Emergent Properties of the Metaphase Spindle

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A metaphase spindle is a complex structure consisting of microtubules and a myriad of different proteins that modulate microtubule dynamics together with chromatin and kinetochores. A decade ago, a full description of spindle formation and function seemed a lofty goal. Here, we describe how work in the last 10 years combining cataloging of spindle components, the characterization of their biochemical activities using single-molecule techniques, and theory have advanced our knowledge. Taken together, these advances suggest that a full understanding of spindle assembly and function may soon be possible.

Because of its prominent geometry, the mitotic spindle was identified under the light microscope as early as the 19th century (Flemming 1882). The central function of this structure, which has fascinated cell biologists ever since, is to accurately segregate chromosomes into two identical sets. The dynamic properties of spindle microtubules are modulated by accessory proteins known as microtubule-associated proteins (MAPs) and motors. These proteins modulate every aspect of a microtubule's life. They help microtubules nucleate, grow, shrink, pause, and switch between all of these states. In recent years, the biochemical activities of these individual proteins have been extensively studied. The advent of single-molecule techniques has allowed unprecedented insight into their detailed activities and the relationship between these activities and the microtubule lattice. However, one question remains. How do spindle morphology and function emerge through the dynamic activities of hundreds of proteins?

"Emergence" describes the way complex properties and patterns of a system arise out of a multiplicity of simple interactions. Examples include the generation of an infinite variety of six-sided snowflakes from frozen water in snow (Libbrecht 2005). Similarly, "flocking," the coordinated motion of animals observed in bird flocks, fish schools, or insects swarms, is considered an emergent behavior (Berdahl et al. 2013). In physics, emergent behaviors are commonly studied to describe complex systems. Physics thus provides a framework for relating the microscopic properties of individual molecules to the macroscopic properties of materials. In this review, we first discuss progress in our understanding of the biochemistry of individual molecules required for modulating microtubule dynamics with a focus on recent quantitative data from biophysical and biochemical

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reconstitution assays. We highlight what we still need to understand to link molecular and collective function. We then discuss theoretical approaches, which integrate molecular details and help to achieve a systems understanding of spindle organization and function. Finally, we discuss forthcoming concepts of cellular scaling, which assure that the spindle adapts its size to the size of the cell.

KEY PLAYERS OF SPINDLE ORGANIZATION

The metaphase spindle is a bipolar array of microtubules assembled from dimeric αβ-tubulin subunits that polymerize in a head-to-tail fashion into polar filaments with β-tubulin facing the plus end and α-tubulin the minus end (Mitchison 1993). Approximately 13 protofilaments associate laterally to form a dynamic microtubule. The de novo formation of microtubules is termed nucleation, which gives rise to a dynamic microtubule. Microtubule dynamic instability can empirically be described by four parameters: (1) the microtubule polymerization velocity, (2) the depolymerization velocity, (3) the catastrophe frequency when microtubules switch from growth to shrinkage, and (4) the rescue frequency when microtubules switch from shrinkage to growth (Mitchison and Kirschner 1984). Microtubule polymerization (Dogterom and Yurke 1997) and depolymerization (Lombillo et al. 1995) produce mechanical forces. In addition, microtubules are subject to passive spindle forces such as elasticity and molecular friction (Dumont and Mitchison 2009b; Itabashi et al. 2009; Shimamoto et al. 2011) and to active force generated by motor proteins, such as kinesins and cytoplasmic dynein, which use the energy from ATP hydrolysis to step along microtubules (Gennerich and Vale 2009). Microtubule nucleation and dynamics as well as spindle forces are controlled by a set of regulatory proteins that specifically interact with distinct regions of the microtubule.

MICROTUBULE NUCLEATION

The centrosome is the classic organelle associated with microtubule nucleation. The γ-tubulin small complex (γ-TuSC) is the conserved, functional unit of the centrosome essential for microtubule nucleation. Multiple γ-TuSCs assemble into a γ-tubulin ring complex (γ-TuRC) in the presence of several other associated proteins (Fig. 1) (Keating and Borisy 2000; Moritz et al. 2000; Wiese and Zheng 2000, 2006; Kollman et al. 2010). The favored model for microtubule nucleation is the template model, in which γ-tubulin assembles into a ring of 13 molecules that form a template for the nucleation of microtubules with 13 tubulin protofilaments (Moritz et al. 1995; Zheng et al. 1995; Pereira and Schiebel 1997). This model is supported by in vitro findings showing that purified γ-TuRC caps microtubule minus ends (Zheng et al. 1995; Moritz et al. 2000), and that the purified yeast γ-TuSC assembles into spiral-like filaments of 13 γ-tubulin molecules per turn (Kollman et al. 2010).

Although centrosomes are considered the classic organelle for microtubule nucleation, spindles readily form in the absence of centrosomes. Plant cell mitosis (De Mey et al. 1982; Zhang and Dawe 2011) and animal egg meiosis occur without centrosomes (Manandhar et al. 2005; Dumont and Desai 2012). In addition, different experimental approaches show that animal cell mitosis can occur normally after centrosomes have been removed (Khodjakov et al. 2000; Hinchcliffe et al. 2001; Megraw et al. 2001; Basto et al. 2006; Mahoney et al. 2006). This implies that nucleation of spindle microtubules does not always rely on centrosomes. Indeed, seminal work in *Xenopus* egg extracts revealed that chromatin can promote microtubule nucleation (Heald et al. 1996). The spatial cue necessary to nucleate microtubules around chromatin is mediated by a diffusion-limited RanGTP gradient (Fig. 1) (Carazo-Salas et al. 1999; 2001; Kaláb et al. 1999; Ohba et al. 1999; Nachury et al. 2001). Ran is a small GTPase that drives nucleocytoplasmic transport during interphase, whereby the high concentration of the guanosine triphosphate (GTP)-bound form of Ran in the nucleus allows the release of newly imported proteins from their binding to importins (Clarke 2008). Similarly, during mitosis, a high-RanGTP gradient, centered around chro-

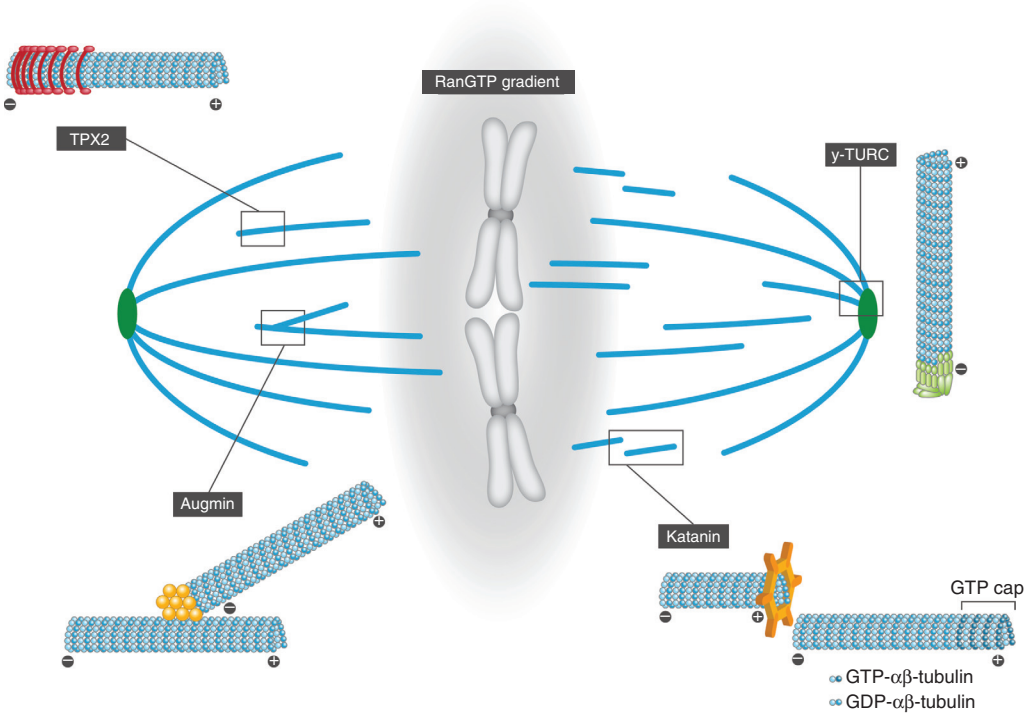

Figure 1. Microtubule nucleation, stabilization, and amplification. The metaphase spindle is a complex structure consisting of microtubules (blue) that nucleate from centrosomes (green) and chromatin (gray). A central centrosomal component is the γ-tubulin ring complex (γ-TuRC), which templates the nucleation of microtubules. The spatial cue necessary to nucleate microtubules around chromatin is mediated by a diffusion-limited RanGTP gradient, the first identified direct effector of which is TPX2. The eight-subunit complex augmin nucleates microtubules parallel to existing microtubules, while katanin severs and disassembles microtubules. GDP, Guanosine diphosphate; GTP, guanosine triphosphate.

matin, releases putative spindle assembly factors (SAFs) from importins, thereby enabling the SAFs to perform their function in spindle assembly. The first identified direct effector of RanGTP in spindle assembly is TPX2 (Fig. 1) (Gruss et al. 2001, 2002). Removal of TPX2 function abolishes spindle assembly (Wittmann et al. 2000; Gruss et al. 2001, 2002; Tulu et al. 2006; Greenan et al. 2010). Although TPX2 induces microtubule nucleation when added to *Xenopus* egg extracts (Gruss et al. 2001) and promotes the assembly of microtubules from pure tubulin in solution (Schatz et al. 2003; Brunet et al. 2004), it remains to be shown whether TPX2 is a true microtubule nucleator. In addition, TPX2 is an activator of the mitotic kinase Aurora A (Kufer et al. 2002; Tsai et al. 2003; Eyers and Maller 2004; Ozlü et al. 2005). Work in HeLa cells suggests that the chromatin nucleation capacity of TPX2 is mediated through Aurora A activation and not by TPX2 directly (Bird and Hymann 2008). Thus, the current model is that the Ran gradient induces downstream gradients, such as an Aurora A phosphorylation gradient, and thereby effects not only microtubule nucleation but also microtubule dynamics and motor activities (Gruss and Vernos 2004).

There is direct evidence for a diffusion-limited RanGTP gradient surrounding chromosomes in mitotic somatic cells (Kaláb et al. 2006). Although chromatin-based microtubule nucleation has been visualized in mammalian cells (Khodjakov et al. 2003), chromatin-dependent nucleation is not essential for spindle bipolarity during human cell mitosis when centrosomes are present (Kaláb et al. 2006; Bird and

Hymann 2008). Thus, the relative contribution of microtubule nucleation by the RanGTP gradient appears to be organism- and cell-type-specific (Sato and Toda 2007). Although this is essential for anastral spindle assembly during female meiosis, it might just provide a kinetic advantage during the early stages of spindle assembly in primarily centrosome-driven somatic cells. Notably, in both somatic cells and *Xenopus* egg extracts, the steepness of the Ran-regulated gradient seems to correlate with spindle size (Kaláb et al. 2002, 2006). Whether the steepness of the Ran gradient or of its effectors actively determines spindle size in these systems is still an open question.

MICROTUBULE AMPLIFICATION

One open question is whether microtubule nucleation by centrosomes and/or the RanGTP pathway can generate a sufficient number of microtubules to account for the total spindle mass. There is experimental evidence that microtubule minus ends are spread throughout the spindle (Burbank et al. 2006; Mahoney et al. 2006; Yang et al. 2007; Brugués et al. 2012), indicative of microtubule nucleation happening within the spindle body. Indeed, the eight-subunit complex augmin (Fig. 1) has been shown to recruit γ-TuRC to the side of pre-existing microtubules and to initiate the nucleation of new microtubules (Goshima et al. 2008; Lawo et al. 2009). This is consistent with the idea of a nucleator that becomes activated once it binds to a microtubule as a kinetic model of autocatalytic microtubule production (Clausen and Ribbeck 2007). Although depletion of augmin by RNAi decreases microtubule density within the spindle (Goshima et al. 2007, 2008; Zhu et al. 2008; Lawo et al. 2009; Uehara et al. 2009; Uehara and Goshima 2010; Petry et al. 2011; Hotta et al. 2012; Nakaoka et al. 2012), the contribution of microtubule amplification seems to differ significantly in different cell types. For example, augmin genes cannot be found in the *Caenorhabditis elegans* genome, making the worm a system in which centrosomes play the dominant role in generating spindle microtubules (Hamill et al. 2002). In *Drosophila* oocytes, augmin is dispensable for chromatin-driven assembly of bulk spindle microtubules (Colombié et al. 2013), whereas, in *Drosophila* S2 cells, augmin depletion significantly reduces microtubule density in spindles (Goshima et al. 2007, 2008). Spindles formed in augmin-depleted *Xenopus* egg extracts show a temporal delay in acentrosomal spindle formation. In the presence of centrosomes, however, defects in spindle morphology are modest (Petry et al. 2011).

So far, it is unclear how chromatin-dependent microtubule nucleation and microtubule-dependent microtubule amplification are interregulated, if at all. A recent study now shows that RanGTP stimulates augmin-dependent microtubule amplification, which is dependent on TPX2 (Petry et al. 2013), thereby linking the two pathways. As augmin nucleates microtubules parallel to existing microtubules, and in this way preserves microtubule polarity (Kamasaki et al. 2013; Petry et al. 2013), this pathway might be important to amplify and stabilize preformed structures once bipolarity is established. Still, the exact mechanism by which RanGTP spatially and temporally controls de novo nucleation on the one hand and microtubule-dependent microtubule nucleation on the other remains to be shown.

MICROTUBULE SEVERING

Although it is clear that regulators of microtubule nucleation, amplification, and growth influence microtubule mass, the cellular consequences of microtubule severing are more complex. Although in vitro severing leads to the complete loss of a microtubule, the in vivo consequences of severing include microtubule amplification, the release of microtubules from nucleation sites, and complete microtubule disassembly (Srayko et al. 2000; McNally et al. 2006; Yu et al 2008; Loughlin et al. 2011). Active microtubule severing was first described as an M phase–specific activity (Vale 1991) and subsequently attributed to the protein katanin (Fig. 1) (McNally and Vale 1993). Katanin is a heterodimeric protein, composed of a targeting subunit (p80) and an enzymatic subunit (p60), with an ATPase activity that severs and disas-

sembles microtubules (Hartman et al. 1998). Together with spastin and fidgetin, katanin represents an AAA (ATPases associated with diverse cellular activities) subfamily with a highly conserved AAA domain at their carboxyl terminus. In *C. elegans*, the loss of katanin results in fewer but longer spindle microtubules (Srayko et al. 2000, 2006; McNally et al. 2006). In contrast, *Dm* katanin was shown to cut stabilized microtubule ends, and thus provide a substrate for kinesin-13-dependent depolymerization (Buster et al. 2002; Zhang et al. 2011). In the two closely related frogs, *Xenopus laevis* and *tropicalis*, katanin was shown to contribute to setting spindle length by differentially accelerating microtubule depolymerization at the spindle poles (see below) (Loughlin et al. 2011).

Although some studies put severing proteins in the context of depolymerases, severing proteins—if at all—are only weak depolymerases (Díaz-Valencia et al. 2011; Zhang et al. 2011) when compared to classic ones such as mitotic centromere-associated kinesin (MCAK). What remains to be understood? Maybe the most important aspect on the molecular level is to understand how severing enzymes identify the regions of microtubules on which they act. Several lines of evidence suggest posttranslational modifications of tubulin to enhance severing activity (Sharma et al. 2007; Lacroix et al. 2010). Although it is clear that severing has an influence on microtubule dynamics, it remains unclear in what way it affects microtubule mass and, thus, spindle organization globally. Severing could induce microtubule depolymerization and thereby increase turnover. Alternatively, severing could create new templates for microtubule growth and thereby influence the effective nucleation rates. This might depend on the cellular context the newly cut microtubule ends encounter and whether they shrink or grow.

MICROTUBULE DYNAMICS

In vitro experiments with purified tubulin show that both microtubule ends exhibit dynamic instability (Walker et al. 1988; Erickson and O'Brien 1992; Desai and Mitchison 1997), in which microtubules coexist in states of growth and shrinkage and interconvert randomly between these two states. The observed in vivo dynamicity of microtubule ends, however, is quite different. Although the microtubule plus end is highly dynamic, the minus end is usually stable. The in vitro reconstitution of physiological microtubule dynamics was first achieved using purified centrosomes, tubulin, and the antagonistic proteins XKCM1 and XMAP215 (Kinoshita et al. 2001). Although these two proteins are among the best-understood MAPs to date, there is a plethora of proteins that regulate microtubule plus-end dynamics. In contrast, only a few proteins that specifically interact and regulate the microtubule minus end have been described so far.

WHAT POWERS THE DYNAMIC BEHAVIOR AT THE MICROTUBULE PLUS END?

The energy required for the dynamicity comes from GTP hydrolysis at the β-tubulin subunit after incorporation of the tubulin dimer into the microtubule lattice. When microtubules are grown with guanylyl-(α,β)-methylenediphosphonate (GMPCPP), a nonhydrolyzable form of GTP, they do not undergo dynamic instability (Hyman et al. 1992), showing that GTP hydrolysis is necessary for the switching behavior. Although the relation of the four parameters of microtubule dynamic instability in pure tubulin solutions is well understood (Walker et al. 1988), it is particularly important to understand how individual proteins influence each of the four parameters (Bowne Anderson et al. 2013).

MICROTUBULE DEPOLYMERASES

XKCM1 is a member of the kinesin-13 family (Fig. 2). Unlike other kinesins, kinesin-13s do not move directionally along microtubules; instead, they employ their ATP-hydrolyzing motor domain to diffuse along the microtubule lattice to target both microtubule ends, and induce conformational changes that lead to microtubule depolymerization in vitro (Hunter et al. 2003; Helenius et al. 2006; Cooper et al. 2010;

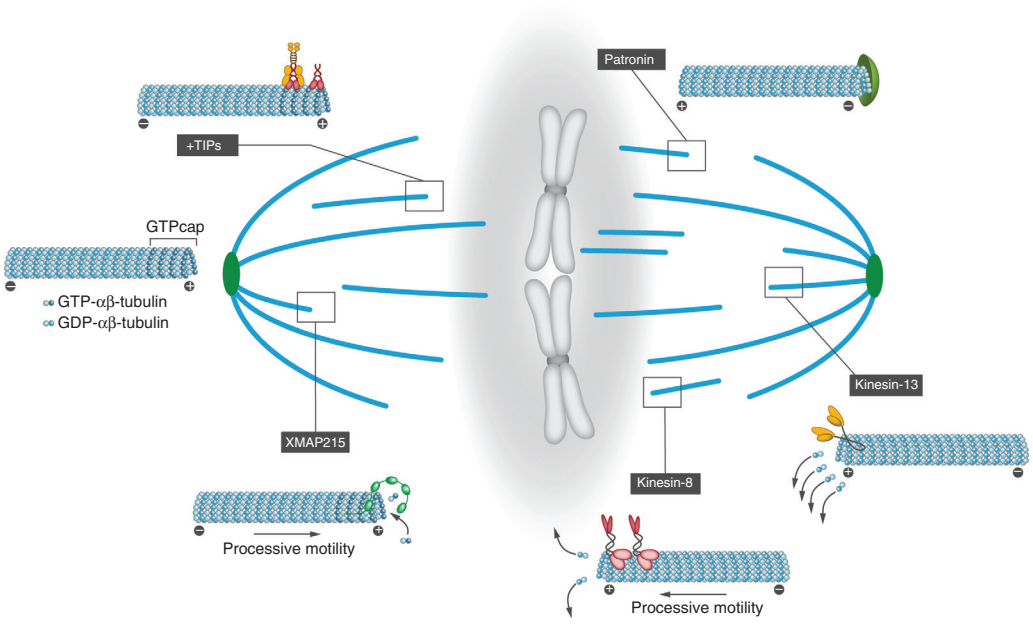

Figure 2. Microtubule dynamics. The intrinsic dynamic instability of microtubules is generated by guanosine triphosphate (GTP) hydrolysis at the nucleotide exchangeable site in β-tubulin. In addition, various proteins regulate the dynamic behavior of microtubules. Although microtubule − ends are specifically stabilized, for example, by patronin, the + ends switch stochastically between growing (regulated by polymerases such as XMAP215) and shrinking phases (regulated by depolymerases such as kinesin-8 and -13). Growing microtubule + ends are further regulated by so-called microtubule plus-end tracking proteins (+TIPs). GDP, Guanosine diphosphate.

Oguchi et al. 2011). The unconventional ATPase cycle of kinesin-13 has optimized this motor protein for microtubule depolymerization (Friel and Howard 2011), explaining how structurally similar motor domains can have different functions. The kinesin-13 depolymerase activity accounts well for the cellular phenotypes caused by loss of its activity leading to a decrease in catastrophe rate and an increase in mitotic spindle length (Walczak et al. 1996; Desai et al. 1999; Tournebize et al. 2000; Rogers et al. 2004). Phenotypes upon loss of kinesin-13 function are very similar to those upon kinesin-8 loss. Inhibition of kinesin-8 activity results in elongated spindles with hyperstable microtubules (Goshima et al. 2005; Mayr et al. 2007; Savoian and Glover 2010). Indeed, kinesin-8 proteins are slow plus-end directed motors with a high processivity that disassemble microtubules exclusively from one end (Fig. 2) (Varga et al. 2006). Surprisingly, kinesin-8 can depolymerize long microtubules faster than short ones. According to the "antenna model," longer microtubules accumulate more kinesin-8, which, because of its high processivity, will allow the motor to reach the microtubule plus end with a high probability. Therefore, kinesin-8 forms length-dependent "traffic jams" at the plus end, where an incoming kinesin-8 will bump off a pausing kinesin-8 molecule together with one or two tubulin dimers (Varga et al. 2009; Leduc et al. 2012). Such a cooperative mechanism leads to a length-dependent depolymerization rate and may serve as a model for how an ensemble of molecules can measure and control microtubule length.

Although experiments with purified proteins show that both kinesin-8 and kinesin-13 can depolymerize stabilized microtubules (Desai et al. 1999; Hunter et al. 2003; Helenius et al. 2006; Varga et al. 2006, 2009; Mayr et al. 2007), a recent study found that they influence catastrophes by quite different mechanisms (Gardner et al. 2011). Catastrophes are thought to result from the loss of a stabilizing GTP-tubulin cap at

the microtubule plus end. Although, for a long time, catastrophes were thought to be a single-step process, Gardner and colleagues show that catastrophe frequency is intrinsically age dependent. The idea is that, during microtubule growth, "catastrophe-promoting events" accumulate over time and increase the likelihood of a catastrophe to happen. While kinesin-8 increases the rate of catastrophe-promoting events, kinesin-13 reduces the number of events necessary for catastrophe (Gardner et al. 2011). Whether catastrophe-promoting events are structural defects in the microtubule lattice remains to be shown. The emerging picture thus is that kinesin-13s promote rapid and global restructuring of microtubules as, for example, required for spindle breakdown at the end of mitosis (Rankin and Wordeman 2010), while kinesin-8 mediates fine tuning of microtubule length as, for example, required during chromosome congression and alignment (West et al. 2001; Mayr et al. 2007; Stumpff et al. 2008). In fungi, however, there is no kinesin-13, only one kinesin-8, Kip3, which does all of the jobs (Varga et al. 2006; Roostalu and Surrey 2013), while flies have three kinesin-13s: KLP10A, KLP59C, and KLP59D (Mennella et al. 2005; Schimizzi et al. 2010). Taken together, although both kinesin-8 and kinesin-13 are catastrophe factors that dramatically affect microtubule lifetime, they will have a different effect on the microtubule length distribution in vivo. How microtubule length ultimately translates into spindle length remains to be shown.

PLUS-END TRACKING PROTEINS

Microtubule growth occurs by the addition of αβ-tubulin heterodimers with GTP bound in the exchangeable site of β-tubulin. Proteins of the XMAP215/Dis1 family catalyze this reaction (Fig. 2). In accordance with their prominent role as microtubule growth promoters, their depletion leads to shorter spindles or defects in spindle morphology in a variety of organisms (Matthews et al. 1998; Cullen et al. 1999; Tournebize et al. 2000; Garcia et al. 2001; Cassimeris and Morabito 2004). Members of the XMAP215/Dis1 family are characterized by tumor overexpressed gene (TOG) domains that function as αβ-tubulin-binding modules (Al-Bassam et al. 2007). The number of TOG domains is species dependent and varies from two to five (Gard and Kirschner 1987; Cassimeris and Morabito 2004; van Breugel et al. 2003). Structure function analyses revealed that TOG domains contribute differentially to the affinity of XMAP215 for the tubulin dimer and, thus, its polymerase activity (Widlund et al. 2011). Our current understanding of XMAP215 function is that it works as a processive polymerase (Brouhard et al. 2008). XMAP215 binds one free tubulin dimer via the TOG domains, interacts with the microtubule lattice via a specific microtubule-lattice-binding domain, and targets the microtubule plus ends by a diffusion-facilitated mechanism, where it persists for numerous rounds of tubulin subunit addition. XMAP215 is suggested to increase the association rate constant of GTP-tubulin by stabilizing a structural intermediate, which may correspond to a "collision complex" whose formation is very fast and diffusion limited (Brouhard et al. 2008).

XMAP215, together with EB1, synergistically reconstitutes physiological microtubule growth velocities (>20 μm/min) in vitro (Zanic et al. 2013). EB1 is a small dimeric, highly conserved plus-end tracking protein (+TIP), which specifically tracks growing, but not pausing or shrinking microtubules, by recognizing the tubulin nucleotide state within the microtubule (Fig. 2) (Zanic et al. 2009; Maurer et al. 2012). In contrast to XMAP215, EB1 does not track microtubule ends processively; instead, it exchanges with fast binding/unbinding kinetics (Bieling et al. 2007). How can we explain the synergistic effect of XMAP215 and EB1 on microtubule growth rates? The release of tubulin bound to XMAP215 was suggested to be dependent on the straightening of tubulin upon incorporation into the microtubule lattice (Ayaz et al. 2012). EB1 might accelerate the polymerase activity of XMAP215 by straightening protofilaments at the microtubule end through enhancement of lateral interactions between neighboring tubulin dimers (Zanic et al. 2013).

EB1 has been shown to mildly accelerate microtubule growth and catastrophe-stimulating

effects in vitro (Bieling et al. 2007; Komarova et al. 2009; Zanic et al. 2013). Its main function, however, might be the regulation of a plus-end tracking proteins (+TIPs) network. EB1 recruits other +TIPs via its carboxy-terminal EB homology domain. The majority of EB1-interacting +TIPs in turn binds EB1 via a short interaction motif residing in basic and serine-rich regions, named "SKIP" (or "SxIP") motif (Honnappa et al. 2009). Prominent examples are adenomatous polyposis coli (APC) (Honnappa et al. 2009), CLASPs (CLIP-associated proteins) (Honnappa et al. 2009; Kumar et al. 2012), SLAIN (van der Vaart et al. 2011), GTSE1 (Scolz et al. 2012), and microtubule depolymerases (Stout et al. 2011; Tanenbaum et al. 2011). In humans, the EB protein family includes three related members, EB1, EB2, and EB3, which are similar in structure and adopt homo- or heterodimeric conformations. The roles of EB2 and EB3 are less well understood. Recent studies, however, imply that differential regulation of EB proteins leads to specific functions throughout mitosis and cytokinesis (Ferreira et al. 2013).

CLASP proteins have emerged as a potential key player at the interface of microtubule and chromosome interactions, potentially by promoting microtubule rescue and suppressing microtubule catastrophe (Akhmanova et al. 2001; Cheeseman et al. 2005; Galjart 2005; Maiato et al. 2005; Mimori-Kiyosue et al. 2005; Drabek et al. 2006; Hannak and Heald 2006; Pereira et al. 2006; Sousa et al. 2007). Only recently, RanGTP, together with CLASP1, was implicated in mitotic spindle positioning (Bird et al. 2013). Although human CLASP1 was originally annotated as having only one TOG domain (Akhmanova et al. 2001), recent structural data report the crystal structure of a cryptic TOG domain (Leano et al. 2013). The identification of a second TOG domain in CLASP supports the idea that TOG domains function in arrays. How CLASPs mechanistically induce rescues is unknown. One possibility is that CLASP reverses microtubule disassembly by incorporating bound tubulin. Alternatively, CLASP could locally stabilize the depolymerizing microtubule lattice, possibly by preventing protofilament curling. Furthermore, it remains to be shown whether rescues play an essential role in spindle organization (Brugués et al. 2012). Direct visualization of rescue events within the metaphase spindle will help to solve these questions.

WHAT KEEPS MICROTUBULE MINUS ENDS STABLE?

Although microtubule minus ends are intrinsically dynamic in vitro (Desai and Mitchison 1997; Goodwin and Vale 2010), they are usually stable in vivo. So far, only very few minus-end-specific proteins have been described. Patronin, initially identified in the RNAi screen for *Drosophila* genes involved in spindle assembly as small spindle phenotype 4 (ssp4) (Goshima et al. 2007), is a capping protein that directly and selectively binds to the microtubule minus end in vitro and protects it from kinesin-13-dependent depolymerization (Fig. 2) (Goodwin and Vale 2010; Wang et al. 2013). It has been speculated that patronin specifically recognizes α-tubulin and protects the minus end by sterically blocking kinesin-13 access. However, the mechanism by which patronin recognizes and protects the minus end remains elusive. In an alternative scenario, patronin could modify the morphology of the minus end by strengthening lateral protofilament interactions and thus reducing kinesin-13 affinity, which is known to prefer curved tubulin protofilaments (Asenjo et al. 2013). Three patronin homologs exist in humans (Baines et al. 2009). Their respective roles, however, are not yet defined, but they may have evolved to interact with distinct partners for localizing microtubule minus-end capping/anchoring activities to distinct subcellular regions (Berglund et al. 2008; Meng et al. 2008). Thus, the three patronin family members might provide new molecular tools for probing the organization and function of microtubules in different vertebrate cell types. Similarly, microspherule protein 1 (MCRS1), a protein that localizes to the microtubule minus-end region, has recently been shown to protect kinetochore fibers from depolymerization (Meunier and Vernos 2011).

Although the studies of microtubule plus-end and minus-end binding proteins developed

largely independent of each other, there is evidence of cross talk between the microtubule ends (Jiang and Akhmanova 2011). For example, it is known that XMAP215 is specifically recruited to the centrosome by the TACC family of proteins indicating that +TIPs function beyond microtubule plus-end regulation (Lee et al. 2001; Peset and Vernos 2008; Hubner et al. 2010). Therefore, studies of microtubule dynamics in the future should shift toward combining plus- and minus-end regulators and analyze their collective behavior.

SPINDLE FORCES

During assembly and function, the spindle passes through several steady states, each relying on a distinct balance of complementary and antagonistic forces. Loss-of-function studies in living cells suggested that a balance of forces generated by antagonistic motor proteins is crucial for spindle assembly and maintenance (Saunders et al. 1997; Mountain et al. 1999; Sharp et al. 1999a, 2000; Dumont and Mitchison 2009b). In addition, numerous theoretical works suggest that spindle size is dependent on the antagonism between motor proteins that slide microtubules in opposite directions (Burbank et al. 2007; Wollman et al. 2008; Ferenz et al. 2009; Loughlin et al. 2010; Brugués et al. 2012). The question of how these forces are integrated, as well as spatially and temporally regulated, to build a structure with a defined length and shape is too complex to be studied as a whole. One approach that helps to shed light on the increasing complexity of spindle forces is the in vitro reconstitution of minimal systems with a defined set of components. Minimal systems, such as antiparallel microtubule overlaps and astral microtubule arrays, have proven valuable systems to study organizational principles of spindle poles and the spindle midzone, respectively (Karsenti et al. 2006; Subramanian and Kapoor 2012; Dogterom and Surrey 2013).

KINESIN-5 AND DYNEIN

In cells lacking kinesin-5 activity, bipolar spindle assembly can be restored when cytoplasmic dynein is inhibited (Mitchison et al. 2005; Tanenbaum et al. 2008; Ferenz et al. 2009). These initial observations led to a model in which dynein-dependent inward forces directly counteract kinesin-5-dependent outward forces. In most organisms, apart from *C. elegans* (Saunders et al. 2007), kinesin-5 is absolutely essential for bipolar spindle assembly, and its loss results in the formation of monopolar spindles (Blangy et al. 1995; Mayer et al. 1999; Sharp et al. 1999b; Kapoor et al. 2000; Goshima and Vale 2003; Kwok et al. 2004). The homotetramer kinesin-5 is a highly conserved plus-end-directed motor (Cole et al. 1994; Kashina et al. 1996), and its unique structure is optimized to cross-link and slide antiparallel microtubules (Fig. 3) (Hentrich and Surrey 2010), thereby producing the necessary outward force that drives centrosome separation during spindle assembly (Splinter et al. 2010; Tanenbaum and Medema 2010). Cytoplasmic dynein, on the other hand, is the major motor responsible for microtubule minus-end-directed movements in most eukaryotic cells. Compared to kinesins, cytoplasmic dynein is unique as it belongs to the AAA^+ family. Dynein is a dimer of two heavy chains, each composed of an AAA ring that binds and hydrolyzes ATP, a microtubule-binding stalk, and a long tail domain (Fig. 3) (Carter et al. 2011). In mitosis, dynein is involved in centrosome separation, chromosome movements, spindle organization in particular pole focusing, kinetochore activity, checkpoint silencing, and spindle positioning (Vaisberg et al. 1993; Gaglio et al. 1996; Heald et al. 1996; Merdes et al. 1996; Busson et al. 1998; Gönczy et al. 1999, 2000; Sharp et al. 1999a; Howell et al. 2001; Grill and Hyman 2005; Varma et al. 2008; Sivaram et al. 2009; Bader and Baughan 2010; Kiyomitsu and Cheeseman 2012; Laan et al. 2012).

This simplified view, in which dynein-dependent inward forces directly counteract kinesin-5-dependent outward forces, has recently been challenged by the observation that kinesin-5 activity is not titratable against dynein activity, suggesting that dynein most likely antagonizes kinesin-5 indirectly by exerting force at different spindle locations (Florian and Mayer 2012). Indeed, both kinesin-5 and cytoplasmic

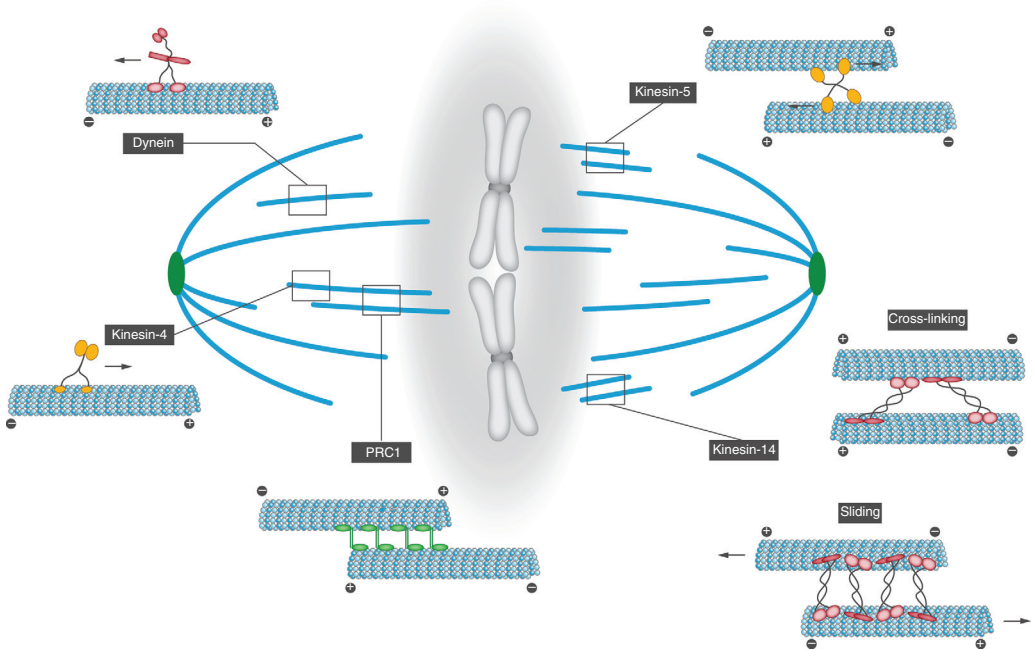

Figure 3. Spindle forces. During assembly and function, the spindle passes through several steady states, each relying on a distinct balance of complementary and antagonistic forces. The homotetramer kinesin-5 is a highly conserved plus-end-directed motor optimized to cross-link and slide antiparallel microtubules, thereby producing outward forces that drive centrosome separation during spindle assembly. Kinesin-4 is a dimeric plus-end-directed motor. Together with PRC1, it forms antiparallel microtubule overlaps with precisely defined lengths; while PRC1 marks the microtubule overlap region and recruits kinesin-4, the motor protein walks processively to microtubule ends in the overlap region, where its accumulation leads to the inhibition of microtubule growth. In contrast to the plus-end-directed motility of other kinesin proteins, kinesin-14 is a minus-end-directed motor that can either slide antiparallel microtubules or cross-link parallel microtubules (adapted from Fink et al. 2009). Cytoplasmic dynein is the major motor responsible for microtubule minus-end-directed movements.

dynein localize to multiple subcellular structures throughout mitosis. At the spindle center, kinesin-5 is proposed to drive microtubule flux by antiparallel microtubule sliding, while the dynein-dependent concentration of kinesin-5 at spindle poles is suggested to contribute to parallel microtubule cross-linking (Uteng et al. 2008). Cytoplasmic dynein localizes to centrosomes, kinetochores, spindle microtubules, and the cell cortex (Pfarr et al. 1990; Steuer et al. 1990; Dujardin and Vallee 2002; Tanenbaum and Medema 2010; Kiyomitsu and Cheeseman 2012). Taking these diverse localizations and functions into consideration, it is not surprising that the depletion of multifunctional proteins results in complex patterns of spindle formation. In the case of dynein, the situation is even more complicated by the fact that several accessory proteins modulate dynein to carry out its many different functions. Prominent examples are the dynactin complexes, LIS1 and NudE (Kardon and Vale 2009; Huang et al. 2012). In the future, it will be interesting to learn how these accessory proteins regulate the detailed function of dynein. Recent exciting advances in the in vitro reconstitution of human dynein (Trokter et al. 2012) and the observation of single dynein molecules in cells (Ananthanarayanan et al. 2013; Rai et al. 2013) will help to advance our understanding of the structural basis of dynein movement and determine how the motor regulation works.

KINESIN-5 AND KINESIN-14

In other systems, kinesin-5 activity is proposed to be antagonized by the inward sliding activity of kinesin-14. Kinesin-14 is a minus-end-directed homodimeric motor (Fig. 3), which uses a pair of motor domains to walk on one microtubule and a nonmotor domain to interact with the second filament. To cross-link two microtubules, kinesin-14 orients stochastically and its motor domains are equally likely to bind either of the two filaments (Braun et al. 2009; Fink et al. 2009). In vitro, kinesin-14 can autonomously induce pole-formation (Hentrich and Surrey 2010), which might be the dominant mechanism by which centrosome-free meiotic spindles are focused in *Drosophila* (Matthies et al. 1996; Sköld et al. 2005). Reconstitution studies combining these two antagonistic motors, however, fail to establish a stable antiparallel microtubule overlap (Tao et al. 2006; Hentrich and Surrey 2010) but generate oscillatory movements, as previously observed in microtubule gliding assays with kinesin-1 and dynein (Vale et al. 1992). Thus, a persistent force balance cannot be achieved by either of these two motor combinations.

KINESIN-14 AND ASE1

Instead, a three-component system consisting of microtubules, kinesin-14, and Ase1, a nonmotor cross-linking protein, was shown to form stable antiparallel microtubule overlaps (Braun et al. 2011). Members of the conserved Ase1/PRC1 family are characterized by their ability to bind to antiparallel microtubule overlaps with high affinity and selectively cross-link them in vitro (Fig. 3) (Janson et al. 2007; Kapitein et al. 2008; Bieling et al. 2010; Subramanian et al. 2010, 2013; Duellberg et al. 2013). In yeast, the spindle midzone is marked by Ase1 localization and defined by the Ase-1-dependent recruitment of all other midzone proteins (Khmelinskii et al. 2007). PRC1, the Ase1 homolog in higher eukaryotes, also selectively binds antiparallel microtubule overlaps (Bieling et al. 2010; Subramanian et al. 2010). Whether PRC1, together with kinesin-14, can set an antiparallel microtubule array with a defined overlap length has not yet been tested. However, it was shown to not substantially oppose kinesin-5 activity (Subramanian et al. 2010).

PRC1 AND KINESIN-4

The question of how antiparallel microtubules are established in metazoan metaphase spindles is still open as PRC1 is not crucial for spindle organization before anaphase (Mollinari et al. 2002). Only after anaphase onset, PRC1 is essential to maintain the overlap length of the central spindle (Kurasawa et al. 2004; Hu et al. 2011), where it recruits kinesin-4. Kinesin-4, a dimeric plus-end-directed motor (Fig. 3), has an inhibitory effect on microtubule growth (Bringmann et al. 2004). Two recent studies show that PRC1, together with kinesin-4, is sufficient to form antiparallel microtubule overlaps with precisely defined lengths in vitro (Bieling et al. 2010; Subramanian et al. 2013). PRC1 and kinesin-4 tag microtubule plus ends. While PRC1 marks the microtubule overlap region and recruits kinesin-4, the motor protein walks processively to microtubule ends in the overlap region, where its accumulation leads to the inhibition of microtubule growth. Importantly, plus-end tagging by PRC1 is microtubule length dependent, and, thus, nicely demonstrates a biochemical mechanism by which the length of antiparallel overlaps can be controlled by suppression of microtubule dynamics.

MODELING SPINDLE ASSEMBLY USING *Xenopus* EXTRACTS

Dynamic spindles assembled in *Xenopus* egg extracts are a powerful way to unravel principles of self-organization. The egg extract is an open system that permits biochemical manipulation and quantitative kinetic studies. In addition, this cell-free system is void of cortical restrictions and spindle material is not limited, which allows studying intrinsic mechanisms of spindle organization. In combination with theoretical and conceptual approaches, it is a particular powerful tool to describe complex dynamic processes. Thus, in recent years, this easily trac-

table system has led to an outpouring of non-mutually-exclusive models that quantitatively describe spindle organization, which we will shortly discuss.

A two-dimensional simulation study by Loughlin and colleagues implemented many processes relevant for *Xenopus* spindle assembly, such as microtubule nucleation and dynamics, steric interactions between microtubules, and motor-induced sliding (Loughlin et al. 2010). This model predicts that microtubule nucleation occurs throughout the spindle and that spindle morphology and, in particular, spindle lengths, are governed by selective microtubule destabilization near the spindle poles. In contrast, in a different model called the "slide-and-cluster" mechanism (Burbank et al. 2007), microtubules nucleate only locally near chromosomes, slide outward by a plus-end-directed motor, cluster by a minus-end-directed motor, and are lost by turnover throughout the spindle. An important feature of the slide-and-cluster model is that the model does not require specific depolymerization of microtubule minus ends at predefined poles. Thus, spindle length primarily emerges as the product of outward sliding velocity and minus-end lifetimes. This, however, requires microtubule lifetimes that are significantly higher than those measured in metaphase spindles (Needlemann et al. 2010).

The above models make distinct predictions for the length distribution and organization of spindle microtubules. Only recently, Brugués and colleagues developed a method to quantitatively measure the length distribution and polarity of microtubules within the spindle. They found that microtubules are shortest at the poles and progressively increase in length toward the center of the spindle. In the spindle center, an equal number of microtubules points in both directions, whereas close to the pole, the majority of microtubules are oriented with their plus end away from the pole (Brugués et al. 2012). Combining these experiments with modeling, the authors suggest that microtubule organization in the spindle is determined by nonuniform microtubule nucleation and local sorting of microtubules by transport. They, however, did not find evidence for spatially varying microtubule stability. The nonuniform nucleation close to chromatin could be consistent with a gradient of microtubule nucleation around chromatin or microtubule-dependent nucleation.

Although all of the above studies predict that microtubule nucleation has a profound influence on spindle organization and length, so far, no one has been able to directly measure microtubule nucleation rates within spindles. It therefore remains unknown how different nucleation mechanisms (i.e., chromatin-mediated and microtubule-dependent microtubule nucleation) contribute to the overall spindle architecture. Thus, measurement of nucleation rates in spindles will be an important topic for the future.

SPINDLE SIZE CONTROL AND SCALING

The metaphase spindle needs to function in cell volumes that vary by several orders of magnitude. Thus, the spindle has to be long enough to span sufficient distance to physically separate chromosomes. Because defects in spindle length result in erroneous cell division (Dumont et al. 2007), robust mechanisms to set the length of a spindle and scale it according to cell size must exist. In its simplest form, spindle length could be constrained by physical cellular boundaries just as the size of asters in frog and fish oocytes (Wühr et al. 2010). Robust size control could also be achieved through so-called dynamic balance models (Chan and Marshall 2012). These models rely on either assembly or disassembly being size dependent such that they balance at one parameter-specified size or balance point. Such a mechanism fits well with a recent mass balance model of spindle length, with the steady-state spindle size effectively set by the balance of microtubule assembly and disassembly (Reber et al. 2013). Alternatively, cell size could control the length of the spindle by providing a finite cytoplasmic volume, where a key component present in limiting amounts is depleted as the structure assembles (Good et al. 2013; Hazel et al. 2013). A number of factors have been suggested to contribute to setting spindle length (see below), and it will be interesting to determine the relevant ones that govern scaling in

different contexts (Goshima et al. 2005; Dumont and Mitchison 2009a,b; Loughlin et al. 2011; Reber et al. 2013; Wilbur and Heald 2013a).

The challenge of scale is particularly apparent during early development, when cell growth and division are uncoupled. During *Xenopus* embryogenesis, for example, cell size dramatically decreases. The 1200-μm-diameter fertilized egg divides and gives rise to approximately 12-μm-diameter blastomeres (Montorzi et al. 2000). During the first mitoses, spindle length is uncoupled from cell size and reaches an upper limit of approximately 60 μm through mechanisms proposed to be intrinsic to the spindle (Wühr et al. 2008). Later in *Xenopus* egg development, a strong correlation between spindle length and cell size emerges. This has been shown in *Xenopus* embryos and extracts from fertilized embryos that recapitulate in vivo spindle size differences (Wühr et al. 2008; Wilbur and Heald 2013b). Two recent studies, which encapsulate extracts from *Xenopus* eggs or embryos in droplets of varying size, confirm that metaphase spindle length and width scale with droplet size in vitro (Good et al. 2013; Hazel et al. 2013), suggesting that cytoplasmic volume could limit the amount of material for assembly. Interestingly, in embryonic extracts from haploid embryos, spindle size is only reduced by approximately 10%. This difference is similar to the DNA-dependent length difference observed previously (Brown et al. 2007; Dinarina et al. 2009) indicating that signaling from chromatin contributes to setting spindle length but is not a major factor. Instead, microtubule stability appears to be a robust mechanism for determining spindle length in *Xenopus* egg extracts, and factors controlling microtubule dynamics are likely to scale spindle length. Indeed, kinesin-13 was shown to be inhibited during early developmental stages by the transport receptor importin α, and activated in later stages when importin α partitions to a membrane pool (Wilbur and Heald 2013b). This mechanism is directly linked to changes in the surface membrane to cell volume ratio and thus suitable for developmental scaling.

Interestingly, the smaller relative of *X. laevis*, *X. tropicalis*, has correspondingly smaller cells, nuclei, and spindles (Levy and Heald 2012). Recent work has shown that the observed differences in spindle size are recapitulated in respective egg extracts. *X. tropicalis* spindles are approximately 30% shorter than *X. laevis* spindles. What is the underlying cause of spindle size difference in the two extracts? Mixed extracts produce spindles of intermediate sizes revealing a dynamic, dose-dependent regulation of spindle size by cytoplasmic factors (Brown et al. 2007). Based on a computational model of meiotic spindle assembly, which predicted that higher localized microtubule depolymerization rates could generate shorter spindles (Loughlin et al. 2010), a single phosphorylation site in katanin was identified as the source of the spindle size differences in the two related frog species. Phosphorylation by the mitotic kinase Aurora B lowers the katanin activity in *X. laevis*, while *X. tropicalis* katanin lacking this phosphorylation site remains active. Consequently, a decrease in microtubule stability causes the shorter spindles in *X. tropicalis* egg extract (Loughlin et al. 2011). This study nicely shows that, in different species, mechanisms have evolved to modulate the intrinsic size of the metaphase spindle. However, it remains to be understood why the *X. tropicalis* spindle needs to be shorter in the first place. Perhaps this is because of later constraints in development that arise as cells become increasingly small, which, in *tropicalis*, may occur sooner given its smaller initial size.

Correlations of spindle length and width with cell size have also been shown in *C. elegans* embryos (Hara and Kimura 2009, 2013; Greenan et al. 2010) and *Mus musculus* (Fitzharris 2009; Courtois et al. 2012). Greenan and colleagues (2010) showed that spindle length correlates with centrosome size through development and that a reduction of centrosome size reduces spindle length. Mechanistically, the authors suggest that centrosome size sets mitotic spindle length by controlling the length scale of a TPX2 gradient along spindle microtubules (Greenan et al. 2010). This is consistent with previous results in human cells, which show that introducing point mutations in TPX2, which abolish the interaction between TPX2 and Aurora A, results in small spindles (Bird

and Hymann 2008). If centrosome size sets spindle size, what then controls the size of the centrosome? Decker and colleagues propose that limiting amounts of centrosome material set the size of the centrosome in *C. elegans* embryos (Decker et al. 2011). The idea is that when centrosomes grow in a finite volume, the cytoplasmic concentration of a limiting (structural) factor will gradually decrease as centrosomes bind and sequester material from the cytoplasm. Such a limiting component system may be a general way of limiting the size of intracellular organelles in systems with fast cell cycles and rapidly changing cell volume (Coyne and Rosenbaum 1970; Stephens 1989; Norrander et al. 1995; Bullitt et al. 1997; Elliott et al. 1999; Brangwynne et al. 2009, 2011; Goehring et al. 2011; Goehring and Hyman 2012; Feric and Brangwynne 2013). The great advantage of the limiting component system is to provide a robust and rapid system that takes advantage of the contribution of a defined amount of maternal cytoplasm to the embryo. Whether similar mechanisms also apply in somatic systems, with longer cell cycles and smaller changes in cell size, is an important direction for future investigation.

OUTLOOK

Here, we have discussed how throughout the last decade, three different directions have converged to suggest that reconstitution of a mitotic spindle might soon be possible. These are the cataloging of spindle components, their in vitro expression and biochemical and physical characterization in minimal systems, and increasingly developed theory. An in vitro reconstitution of the metaphase spindle from purified components will likely begin as a spindle similar to a *Xenopus* oocyte spindle, in which the dynamics of microtubules are dominated by chromatin. However, the increasingly sophisticated reconstitution of kinetochores and centrosomes suggest that a full reconstitution of a functional spindle will be possible. This will indeed be a triumphant conclusion to the work of Walther Flemming almost 150 years ago, who could hardly have conceived of such an achievement.

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