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Mechanisms of spindle assembly and size control

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Abstract

The spindle is crucial for cell division by allowing the faithful segregation of replicated chromosomes to daughter cells. Proper segregation is ensured only if microtubules and hundreds of other associated factors interact to assemble this complex structure with the appropriate architecture and size. In this review, we describe the latest view of spindle organization as well as the molecular gradients and mechanisms underlying microtubule nucleation and spindle assembly. We then discuss the overlapping physical and molecular constraints that dictate spindle morphology, concluding with a focus on spindle size regulation.

Running title

Spindle assembly and size control

Keywords

Spindle, Mitosis, Meiosis, Microtubules, Spindle scaling.

Introduction

One process essential to life, from organism development and homeostasis to reproduction, is cell division, which allows the faithful segregation of duplicated genetic material from mother to daughter cells. Any dysfunction in this process can potentially lead to an abnormal number of chromosomes in daughter cells, called aneuploidy, and is associated with numerous chromosomal disorders and many pathologies in humans, such as cancers (Chunduri and Storchová, 2019). To ensure the accurate segregation of chromosomes in both meiosis and mitosis, the spindle, a dynamic structure mainly composed of microtubules (MTs), is assembled. This structure provides the necessary forces to align the chromosomes at its center, known as the metaphase plate, and then physically separate the sister chromatids to each daughter cell during anaphase.

Although this MT-based assembly was first described at the end of the 19th century by pioneers in mitosis research, in particular Walther Flemming (Flemming, 1882), the precise mechanisms of spindle assembly and chromosome segregation are still not fully understood. In fact, these questions remain very challenging due to the complexity of this structure which is composed of thousands of MTs and hundreds of proteins (Sauer *et al.*, 2005) or non-protein factors (Chang *et al.*, 2004; Groen *et al.*, 2011) that can assemble together through various mechanisms and pathways. The substructure of the spindle is in constant flux (Mitchison, 1989; Rogers *et al.*, 2005) due to the dynamic instability of MTs (Mitchison and Kirschner, 1984), which cycle through phases of polymerization and depolymerization through addition and loss of tubulin subunits at their ends, stochastically undergoing transitions between growth and shrinkage (catastrophe) and shrinkage to growth (rescue) (Walker *et al.*, 1988). In addition, a variety of motor proteins translocate MTs within the spindle (Yang *et al.*, 2008; Gatlin and Bloom, 2010). Notably, despite its universal function in eukaryotes, spindle size and morphology vary dramatically across species as well as during the development of an organism (Crowder *et al.*, 2015).

Studies over the past few decades have provided new insight into some of the key conserved mechanisms regulating spindle size and architecture. In this review, we first describe the main pathways responsible for spindle MT nucleation and assembly, focusing on activity gradients and mechanisms of MT amplification. Next, we discuss the physical properties and constraints that influence metaphase spindle size. Finally, we address the role of specific molecular actors that have recently emerged as key regulators of spindle size and morphology in metazoans.

Spindle microtubule organization

During interphase, MTs, as part of the cytoskeleton, play a structural role and serve in particular as tracks for vesicles and organelle transport. When the cell transitions into mitosis, the MT network is completely disassembled and spindle MTs are generated *de novo* (Zhai *et al.*, 1996; Petry, 2016). Although MTs are the main component of spindles, not all MTs are equal in terms of function within the spindle and three main populations can be distinguished: astral MTs, k-fibers, and non-kinetochore MTs (Figure 1).

Astral MTs, found at the spindle poles, were first described as “astral rays” (Inoué and Sato, 2008) that project filaments from the spindle poles toward the plasma membrane. By interacting with molecular factors at the cell cortex, they provide the necessary forces to orient and position the spindle in dividing cells (Pietro *et al.*, 2016). Even though astral MTs were shown to be important for spindle orientation and asymmetric cell division (Siller and Doe, 2009), they are absent or not long enough to extend from pole to cell membrane in cells without centrosomes or in very large embryonic cells, respectively. In such cases, other players, such as for instance acentriolar MT organizing centers or the actin network, function to position the spindle (Moorhouse and Burgess, 2014; Bennabi *et al.*, 2016; Mogessie *et al.*, 2018). Bundles of MTs, called k-fibers, connect the spindle to the chromosomes through a macromolecular complex termed the kinetochore (Cheeseman and Desai, 2008), which assembles at the centromere of each sister chromatid at the onset of mitosis. K-fibers connect sister chromatids to opposite spindle poles and drive the alignment of chromosomes at the metaphase plate. These fibers are the most stable population of MTs in the spindle, are resistant to low temperature-induced depolymerization and possess distinct post-translational modifications of their tubulin subunits (Janke and Bulinski, 2011; Barisic *et al.*, 2015). Of note, it has been shown that the formation of a spindle composed only of k-fibers could be induced using a gentle method of cooling (Rieder, 1981). Interestingly, non-kinetochore MTs linking sister chromatids, called bridging fibers, were recently discovered in HeLa and PtK1 cells (Kajtez *et al.*, 2016). Using laser-cutting, the authors demonstrated that these fibers contribute to the tension between sister kinetochores, and that their thickness defines the forces at the spindle poles and kinetochores. The third category of MTs structuring the spindle is generally termed non-kinetochore MTs, and can be further classified into polar and interpolar MTs. Whereas polar MTs are short and present only within half the spindle, interpolar MTs bind to MTs extending from the opposite pole in an antiparallel manner to form overlapping bundles (Brinkley and Cartwright, 1971). In addition, interpolar MTs have

been demonstrated to play a key role in the spindle elongation that occurs in late anaphase as MTs grow and slide along each other (Masuda *et al.*, 1990; Scholey *et al.*, 2016).

Within the spindle, these populations are intertwined and cooperate to ensure that the spindle is fully functional. Nonetheless, this complex assembly is only possible thanks to the cooperative and coordinated action of MT molecular motors (Figure 1, insets 1-4). While plus-end-directed antiparallel cross-linking motors, in particular the Kinesin-5 Eg5, sort MTs into antiparallel arrays and maintain the association of interpolar MTs, minus-end-directed cross linking motors, including the Kinesin-14 HSET/XCTK2 and Dynein, act to focus of MTs into spindle poles (Walczak *et al.*, 1998; Walczak and Heald, 2008; Ems-McClung *et al.*, 2020). In addition, CENP-E at kinetochores, and chromokinesins on chromosome arms, walk along stable detyrosinated MTs of k-fibers to move chromosomes and maintain their position at the metaphase plate (Barisic *et al.*, 2015). Strikingly, the relative proportions of MTs within each subpopulation varies among cell type and species, but the fact that all three populations are present in most species suggests a strong evolutionary constraint on overall spindle architecture (Crowder *et al.*, 2015). One possible mechanism to explain the differing proportions of MT subpopulations observed in varying cell types could be related to changes in the pathways of MT nucleation.

Mechanisms of spindle microtubule nucleation

The first and best studied MT organizing center is the centrosome. This organelle is comprised of two centrioles surrounded by an amorphous mass of proteins termed pericentriolar material (PCM) (Urbani and Stearns, 1999). One important component of the PCM is γ -tubulin, which, together with additional subunits named gamma-tubulin complex proteins (GCPs), form a macromolecular assembly called the γ -tubulin ring complex (γ -TuRC). The γ -TuRC acts as an active MT-nucleator, capping, stabilizing and anchoring the minus-ends to the centrosome (Zheng *et al.*, 1995). With the discovery of MT dynamic instability, Kirschner and Mitchison proposed in 1986 a model for spindle assembly known as “search-and-capture” (Kirschner and Mitchison, 1986). This model suggested that centrosome-nucleated MTs probe space within the cytoplasm until they are captured and stabilized by one of the kinetochores on a chromosome. Thus, chromosomes are firstly captured by only one pole, and are thus “mono-oriented”. Then, when the opposite kinetochore captures MTs from the opposite pole, they become “bi-oriented”. Search-and-capture was actually directly visualized in live newt lung cells only four years after its

formulation (Hayden *et al.*, 1990; Rieder and Alexander, 1990). This mechanism relies on dynamic instability, without which the search time would be too long compared to the time it takes a spindle to assemble (Holy and Leibler, 1994). However, because the density of MT ends decreases as the distance between the centrosome and chromosomes increases, search-and-capture of 46 chromosomes in human cells would take much longer than the observed duration of prometaphase, if not biased towards chromosomes (Wollman *et al.*, 2005). This observation implied the existence of mechanisms facilitating the encounter between microtubules and kinetochores to promote spindle assembly.

With the development of cell-free *Xenopus* egg extracts (Lohka and Masui, 1983), spindle assembly could be observed in an in vitro system through the addition of demembrated *Xenopus* sperm nuclei (Lohka and Maller, 1985). This system was then used to discover and study MT nucleation pathways and mechanisms of spindle assembly in a vertebrate model. In this system, addition of plasmid DNA-coated beads lacking both centrosomes and kinetochores was sufficient to form bipolar spindles (Heald *et al.*, 1996). This was consistent with the previous observation that injection of viral DNA into a frog egg promoted microtubule assembly (Karsenti *et al.*, 1984). These studies thus revealed the existence of mechanisms allowing for the nucleation of MTs in the vicinity of the chromosomes. By comparing spindles assembled around sperm nuclei containing centrosomes with those assembled around chromatin beads, Heald and colleagues suggested that, while centrosomes are dispensable for spindle assembly, they regulate the organization of spindle MTs by remaining an important site for spindle pole formation (Heald *et al.*, 1997). Assembly of acentrosomal spindle was described as early as 1972 in mouse oocytes (Szollosi *et al.*, 1972). In *Drosophila*, spindle assembly was also observed in meiotic female cells in which centrosomes are absent (Theurkauf and Hawley, 1992). Using laser microsurgery to destroy centrosomes in CVG-2 cells (green monkey fibroblasts), Khodjakov and colleagues revealed that spindles were able to self-assemble in the absence of centrosome (Khodjakov *et al.*, 2000). It was moreover demonstrated that cell division and development progressed without defect in fly embryos that contained no centrosomes (Basto *et al.*, 2006). Therefore, chromatin-derived MT nucleation is central to spindle assembly, particularly in the absence of centrosomes, and facilitates search-and-capture (Heald and Khodjakov, 2015). Furthermore, acentrosomal spindle assembly highlights the role of MT motors in driving spindle self-organization and bipolarity (Walczak *et al.*, 1998; Gatlin and Bloom, 2010) (Figure 1).

Several factors are involved in chromosome-dependent MT nucleation and spindle assembly, but one of the key proteins is the small guanosine triphosphatase (GTPase) Ran. While its role in the nuclear import and export machinery is well established (Görllich, 1997), Ran has been demonstrated over the last 20 years to be a central actor in spindle assembly. Ran activity depends on its nucleotide-binding state (GDP or GTP). It is regulated by the GTPase activating protein (GAP) RanGAP1 in the cytoplasm, and by the guanine nucleotide exchange factor (GEF) RCC1, which is bound to chromatin. RanBP1 (a guanine nucleotide dissociation inhibitor) acts as a cofactor for RanGAP1 by increasing its GTP hydrolysis activity. Kalab, Pu and Dasso showed that spindle assembly is disrupted by the addition of RanBP1 to *Xenopus* egg extract, showing a role of Ran in spindle assembly, independently of its role as a nuclear transporter (Kalab *et al.*, 1999). Concomitantly, other studies using egg extracts led to the proposition that RCC1 generates a high concentration of RanGTP around chromosomes (Carazo-Salas *et al.*, 1999), which acts as a mechanism for chromosome-dependent stimulation of MT nucleation and spindle assembly (Wilde and Zheng, 1999). This hypothesis was then confirmed by the direct visualization of a RCC1 derived RanGTP gradient surrounding chromosomes throughout the cell cycle (Kalab *et al.*, 2002). RCC1 binds directly to histones H2A and H2B of the nucleosomes that form chromatin. This interaction was suggested to stimulate the activity of RCC1, and thus to establish the polarity of the RanGTP gradient (Nemergut *et al.*, 2001). Remarkably, RCC1-coated beads were sufficient to promote bipolar spindle assembly in *Xenopus* egg extracts without chromatin or kinetochores (Halpin *et al.*, 2011).

The mechanism by which RanGTP promotes MT nucleation at the proximity of chromosomes involves the release of various spindle assembly factors (SAFs) from sequestration by importins α and β . RanGTP was shown to act as a spindle assembly stimulator by increasing the rescue frequency of MTs, and by altering the balance of motor activities (Wilde *et al.*, 2001). To date, more than twenty SAFs have been shown to be regulated by the RanGTP gradient and are listed elsewhere (Cavazza and Vernos, 2016). One example of RanGTP regulated SAFs is the spindle MT cross-linking kinesin XCTK2, which requires a RanGTP gradient for proper localization and motility (Weaver *et al.*, 2015). Another important SAF regulated by RanGTP is TPX2, which will be discussed later in more detail. TPX2 is inactivated by importin α and is required for RanGTP activity and chromosome-dependent spindle self-assembly (Gruss *et al.*, 2001). TPX2 is also involved in a process called “MT amplification” that is crucial for the generation of the high density of MTs within the spindle (Figure 1, inset 5) and which is controlled by the Ran gradient (Petry

et al., 2013). By employing a genome-wide RNAi screen in *Drosophila* S2 cells for spindle morphology (Goshima *et al.*, 2007), Augmin was identified as a protein complex involved in the recruitment of γ -TuRC to the side of pre-existing MTs and required for centrosome-independent MT nucleation within the spindle (Goshima *et al.*, 2008). However, due to the high MT density within the spindle, branching MT nucleation could not be observed. It is only when total internal reflection (TIRF) microscopy was used in *Xenopus* egg extract that MT branching could be observed for the first time. These experiments showed that MTs grow at a low branch angle that conserves the polarity of the MT from which it is branching, forming parallel MT structures (Petry *et al.*, 2013). More recently, it was demonstrated that TPX2 is first deposited on pre-existing MTs, followed by Augmin and γ -TuRC, which then nucleates branching MTs (Thawani *et al.*, 2019).

In addition to RanGTP, another factor, whose localized activity functions in a gradient to regulate spindle assembly, is Aurora B (Xu *et al.*, 2010). This kinase is part of the Chromosome Passenger Complex (CPC), which is required for chromatin-dependent MT stabilization and spindle assembly (Sampath *et al.*, 2004). While RCC1 localizes throughout the chromatin, the CPC is concentrated at the inner centromere, and is thus responsible for the generation of most of the MTs near the kinetochores. This complex is also composed of INCENP, survivin, and Borealin/DasraA (Kelly *et al.*, 2007). Aurora B locally phosphorylates and inactivates MT-destabilizing proteins including the kinesin-13/MCAK (Andrews *et al.*, 2004; Lan *et al.*, 2004; Ohi *et al.*, 2004) and the heat-stable oncoprotein Stathmin/Op18 (Gadea and Ruderman, 2006). The importance of the CPC for spindle assembly in the absence of centrosomes was first demonstrated in *Drosophila* female meiosis (Colombié *et al.*, 2008). Using *Xenopus* egg extracts in the absence of RanGTP-gradient, the activity of the CPC was shown to be sufficient to assemble bipolar spindles (Maresca *et al.*, 2009). These two gradients are thus essential for bipolar spindle formation and originate from mitotic chromatin in the case of Ran, or from centromeric chromatin in the case of CPC. While these pathways seem to be distinct (Sampath *et al.*, 2004; Maresca *et al.*, 2009), relationships exist between the two. For instance, RCC1 acts at the kinetochore to modulate the spindle assembly checkpoint (Arnautov and Dasso, 2003), where it regulates the metaphase-anaphase transition through the displacement of Sgo1 and CPC (Zhang *et al.*, 2018). In addition, the stabilization of k-fiber minus-ends is ensured by a Ran-GTP-dependent mechanism (Meunier and Vernos, 2011) and Ran-GTP is crucial for the maintenance of stable kinetochore-microtubule attachments through the regulation of Aurora

B (Lee *et al.*, 2012). It is also interesting to note that RanBP2 is a regulator of Borealin sumoylation, a modification peaking in early mitosis (Klein *et al.*, 2009).

Physical constraints influencing spindle size

While there have been numerous studies regarding mechanisms of spindle assembly, it is only recently that the range of spindle sizes and morphologies and their regulation have begun to be appreciated. By performing a comparative analysis of spindle morphometrics across metazoans, Crowder *et al.* revealed a linear scaling of spindle size to embryonic cell size conserved across phyla (Crowder *et al.*, 2015). Interestingly, they also revealed a moderate contribution of genome size to spindle size. One interesting question is whether the scaling of spindle size to cell and genome size is the consequence of direct physical constraints, or is due to evolutionary driven molecular adaptations.

In the 1980's, Nicklas *et al.* demonstrated for the first time a direct relationship between chromosome number and spindle length (Nicklas and Gordon, 1985). Using micromanipulation and electron microscopy on spermatocytes from *Melanoplus differentialis*, they showed that the number of chromosomes determines spindle length, as the one by one removal of chromosomes resulted in a gradual decrease in spindle length, until it reached ~40% of its original size after all chromosomes were removed. Consistent with a scaling relationship between genome and spindle size, a strong correlation between organism, cell, spindle, and genome size exists in two *Xenopus* species. *X. laevis* possesses an allotetraploid genome and a large spindle, while *X. tropicalis* has a diploid genome and a smaller spindle. Using chromatin-coated beads with a controlled quantity of DNA per bead and *Xenopus* egg extract to assemble spindles, it was shown that the chromatin mass influences in a direct manner the size of the spindle, but only minimally with a size increase of only 10% despite a 400% increase in the amount of DNA (Dinarina *et al.*, 2009). Consistent with this observation, it was found that *X. laevis* spindles assembled around *X. tropicalis* chromosomes (half the genome size) were only 10% shorter in length (Brown *et al.*, 2007). Similarly, by generating haploid *X. laevis* embryos, it was shown that mitotic spindle size can be reduced by about only 10% by halving the amount of DNA in embryos (Wühr *et al.*, 2008). Finally, in *C. elegans*, spindle morphology adjusts to ploidy, with the exception that an increase in ploidy is accommodated by an increase in spindle width rather than length (Hara and Kimura, 2013). Therefore, while genome size seems to directly influence spindle size, its contribution is quite

limited, is not linear, and does not explain the total spindle length differences between species.

Another physical factor that might contribute to spindle scaling is cell size. Using microfluidic technology to encapsulate cytoplasm in defined volumes and geometries, it was demonstrated that varying the cytoplasmic volume is sufficient to change spindle length, and to mimic the scaling observed in *Xenopus* cells during development (Good *et al.*, 2013; Hazel *et al.*, 2013). Notably, these experiments also reproduced the upper size limit observed earlier in *Xenopus* (Wühr *et al.*, 2008) and in other metazoans (Crowder *et al.*, 2015), so that the spindle starts to scale only when compartment size is reduced to ~140 μm in diameter. These studies confirmed the existence of two regimes of spindle scaling to cell size: one in which spindle size is physically coupled to cell diameter, and a second in which they are uncoupled. To explain the coupling of spindle size to cell size, Good *et al.* proposed the limiting component model: by reducing cytoplasmic volume, one component becomes quantitatively limiting to form the spindle, leading to a decrease in spindle size (Good *et al.*, 2013). A previous report suggested that the amount of available tubulin determines spindle length (Sluder, 1976). Good *et al.* tested tubulin as a possible limiting factor but supplementing the extract with brain tubulin did not alter spindle scaling in droplets, which led to the suggestion that the levels of several factors could be limiting for spindle assembly (Good *et al.*, 2013). Interestingly, experimental addition of the MT polymerase XMAP215 (factor discussed below) to *Xenopus* egg extracts and embryos was enough to increase spindle size increase (Reber *et al.*, 2013; Milunovic-Jevtic *et al.*, 2018). This suggests that while other spindle components, such as tubulin, are not limiting, XMAP215 might be. This is actually interesting to note that MT growth velocity was shown to correlate with embryonic mitotic spindle length in *C. elegans* and sea urchin. In these systems, MT growth speed decreased with decreasing cell size, supporting a model in which spindle size could be set by a limiting MT polymerase (Lacroix *et al.*, 2018). In apparent contradiction with the idea of tubulin not being limiting, it was recently shown that addition of *Xenopus* tubulin, but not brain tubulin, is able to increase spindle size and mass in egg extract (Hirst *et al.*, 2020).

While physical constraints appear to contribute to spindle scaling, it has become clear that these mechanisms are superimposed upon molecular controls. Indeed, different spindle scaling was observed in droplets of the same size with different stage *X. laevis* embryo extracts (early vs later stages), revealing that cytoplasmic composition also contributes to spindle scaling (Good *et al.*, 2013). Helping to explain this phenomenon, importin α has recently been shown to be a sensor of the surface area and volume of the cell in order to

regulate organelle size scaling, including the spindle, by modulating the activity of regulator proteins through the interaction with their nuclear localization sequences (Brownlee and Heald, 2019).

Molecular control of spindle length through modulation of MT dynamics, transport and nucleation

Interest in spindle length regulation started gaining traction through multiple studies in the early 2000s. With the first systematic evaluation of MT dynamics factors on metaphase spindle length control performed in a *Drosophila* S2 cell line, Goshima and colleagues showed that spindle length was sensitive to alterations in MT dynamics with a decrease in length with the depletion of MT stabilizers (EB1, Msps/chTOG, Mast/CLASP) or an increase in length with the depletion of depolymerizers (Kinesin-13 Klp10A, Kinesin-8 Klp67A) as well as opposite effects through the overexpression of these factors (Goshima *et al.*, 2005) (Figure 2A). Using computer simulations, more recent studies clearly exemplified that modulating MT dynamics is sufficient to control spindle length and to recapitulate observed variations (Loughlin *et al.*, 2010; Lacroix *et al.*, 2018; Hirst *et al.*, 2020). One interesting example of MT regulators controlling spindle size is XMAP215/chTOG. XMAP215 was first described as a *Xenopus* MT-Associated Protein (XMAP) that promotes an increase in the MT growth rate *in vitro* (Gard and Kirschner, 1987), and then, more precisely, as a processive polymerase able to directly add up to 25 tubulin dimers at a time to the MT plus-end (Brouhard *et al.*, 2008). Moreover, it was shown in *Xenopus* egg extracts to control spindle length, with increasing MT growth velocity being linearly correlated with an increase in spindle length and total MT spindle mass (Reber *et al.*, 2013). However, because MT density, lifetime and spindle shape remained unchanged, the authors proposed the idea that spindle size is determined by a mass balance, whereas shape is determined by a force balance (Reber *et al.*, 2013). Consistent with this work in egg extract, microinjection of XMAP215 in *X. laevis* embryo cells demonstrated that it is sufficient to induce larger spindles in cells, confirming its role in spindle length control (Milunovic-Jevtic *et al.*, 2018). Yet, this MT-polymerizer does not seem to act alone. Indeed, its interaction with EB1 (end-binding protein 1) appears to be necessary for spindle organization as its disruption results in shorter and multipolar spindles, with reduced MT density, as well as failure in chromosome segregation (Kronja *et al.*, 2009). Other factors that stabilize MTs were shown to participate to spindle length control. This is the case of the chromokinesin Kid that bundles and stabilizes MTs

(Tokai-Nishizumi *et al.*, 2005; Bisht *et al.*, 2019) and of other factors of the TOG-like domain containing-protein family CLASP/Mast/Orbit that act at the MT plus-end to stabilize them (Maiato *et al.*, 2005; Hannak and Heald, 2006; Laycock *et al.*, 2006; Reis *et al.*, 2009; Espiritu *et al.*, 2012; Young *et al.*, 2014), both of these families being in fact primarily important for spindle integrity. In addition, MT depolymerases or destabilizers, such as the Kinesin-13 MCAK (Mitchison *et al.*, 2005), the MT destabilizing protein Stathmin/Op18 (Budde *et al.*, 2001), and of various MT depolymerases of the Kinesin-8 family (Savoian and Glover, 2010; Wang *et al.*, 2010; Stout *et al.*, 2011; Weaver *et al.*, 2011) were also shown to be involved in spindle length control in particular in *Xenopus* and invertebrate systems.

Another important mechanism through which spindle size can be adjusted is MT translocation and sliding by molecular motors (Figure 2B). Several studies in yeast and drosophila revealed the role of antagonistic MT sliding mechanisms of in particular Kinesins -5 and -14 in controlling spindle length (Saunders *et al.*, 1997; Goshima *et al.*, 2005; Brust-Mascher *et al.*, 2009; Goshima and Scholey, 2010; Syrovatkina *et al.*, 2013). In *Xenopus*, the minus end-directed Kinesin-14 motor XCTK2 was shown to control metaphase spindle length (Cai *et al.*, 2009). This motor cross-links and slides both parallel and antiparallel MTs (Tao *et al.*, 2006; Hentrich and Surrey, 2010) and contributes to proper spindle assembly (Walczak *et al.*, 1997, 1998). XCTK2 MT binding is regulated by the Ran-GTP gradient through the association of importin α/β with a bipartite nuclear localization sequence located in the tail of XCTK2 (Ems-mcclung *et al.*, 2004). This association allows the Ran-GTP gradient to spatially coordinate the motility and the localization of XCTK2 within the spindle (Weaver *et al.*, 2015). In addition, while XTCK2 is able to cross-link both parallel and antiparallel MTs, the Ran-GTP gradient generates an effector gradient of XCTK2 that leads to the preferential parallel MT cross-linking and sliding near the spindle poles, thus participating to their focusing (Ems-McClung *et al.*, 2020). Interestingly, the cross-linking and sliding activities of HSET/XTCK2 are required to regulate spindle length and the Ran-GTP regulation of these activities is important for proper spindle morphology (Cai *et al.*, 2009). This led the authors to propose that the Kinesin-14 motors would cross-link and transport parallel MTs in a half spindle, not only to facilitate pole focusing, but also for spindle elongation.

Finally, recent studies suggest that MT nucleation may also regulate spindle length with a key role of the TPX2 protein (Figure 2C). In *C. elegans*, centrosome size was shown to scale with spindle size. While this was independent of γ -tubulin levels, centrosome size determined the length of a TPXL-1 (TPX2-Like-1) gradient along MTs, resulting in the

control of spindle length (Greenan *et al.*, 2010). TPX2 was discovered as being responsible for the localization of Xklp2, a plus-end-directed kinesin-like needed for centrosome separation at the onset of spindle formation (Wittmann *et al.*, 1998). The interest in TPX2 (targeting protein for Xklp2) increased with the discovery of its role in the organization of spindle poles (Wittmann *et al.*, 2000). Recent findings highlighted TPX2 as a determinant of spindle shape in murine neural stem cells, contributing to robustness and correct chromosome segregation (Vargas-Hurtado *et al.*, 2019). In HeLa cells, TPX2 is essential for the formation of a stable bipolar chromosome-induced spindle (Gruss *et al.*, 2002), and its reduced expression by siRNA leads to multipolar spindles and fragmented centrosomes (Garrett *et al.*, 2002). Notably, TPX2 has been shown in this system to bind to CLASP and the kinesin-13 Kif2a, what appears to control spindle length (Fu *et al.*, 2015). Importantly, TPX2 functions in spindle size control through a central role in MT nucleation from pre-existing MTs via the augmin pathway and this nucleation potential is increased by Ran-GTP (Petry *et al.*, 2013; Alfaro-Aco *et al.*, 2017; Thawani *et al.*, 2019). It was recently shown that in *Xenopus* egg extract the upper spindle size limit is determined by the spatial regulation of this MT-stimulated MT nucleation (Decker *et al.*, 2018). Yet, while TPX2 is regulated by Ran-GTP, it was shown that the interaction between SAFs and microtubules leads to a feedback mechanism that amplifies spindle assembly factors to make spindle size independent of the RanGTP gradient (Oh *et al.*, 2016).

Molecular mechanisms of spindle size scaling in *Xenopus*

MT dynamics, transport and nucleation are all accepted to be potentially involved in spindle scaling. *Xenopus* frogs have been instrumental over the past 10 years in investigating mechanisms of interspecies and developmental scaling. These studies revealed mechanisms of vertebrate spindle scaling through the regulation of MT dynamics by the severing factor Katanin (Loughlin *et al.*, 2010, 2011) and the depolymerizing kinesin Kif2a (Wilbur and Heald, 2013; Brownlee and Heald, 2019; Miller *et al.*, 2019) as well as regulation of MT nucleation and translocation by a TPX2/Eg5 module (Helmke and Heald, 2014) (Figure 3).

Katanin was first described as an enzyme that uses the energy of ATP hydrolysis to sever MTs by breaking tubulin-tubulin bonds (McNally and Vale, 1993). Subsequent studies have revealed its role to be more complex, and Katanin has proposed to regulate the density of MTs by increasing their number and decreasing their length, as well as by increasing the number of non-centrosomal MTs through its severing activity (Buster *et al.*, 2002). In *C. elegans* meiotic cells, katanin was shown to convert long MTs into shorter ones near meiotic

chromosomes, while a decrease in katanin in mutant cells inhibited the self-organization of MTs into a bipolar spindle (Srayko *et al.*, 2006). A role for katanin in interspecies spindle scaling was revealed by Loughlin *et al.*, who found that an inhibitory Aurora-B phosphorylation site in the catalytic subunit contributes to the spindle size difference observed between two *Xenopus* species. Indeed, this site is present in *X. laevis*, while absent from *X. tropicalis*. By combining in silico simulations with inhibition of katanin in egg extracts, they concluded that increased katanin activity in *X. tropicalis* results in a shorter spindle than in *X. laevis* (Loughlin *et al.*, 2010, 2011).

Kif2a is a kinesin-13 MT-depolymerizing protein that interacts with MTs. Its activity results in shorter MTs, and is inhibited by importin α binding (Wilbur and Heald, 2013). During development, with decreasing cell size directly leading to an increased surface area to cell volume ratio, importin α membrane sequestration increases, resulting in the release of Kif2a inhibition and thus smaller spindles. This process was shown to regulate the developmental scaling of *X. laevis* spindles and nuclei as well as spindle and nuclear scaling in somatic human cells (Brownlee and Heald, 2019). Interestingly, Kif2a was also shown to participate in spindle scaling in a distantly related Pipid frog, *Hymenochirus boettgeri*. In this species, Kif2a possesses a Plk1 phosphorylation site, not present in *X. laevis*, which allows its activation (Miller *et al.*, 2019), thus participating in interspecies scaling. Moreover, kif2a has also been shown to be involved in spindle self-organization by being spatially-regulated by the Aurora B gradient (Uehara *et al.*, 2013), and to be targeted to spindle poles by dynein/dynactin to promote local MT depolymerization and spindle length regulation (Gaetz and Kapoor, 2004).

Remarkably, in addition to such molecular controls of MT dynamics that regulate spindle size, a recent study revealed that differences in intrinsic dynamic properties of tubulin itself contribute to spindle length difference between *X. laevis* and *X. tropicalis* (Hirst *et al.*, 2020).

In addition to its role in MT nucleation, the TPX2 protein interacts with Aurora A by its N-terminal domain (Brunet *et al.*, 2004) and with the kinesin-5 Eg5 with its C-terminal domain (Eckerdt *et al.*, 2008). Bacterial Artificial Chromosome (BAC) technology (Kittler *et al.*, 2005) has been used to investigate the interaction between these factors by generating TPX2 lacking its Eg5 interacting domain. This work showed that this interaction is involved in k-fiber formation and spindle organization, but more importantly, that TPX2 localizes Eg5 to spindle poles and regulates its activity in MT sliding (Ma *et al.*, 2011). In fact, Eg5 was first described in *Aspergillus nidulans* as a kinesin-like involved in nuclear division (Enos

and Morris, 1990). While its role in MT sliding at the spindle midzone is well known (Kapitein *et al.*, 2005), its involvement in spindle architecture is still being investigated. Spindle flux, which corresponds to the continuous poleward movement of tubulin, relies on the constant addition of tubulin subunits at microtubule plus ends and their corresponding removal from microtubule minus-ends at spindle poles as well as MT sliding towards the poles. This latter aspect is thought to be regulated by Eg5 since its inhibition or its depletion result in decreasing flux rates (Miyamoto *et al.*, 2004). Interestingly, careful analysis of the flux velocity revealed that it decreases by about 20% near the poles and that the inhibition of either or both Eg5 and dynein/dynactin leads to the suppression of this slow-down (Yang *et al.*, 2008). It was recently proposed that a specific array of MTs in the mid half-spindle possesses less mechanical rigidity than elsewhere, behaving in a fluid-like manner, and that this heterogeneity relies upon Eg5 and dynein (Takagi *et al.*, 2019). Importantly, a TPX2/Eg5 interaction was shown to be involved in controlling spindle length and architecture and responsible for the differences observed between *X. laevis* and *X. tropicalis* spindles. Indeed, Helmke and Heald found that TPX2 levels were three times higher in *X. tropicalis* cytoplasm compared to *X. laevis*. Sufficiently, addition of TPX2 in *X. laevis* extracts reduced spindle length by about 20% and relocalized Eg5 to the spindle poles similar to *X. tropicalis* spindles. Interestingly, TPX2 contains a stretch of 7 amino acids only present in *X. laevis*, and while its deletion in *X. laevis* increases MT nucleation, its insertion in *X. tropicalis* decreases MT nucleation (Helmke and Heald, 2014). Therefore, TPX2 appears to be a key factor which modulates MT nucleation, organization, and spindle assembly, the total sum of which is used to scale spindle size.

Conclusion

One hundred and forty years after the first description of mitosis, we are finally beginning to obtain a clearer picture of the organization of the spindle and are even approaching a mechanistic understanding of the establishment of spindle architecture. The study of the spindle at the molecular level benefited greatly from the development of new molecular and imaging technologies during the past few decades. In particular, *Xenopus* extract systems have increasingly been instrumental in discovering mechanisms of spindle assembly, while recent interspecies studies have allowed for a deeper understanding of spindle architecture variations. Yet, it remains to be assessed what molecular players are universally at work or specific to a particular species or cell. Moreover, in addition to responding to numerous molecular factors, it becomes clear that spindle assembly also responds to genome and cell

size to scale and optimize its function to a particular cellular environment. Overall, as we decipher mechanisms by which spindles assemble and organize, understanding the interplay between the different mechanisms and pathways, and how they contribute to faithful chromosome segregation will likely require many more years of investigation.

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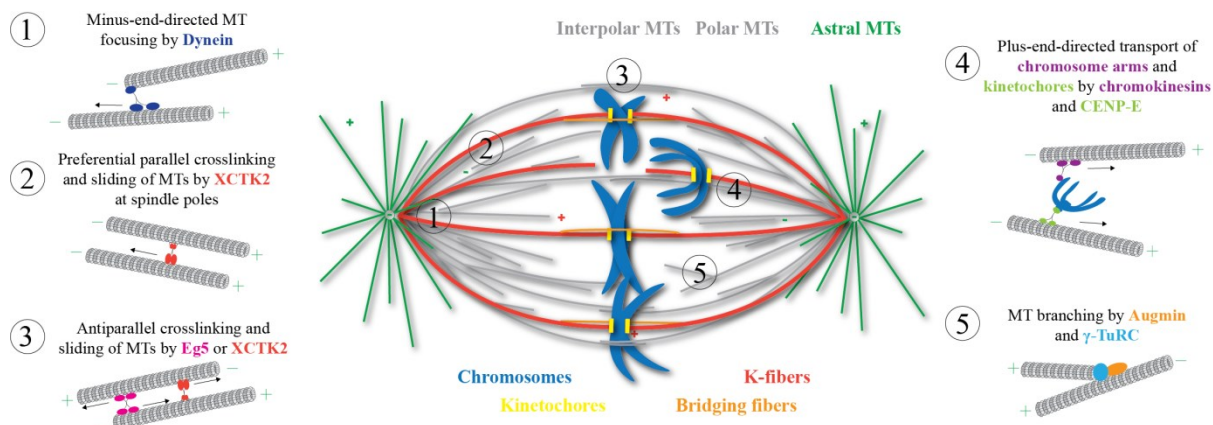


Figure 1. Spindle, MT subpopulations and motor activities.

Different MT populations compose the spindle. Astral MTs (green) emanating from MT organizing centers, such as the centrosome, have their plus-ends radiating outward and toward the cell cortex. K-fibers (red) link with their plus-ends the kinetochores (yellow) assembled on centromeres of chromosomes (blue) to the spindle poles together with their bridging fibers linking sister chromatids (orange). Other, non-kinetochore MTs (gray), include short polar MTs within half of the spindle, and long interpolar MTs extending past the spindle equator and crosslinking with antiparallel MTs extending from the opposite spindle pole. Assembly of these different MT subpopulations relies on motor proteins. (1) Dynein, minus-end directed motor, moves MTs poleward to focus spindle poles. (2 and 3) XCTK2, minus-end directed motor, crosslinks and slides both parallel and antiparallel MTs but participates to pole focusing due to preferential parallel crosslinking at spindle poles. (3) Eg5, plus-end directed motor, crosslinks antiparallel MTs in metaphase and slide them for pole separation during anaphase. (4) CENP-E and Chromokinesin, plus-end directed motors, transport chromosomes, via their kinetochores or arms, toward spindle equator. (5) Augmin complex recruits γ -TuRC on MTs to nucleate MTs, leading to spindle MT amplification.

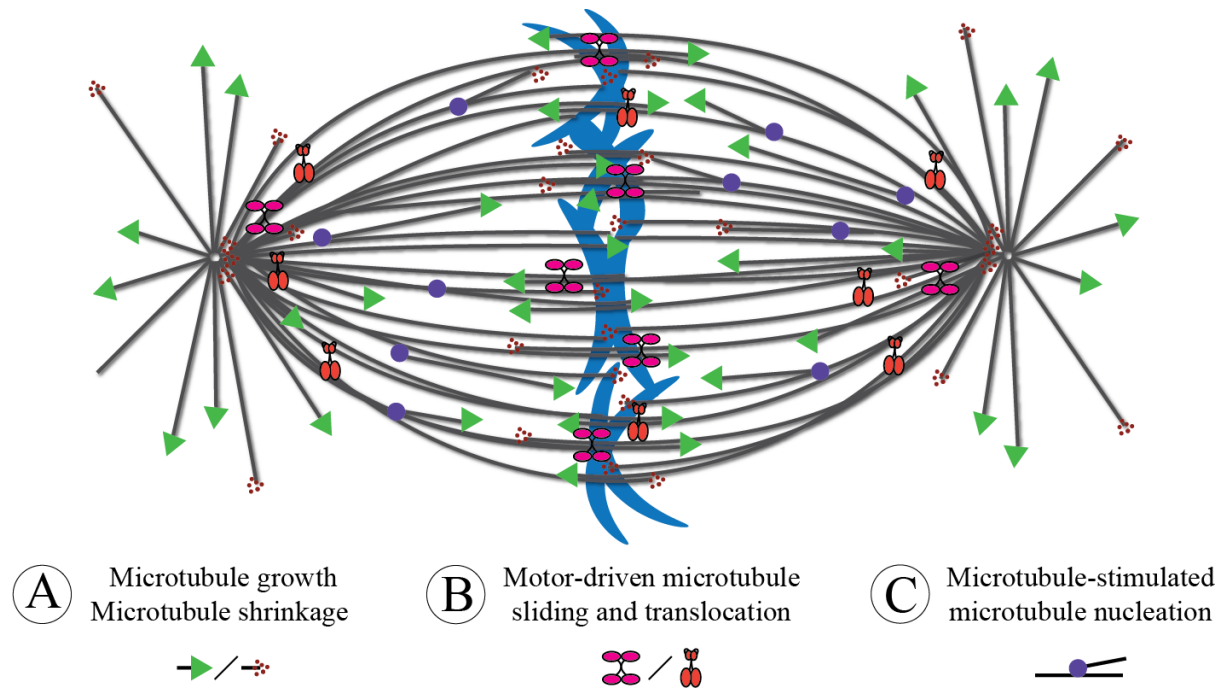


Figure 2. Activities involved in metaphase spindle length control

Different activities are responsible for controlling metaphase spindle size in metazoans. (A) MT dynamics is controlled by many different stabilizers/polymerizers (such as TOG/TOG-like domain containing proteins of the chTOG/XMAP215 and CLASP/Mast/Orbit families, as well as bundling chromokinesins of the Kid family) and destabilizers/depolymerizers (including the Kinesin-8 and Kinesin-13 families as well as Statmin/Op18 proteins) to establish and maintain spindle length. (B) MT translocation and sliding by the antagonistic action of, in particular, the Kinsein-5 and Kinesin-14 motors control spindle length. (C) MT-dependent MT nucleation by the TPX2-Augmin- γ TuRC branching complex controls spindle microtubule number and thus spindle length.

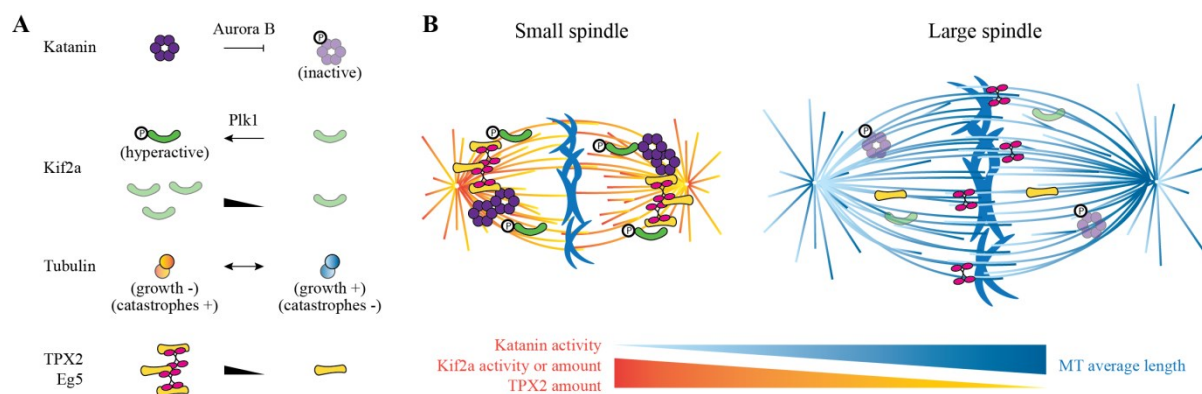
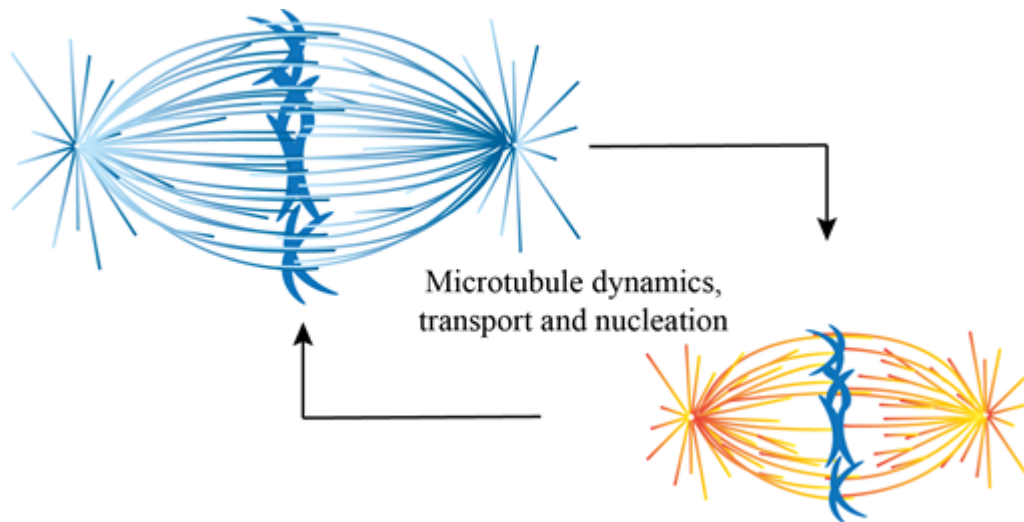


Figure 3. Factors involved in interspecies and developmental metaphase spindle scaling in related frog species.

Katanin MT severing activity is downregulated by Aurora B-dependent phosphorylation in *X. laevis*. Absence of this inhibitory site is responsible for small spindles in *X. tropicalis*. Kif2a MT depolymerizing activity is upregulated by Plk1-dependent phosphorylation and is responsible for small spindles in *H. boettgeri*. In addition, an increasing amount of non-phosphorylatable Kif2a on spindles with decreasing cell size is responsible for the developmental spindle scaling in *X. laevis*. Differences in intrinsic dynamic properties of tubulin itself contribute to spindle length difference between *X. laevis* and *X. tropicalis*. Higher wild-type amount of the spindle assembly factor TPX2 in *X. tropicalis* increases the recruitment of Eg5 to spindle poles what is involved in small spindle length. (A) Summary of scaling factors' activity regulations that account for small (left) and large (right) spindles. (B) Scheme of combined molecular regulation of spindle scaling onto small (left) and large (right) spindles.



Outstanding discoveries were made over the past few years and changed our view of spindle assembly and organization. In particular, we now better understand how spindle size is regulated by physical constraints superimposed with molecular controls that spread from cell size sensing to the fine regulation of microtubule dynamics. Here, we review these latest studies and provide an overview of spindle architecture and size control mechanisms.