1, and SPD-5 (a CDK5RAP2 homolog) [52–54]. *in vitro*, SPD-5 undergoes phase transitions and the addition of PLK1 and SPD-2 enhance condensate formation [51]. Strikingly, when TPX2 and XMAP215 homologs, TPXL-1 and ZYG-9, were added along with soluble tubulin, tubulin was concentrated and **MT asters** formed, representing a minimal centrosome. In a separate study, it was shown that these proteins can recruit soluble tubulin to the centrosome, which results in a tenfold increase in tubulin concentration compared with the cytoplasm in mitosis [55]. Thus, LLPS provides a means to further concentrate tubulin, required for the rate limiting step in nucleation, as well as concentrate proposed activation factors such as CM1-containing proteins.

Acentrosomal MT nucleation

In some cell types, including oocytes undergoing meiotic division, spindle poles are organized in the absence of centrosomes. In fact, it was recently shown that 17 centrosomal proteins form a **liquid-like spindle domain (LISD)** that is distinct from the cytoplasm (Figure 3B) [56]. Unlike other centrosomal proteins that exhibit phase-separating behavior, the LISD does not concentrate soluble tubulin, but only enriches different MT regulatory factors in acentrosomal spindles. Notably, disruption of the LISD causes the release of MT regulatory factors into the cytoplasm. This in turn leads to severe spindle defects, including reduced MT density within the spindle and the midzone, as well as depleted KT fibers

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[**kinetochore-fibers (K-fibers)**]. An interesting observation in this study was that HAUS6, a subunit of the augmin complex, was recruited to the LISD [52]. The augmin complex mediates a MT-dependent nucleation pathway, termed branching MT nucleation, and the connection between these pathways is an exciting area for further investigation. Finally, while the presence of the LISD was reported in mammalian oocytes only, it remains an open question whether similar structures or mechanisms exist in other cell types.

Chromosomal MT nucleation: supplying MTs to the right place at the right time

Chromosomes were originally thought to be merely passive cargo during spindle assembly. Yet, several pieces of evidence demonstrated a critical role for chromatin itself in spindle assembly [57,58]. A key breakthrough in the field came from the discovery that DNA-coated beads can assemble a bipolar spindle in *Xenopus laevis* egg extract [58]. This remarkable discovery showed that spindle assembly needs neither centrosomes ('search') nor KTs ('capture'). In cell types that typically contain centrosomes, spindle assembly can proceed even when centrosomes are chemically inactivated or ablated with laser irradiation [59–61]. Even more strikingly, *Drosophila* can be born without centrosomes entirely [61]. In all these cases, MT formation occurs via several MT nucleation pathways originating from chromosomes.

The Ran pathway

Perhaps the most well-studied pathway depends on the small GTPase Ran. Its active Ran GTP form releases so-called **spindle assembly factors (SAFs)** that conduct spindle assembly. RanGTP, and consequently SAFs, form a radial gradient around chromosomes because chromosome-bound guanine nucleotide exchange factor RCC1 converts Ran GDP into Ran GTP, thereby creating a concentrated source of active Ran [62] (summarized in Figure 4A). Cytoplasmic Ran GTPase activating protein 1 (Ran GAP1) away from chromosomes converts Ran GTP back into inactive Ran GDP. Thus, a biochemical gradient is established to promote spindle assembly spatially near chromosomes. Indeed, a minimal system containing RCC1-bound beads and *X. laevis* egg extract is sufficient to induce spindle assembly [63].

In this gradient, active Ran GTP binds to the karyopherin importin β, which is part of the importin α/β heterodimer, thereby releasing the sequestered SAFs (Figure 4A). Simultaneously, the Ran gradient is enhanced by a positive feedback loop as RCC1 itself is sequestered by karyopherins and liberated by its product RanGTP, which likely reduces the time to establish the gradient [64,65], although the gradient's role in spindle assembly has also been debated [66]. How SAFs exactly conduct spindle assembly is an active area of investigation, even today, as new SAFs are still being discovered. The most prominent Ran-regulated SAF is Targeting Protein for Xklp2 (TPX2), which is a major effector of branching MT nucleation, whereby MT nucleation is off the side of a pre-existing MT lattice (Figure 4B) [67]. Surprisingly, it was recently shown that the MT binding activity of another major branching factor, augmin (HAUS in humans), is also subject to Ran regulation [68,69].

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Branching MT nucleation

In addition to TPX2, branching MT nucleation [70] requires the eight subunit complex, augmin and the nucleation module defined by γ-TuRC and XMAP215 [33,70,71]. Critically, branching MT nucleation autocatalytically increases spindle MTs number, while conserving their polarity. The absence of branching MT nucleation leads to severe spindle defects and defective K-fiber formation [71–75]. In fact, a recent study in Indian muntjac fibroblasts (i.e., cells with unusually large KTs, ideal for studying K-fiber maturation) showed that loss of augmin is the most deleterious condition for mitosis, even among other essential proteins tested, including Ndc80 complex, the **chromosome passenger complex (CPC)**, Aurora A, and chTOG [76]. TIRF microscopy in *Xenopus* egg extract revealed that branching MT nucleation is stimulated by TPX2 and is dependent on augmin to recruit γ-TuRC to existing MTs in order to initiate new MT branches (Figure 4B) [70].

Building upon many cell biological studies, *in vitro* reconstitutions using purified proteins from *X. laevis, Drosophila melanogaster*, and HeLa cells recently defined the minimal components for branching nucleation and their hierarchy [77,78]. In *X. laevis*, first, branching MT nucleation is spatially regulated by Ran, as it releases the primary branching factor, TPX2, from importins around chromosomes. Once released by Ran, TPX2 locally initiates the branching reaction by binding to the MT lattice [79]. Critically, TPX2 binds to MTs as a disordered, liquid-like condensed phase capable of forming discrete droplets along the MT [80], which is governed by Rayleigh-Plateau instability [81]. Such hydrodynamic features could be broadly applicable to many biomolecular condensates that interact with MTs [82]. TPX2 defines the branch sites and, subsequently, augmin and γ-TuRC localize to the TPX2-coated lattice, allowing nucleation of branched MTs to form the mother MT [77,83]. In contrast to frogs, *in vitro* reconstitutions of branching MT nucleation using proteins from *D. melanogaster* and HeLa cells demonstrated that only augmin and γ-TuRC, but not TPX2, were required [78], which may illustrate species-specific differences [84]. Moreover, a study performed in HeLa cells elucidated post-translational modifications as yet another regulatory mode of branching. Specifically, phosphorylation of NEDD1 is essential for the augmin-g-TuRC interaction and, importantly, phosphorylation of both augmin and NEDD1 is essential for the formation of branched MTs [85]. It is likely that post-translational modifications play important roles in other MT nucleation pathways as well, opening up future areas of research.

Unlike branched structures formed by Arp2/3 and actin, which form a stereotypic 60° branch angle, branched MTs exhibit a shallow range of branched angles that result in nearly parallel daughter MTs with the same polarity [70,77,78]. This orientation is primed for quickly building a spindle, since MTs attach to the KTs via their plus ends and form bundles of parallel MTs (K-fibers) that span to the centrosomes. In addition, non-KT MTs in the spindle exhibit the same orientation, with their plus ends pointing toward chromosomes. However, how this polarity bias is maintained on a molecular level is unknown [86].

The chromosome passenger complex

Independent of Ran, another chromatin-induced spindle assembly pathway exists, namely via the CPC (Figure 4D) [87]. The CPC is perhaps best known for its role in preventing

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aberrant KT-MT attachments and its role in the **spindle assembly checkpoint** [88,89]. However, new work points to the CPC also playing a role in MT nucleation. Indeed, the CPC promotes MT assembly by targeting and inhibiting the function of MT destabilizers such as the mitotic kinesin, MCAK, and stathmin near KTs [87,90–94]. It is unclear if it plays a more direct role through the recruitment of other nucleation factors.

The CPC is located at the inner KT during spindle assembly and contains four components: an inner centromeric scaffold (INCENP), Aurora B kinase, survivin, and borealin [95]. Depending on the cell cycle stage, the CPC can be targeted either to chromatin, the inner KT, or to MTs [96–100]. There is recent evidence that the CPC is a biomolecular condensate and undergoes LLPS both *in vitro* and *in vivo* [101]. The centromere-targeting region of the CPC containing survivin, borealin, and the N terminus of INCENP phase separates in a manner dependent on an intrinsically disordered region of borealin, and other components of the centromere are concentrated within the liquid droplets. Strikingly, the CPC droplets concentrated α/β tubulin and nucleated MTs at 20-fold below the critical concentration *in vitro* [101]. With the possibility of the CPC more actively contributing to MT nucleation, further research is required to determine how the CPC contributes to spindle assembly from chromosomes.

MT nucleation at KTs

The easiest way to construct the critical K-fibers would be if KTs made them themselves (Box 1). Indeed, MT nucleation directly at the KT could explain the high accuracy and short capture times observed during cell division with centrosomes [102] and, more importantly, explain how K-fibers are made in acentrosomal spindles. However, it was unclear if MT nucleation at the KT is a universally conserved nucleation pathway with a dedicated set of proteins to target and activate γ-TuRC, or if it was only utilized as a backup or in specific systems. Moreover, it was not understood how the proper MT polarity would be achieved if the plus ends were initially in the wrong orientation, pointed away from KTs towards the poles.

Several lines of evidence have now addressed this dilemma and shown that MT nucleation near the KT helps to capture chromosomes. Early studies showed that Stu2 (the chTOG/XMAP215 analog) is required for generating MTs at the KT in budding yeast, whereas γ-tubulin is not. Moreover, Stu2 alone is sufficient for MT nucleation when artificially tethered to chromosome arms [103]. However, it was not clear if this mechanism was relevant for metazoans that undergo open mitosis.

At the beginning of metaphase in mammalian cells, short MTs are generated near the KT that attach side-on before being converted to the proper end-on attachment. Recently, two studies used a combination of cryo-electron tomography (ET) reconstructions, biophysical tools, and mathematical modeling to determine KT MT distributions in HeLa cell spindles [104,105]. Their data supported a model where MTs bound to the KT originated through the *de novo* nucleation of MTs near KTs and not through recruitment of spindle or centrosomal MTs. Branching MT nucleation is also active in building MTs at the KT, particularly K-fibers, by amplifying parallel MT bundles [70]. Augmin amplification of MTs via branching nucleation indeed explains the plus end distribution observed during live-cell imaging [75].

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Using *Xenopus* egg extract, branching MT nucleation could be directly visualized and shown to occur near and towards KTs, which further explains how the proper MT polarity is achieved [106].

Molecularly, *Drosophila* augmin subunit Dgt6 (HAUS6) interacts with Ndc80 complex, which connects MTs with the KT at the KT [107]. Indeed, the MT nucleator γ-TuRC can be recruited to the KT during spindle assembly to directly nucleate MTs, a function important for K-fiber assembly [108]. The γ-TuRC recruitment role to the KT is executed by the nuclear pore protein complex Nup107–160 in a RanGTP-dependent manner [109] (Figure 4C). Whether and how the Nup107–160 complex is connected to branching MT nucleation still needs to be investigated.

Regulation of MT nucleation pathways to build a spindle

How are pathways scaled with respect to one another?

In order to assemble a spindle, each MT must not only be nucleated from its required location, but also at a specific time and with a specific MT number output with respect to the other pathways.

Besides quantifying MT number contribution from each pathway, studies where one pathway is reduced or eliminated highlights how other MT nucleation pathways can compensate for one another.

It is known that centrosomes and chromosomes start nucleating MTs around the same time [110]. Moreover, centrosomal and non-centrosomal MT nucleation act synergistically to promote spindle assembly [111]. Interestingly, reducing the contribution from one nucleation pathway causes the other pathways to increase their contributions, pointing to inter-pathway crosstalk that ensures robust spindle assembly. Other studies in *Drosophila* have used laser ablation and siRNA to inactivate centrosomal MT nucleation and show that spindle formation can still proceed normally [59,112], establishing the compensatory nature of spindle assembly pathways.

Recently, there has been a focus on quantifying the role of branching MT nucleation on spindle mass. One study examined MT nucleation related to aster formation in *X. laevis* using laser ablation techniques and mathematical modeling [113]. The authors found that branching MT nucleation was the main physics at play in controlling the steady state size of bounded asters. Moreover, a separate study measured MT diffusion from spindle boundaries and used computational modeling to confirm that branching provides the main source of spindle MTs in *Xenopus laevis* [114].

How are MT nucleation pathways intercalated to build the spindle?

While progress has been made to identify individual MT nucleation pathways and their molecular players, we must now determine how MT nucleation pathways intersect with one another to create a diamond-shaped spindle with a uniform appearance. A key role of organizing different populations of MTs, once nucleated, is fulfilled by molecular motors (Figure 5). The kinesin-5 (Eg5) crosslinks MTs and participates in antiparallel MT sliding

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to achieve bipolarity [115,116]. Recently, Eg5 was shown to promote MT nucleation and increase polymerization rates by affecting the straight-to-curved transition of tubulin [117], the role of which still needs to be confirmed *in vivo*. Interestingly, Eg5 also interacts with TPX2 via its C terminus, separate from its MT binding domains [118]. One could speculate that TPX2 initiates branching near chromosomes and is then transported by Eg5 throughout the spindle body to target branching to different locations.

Dynein motors are also essential for spindle assembly and are involved in minus-end directed pole focusing in a way that depends on the adapter dynactin and the crosslinker NuMA [119–121]. Depletion of either Eg5 or dynein results in a collapsed spindle, whereas simultaneous depletion of both motors results in normal spindle assembly, emphasizing the importance of force balancing by each motor as well as the redundant abilities of other motors to pick up the slack [122]. A pioneering simulation study used Langevin dynamics, in which the Brownian and active motion of MTs in response to both conservative and motor forces are explicitly computed, to model acentrosomal spindle assembly in two dimensions [123]. They concluded that MTs organized by kinesin and dynein motors only assembled into an accurate bipolar structure when MT nucleation took place throughout the spindle, emphasizing the interplay between motor transport and MT nucleation.

Concluding remarks

During the past decades, we have learned that MTs can nucleate from a variety of locations in the spindle, including centrosomes, near chromosomes, and within the spindle body itself. It is now known that most organisms use all of these pathways in combination with each other, while others rely on only acentrosomal MT networks to build a spindle. Common to all these pathways is the γ-TuRC/XMAP215 nucleation module, which is targeted throughout the spindle to be activated for nucleating new MTs at the right time and place. With technical advances in cell biology, biochemistry, and structural biology, MT nucleation and its regulation will be understood down to the atomic level (see Outstanding questions). In addition, work done in the last years offers a glimpse into the future, where MT nucleation will be studied in the context of a functional spindle and not just via isolated biochemical pathways. Ultimately, understanding when and where MTs are made in the spindle will help explain how the spindle machinery is built and maintained to orchestrate cell division.

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Glossary

Centriole

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a eukaryotic organelle that acts as the basis for the formation of cilia and flagella. It consists of microtubules that are organized in a cylinder containing nine bundles of triplet microtubules.

Chromosome passenger complex (CPC)

a complex containing the proteins Aurora B, INCENP, Borealin, and Survivin, which localize to the inner centromere. The CPC regulates attachment of microtubules to chromosomes during mitosis.

γ-Tubulin ring complex (γ-TuRC)

the 2.2 MDa protein complex responsible for nucleating microtubules synergistically with XMAP215 in cells. In metazoans, the γ-TuRC is composed of five copies of γ-TuSC, additional GCP homologs, and other accessory proteins.

γ-Tubulin small complex (γ-TuSC)

a tetramer composed of two copies of γ-tubulin, one copy of γ-tubulin complex protein 2 (GCP2), and one copy of γ-tubulin complex protein 3 (GCP3).

Kinetochore (KT)

a complex of proteins assembled at the centromeres on chromosomes. The kinetochore forms the bridge between spindle microtubules and chromosomes, allowing the segregation of genetic material into daughter cells.

Kinetochore-fibers (K-fibers)

parallel bundles of microtubules and associated proteins that connect the kinetochore with the spindle poles.

Liquid-like spindle domain (LISD)

a liquid-like, condensed protein region containing microtubule nucleation and centrosomal proteins that promotes spindle assembly in mammalian oocytes.

Liquid–liquid phase separation (LLPS)

the process during which biomolecules undergo a phase transition to form a concentrated and biochemically distinct compartment, often in the form of liquid-like droplets.

Microtubule organizing centers (MTOCs)

cellular sites where microtubules are nucleated and then organized. Examples include the centrosome, the basal body, and the nuclear envelope, among others. New MTOCs are still being discovered.

MT asters

radial arrays of microtubules in which the microtubule minus ends are centrally anchored and the plus ends symmetrically extend outward.

Pericentriolar material (PCM)

a dense, organized matrix of proteins that surrounds the centrioles within the centrosome. During metaphase, the PCM is expanded and organized into protein layers, including γ-tubulin, which is responsible for nucleating new microtubules.

Spindle assembly checkpoint

a signaling pathway that monitors the attachment of kinetochores to the spindle, thus acting as a safety feature for chromosome segregation.

Spindle assembly factor (SAF)

a protein downstream of the GTP-loaded protein Ran, which contains a nuclear localization sequence via which the importin α/β heterodimer binds and thereby inhibits the SAF. Ran GTP releases the SAF from importin α/β and thereby initiates spindle assembly.

Spindle pole body (SPD)

the major microtubule organizing center in yeast, analogous to the centrosome in higher organisms.

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Box 1.

How to make kinetochore (KT) fibers via different microtubule (MT) nucleation pathways

A key element of the spindle is the KT fibers which connect each sister KT to opposite poles and which is responsible for physically segregating sister chromatids. KT fiber formation illustrates how multiple MT nucleation pathways come together to form specialized spindle structures. Studies performed in *Drosophila melanogaster* S2 cells and human cells showed that at the beginning of spindle assembly, short non-centrosomal MTs are nucleated near KTs. These MTs, nucleated at the KT, begin as lateral attachments that are then converted to end-on capture in a manner dependent on the kinesin motor, CENP-E. The K-fiber is eventually captured by its minus ends by astral MTs and transported to spindle poles via dynein. Recent cryo-ET reconstructions are consistent with this and describe an augmented search and capture model, where MTs incorporated into K-fibers originate from both centrosomal and non-centrosomal sites. Augmin-based amplification is also particularly convenient for K-fiber formation and, in fact, was shown to be important for K-fiber maturation. Branching factors targeted to KT MTs provide a spatial bias allowing for the rapid and directional generation of MT density towards KTs, thereby facilitating KT capture.

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Highlights

For a long time, it was unclear how the myriads of microtubules are generated in a spindle to orchestrate cell division.

In recent years, many microtubule nucleation pathways that generate spindle microtubules have been identified and shed light on this question.

Moreover, the γ-tubulin ring complex (γ-TuRC), once thought to be the cell's sole nucleator, is now known to work synergistically with the protein XMAP215/ch-TOG.

Recent high resolution cryogenic electron microscopy structures and *in vitro* single molecule studies of γ-TuRC provided clues towards its mode of action.

It is an emerging concept that biomolecular condensation plays a role in microtubule nucleation in the spindle.

The next challenge will be to determine how γ-TuRC is targeted to the right location in the spindle at the right time and subsequently turned on to generate the spindle framework.

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Outstanding questions

How does γ-TuRC change its conformation during the process of nucleation and how do these changes correspond to the execution of MT nucleation? How does this work together with factors that activate this reaction?

How do MTOCs affect the structure and function of γ-TuRC, the MT nucleation reaction, and, subsequently, MT organization?

How are MT nucleation pathways coordinated with one another in space and time to assemble the spindle? How does this differ between cell types and organisms?

How do physical properties such as LLPS and network rheology affect spindle assembly?

How do motors intersect with each MT nucleation pathway and how do they merge spindle MTs originating from different pathways in order to achieve the self-organization of the spindle?

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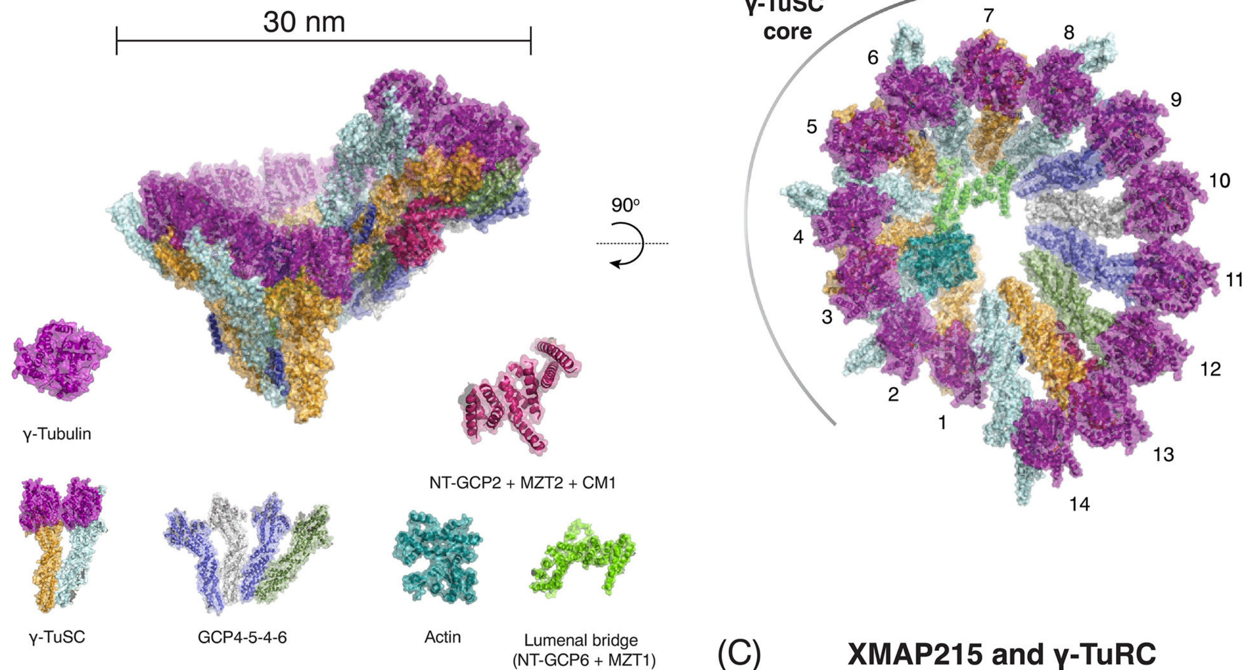

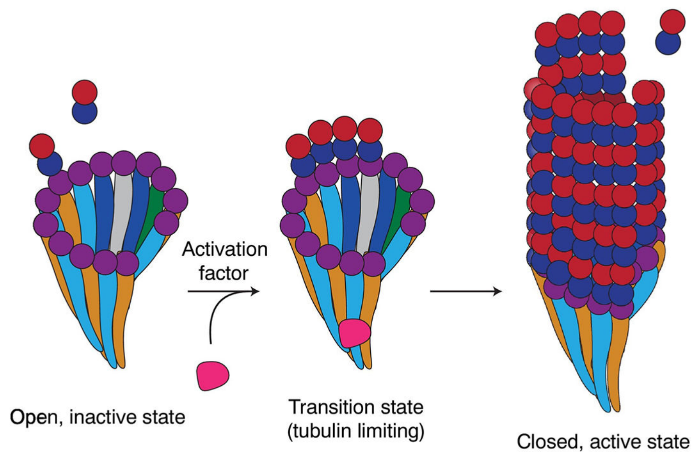

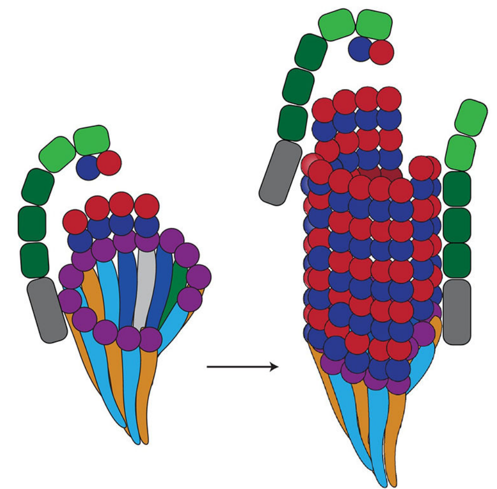

Figure 1. Microtubule (MT) nucleation by the γ-tubulin ring complex (γ-TuRC).
(A) Side view (left) and top down view (right) of the human γ-TuRC structure determined by cryogenic electron microscopy (cryo-EM) [25] PDB ID: 6V6S. In the human γ-TuRC structure, cryo-EM density has been assigned to 14 copies of γ-tubulin with a γ-TuSC core, GCPs 4–6, globular actin, and MZT 1/2. (B) γ-TuRC is in an open state and has been proposed to get activated and closed via γ-TuRC binding proteins (pink) and binding of tubulin dimers. (C) XMAP215 and γ-TuRC form the essential MT nucleation module, where XMAP215 binds to γ-TuRC via its C-terminal domain and facilitates binding of tubulin dimers through its TOG domains.

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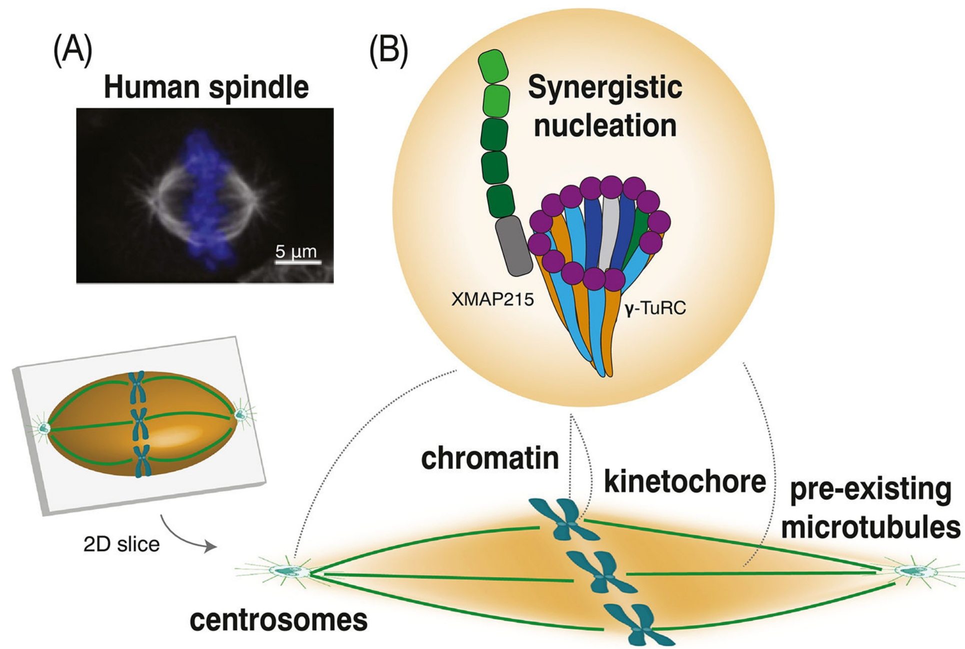

Figure 2. γ-Tubulin ring complex (γ-TuRC) and XMAP215 synergistically nucleate microtubules (MTs).

(A) A somatic spindle from human HeLa cells. MTs are shown in gray and DNA is shown in blue. Image reproduced, with permission, from Helmke, Heald, & Wilbur, *Int. Rev. Mol. Cell Biol.* 2013. (B) γ-TuRC and XMAP215 (top) are targeted to centrosomes, chromosomes, and the sides of pre-existing MTs within the spindle (bottom; shown as a 2D slice).

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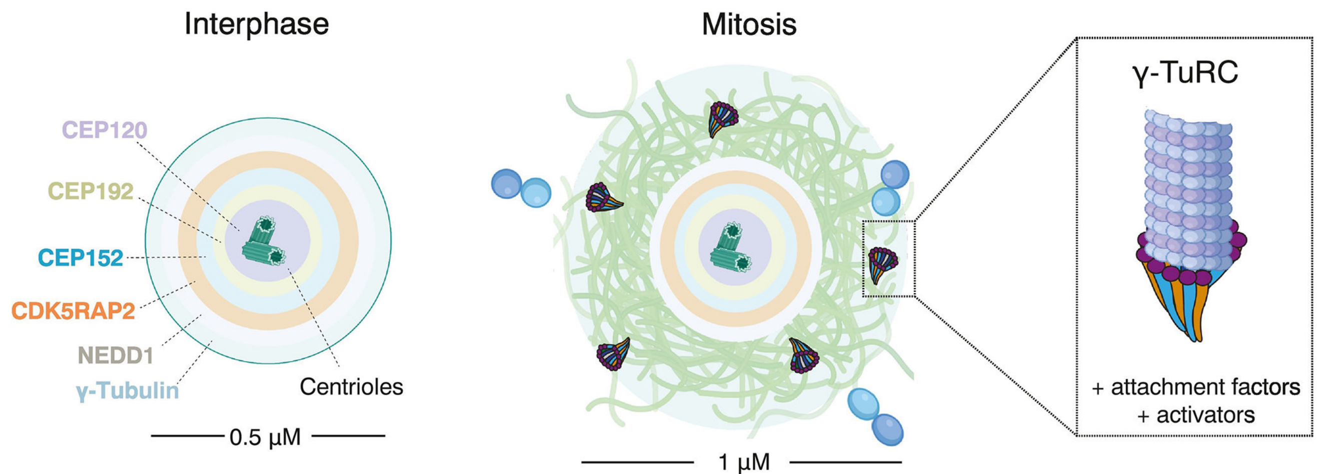

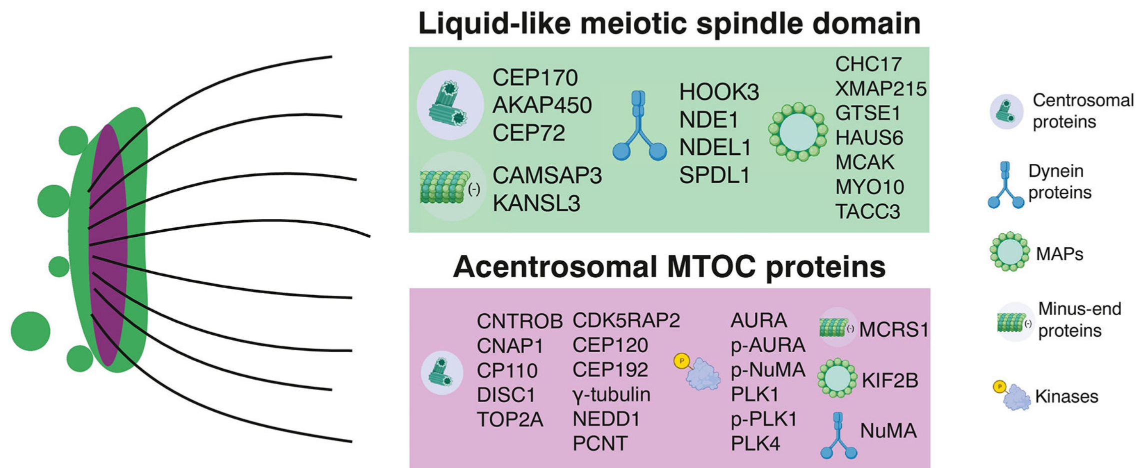

Figure 3. Spindle pole organization and microtubule nucleation in centrosomal (A) and acentrosomal spindles (B).

(A) Centrioles are surrounded by concentric layers of proteins during interphase (left). During mitosis, the pericentriolar material (PCM) undergoes maturation and expansion (middle), which recruits γ-tubulin and potently increases microtubule (MT) nucleation capacity via γ-tubulin ring complex (γ-TuRC) (right). (B) In acentrosomal spindles, poles contain many proteins that also localize at centrosomes (shown in purple). In human oocytes, a liquid-like meiotic spindle domain was discovered, which concentrates centrosomal proteins in a distinct phase with specific proteins (green). Abbreviations: MAP, microtubule-associated protein; MTOC, microtubule organizing center.

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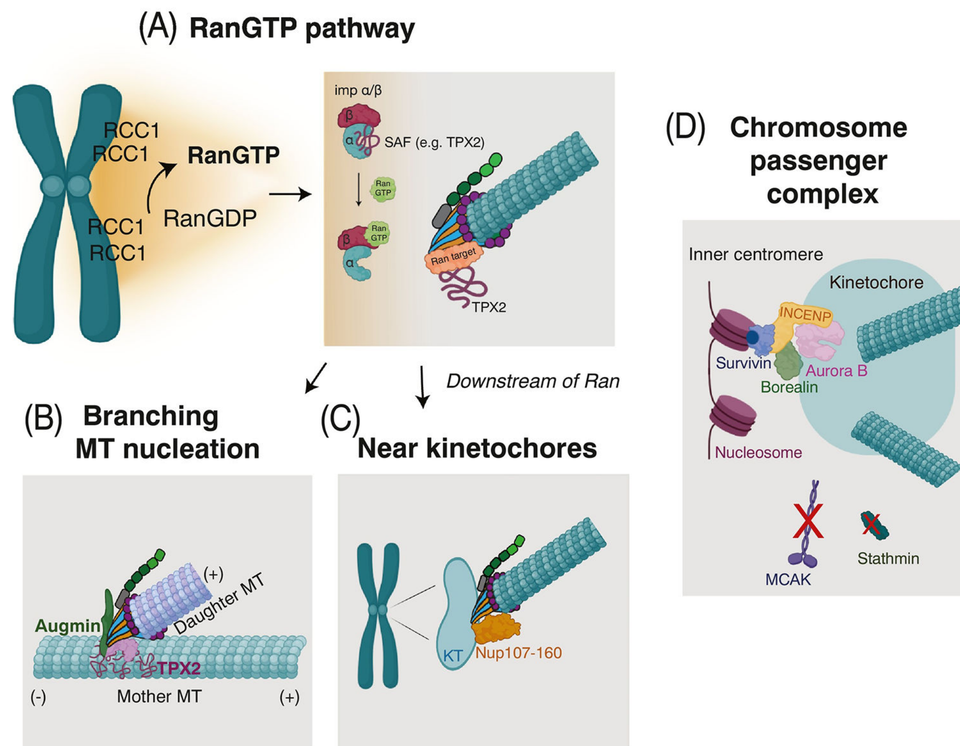

Figure 4. Chromatin-mediated microtubule (MT) nucleation pathways.

(A) RCC1 generates active Ran GTP near chromatin. Active Ran GTP binds to the importin α/β heterodimer and releases spindle assembly factors (SAFs), including TPX2, which can target γ-tubulin ring complex (γ-TuRC) for MT nucleation. (B) In *Xenopus laevis*, TPX2 localizes to pre-existing MTs and targets augmin and γ-TuRC to initiate the formation of branched MT networks. (C) The γ-TuRC nucleation module can be targeted to the kinetochore (KT) via the Nup107–160 complex in a manner dependent on the Ran pathway. (D) Independently of Ran, the chromosome passenger complex (CPC) at the KT promotes MT assembly by inhibition of MT destabilizers like MCAK and stathmin.

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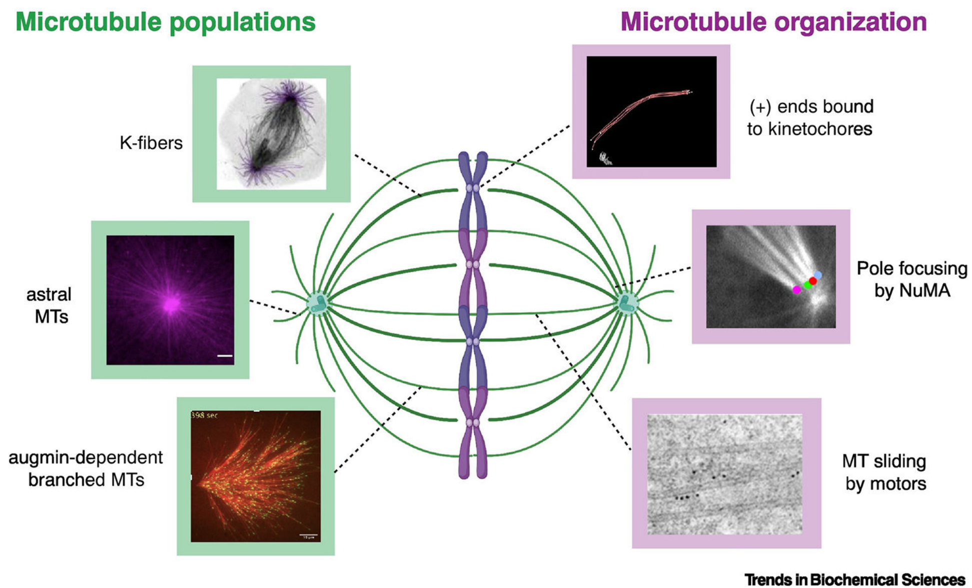

Figure 5. Organization of the mitotic spindle.

The metaphase spindle contains several distinct populations of microtubules (MTs, shown in green) that are nucleated synergistically by γ-tubulin ring complex (γ-TuRC)/XMAP215. The MTs are organized by various microtubule-associated proteins (MAPs), including motor proteins and crosslinkers, which coordinate MT polarity, and the formation of MT bundles, including kinetochore-fibers (K-fibers), which focus to spindle poles via NuMA and dynein motors as well as bridging fibers, which bridge sister K-fibers attaching to opposite spindle poles. These various activities are shown in purple. Images reprinted with permission from references [44] (astral MTs), [72] (K-fibers), [98] and [99] (plus ends bound to kinetochores), [110] (MT sliding by motors), and [115] (pole focusing).