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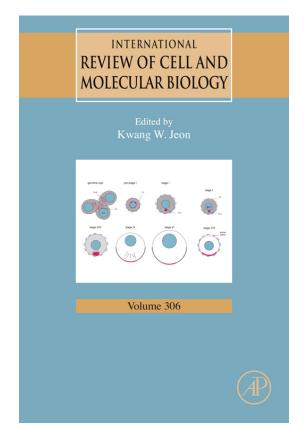
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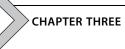
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# Interplay Between Spindle Architecture and Function

## Kara J. Helmke, Rebecca Heald<sup>1</sup>, Jeremy D. Wilbur<sup>1</sup>

Department of Molecular and Cell Biology, University of California, Berkeley, California, USA <sup>1</sup>Corresponding authors: e-mail address: bheald@berkeley.edu; Jwilbur@berkeley.edu

## Contents

1.	Introduction	84
2.	Conserved Features of the Metaphase Spindle	85
	2.1 Microtubules and their dynamics	85
	2.2 Spindle bipolarity	86
	2.3 Microtubule populations within the spindle	87
	2.4 Microtubule dynamics and organization in the spindle	90
3.	Nucleation and Origin of Microtubules in the Spindle	93
	3.1 Centrosome-mediated search-and-capture	94
	3.2 Mitotic chromatin-mediated nucleation	96
	3.3 Kinetochore-driven nucleation	98
	3.4 Microtubule-branching nucleation	99
4.	Molecular Mechanisms Defining Spindle Architecture: Microtubule Stability and	
	Transport	101
	4.1 Microtubule-stabilizing proteins	101
	4.2 Microtubule-destabilizing proteins	103
	4.3 Motor proteins and microtubule transport	104
5.	Variations on a Theme: Tailoring Spindle Architecture	106
	5.1 Spindle features and phylogeny	108
	5.2 Spindle size	110
	5.3 Spindle architecture and compartmentalization	112
	5.4 Simulations of microtubule arrangement in the spindle	115
	5.5 Future prospects for determining spindle architecture	117
6.	Conclusions	118
Ac	knowledgments	119
Re	ferences	119

## Abstract

The mitotic spindle performs the universal and crucial function of segregating chromosomes to daughter cells, and all spindles share common characteristics that facilitate this task. The spindle is built from microtubule (MT) polymers and hundreds of associated factors that assemble into a dynamic steady-state structure that is tuned to the cellular environment. In this review, we discuss the phenomenology and underlying mechanisms that describe how spindle architecture is optimized to promote robust chromosome segregation in diverse cell types. We focus on the role of MT dynamics, stabilization, and transport in an effort to understand how the molecular mechanisms governing these processes lead to the formation of the functional, steady-state spindle structure. Finally, we investigate the basis of spindle variation and discuss why spindles take on certain forms in different cell types. The recent advances in understanding spindle biology have shown that spindle assembly utilizes multiple but common pathways weighted differently in different cells and organisms. These assembly differences are correlated with variations in spindle architectures that may influence the regulation of molecules in the spindle. Overall, as architectural features of different spindles are elucidated, the available comparative genomic data should provide structural and mechanistic insight into how a spindle is built, how dynamic interactions lead to a steady-state structure, and how spindle function is disrupted in disease.

# 1. INTRODUCTION

The generation and continuation of life depends on cell division through gametogenesis, development, and homeostatic maintenance of tissues and organisms. Accurate chromosome segregation is essential for genome stability, and deviations in the process can generate chromosomal changes associated with evolution or cause cellular transformation and cancer (Chen et al., 2012; Gordon et al., 2012; Holland and Cleveland, 2012). To achieve high fidelity genome transmission, a dynamic architectural arrangement of the microtubule (MT) cytoskeleton called the spindle is assembled at the onset of mitosis or meiosis. The spindle provides the scaffold and mechanism for force generation to physically separate chromosomes to daughter cells, and also determines the position of the cytokinetic furrow (Glotzer, 2003). Across eukaryotic biology, cell size and shape vary dramatically, requiring complex spatial information to be integrated by the spindle, which may adjust by altering spindle factors and their organization. Genomewide screening and proteomic studies have provided comprehensive lists of spindle-associated proteins and there is evidence that nonprotein components other than chromosomes such as RNA also function as integral spindle components (Blower et al., 2005; Bonner et al., 2011; Chang et al., 2004; Gache et al., 2010; Goshima et al., 2007; Groen et al., 2011). Together with functional investigation of individual factors, these studies provide a critical foundation for investigating the physiological mechanisms that tune spindle size, morphology, and function.

The catastrophic potential of chromosome segregation errors provides a strong selective pressure to form a functionally robust spindle. The specific characteristics of a given cell type, including its size, shape, and contents, will drive diversification of the spindle to suit the particular needs of the cell. In this review, we first describe the architectural features common to all spindles and the fundamental mechanisms of MT dynamics and organization. We then expand upon the key activities that generate spindle architecture, including MT nucleation, transport, and stability within the spindle. Elaborations on the basic spindle theme are then discussed. Throughout, we utilize specific examples to illustrate general principles of spindle biology and describe the experimental and computational results that are generating a clearer picture of spindle assembly, architecture, and function.

# 2. CONSERVED FEATURES OF THE METAPHASE SPINDLE

Chromosome segregation, the common function of all spindles, is preceded by the formation of a steady-state structure at metaphase in which duplicated chromosomes are attached to spindle MTs and poised to separate to opposite ends of the cell at anaphase. While the details of spindle assembly and architecture vary across species and cell types, universal principles operate to ensure chromosome segregation.

## 2.1. Microtubules and their dynamics

The fundamental structural unit of the spindle is the MT (Fig. 3.1), whose dynamics and organization are modulated by hundreds of MT-associated proteins (MAPs) and motors. MTs are polymers made of  $\alpha$ - and  $\beta$ -tubulin heterodimers that bind head-to-tail to form polarized protofilaments, and, in turn, ~13 protofilaments associate laterally and in the same orientation to form a hollow rigid tube about 25 nm in diameter (Chretien et al., 1992; Downing and Nogales, 1998). The asymmetry of the tubulin dimer confers polarity to the polymer—the end with exposed  $\alpha$ -tubulin is called the minus-end, while the  $\beta$ -tubulin end is called the plus-end—leading to different polymerization and depolymerization reactions at each end.

MTs may be growing or shrinking and can abruptly switch between the two states, a behavior termed dynamic instability (Mitchison and Kirschner, 1984a,b). Following polymerization, hydrolysis of GTP bound to  $\beta$ -tubulin occurs rapidly within the lattice of the MT, and MTs possessing a terminal "cap" of tubulin dimers that have not yet hydrolyzed their GTP can

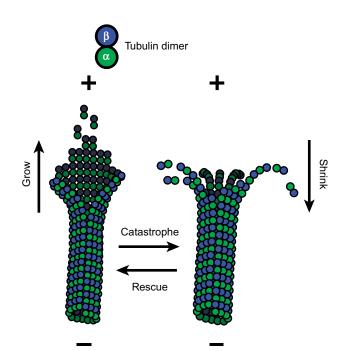
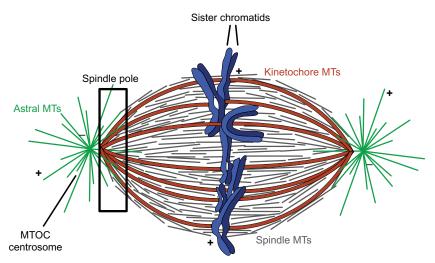


Figure 3.1 MT structure and dynamics. A microtubule is a hollow cylindrical structure built from ~13 parallel protofilaments of  $\alpha\beta$ -tubulin heterodimers. The subunits in each protofilament all point in the same direction, giving the MT lattice a distinct structural polarity, with  $\alpha$ -tubulins exposed at the minus-end and  $\beta$ -tubulins exposed at the plusend. Following polymerization, GTP hydrolysis within  $\beta$ -tubulin destabilizes the MT, but if the polymerization rate is sufficiently high, a GTP cap is maintained and the microtubule continues to grow. If hydrolysis catches up to the end, the GTP cap is lost, and the MT undergoes a catastrophe and shrinks as its protofilaments peel apart. If the GTP cap is regained, a rescue occurs and MT growth resumes.

maintain the growing state. If the terminal tubulin dimers hydrolyze their  $\beta$ -tubulin GTP, the MT loses its cap and switches to a depolymerizing state, a transition called "catastrophe." Conversely, a shrinking MT that regains its GTP tubulin cap can resume growing, a so-called "rescue" (Fig. 3.1).

## 2.2. Spindle bipolarity

The key feature of spindle structure that facilitates its function is its bipolar, antiparallel arrangement of MTs, which ensures that replicated chromosomes are separated into exactly two complete sets (Fig. 3.2). Several elements reinforce the twofold symmetry of the spindle, starting with the linear and polarized MTs themselves, which when bundled generate a bipolar axis. In addition, duplication of the chromosomes and of the MT-organizing center (MTOC) or centrosome prior to mitosis provides



**Figure 3.2** Metaphase spindle anatomy and constituent MT populations. MTs are arranged in an antiparallel array with their plus-ends oriented toward the center of the spindle and their minus-ends toward the poles. MTs that connect to the chromosomes at their kinetochores are called kinetochore fibers or k-fibers (red) and may consist of a single MT or bundles of MTs, depending on the cell type. At metaphase, the sister chromatids of each duplicated chromosome are attached to opposite spindle poles. Spindle MTs (gray) can be long, extending from the pole across the middle of the spindle, or short, forming a tiled array. Astral MTs (green) emanate from the poles or associated centrosomes with their plus-ends extending outward where they may interact with the cell cortex.

cues for bipolarity. Even in the presence of multiple centrosomes, MTs of the spindle frequently coalesce into a bipolar structure, indicating a high degree of MT self-organization during spindle formation (Godinho et al., 2009). The spindle poles can be defined as the two ends of the spindle and are where the highest density of MT minus-ends is found. In many cell types, this focused arrangement of MT minus-ends is reinforced by the presence of a MTOC or centrosome that nucleates MTs so that their minus-ends remain tethered at or near the spindle pole. The organization of MTs at the spindle pole and their regulation is a point of architectural variation among different spindles that likely has important functional consequences for controlling spindle size, chromosome segregation, and interactions with the cell cortex.

#### 2.3. Microtubule populations within the spindle

Three different populations of MTs define the most basic features of metaphase spindle architecture (Fig. 3.2). The most crucial population is termed kinetochore or k-fibers. K-fiber MTs connect to duplicated chromosomes so that one copy (called a sister chromatid) is attached to each pole of the spindle. The connection occurs at the kinetochore, a macromolecular MT attachment site formed during mitosis at the centromere of each sister chromatid (Jensen, 1982; Rieder, 2005). An examination of a small variety of spindles using electron microscopy, and more recently electron tomography, has identified some structural features of k-fibers, while cell-based observations and perturbations have provided insight into the dynamics of k-fiber MTs. In some fungi, such as Saccharomyces cerevisiae and Ashbya gossipi, k-fibers consist of one or two MTs that extend from the spindle pole to the kinetochore (Gibeaux et al., 2012; Winey et al., 1995), while in most species, k-fibers are bundles of 20-30 MTs (McEwen et al., 1997; Rieder, 1981). In Ptk1 cells, individual k-fiber MTs may span the entire half spindle length but are bundled with many shorter overlapping MTs (Rieder, 1981). Holocentric chromosomes have the unusual property of assembling kinetochore complexes and attaching to MTs along their entire length. Although best characterized in the nematode Caenorhabditis elegans, holocentric chromosomes are found in a broad range of plant and animal species (Melters et al., 2012). Despite variation in their number and organization, a common feature of k-fiber MTs is that they are resistant to treatments that depolymerize other spindle MTs, such as cold temperature or low doses of MT-depolymerizing drugs. The extensive bundling of k-fibers by MT cross-linking proteins likely increases the stability of their component MTs. The functional effect of k-fiber MT stability may be to provide a rigid connection to chromosomes so that forces are transmitted efficiently and not lost on bending or splaying of k-fiber MTs. A fascinating feature of k-fiber MTs is that their dynamics appear coordinated, and end-on kinetochore attachment is maintained even though the associated MTs are growing or shrinking (Guo et al., 2013). In vertebrate somatic cells, once sister chromatids are attached to opposite poles, the sister chromatid pair oscillates as one k-fiber grows and the other shrinks, congressing to build a "metaphase plate" in which chromosomes are aligned in the center and poised to segregate. K-fiber MTs are therefore absolutely essential for spindle function, and this population of MTs is found in some form in all spindles.

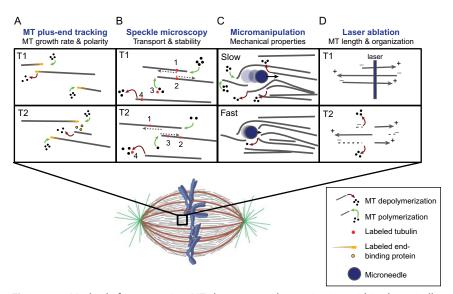
A second population of MTs within the spindle constitutes those that are not part of a k-fiber, referred to here as *spindle MTs*. These MTs may be short and reside within a single half of the spindle or long enough to cross over the center of the spindle to interact with oppositely oriented MTs in an antiparallel fashion (termed interpolar MTs). Spindle MTs are less stable than k-fiber MTs and may be more or less numerous. They appear to increase the rigidity of the spindle to resist to pole-to-pole compression and may provide a pool of MTs to help maintain k-fibers. Because kinetochores and chromosome arms can move via motor proteins along spindle MTs toward the center of the spindle, spindle MTs provide a k-fiber-independent mechanism that promotes chromosome congression to the metaphase plate (Cai et al., 2009; Guo et al., 2013). However, the role of spindle MTs has infrequently been studied explicitly. In one case, fission yeast spindle MTs extending from each pole were shown to be dispensable for meiotic spindle function (Akera et al., 2012); however, in other systems, spindle MTs have a more pronounced role. Depletion of spindle MTs from Xenopus egg extract spindles by addition of a tubulin-sequestering protein revealed a role for spindle MTs in establishing proper MT dynamics within the k-fiber as well as for maintaining spindle size (Houghtaling et al., 2009). In general, larger spindles appear to contain a higher proportion of spindle MTs compared with k-fibers, indicating that they primarily play a structural role.

In addition to the k-fiber and spindle MTs that make up the body of the spindle, a population of astral MTs projects outward toward the cell cortex from the MTOC or centrosome at or near the spindle poles. Astral MTs appear less tightly bundled than MTs of the spindle body and, though found in most spindles, they appear to be absent from female meiotic spindles and plant spindles that lack centrosomes. Astral MTs may span the distance from the spindle pole to the cortex and thus provide the cues and forces to properly orient and position the metaphase spindle, which is required for asymmetric cell divisions important for cell fate determination, tissue organization, and development (Noatynska et al., 2012). However, in very large embryonic cells, single astral MTs are not long enough to reach the cell periphery, and the mechanism by which astral MTs contribute to metaphase spindle positioning is unclear (Mitchison et al., 2012). A prominent feature of astral MTs is their increase in length and density at the metaphase to anaphase transition in both somatic and early embryonic cells, which likely contributes to their function in positioning the spindle and cleavage furrow (Wuhr et al., 2010). If astral MTs were induced to lengthen inappropriately in HeLa cells, however, spindles oscillated dramatically in early anaphase due to blebbing of the cell cortex at the poles, indicating that proper astral MT dynamics is essential to coordinate spindle position with cortical contractility (Rankin and Wordeman, 2010).

The relative partitioning of MTs among the different populations varies in different cell types and species, but the high frequency at which all three populations of MT appear suggests a strong evolutionary constraint on overall spindle architecture. Interestingly, k-fiber, spindle, and astral MTs each appear to possess unique features and regulation. The identification of MAPs that are specific to certain MT populations (discussed in Section 4) supports the idea that these are truly functionally distinct; however, an important line of investigation will be to understand how crosstalk among populations contributes to spindle function.

#### 2.4. Microtubule dynamics and organization in the spindle

The dynamic properties of MTs have been studied extensively, and a variety of biochemical conditions and protein factors have been shown to modulate parameters of MT growth, pause, catastrophe, shrinkage, and rescue (Al-Bassam and Chang, 2011; Amos, 2004, 2011; Amos and Schlieper, 2005; Desai and Mitchison, 1997; Drummond, 2011). One question is whether the densely packed spindle MTs are similarly modulated. Techniques marking a subset of MTs in the spindle, such as fluorescence recovery after photobleaching (FRAP), have established that MTs are very dynamic with recovery half-times in the tens of seconds (Wadsworth and Salmon, 1986). Imaging individual MTs in the spindle is extremely difficult, but two dynamic properties have been well characterized: MT growth within the spindle and their continuous transport toward the spindle pole, known as poleward MT flux (Ganem et al., 2005; Sawin and Mitchison, 1991, 1994; Tirnauer et al., 2004). The identification and fluorescent labeling of proteins such as EB1 that remains associated with the growing MT plus-end have allowed the tracking of individual MTs and established that growth rates of MTs both within and outside of the spindle are similar at metaphase (Tirnauer et al., 2004). In addition, plus-end tracking provides information about where MTs are polymerizing in the spindle and their polarity (Fig. 3.3A). Although dynamic instability largely accounts for the high rate of MT turnover in the spindle, the use of FRAP, photoactivation, and, especially, fluorescent speckle microscopy (FSM) to generate fiduciary marks has provided measurements of MT flux (Fig. 3.3B), which also promotes turnover through the net assembly at MT plus-ends and disassembly at MT minus-ends. However, the extent to which these movements are representative of all spindle MTs or particular MT populations remains unclear (Maddox et al., 2003; Sawin and Mitchison, 1991, 1994; Wadsworth and Salmon, 1986; Waterman-Storer et al., 1999).



**Figure 3.3** Methods for measuring MT dynamics and organization within the spindle. (A) MT end-binding proteins can be fluorescently labeled and used to track the growing ends of MTs in the spindle. These do not track with the depolymerizing ends of a MT. (B) MT flux can be measured when fluorescently labeled tubulin "speckles" are incorporated into a MT. Speckle movement can then be tracked or lifetime within a MT can be assessed. (C) Micromechanical properties of the spindle can be measured using force-calibrated microneedles to infer mechanistic and architectural features of the spindle. (D) Femtosecond laser ablation cuts subsets of MTs in the spindle, leading to depolymerization from the new plus-end. Organization and architecture of the spindle can be inferred from the depolymerization dynamics.

In *S. cerevisiae*, spindle MTs were observed to turn over from their plus-ends within the spindle body, but flux through the spindle as in most metazoan spindles was not observed (Maddox et al., 2000). This suggests that dynamic MTs are a property of all spindles, while poleward MT flux is not. Furthermore, experimental conditions that block poleward MT flux in cultured cells by inhibiting two MT-depolymerizing proteins did not prevent spindle assembly or chromosome alignment (Ganem et al., 2005). However, chromosome segregation errors occurred under these conditions, indicating that MT flux may play a role in promoting robust spindle function, perhaps by promoting correct k-fiber–chromosome connections. Another more speculative possibility is that poleward flux is critical, particularly in large

spindles, to drive spatial distribution of spindle-associated factors or even the MTs themselves.

Measurements of MT transport and lifetime through single molecule and FSM imaging have provided insight into how MT dynamics may establish and respond to the spindle environment. By tracking correlated movements of pairs of fluxing tubulin dimers in a Xenopus egg extract spindle, the arrangement of MTs was predicted to be a tiled-array in which many MTs overlap to span the distance between the spindle pole and the metaphase plate (Fig. 3.3B; Yang et al., 2008). This suggests that spindle MTs must be highly cross-linked to form a robust yet dynamic structure. However, chromosomes must be able to move within the spindle to align at the metaphase plate. An investigation into the mechanical properties of the Xenopus egg extract spindle indicates how MT cross-linking provides structural integrity to the spindle and yet allows chromosome movement (Fig. 3.3C). The viscoelastic properties were probed using force-calibrated microneedles inserted into the spindle and were shown to depend on the amount of MT cross-linking (Shimamoto et al., 2011). Moreover, the viscoelastic behavior was timescale dependent and indicated that chromosome movements occur on a timescale in which elastic strain can be dissipated through remodeling of the spindle structure, possibly by MT depolymerization and repolymerization or flux (Shimamoto et al., 2011; Fig. 3.3C). Thus, MT dynamics within the spindle drive the size, shape, and organization of the spindle as well as determine its micromechanical properties.

Despite the highly cross-linked nature of the spindle, there is evidence that the spindle environment is not uniquely stabilizing or destabilizing for MTs (Needleman et al., 2009). The stability of MTs within the spindle was determined using single molecule lifetime measurements of labeled tubulin in spindle MTs, similar to a FSM experiment. Instead of measuring the displacement of the fluorescent speckle, these measurements assess how long a labeled tubulin dimer resides in a spindle MT before a catastrophe releases it. In Xenopus egg extracts, the residence lifetimes were very similar within a spindle compared to much sparser MT arrays nucleated from Tetrahymena pellicles (Needleman et al., 2009). This observation indicates that the Xenopus cytoplasm is loaded with a variety of MT-stabilizing/ destabilizing proteins that can act on MTs, regardless of their density or cross-linking. Perhaps more interestingly, tubulin lifetime measurements did not vary across different regions of the spindle, suggesting that despite specific localization of factors that alter MT dynamics within the spindle, overall MT stability is uniform.

Further support for a model of spindle organization with homogenous MT stability in all regions stems from using a femtosecond laser ablation technique to cut a subset of MTs within a metaphase *Xenopus* egg extract spindle (Brugues et al., 2012; Fig. 3.3D). The cut MTs depolymerized from their newly generated plus-ends at the site of laser irradiation, generating waves of depolymerization moving toward each spindle pole, which could be observed by tracking the changes in MT intensity over time. Quantitative information was derived about the MT population from the wave decay, such as the density of minus-ends. By comparing the two waves, information about the polarity of MTs could be obtained, and irradiating different regions of the spindle showed that MTs were longer near the center of the spindle and shorter toward the poles. Interestingly, inhibition of poleward MT flux homogenized spindle MT lengths, supporting a model in which spindle architecture depends on both the position of MT nucleation near the center of the spindle and poleward MT transport (Brugues et al., 2012).

Together, these studies provide clues as to how global features such as spindle size and shape are established and maintained over timescales much longer than the MT lifetime and over distances much longer than the average spindle MT. Due to its large size and accessibility for perturbation, the *Xenopus* egg extract spindle has been investigated most intensively, and in the future, it will be critical to elucidate how MT nucleation, dynamics, and flux operate to produce a functional spindle and how these properties are modulated in different spindle types.

# 3. NUCLEATION AND ORIGIN OF MICROTUBULES IN THE SPINDLE

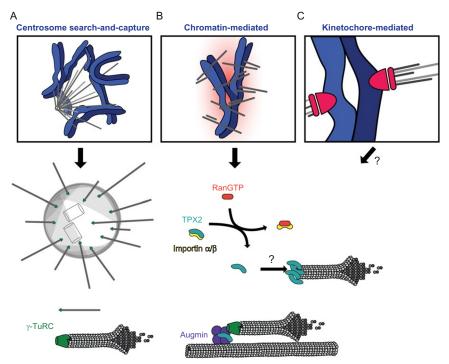
There are two recognized spindle assembly pathways, "search-and-capture" and "spindle self-organization," which are not mutually exclusive and are thought to operate simultaneously in most cases to promote robust spindle formation. These two pathways are broadly defined by where MTs nucleate. Search-and-capture occurs by selective stabilization of MTs nucleated at the MTOC or centrosome and self-organization describes any number of modes of nucleation followed by incorporation of the MTs into a bipolar array by the activity of associated proteins including MT-based motors. Less well understood, however, is what determines the relative role of a pathway or the preferred types of MT nucleation in a particular cell type and how these pathways are interregulated and contribute to spindle architecture. Depending on where and how MTs are nucleated, the proportion of MTs in each population may change, thereby altering the organization of the spindle.

#### 3.1. Centrosome-mediated search-and-capture

Since MT dynamic instability was first observed, Mitchison and Kirschner (1985) proposed that spindle assembly could occur through the selective stabilization of centrosome-nucleated MTs at kinetochores. By frequently switching between growing and shrinking states, MTs can "probe" the cytoplasm randomly until captured by a chromosome, allowing the cell to gradually form a bipolar MT array from two centrosomes and pairs of sister chromatids (Holy and Leibler, 1994).

When present, centrosomes appear to be the dominant MT-generating and -organizing centers of the cell. A typical centrosome consists of a pair of centrioles surrounded by pericentriolar material that recruits the tubulin isoform  $\gamma$ -tubulin in a complex called the  $\gamma$ -tubulin ring complex ( $\gamma$ -TuRC) that provides a template for MT growth by stabilizing and anchoring the minus-ends of MTs at the centrosome (Guillet et al., 2011; Hannak et al., 2002; Kollman et al., 2010, 2011; Fig. 3.4A). Upon entry into mitosis, centrosomes greatly increase their capacity to nucleate MTs, a process termed centrosome maturation.

While a simple and appealing hypothesis, computational simulations indicated that search-and-capture would be rather inefficient on its own and require hours longer than the time period of a normal mitosis (Wollman et al., 2005). However, the time could be reduced if geometric constraints were added to the model that optimized the size and spatial exposure of target kinetochore-binding sites to centrosome-nucleated MTs, specifically by increasing chromosome mobility and by incorporating chromosome-dependent MT-nucleating pathways discussed below (Paul et al., 2009). Remarkably, in vivo observations have revealed that spindle components are spatially positioned at early stages of spindle assembly to facilitate chromosome–MT interactions, fulfilling the hypothetical parame– ters necessary for rapid spindle assembly posited in simulation. By implementing 3D imaging with high temporal and spatial resolution of MTs, kinetochores, and chromosomes during spindle assembly in human RPE1 cells, a toroidal distribution of chromosomes could be resolved in which kinetochores reside in an area with extremely high MT density and are not shielded by chromosomes (Magidson et al., 2011; Fig. 3.4A). This transient prometaphase arrangement is promoted by the polar ejection force due to chromokinesins (discussed in Section 4) as well as by lateral



**Figure 3.4** Spindle assembly pathways and mechanisms of MT nucleation. (A) Centrosome-mediated search-and-capture proceeds by nucleation of MTs from the  $\gamma$ -tubulin ring complex embedded in the centrosome. Orientation of chromosomes in a toroidal arrangement around the center of the nascent spindle, with exposed kinet-ochores, facilitates connections between these centrosomal MTs and kinetochores. (B) The chromatin-mediated self-assembly pathway is governed by a spatial gradient of RanGTP, which stimulates the release of MAPs in the vicinity of the chromosomes. One confirmed cargo, TPX2, has a role in nucleation of MTs in this pathway, but the mechanism is unknown. The Augmin complex, which recruits  $\gamma$ -TuRC, amplifies MTs nucleated around the chromosomes in this pathway. (C) MTs have been shown to nucleate directly from kinetochores; however, the mechanism is not yet elucidated but might share some commonalities with the chromatin-mediated pathway.

interactions between MTs and kinetochores. Orienting kinetochores in this way predisposes them for MT interactions, facilitating the formation of end-on MT attachments and biorientation, both *in vivo* and *in silico* (Magidson et al., 2011).

Despite the prevalence of centrosomes, the existence of meiotic or plant spindles that lack them, together with studies employing centrosome ablation, clearly demonstrate that they are not essential for bipolar spindle assembly (Khodjakov et al., 2000). However, if the centrosome was removed by microsurgery early in the cell cycle, a MTOC-lacking centriole was observed to reform and generate an astral MT array (Hornick et al., 2011). Separation of the *de novo* MTOC into two MT asters was required for bipolar spindle assembly and failed in 35% of cells, which then formed monopolar spindles. This result demonstrates that *de novo* MTOCs, like centrosomes, form dominant sites of MT nucleation that strongly influence spindle assembly. When present, centrosomes may promote more robust spindle bipolarity. Although dispensable for much of *Drosophila* development, centrosomes are essential for the syncytial nuclear divisions during early embryogenesis and function in spindle positioning through astral MT interactions with the cell cortex, which is crucial for asymmetric, polarized divisions in cells such as neuroblasts (Basto et al., 2006). Thus, centrosomes are not necessarily required for all cell divisions but appear to be essential in animals.

In addition to promoting bipolarity, centrosomes and associated factors can regulate spindle length. In the *C. elegans* embryo, spindle size correlates with centrosome size and molecular perturbations that altered centrosome size proportionally changed spindle length (Greenan et al., 2010). How centrosome size is established is not understood, but a spindle-associated TPX2-like protein (discussed in Section 4.1) in *C. elegans* has been implicated and might provide a molecular link between the centrosome and spindle architecture. In light of these examples, spindle-pole-associated centrosomes and their astral MT arrays might be considered an architectural feature of different spindles rather than an absolute requirement for spindle assembly or function.

#### 3.2. Mitotic chromatin-mediated nucleation

Experiments using *Xenopus* egg extracts demonstrated that MTs nucleated around chromatin-coated beads could organize into a bipolar spindle structure in the absence of both centrosomes and kinetochores, illustrating the self-organization pathway of spindle assembly (Heald et al., 1996). In both egg extracts and human tissue culture cells, chromatin effects are mediated primarily by a gradient of GTP-bound Ran, which peaks at the chromosomes. Single glass beads coated with RCC1, the guanine nucleotide exchange factor that loads Ran with GTP, could induce bipolar spindle assembly in *Xenopus* egg extracts, indicating that the generation of RanGTP is sufficient for spindle formation (Halpin et al., 2011). However, RCC1 bead spindles were unstable and displayed abnormal MT density, indicating that other chromatin factors are required to generate an architecturally normal spindle. The RanGTP gradient induces spindle formation by triggering

the release of spindle assembly factors (SAFs) from inhibitory interactions with the transport molecules, Importin  $\alpha/\beta$ , resulting in MT nucleation and organization around mitotic chromatin (Fig. 3.4B). Motor proteins organize the MTs into an antiparallel array by sliding them away from the chromatin and focusing their minus-ends into spindle poles. In cell types with centrosomes, clustering of MT minus-ends may also be facilitated by enriching MT binding or motor proteins at the centrosomes or by the astral MTs, which provide tracks on which to focus the antiparallel array (Compton, 1998).

How MTs are nucleated downstream of RanGTP is not yet clear. Immunodepletion of the MAP and Importin  $\alpha$ -cargo TPX2 completely abrogated RanGTP-dependent MT nucleation in *Xenopus* egg extracts, and recombinant TPX2 could induce MT nucleation in solutions of pure tubulin *in vitro* (Gruss et al., 2001; Schatz et al., 2003; Fig. 3.4). This protein is predicted to have little tertiary structure and may oligomerize, but it remains unclear whether TPX2 nucleates MTs directly or if it acts to stabilize small MT assemblies or requires other cellular factors such as the  $\gamma$ -TuRC for activity.

In addition to activating SAFs by liberation from importins, new evidence has revealed a role for Ran in regulating the anaphase promoting complex/cyclosome (APC/C)-induced degradation of certain SAFs during mitosis and spindle assembly. The APC/C has been localized to the spindle poles, and its inhibition resulted in aberrant spindle phenotypes, suggesting a role for the APC/C in spindle maintenance before the spindle checkpoint is satisfied and prior to anaphase (Ban et al., 2007; Goshima et al., 2007; Kraft et al., 2003; Somma et al., 2008; Tugendreich et al., 1995; Williamson et al., 2009). Two SAFs, NuSAP and Hepatoma upregulated protein (HURP), are protected from APC/C recognition and degradation by binding to Importin  $\beta$ . One model is that an additional layer of spatial and temporal regulation on Ran cargoes is generated: RanGTP liberates them from Importin  $\beta$  to perform their assembly and MT-stabilizing tasks but limits their lifetime in the spindle by rendering them susceptible to ubiquitylation by the APC/C (Song and Rape, 2010). This subtle mode of regulation implies that Ranregulated SAFs are powerful modulators of spindle architecture that require precise temporal and spatial regulation to generate a functional spindle.

In addition to the factors regulated by Ran/importin binding, the chromatin-mediated pathway includes additional signals generated by the chromosome passenger complex (CPC; Ruchaud et al., 2007). In most organisms, the CPC consists of the mitotic kinase Aurora B and associated

factors that regulate its activity and targeting: INCENP, Survivin, and Borealin/Dasra. CPC activity is essential in some cases of acentrosomal spindle assembly, such as *Drosophila* meiosis and in *Xenopus* egg extracts (Colombie et al., 2008; Gadea and Ruderman, 2005; Sampath et al., 2004). The contribution of the CPC to chromatin-mediated spindle assembly is primarily through Aurora B-dependent phosphorylation and inhibition of MT-destabilizing proteins Op18/Stathmin and the kinesin-13 MCAK, thereby promoting MT stability in the vicinity of chromosomes (Gadea and Ruderman, 2006; Ohi et al., 2004).

#### 3.3. Kinetochore-driven nucleation

Early studies from Brinkley and McIntosh described MTs polymerizing from kinetochores, but until more recently this was not demonstrated in live cells (Khodjakov et al., 2003; Maiato et al., 2004; Pepper and Brinkley, 1979; Snyder and McIntosh, 1975). While the molecular mechanism of MT nucleation at the kinetochore is unknown, there is evidence that this process, like chromatin-mediated spindle assembly, requires the small GTPase Ran (Torosantucci et al., 2008; Tulu et al., 2006).

However, experiments with cells proceeding through mitosis with unreplicated genomes (MUG) showed that spindles formed around kinetochores while the fragmented chromatin was excluded, even though it had established a gradient of RanGTP (O'Connell et al., 2009). If kinetochore assembly was inhibited, the mitotic chromatin attracted astral MTs but failed to form a bipolar spindle. These results suggest that kinetochore MT nucleation is dominant in somatic cells and does not require a RanGTP gradient, which can direct the growth of MTs from centrosomes, but on its own is insufficient for spindle formation.

Like RanGTP, Aurora B kinase has been observed to generate a gradient around chromosomes, but in this case, the gradient consists of phosphorylated substrates, which are produced by concentration and activation of the kinase at centromeres/kinetochores in association with the CPC, followed by release and diffusion to reach substrates at a distance (Wang et al., 2011). The relative role of the two gradients and their ability to promote spindle assembly has been examined in *Xenopus* egg extracts (Maresca et al., 2009). Interestingly, by adding an inhibitor of RCC1 together with a Ran mutant locked in the GTP form, the RanGTP gradient was abolished, but spindle assembly still occurred in the presence of replicated mitotic chromosomes containing centromeres, but not around chromatin-coated beads, even though both bound equivalent amounts of CPC. These results indicate that concentration and activation of Aurora B is required for full activity. However, the downstream activities involved in MT nucleation are unknown.

Whatever the mechanism for kinetochore-mediated nucleation of MTs, it is clear that establishing close contact of MTs with kinetochores early during spindle assembly likely improves the kinetics of assembly by increasing the effective size of the kinetochore target during search-and-capture. Furthermore, kinetochore-mediated nucleation may contribute directly to k-fiber formation.

#### 3.4. Microtubule-branching nucleation

The idea of existing MTs acting as templates for new MT nucleation and growth stems from observations in *Drosophila* S2 cells and *Xenopus* extracts showing that MT plus-ends, as visualized by fluorescently labeled EB1, appear throughout the body of the spindle (Mahoney et al., 2006; Tirnauer et al., 2004), indicating nucleation at points distinct from centrosomes or chromosomes. Furthermore, plant cortical arrays clearly generate branched MT arrays, though the molecular mechanism of this remains unknown. In animals, this MT-nucleating activity is mediated by an eight-member complex called Augmin originally identified in *Drosophila*, and its depletion resulted in severe loss of spindle MTs (Goshima et al., 2008; Uehara et al., 2009). An interaction between the Augmin complex and  $\gamma$ -TURC was shown to be essential for its MT nucleation in human cells, and a similar interaction is predicted for *Drosophila* (Uehara et al., 2009). This suggests a model in which Augmin binds to preexisting MTs and recruits  $\gamma$ -TURC to nucleate a new MT (Fig. 3.4).

The nucleation of MTs by Augmin could function to promote spindle assembly in two ways. First, nucleation of MTs off preexisting MTs could quickly bolster MT mass and increase the kinetics of spindle formation, which has experimental support in *Xenopus* egg extracts (Petry et al., 2011). Second, MT-branching nucleation could assist in establishing bipolarity by generating antiparallel overlap through nucleation of MTs along the same axis as the existing spindle array, as polarity of newly branched MTs is maintained (Petry et al., 2013; Fig. 3.5A). Further cross-linking of these MTs would then provide structural stability to the spindle.

MT-branching nucleation has interesting implications for the architecture of the spindle. Due to the low angle of branching and the maintenance of MT polarity, nucleation occurring anywhere along an existing MT would

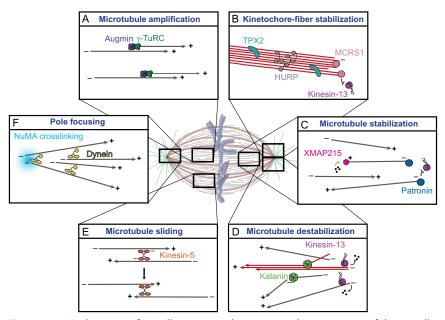


Figure 3.5 Mechanisms of spindle-associated proteins and organization of the spindle. (A) The Augmin complex stimulates amplification of MTs through branched nucleation in the same polarity as existing MTs, enhancing the formation kinetics and density of the overlapping tiled array of spindle MTs. (B) Kinetochore fibers are formed by the action of bundling proteins, such as HURP and TPX2, and are provided additional stability by proteins that protect the minus-ends, such as MCRS1, from depolymerization by kinesin-13 motors. (C) Microtubule stabilization in the spindle structure is affected by many different functional mechanisms, such as promoting polymerization (XMAP215) or by protection from destabilization (Patronin). (D) Microtubule destabilization, on both spindle MTs and kinetochore fibers, can occur through severing by enzymes such as Katanin or by end-depolymerization via kinesin-13s. Destabilizing proteins have specific and varied locations throughout the spindle, suggesting the importance of local destabilizing activity in the spindle. (E) In the antiparallel overlap zone, the tetrameric kinesin-5 motor walks to the plus-ends of neighboring MTs, effectively sliding the minus-ends poleward. (F) Pole focusing occurs through the concerted effort of minus-end directed motors, such as dynein, which carry MTs as cargo toward minusends. Additionally, cross-linking activity by NuMA is directed to the poles by dynein transport, where large oligomers create a MT clustering force.

likely produce a structure akin to a tiled array, held together by cross-linking factors and molecular motors. In *Xenopus* egg extract containing spindles with tiled-array architecture self-organized around chromatin-beads, Augmin depletion defects were severe and resulted in abnormally long, multipolar spindles (Petry et al., 2011). In contrast, the major mitotic phenotype

seen upon Augmin depletion in *Drosphila* were defects in the establishment of the anaphase MT arrays, suggesting that branching MT nucleation is most important when antiparallel arrays of MTs are prominent (Uehara et al., 2009). Consistent with this idea, Augmin was found to be dispensable for bipolar spindle formation in human cells whose spindles normally form in a centrosome-driven pathway, and the presence of centrosomes in *Xenopus* spindles largely mitigated the effects of Augmin depletion (Petry et al., 2011). Together, these data imply that unique spindle architectures are likely established through different modes of MT nucleation and organization, although we do not yet understand how branching MT nucleation sites are positioned and regulated.

#### 4. MOLECULAR MECHANISMS DEFINING SPINDLE ARCHITECTURE: MICROTUBULE STABILITY AND TRANSPORT

The metaphase spindle maintains its steady-state morphology for minutes to hours through the dynamic activities of a hundred or more proteins. To understand how common features of spindle architecture arise and points at which the basic spindle structure can be elaborated, a complete understanding of the individual reactions within the spindle must be achieved. This effort, of course, has yet to be realized, but we will next summarize known mechanisms necessary for robust spindle assembly with a focus on MT organization. We will examine the details of some MT-stabilizing, –destabilizing, and –transporting proteins. Most spindle proteins fit into one of these categories, some fit into more than one, while some have activities that are poorly understood. We focus on examples from a structure—function perspective, describing how specific proteins and activities contribute to spindle assembly and architecture.

# 4.1. Microtubule-stabilizing proteins

As described earlier, several pathways of MT nucleation during spindle formation have been characterized. However, the precise mechanism(s) by which MTs nucleate at sites other than the centrosome remains unclear, specifically whether or not all modes require  $\gamma$ -TuRC, or if other factors such as TPX2 or Augmin are capable of nucleating MTs independently. At the biochemical level, it is extremely difficult to distinguish between stabilization of very small tubulin assemblies and true nucleation, although the need to delineate between alternate mechanisms may not be crucial if they are functionally redundant. However, a molecular understanding of the factors generating MTs remains important to reveal regulatory mechanisms and the structure–function relationships of these proteins.

In addition to potential effects on MT nucleation, an increase in MT stability can stem from at least three mechanisms: the stabilization of tubulin dimer or protofilament interactions, an increase in MT growth, or suppression of MT catastrophes. There are a large number of proteins that appear to operate through these mechanisms within the spindle, but we will focus on representative examples from each of the three classes, including HURP, XMAP215/ chTOG, Patronin, and MCRS1. These factors are either required for spindle assembly or have severe spindle defects when absent, indicating the importance of MT stabilization in determining spindle architecture in general.

The function of HURP in the spindle seems to be to stabilize dimer or protofilament interactions (Fig. 3.5B). Interestingly, HURP associates specifically with k-fiber MTs in the spindle and has been studied most extensively in *Drosophila*, where the HURP ortholog is called Mars. Mars preferentially binds to k-fibers and promotes resistance to MT-destabilizing conditions (Yang and Fan, 2008). Electron microscopy studies have suggested that HURP facilitates wrapping of a MT sheet around existing MTs in a sheath-like structure to provide extra stability, but whether this occurs *in vivo* is not known (Santarella et al., 2007). This is an example of a protein that stabilizes dimer or protofilament interactions, but it may also lead to increased cross-linking of MTs within the k-fiber bundle if HURP molecules can bridge MTs or interact with multiple other proteins concurrently. Elucidating HURP's mechanism of action in detail may contribute to our understanding of the architectural requirement for different MT populations in the spindle.

An alternative mode of MT stabilization is the promotion of MT growth. This can negate the effects of MT destabilization across a population, and XMAP215 is an established MT-polymerizing protein that has been shown to antagonize the activity of known MT-destabilizing proteins (Brouhard et al., 2008; Tournebize et al., 2000; Fig. 3.5C). Depletion of XMAP215 from *Xenopus* egg extracts decreased MT aster size and led to disorganized spindles. Interestingly, XMAP215 can rescue the formation of MTs under conditions of decreased nucleation/stabilization induced by depleting TPX2 or  $\gamma$ -tubulin (Groen et al., 2009). This clearly shows that promoting MT growth is an important requirement of spindle assembly, but it remains to be seen how specific spindle architectural features are modulated in response to different types of MT stabilization.

Finally, some classes of MT-stabilizing proteins seem to protect MT ends from the activities of depolymerizing proteins. While many of the known proteins are plus-end binding, historically, it has been unclear what activities are able to regulate MT minus-end dynamics in cells. Minus-end-binding proteins, such as Patronin and MCRS1, have now been identified as important for regulating MT stability from the minus-end (Fig. 3.5C). Cell-based assays in Drosophila showed that knockdown of Patronin increased depolymerization rates from the MT minus-end mediated by kinesin-13s (discussed below). In vitro, Patronin could compete with kinesin-13s for MT binding and reduce the depolymerization rate (Goodwin and Vale, 2010). Similarly, MCRS1 is also thought to counteract the kinesin-13 MCAK but instead displays preferential binding to MT minus-ends in k-fiber bundles (Meunier and Vernos, 2011; Fig. 3.5B). Pull-down assays in Xenopus egg extracts showed indirect interaction between MCRS1 and MCAK, and MCRS1 decreased in vitro MCAK activity on centrosome-nucleated MTs in a dose-dependent manner (Meunier and Vernos, 2011). Knockdown of either Patronin or MCRS1 caused severe spindle defects-a small spindle phenotype or loss of chromosomally nucleated MTs and constitutive spindle checkpoint activation, respectively-suggesting that modulating minus-end stability throughout the spindle is important for spindle architecture and function.

## 4.2. Microtubule-destabilizing proteins

MT destabilization is necessary to disassemble the interphase MT array at the transition from interphase to mitosis and thus provides the substrate for building the spindle. Importantly, within the spindle, MT-destabilization rates determine the lifetime of MTs and therefore can have a profound effect on spindle size and organization. MT-destabilization activities have a strong effect on spindle size when overexpressed or depleted and have been identified as effectors utilized under different physiological conditions to control meiotic spindle size between different species and mitotic spindle size during development.

There are three major classes of MT-destabilizing proteins: destabilizing kinesin family members (kinesin-13, kinesin-14, and kinesin-8), MT-severing enzymes of the AAA ATPase family (Katanin, Spastin, Fidgetin), and tubulin dimer-sequestering proteins (OP18/Stathmin, RB3; Fig. 3.5D). Kinesin family molecules have complex regulation through phosphorylation, protein interaction partners, and localization and are

important modulators of MT density and dynamics. Tubulin-sequestering proteins seem to have decreased activity during mitosis due to inhibitory phosphoregulation and therefore will not be further discussed (Budde et al., 2001; Cassimeris, 2002; Gadea and Ruderman, 2006). The opposite is true for the MT-severing protein Katanin, which has increased activity during mitosis (McNally and Thomas, 1998). Severing proteins physically cleave MTs using the energy of ATP hydrolysis at points within the MT polymer and may also depolymerize MTs from the ends (Diaz-Valencia et al., 2011; Mukherjee et al., 2012; Zhang et al., 2011). Given the regulated changes in activity during mitosis, it appears that the length distribution of MTs and mechanism of MT destabilization generated by kinesin family members and severing proteins are better suited to mitotic spindle assembly and function than tubulin-sequestering proteins.

Kinesin-13 family members are known to play important roles in chromosome attachment, MT flux, and MT density and length. It is well established that MCAK, a kinesin-13 family member, provides global control of MT lengths in Xenopus egg extract, as its inhibition or depletion caused the formation of large, poorly organized assemblies of MTs rather than spindles (Ohi et al., 2004; Walczak et al., 1996). This simple observation also demonstrates that disrupting a single activity that contributes to spindle architecture can lead to dramatic morphological changes. The kinesin-13 family members have some overlapping functionality and localization, indicating that spindles need a certain level of MT destabilization, but where destabilization is localized to build and regulate the organization of the spindle is unknown. Moreover, specific regions of the spindle are enriched for some family members but not others, and although actual activity levels have yet to be measured in different regions of the spindle, the localization differences imply diversity in the requirement for controlling distinct spindle MT populations (Welburn and Cheeseman, 2012).

#### 4.3. Motor proteins and microtubule transport

In addition to those factors that contribute to the stability or instability of MTs, motors are necessary to sort and organize these MTs into a steady-state spindle structure. Motor activities such as cross-linking and pole-coalescence contribute broadly to spindle architecture, including the establishment of bipolarity. Changes in these motor activities, or the balances between them, have been implicated in regulating spindle size computationally and experimentally, and modulation of their activities could contribute to spindle morphology and the establishment of different MT populations in the spindle.

The major plus-end-directed motor in the spindle is the kinesin-5 member Eg5/BimC/Kinesin spindle protein. Eg5 is a homotetramer capable of binding and cross-linking two MTs (Cole et al., 1994). For antiparallel MTs, which Eg5 seems to preferentially bind, the motor functions to slide both MTs with their minus-ends outward away from the center of the spindle (Fig. 3.5E). This establishes a basic plus-end in, minus-end out organization of MTs as well as aligning and cross-linking them in an orientation along the same axis. Disruption of Eg5 by the small molecule inhibitor monastrol results in collapse of the spindle into a monopole structure in *Xenopus* egg extracts (Kapoor et al., 2000). In mammalian cells, Eg5 is required to establish bipolarity of the spindle but not to maintain it (Kollu et al., 2009), since the kinesin-12 HKlp2 shares this function (Vanneste et al., 2009). Additionally, Eg5 sliding of MTs toward the pole is thought to contribute to MT flux, as the inhibition of Eg5 eliminates flux in Xenopus egg extract spindles (Miyamoto et al., 2004). However, Eg5 inhibition in mammalian cells does not drastically change the flux rate (Cameron et al., 2006). This difference may reflect alternative spindle architectures in which the extent of the antiparallel MT overlap in Xenopus is much higher. Clearly, Eg5 and its orthologs play a critical role in establishing the antiparallel array of spindle MTs and therefore the spindle architectural features and functions associated with it.

The dominant minus-end-directed motors in the spindle are dynein and kinesin-14 class members (including Ncd, HSET, XCTK2). Between two antiparallel MTs, minus-end movement functions to oppose the MT motion of kinesin-5s. However, one MT could also act as cargo, transported toward the minus-end of another MT, thereby clustering the minus-ends of MTs together. These motors therefore have a critical role in focusing MTs into a coalesced pole (Fig. 3.5F). *Drosophila* containing disruptions in Ncd, a kinesin-14, have defects in spindle pole integrity (Endow et al., 1994), and pole formation is completely abrogated upon dynein inhibition in the centrosome-independent, self-organizing pathway of spindle assembly around DNA-coated beads in *Xenopus* egg extracts (Heald et al., 1996).

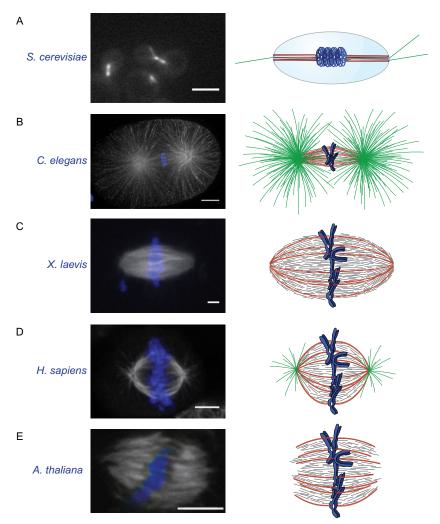
In addition to its role in MT organization, dynein also retains its capacity to carry cargoes to minus-ends and the spindle poles, spatially localizing certain activities. Some dynein cargoes are thought to help form and maintain the spindle pole, such as NuMA, which stabilizes the pole structure through assembly of multiple MT-binding domains upon formation of higher order oligomers (Merdes et al., 2000; Fig. 3.5F). Dynein also carries a number of motors to the pole, like Eg5 and Xklp2, the outcome of which could alter motor activity depending on local MT organization. For example, Eg5 or other motors that typically slide against antiparallel MTs will encounter a much higher density of parallel MTs at the poles, where it may act instead as a cross-linker (Uteng et al., 2008; Wittmann et al., 1998). By promoting dynamic transport of factors throughout the spindle, motors provide an additional layer of regulation of spindle assembly and maintenance factors.

Finally, a large number of motor proteins have been identified that bind not only to MTs but also to chromosomes. Despite common localization, the functions of these varied proteins appear to be nonredundant. The kinesin-10 member Xkid facilitates the congression of chromosomes and their alignment at the metaphase plate (Funabiki and Murray, 2000). While some kinesin-4 members also contribute to chromosome alignment, interestingly, Xklp1 has also been shown to regulate MT density in the spindle (Castoldi and Vernos, 2006; Vernos et al., 1995). The many roles of chromokinesins indicate that MT-chromosome attachments are not only important for physically tethering and separating the chromosomes, but they actually help establish and maintain the spindle structure as well.

# 5. VARIATIONS ON A THEME: TAILORING SPINDLE ARCHITECTURE

Subcellular organization must accommodate the variety of cellular structures and lifestyles found among eukaryotic species. Similarly, within an organism, specialization of cell types involves changes to cell shape and function that may require alteration of subcellular structures. To maintain accurate chromosome segregation in a variety of contexts, the spindle has adapted to accommodate cell size, shape, dynamics, and contents (Fig. 3.6). How then does the underlying architecture of morphologically distinct spindles facilitate chromosome segregation and yet allow unique functional elaborations? Can the presence or levels of specific spindle proteins, domains, or activities be correlated with spindle architectures across phylogenies or upon cellular specialization? While genomic and expression data that can assess changes in individual spindle-related proteins is pouring in, cell biological assessment of spindle architectures is lacking to generate comprehensive comparisons. However, some correlations of morphology with genomic data are available and can provide some insight into unique features of spindle architecture across phylogeny, and development of systems to specifically study the molecular mechanisms driving different spindle architectures is progressing.

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**Figure 3.6** Spindle morphology has changed concomitantly with the diversification of eukaryotic species and with specialization of cells within an organism. All scale bars 5  $\mu$ m. In the schematics, spindle MTs are indicated in gray, kinetochore fiber MTs in red, and astral MTs in green. (A) Spindles in budding yeast contain one MT per kinetochore fiber in a closed mitosis, with few astral MTs that extend to the cell cortex. (B) The *C. elegans* one-cell embryo spindle contains large astral arrays of MTs that extend to both chromosomes and the cortex. Single kinetochore fiber MTs bind at intervals along the length of the entire holocentric chromosome, which contains kinetochore features throughout. (C) *X. laevis* meiotic spindles formed in cytoplasmic egg extracts contain many short MTs that are held together by motors and cross-linking activity into a large tiled array. (D) Somatic spindles in HeLa cells contain dominant and robust kinetochore fibers with a moderate number of astral MTs which contact the cortex *Continued* 

#### 5.1. Spindle features and phylogeny

In small and genetically tractable organisms such as yeast, spindle morphology and molecular mechanisms can more easily be correlated because serial section electron microscopy and electron tomography have allowed detailed reconstructions of whole spindles in the evolutionarily divergent fungi S. cerevisiae, Schizosaccharomyces pombe, and Ashbya gossypii (Ding et al., 1993; Gibeaux et al., 2012; Winey et al., 1995). Interestingly, S. cerevisiae has one MT per kinetochore, A. gossypii has two, and S. pombe has two to four MTs per kinetochore. One might imagine that these differences could help elucidate important mechanisms controlling the number of MT-kinetochore connections and test hypotheses as to whether certain factors correlate with particular aspects of spindle architecture. However, morphological similarity does not necessarily indicate common mechanisms. S. cerevisiae and A. gossypii both possess point centromeres in which a DNA-binding sequence is necessary and sufficient to drive kinetochore assembly (Meraldi et al., 2006). In contrast, other fungi, plants, and mammals form regional centromeres that usually occupy repetitive DNA and are inherited epigenetically (Burrack and Berman, 2012b). Nevertheless, the functional MT-binding unit of the kinetochore is conserved and allows remarkable flexibility to maintain accurate chromosome segregation, despite differences in centromeric sequences, kinetochore protein levels, and the number of MT-binding sites (Burrack and Berman, 2012a).

Recently, by comparing genetic interactions within *S. cerevisiae* and *S. pombe*, it has been possible to confirm widespread conservation of genetic relationships and also to identify repurposing of orthologous complexes that have evolved divergent functions and partnerships that alter mitotic mechanisms (Frost et al., 2012). For example, despite striking morphological differences between the yeast spindle pole body and mammalian centrosome, the endosomal sorting complex required for transport (ESCRT) plays a role in their duplication in both *S. pombe* and in human cells (Frost et al., 2012). However, this ESCRT function is not conserved in *S. cerevisiae*.

Figure 3.6—Cont'd for positioning cues. (E) Plant spindles, like this example from *Arabidopsis thaliana*, are acentrosomal and typically take on a barrel-shaped architecture with less-focused poles and no astral MTs. *Plant spindle image adapted from Smertenko et al.* (2008), (www.plantcell.org). Copyright American Society of Plant Biologists.

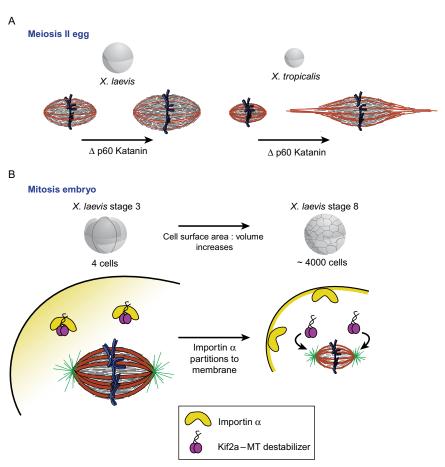
In metazoans, the details of spindle architecture are not as well understood, and their striking diversity implies significant architectural differences (Fig. 3.6). Serial section electron microscopy has provided insight into spindle architecture in a Ptk1 cell, revealing well-defined populations of astral, spindle, and k-fiber MTs (Mastronarde et al., 1993). In *C. elegans*, chromosomes are holocentric, meaning that the entire face of each chromosome functions as a kinetochore, leading to a different partitioning of MT populations (Maddox et al., 2004). Embryo spindles of sea urchin and related echinoderms possess prolific astral MTs with centrosomes closely associated with spindle poles. The distance between centrosomes and spindle poles varies considerably among cell types and species. Other variations on spindle size and morphology appear with every comparative analysis of spindle morphology, and correlations with genomic sequence and expression data may provide clues to the molecular basis of these variations.

TPX2, a mediator of chromatin-directed spindle assembly discussed earlier, provides one example of a spindle factor whose sequence and precise function vary among species. TPX2 homologs have been investigated in C. elegans (TPXL-1), Drosophila melanogaster (D-TPX2/Mei38/Ssp1), as well as in Xenopus species, but in the absence of EM data, only spindle morphology and not detailed architectural features can be compared. In all cases, TPX2 is important to assemble a properly organized spindle. In Drosophila, D-TPX2 is nonessential and its deletion/knockdown leads to a short spindle phenotype. D-TPX2 localizes to k-fibers in meiotic and somatic Drosophila cells, implicating its function on this population of MTs (Goshima, 2011). In C. elegans, knockdown of TPXL-1 also decreases spindle size but the protein is enriched at the poles of the spindle and its abundance correlates with centrosome size and spindle length (Greenan et al., 2010). In contrast, TPX2 is essential for spindle formation in *Xenopus* egg extracts, where it is an important component of the Ran pathway of chromatin-dependent spindle assembly (Gruss et al., 2001; Wittmann et al., 2000), and RNAi studies in human cells further indicate a requirement for TPX2 for spindle formation (Gruss et al., 2002). Interestingly, the C. elegans and Drosophila TPX2-like proteins possess only a subset of the functional domains found in vertebrate TPX2, and each contains domains not found in the vertebrate homologs (Goshima, 2011). Evolutionarily, one could imagine this protein may have adapted to perform different spindle-related functions in each of these organisms, potentially indicating one mechanism by which spindle architecture is tuned to each species.

#### 5.2. Spindle size

Although manipulation of factors such as TPX2 can alter spindle size (Bird and Hyman, 2008; Goshima and Scholey, 2010), mechanisms that function physiologically to adjust spindle size and architecture to cells of different sizes and shapes have only recently come to light, and thus far are limited to Xenopus in vitro systems. The first was found in a comparison of Xenopus laevis and its smaller relative Xenopus tropicalis, which produces smaller eggs. Spindles formed in X. tropicalis egg extracts are also smaller, and mixed extracts produced spindles of intermediate sizes, indicating that intrinsic, dosedependent cytoplasmic activities operate (Brown et al., 2007). This established a system to investigate mechanisms of spindle scaling by identifying differences in spindle MT behavior in the two extracts and then determining whether the proteins responsible function as regulatory factors. Computational simulation of a steady-state spindle (described below) predicted that changes in MT stability could robustly change spindle length (Loughlin et al., 2010) and greater activity of the MT-severing enzyme Katanin, a hexameric AAA ATPase was observed in X. tropicalis egg cytoplasm compared with X. laevis. Consistent with Katanin functioning as a spindle scaling factor, greater amounts were observed at X. tropicalis spindle poles and its inhibition increased spindle length to a greater degree in X. tropicalis compared with X. laevis. Protein levels in egg extracts and activity of recombinant catalytic p60 subunit of Katanin were similar for the two species. Instead, a posttranslational mechanism was found to be responsible for spindle length scaling by Katanin, as X. tropicalis p60 lacks an inhibitory Aurora B kinase phosphorylation site that is present in X. laevis p60 (Loughlin et al., 2011; Fig. 3.7A).

The second *Xenopus* system to address size scaling is the early *X. laevis* embryo, which, following fertilization, undergoes rapid cleavages in the absence of growth or transcription, undergoing an exponential decrease in cell size. At early developmental stages (between two and several hundred cells), an upper limit to spindle size is observed (Wuhr et al., 2008). Once cell diameter decreases to near twice the length of the spindle, linear scaling is observed between spindle length and cell diameter through the ~4000 cell stage, when zygotic transcription initiates and cell cycles become asynchronous. By preparing extracts from embryos at stage 3 (4 cell) and stage 8 (~4000 cell), spindle size differences could be recapitulated *in vitro*, again allowing a molecular dissection of underlying mechanisms (Wilbur and Heald, 2013). As for interspecies spindle scaling, higher MT destabilization



**Figure 3.7** Microtubule destabilization regulates spindle size in two *Xenopus* systems. (A) MT severing by p60 Katanin regulates spindle length and architecture differently in two *Xenopus* species. Depletion of p60 Katanin leads to larger spindles in *Xenopus laevis*. In *Xenopus tropicalis*, p60 has an important role in establishing proper spindle length and coordinating k-fiber and spindle MT populations such that k-fibers terminate at the pole. (B) In early embryonic development, spindle size decreases by 30–40 µm and is associated with changes in MT density, indicating differences in MT architecture. Spindle size is controlled in part by regulation of the MT-depolymerizing kinesin-13 Kif2a by Importin  $\alpha$ . Importin  $\alpha$  can be membrane-bound, releasing Kif2a to act on the spindle, or in the cytoplasm where it inhibits Kif2a. Therefore, the regulation of Kif2a, and ultimately spindle size, is dependent on the membrane-to-cytoplasm or surface area-to-volume ratio of the cell.

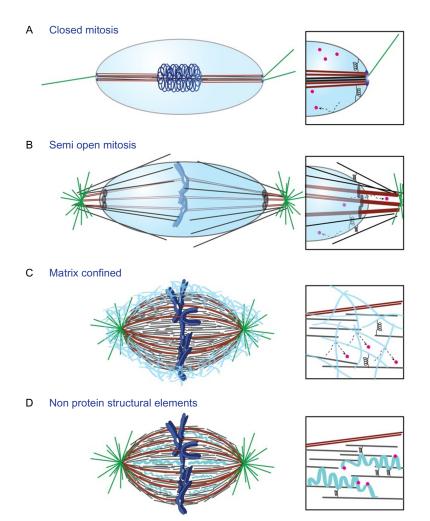
activity was present in smaller spindles, but in this case, differences in a depolymerizing kinesin, Kif2a, were responsible. Kif2a is inhibited by the import receptor Importin  $\alpha$ , whose cytoplasmic levels decrease during development as more of it partitions to a membrane fraction (Wilbur and Heald, 2013; Fig. 3.7B). This allows cells to autonomously coordinate spindle size independent of developmental stage, a need that arises due to asymmetric cell divisions that occur in the embryo.

Thus, in both interspecies and developmental scaling systems, spindle size differences are mediated, at least in part, by changes in MT stability. Interestingly, size changes are accompanied by architectural differences. In response to Katanin inhibition, the kinetochore fibers of *X. tropicalis* spindles protrude through the spindle poles, indicating that Katanin not only contributes to spindle length differences but is also required to coordinate k-fiber and spindle MT stability in *X. tropicalis* (Fig. 3.7A; Loughlin et al., 2011). Spindle assembly pathways also differ, with smaller spindles less dependent on the RanGTP pathway (Wilbur and Heald, 2013). Recent work has also identified unique features of MT asters in the very large cells of amphibian embryos (Mitchison et al., 2012). The reworking of architectural features of large MT assemblies is therefore a commonly utilized mechanism to adapt to varying cellular environments.

#### 5.3. Spindle architecture and compartmentalization

Observations of the different spindle architectures that occur across phylogeny can be used to understand how different molecular mechanisms function in concert to establish an emergent dynamic structure such as the spindle. These same observations can also be used to understand how subcellular structures are coordinated with the cellular context to provide a critical function under different physiological conditions. Comparative analysis may facilitate integration of molecular, cellular, and organizational information of spindle function.

In many fungi and other nonmetazoan eukaryotes, mitotic spindle formation occurs enclosed within a nuclear envelope that does not break down during mitosis (De Souza and Osmani, 2007; Fig. 3.8A). Maintenance of the compartmentalization of the cell provides a way to localize and concentrate molecules important for spindle assembly and chromosome segregation and prevents the need to sort the cytoplasm after mitosis. It may also provide a structural component that resists forces and provides anchoring sites for proteins and thereby contributes to spindle assembly. However, having spindle



**Figure 3.8** Components of the nucleus or nonprotein macromolecules may provide structural support or sequestration of spindle components from the surrounding cytoplasm. (A) Closed mitosis occurs completely within the nuclear envelope. (B) In semi-open mitosis, the nuclear envelope has openings sufficient to allow large structures like MTs to pass through, or has broken down into fragments but is not completely disassembled. (C) A proteinaceous matrix or meshwork interacts with and surrounds the spindle, providing a means for sequestering some large protein complexes or resisting forces. (D) Nonprotein macromolecules contribute to spindle assembly by interacting with spindle components either internally or externally like the proteinaceous matrix.

assembly occur in a closed compartment requires the transport of normally cytoplasmic contents into the nucleus, such as tubulin and many spindleassociated proteins. Both Giardia intestinalis and C. elegans, undergo a semiclosed (or semiopen depending on how optimistic you are) mitosis, in which chromosomes remain within a permeabilized or partially broken down nuclear envelope (Fig. 3.8B). In Giardia, MTs external to the nucleus penetrate through fenestrae to form the mitotic spindle (Sagolla et al., 2006), while in C. elegans cytoplasmic proteins can penetrate the more permeable nuclear envelope (Hayashi et al., 2012). Again, in these cases, the various non-MT structures may physically support spindle assembly and spatial localization of spindle components. To speculate, this external support from the nuclear envelope and relatively small genomes may preclude the need for large arrays of MTs to organize around the chromatin to establish a mechanically sound structure. However, this remains to be explicitly tested. The development of new systems and a comprehensive assessment of spindle architecture in the context of different nuclear envelope structural support systems and different genome sizes are needed.

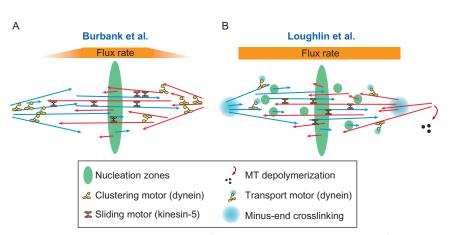
In a variation on closed mitosis, spindles formed in a Drosophila embryo appear to have a compliant meshwork of large proteins that surround the spindle, termed the spindle matrix (Johansen and Johansen, 2009; Fig. 3.8C). Disruption of the spindle matrix protein Chromator can increase mitotic index and is homozygous lethal (Ding et al., 2009). The spindle matrix may provide structural support similar to remnant or full nuclear envelopes of nonmetazoans, but since the Drosophila embryo spindle architecture appears to be large and robust, an alternative function may be to maintain some level of compartmentalization of the spindle away from the rest of the cytoplasm. In the context of a *Drosophila* embryo syncytium, a means of compartmentalizing the components of adjacent spindles may be critical prior to cellularization. In *Xenopus* egg extract spindles, there appears to be no isotropic strong spindle matrix (Gatlin et al., 2010). However, a meshwork of lamin proteins is associated with the spindle and remains intact after MT depolymerization (Tsai et al., 2006). Whether the lamin meshwork is necessary or sufficient to sequester cytoplasmic components or to provide force resisting anchor sites remains to be elucidated in detail, but there is support for both hypotheses (Goodman et al., 2010).

Finally, at least three nonprotein macromolecules are implicated in spindle assembly in *Xenopus* egg extracts. These are RNA, poly-ADP ribose, and glycogen (Blower et al., 2005; Chang et al., 2004; Groen et al., 2011). While the molecular mechanisms by which these factors act to promote spindle assembly remain to be established, given the long polymeric nature of these molecules, it is possible that they can function to stabilize or confine spindle components (Fig. 3.8D). Understanding the architectural and structural features of these different spindles and the different force requirements for chromosome segregation by comparative analysis will be necessary to determine whether confinement or structural support is necessary for the function of the spindle and whether this comes from the nuclear envelope or polymeric meshworks of macromolecules.

#### 5.4. Simulations of microtubule arrangement in the spindle

Despite the significant insights stemming from years of experiments on spindle structure, the detailed underlying architectures of most spindles are yet to be determined. This is primarily due to the technical challenge of imaging a densely packed structure made up of bundled MT polymers by either electron microscopy or light microscopy. Recently, to gain insight into arrangements of MTs compatible with the organizational properties and defined steady-state structure of the spindle, several computational models have been developed. While many models have provided quantitative insight into specific parts of spindle form or functions, such as the dynamics of individual MTs and rates of spindle length changes, two complementary spindle simulation studies are most related to spindle architecture.

Both simulations were designed to understand the complex interactions, forces, and structures that emerge from many factors acting in concert. Though the fundamental implementations were different, these simulations used combinations of MTs and various motor proteins or regulatory activities to elucidate how the basic features of the spindle, such as antiparallel arrays and spindle poles, might be attained (Fig. 3.9). Many different simplifications were used to minimize computational complexity and study different aspects of spindle architecture, yet the two simulations found qualitatively similar results. In each case, a sliding motor (modeled after kinesin-5 proteins) driving outward sliding of MTs and a clustering force were necessary to form the bipolar MT array and spindle poles. In the simulation of Burbank et al. (2007), clustering was produced by a motor that bound to two MTs and walked toward their minus-ends, modeled after dynein (Fig. 3.9A). In the simulation by Loughlin et al. (2010), clustering was introduced through an activity that was transported to the minus-end of MTs and could cross-link within  $5 \,\mu m$  from the end but did not apply a force, modeled after NuMA (Fig. 3.9B). In both models, MT nucleation



**Figure 3.9** Computational simulations of spindle architecture establish MT lifetime as a critical factor for setting spindle size and two complementary mechanisms of pole formation. (A) The model developed by Burbank et al. utilized an outward sliding force on antiparallel MTs, similar to the known functions of the kinesin-5 proteins, and a clustering force, similar to dynein, to generate a system where spindle length was regulated and poles formed at the balance point of outward sliding and clustering forces. Nucleation of MTs in this simulation was near the center of the spindle. (B) The model by Loughlin et al. utilized an outward sliding force on antiparallel MTs, a dynein-like transport molecule with minimal clustering capability, and a MT-depolymerizing activity similar to kinesin-13s. Again, spindle length could be regulated and poles formed. However, pole formation occurred at the point where outward sliding and depolymerization were balanced. Nucleation in this model was both in the center of the spindle but also distributed throughout the body in a MT-branching type nucleation.

occurred near the center of the spindle; however, the model by Loughlin et al. also implemented nucleation throughout the spindle similar to that expected for branching MT nucleation. With common approaches to simulating minimal spindle systems, both models led to a similar conclusion about spindle architecture. Qualitatively, both models (1) produced a high concentration of minus-ends defining the spindle poles, with some still distributed throughout the spindle, (2) did not require a complex restoring force beyond the few motors (or other enzymes) in the simulation, and (3) showed that the lifetime of the MT within the spindle primarily controlled spindle length.

The major difference that emerges from these two models, and one that predicts significant architectural differences in the spindle, is the difference in the transport rate of MTs. In the model by Burbank et al., MTs slow as they near the pole due to force generated by the clustering motor that opposes the sliding motor. This is consistent with measurements of MT flux by speckle microscopy (Yang et al., 2008) but leaves open the role of MT depolymerases of the kinesin-13 family in driving flux that has been indicated in human cell lines (Ganem et al., 2005). In the model by Loughlin et al., the transport of MTs is homogeneous throughout the spindle, and MTs depolymerize as they reach the pole through the action of a specific MT-destabilizing activity, replicating the proposed involvement of kinesin-13's in MT flux but not recapitulating the slowed flux near the poles. The slightly different clustering and nucleation mechanisms could account for the different requirements for spindle pole formation and therefore spindle length control. Ultimately, the models are not mutually exclusive and both rely on modeling of a minus-end-directed motor based on dynein. It is likely that both mechanisms work to drive spindle architecture into a robust regime capable of maintaining steady-state structure and generating precise function, despite fluctuating conditions.

#### 5.5. Future prospects for determining spindle architecture

With the development of super-resolution light microscopy and additional technical advances, it may soon become feasible to distinguish individual MTs and small bundles within the densely packed spindle. Even if MT organization cannot be resolved precisely, other spindle components should be amenable to super-resolution techniques. Analysis of components that are crucial to spindle MT organization will make it possible to infer important aspects of spindle architecture. For instance, several proteins have recently been identified that bind the minus-ends of MTs in addition to the wellestablished y-TuRC (Goodwin and Vale, 2010; Meunier and Vernos, 2011). Super-resolution investigations with these proteins will test current predictions of the distribution of MT minus-ends in the spindle, which have not yet been observed directly. Quantification of the number of MTs in the kinetochore fiber has already been estimated through electron microscopy data, but the dynamic process of partitioning k-fiber MTs from the rest of the spindle MTs remains unknown. Finally, measurements of strength and stability of the spindle—the functional consequences of different architectural arrangements of MTs—are only just beginning and should provide substantial insight into the mechanisms of chromosome segregation.

Other novel methods, especially those based on quantitative analyses, may provide unique insights into spindle architecture, much like the laser ablation studies that were used to probe MT length and organization. However, direct imaging of spindle organization through electron microscopy remains the gold standard for determining spindle architecture. Studies of various small fungal spindles through electron tomography have establish the groundwork for larger, more complex spindle structures and provided an important structural context for studies in those systems, as well as insight into general principles of spindle architecture and evolution. Even reconstructions or EM images of relatively small slices of larger complex spindles will provide important constraints on MT length distributions and spatial organization that could be incorporated into studies of MT dynamics and simulations.

# 6. CONCLUSIONS

By altering the basic spindle plan, cells have evolved mechanisms that promote robust chromosome segregation and cell division that accommodates their diverse morphologies and functions. Given the critical role of the spindle, it is not surprising that many associated factors have been linked to diseases in which chromosome segregation is disrupted (Noatynska et al., 2012). For example, a significant percentage of MAPs that affect spindle assembly or function have altered expression levels at the mRNA or protein level in a variety of cancers (Bieche et al., 2011; Chang et al., 2012; Wang et al., 2010). Chromosome segregation errors in female meiosis also pose a significant problem and lead to the high incidence of human miscarriage and developmental diseases such as Down syndrome (trisomy 21; Hassold and Hunt, 2001). The unique architecture of the meiotic spindle, which must maintain its structure and function for long periods in oocytes prior to fertilization, provides an interesting example of the role and effects of spindle structure and the importance of understanding the underlying mechanisms.

We are now approaching a more mechanistic understanding of how spindle architectures are established. Work from a variety of model systems continues to fill gaps in our current knowledge and importantly provides the basis for comparative analysis that allows the identification of general principles and specific elaborations of the spindle plan. Specifically, insights into the biology of the spindle matrix have been gleaned from studies in *Drosophila*. The complete MT structure of spindles in fungi by electron tomography have provided high-resolution views of spindle architectures and are poised to leverage this information in combination with the vast amounts of genomic data now available. *Xenopus* extract systems have been particularly useful for studying female meiotic spindle architecture and mechanisms that control spindle size, the partitioning of different MT populations, and the roles of different MT nucleation pathways, among other features. Robust spindle assembly and control of cell cycle and developmental states, the ease of spindle manipulation in this biochemically tractable system, and the high-resolution light microscopy approaches available all contribute to the power of *Xenopus in vitro* systems. Although much of what has been learned from *Xenopus* extracts is yet to be generalized, we expect future experiments to validate common principles of spindle organization and function across many different species and disease states. Overall, as the mechanistic roles of known spindle components are elucidated, we will understand how specific MT organizations emerge and how different spindle architectures contribute to the common function of chromosome segregation.

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