Cooperative mechanisms of mitotic spindle formation

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Summary

Cooperativity is well known to promote the speed of some biochemical reactions by accelerating the activity of enzymes. Recent studies have shown that cooperative interactions also function during the formation of a complex cellular structure, the mitotic spindle. Capture of kinetochores by dynamic astral microtubules was originally proposed as the basis of spindle formation. However, mounting evidence indicates that a more complex series of events occurs. It is now clear that there are multiple microtubule nucleation and capture sites throughout the spindle. Kinetochores, centrosomes and microtubules play multiple roles in establishing connections between spindle components and integrating them into a common structure. These data support a modified search-and-capture model that incorporates additional assembly pathways coordinated by a RanGTP gradient.

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Introduction

The cell cycle culminates in mitosis, when replicated molecules of DNA compacted into chromosomes are equally partitioned between the two daughter cells. This distribution needs to be precise since the consequences of a single error are grim: aneuploid cells are either non-viable or, worse, they can become the first step on the road to cancerous transformation. Considering that millions of cells undergo mitosis at any given time in an average human body, the overall fidelity of mitotic mechanisms becomes apparent.

Chromosome movements during mitosis rely upon a complex macromolecular machine known as the mitotic spindle. The spindle has three principal components: centrosomes, chromosomes and microtubules. It forms as chromosomes become connected to the two centrosomes (spindle poles) by microtubule bundles that link each pole to a specialized macromolecular assembly on the chromosome body termed the kinetochore. If we are to understand the principles of mitotic spindle formation, the exact mechanisms that govern how sister kinetochores on individual chromosomes establish connections with the opposite spindle poles must be elucidated.

The search-and-capture hypothesis

In 1984 Mitchison and Kirschner discovered an intriguing property of microtubules they termed 'dynamic instability' (Mitchison and Kirschner, 1984). In essence, microtubule plus ends interconvert between polymerization and depolymerization states. Thus, at any given time an individual microtubule grows or shrinks before it ultimately disassembles. This behavior became the foundation of the first robust hypothesis of mitotic spindle formation (Kirschner and Mitchison, 1986). The core of this model was the replicated centrosomes, each of which nucleates a radial array of highly dynamic astral microtubules. In this model, the plus ends of microtubules nucleated at centrosomes grow, shrink and re-grow, randomly exploring space. Contact with a kinetochore results in 'capture' of a chromosome and suppression of the microtubule's dynamics. As a result, the kinetochore establishes a relatively stable connection to the pole. Kirschner and Mitchison suggested that, over time, more and more microtubules become stabilized by repetitive capture so that the number of microtubules extending from centrosomes to kinetochores (K-MTs) increases while the number of astral microtubules decreases proportionally, leading to the formation of a typical fusiform spindle-like structure (Fig. 1A). Four years after the hypothesis was formulated, capture of microtubules by the kinetochore was directly visualized (Hayden et al., 1990; Rieder and Alexander, 1990), thereby validating the major postulate of the search-and-capture mechanism.

The classic formulation of search-and-capture assumes that all microtubules are nucleated from the centrosomes and that the sole function of the kinetochore is to establish multiple stable contacts with the plus ends of astral microtubules. In this scenario, kinetochores simply lie in wait until capture of both sister chromatids results in bi-orientation and proper alignment of the chromosome (Fig. 1B). Thus, the role of the chromosomes is passive and the spindle develops into a unified structure as centrosomes incorporate chromosomes one by one.

Although the search-and-capture hypothesis readily explained several key features of mitotic spindle formation, it also presented several conceptual difficulties. First, random probing of the cytoplasm for multiple small targets is likely to have a low probability of success and, therefore, is an inefficient mechanism. Indeed, recent mathematical modeling of kinetochore capture predicted that several hours would elapse before each of 92 kinetochores in a human cell had captured microtubules (Wollman et al., 2005). Thus, unbiased search-and-capture cannot explain the typical observed

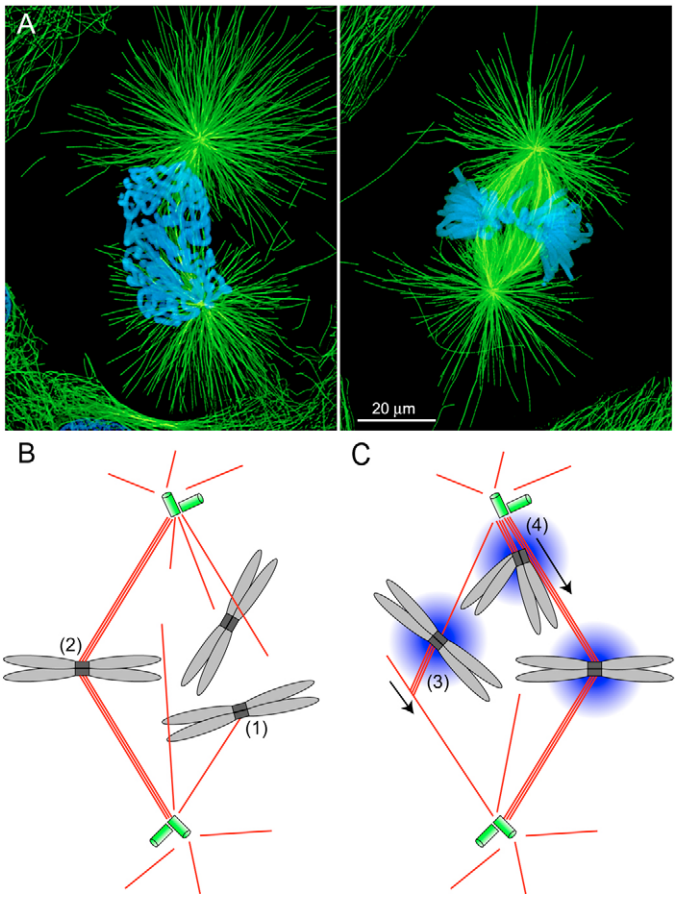

Fig. 1. The process of mitotic spindle formation. (A) Newt lung cells fixed and stained for microtubules (green) and DNA (blue); images reprinted from Current Biology, 1999, 9:R193 with permission from Elsevier (left image) and from Cell Motility & Cytoskeleton, 1999, 43(3) with permission from Wiley (right image). The transition from two arrays of astral microtubules organized by centrosomes (left) to typical fusiform metaphase spindle (right) requires the establishment of connections between chromosomes and spindle poles via dynamic microtubules. (B,C) Search-and-capture models of chromosome congression. In the original formulation of search-and-capture (B), the space around passive chromosomes is probed by growing and shrinking microtubules (red) nucleated from centrosomes (centrioles in green). Successive capture events (1) on sister kinetochores result in bi-orientation and congression (2). Additional mechanisms of search-and-capture (C) initiated by the chromosomes. K-fibers nucleated at kinetochores via a RanGTP gradient (blue) elongate toward the periphery and capture astral microtubules (3). They are subsequently incorporated into the pole by dynein-based transport. Mono-oriented chromosomes can also use K-fibers of other, bi-oriented, chromosomes resulting in congression before bi-orientation (4).

duration of prometaphase (15-30 minutes). Another conceptual difficulty arises from the behavior of chromosomes known as 'mono-orientation' (Hayden et al., 1990; Skibbens et al., 1993). As soon as one of the two sister kinetochores has captured an astral microtubule, the chromosome is transported toward the centrosome from which this microtubule emanates. Consequently, it becomes positioned close to one pole, where the concentration of microtubules emanating from the other centrosome is negligible. According to the original model, the unattached kinetochore on such a mono-oriented chromosome would not be able to capture microtubules and become connected to the other spindle pole. Yet, time-lapse recordings of mitosis reveal that chromosome mono-orientation and subsequent congression (movement back to the spindle equator) is a common phenomenon observed in many cells. Finally, search-and-capture of astral microtubules from spindle poles is clearly not applicable to cells that lack centrosomes, such as those of higher plants and many meiotic eggs. Therefore, alternative and/or supplementary spindle assembly mechanisms must exist. In fact, as early as 1988 it was noted that "a mixed origin of kinetochore microtubules by both capture and nucleation is a real possibility" (Nicklas and Kubai, 1985).

Recent work in a variety of experimental systems provides evidence for additional search-and-capture pathways that function during spindle assembly. These are augmented by cooperative mechanisms in which the activity of a given spindle component facilitates or accelerates the process to increase the overall efficiency of mitosis.

Chromosome-driven mitotic spindle formation

An alternative mode of spindle assembly during cell division was suggested by experiments in which microinjection of nuclei and DNA into *Xenopus* eggs induced the formation of spindle-like structures (Karsenti et al., 1984). This was the first indication that chromatin, and not centrosomes, might drive formation of the spindle. Such a chromatin-based model gained support following the identification of several acentrosomal animal systems capable of successfully organizing a spindle (Heald et al., 1996; Khodjakov et al., 2000; Hinchcliffe et al., 2001; Basto et al., 2006). Furthermore, a pathway for microtubule nucleation in the vicinity of chromatin was discovered that depends on the activity of the small GTPase Ran.

RanGTP promotes microtubule nucleation and stabilization (Carazo-Salas et al., 2001; Wilde et al., 2001) and is present in a concentration gradient around mitotic chromosomes (Kalab et al., 2002). The gradient is established through the activity of the chromosome-associated guanine-nucleotide-exchange factor (GEF) RCC1 (Carazo-Salas et al., 1999) and has been proposed to effect spindle formation through two mechanisms. The first involves direct stimulation of microtubule nucleation by chromatin (Heald et al., 1996; Karsenti and Vernos, 2001); the second acts by creating a local concentration of microtubule stabilizing factors around the chromosomes to promote the capture of astral microtubules (Bastiaens et al., 2006). The importance of this gradient was highlighted in experiments probing the effects of its perturbation on spindle assembly in *Xenopus* extracts (Caudron et al., 2005). By altering the ratio of key controllers of the system, Caudron and co-workers could modulate the size of the gradient. Reduction of the distance the gradient spans results in a loss of spindle asymmetry and fewer attached chromosomes.

Because of the much smaller size of somatic cells relative to large invertebrate eggs or extracts, it was not clear whether a similar RanGTP gradient has sufficient space to become established in all cell types. Modeling of the gradient span in somatic cells, taking into account the size of the chromosomes, the volume of cytoplasm, and the concentrations and rate constants of individual components, suggests it cannot

(Gorlich et al., 2003). However, direct visualization of a RanGTP gradient in mitotic HeLa cells indicates that the distribution of its components is somehow normalized to the volume of cytoplasm. Attenuation of this gradient disrupts only the early steps of spindle formation and RanGTP does not appear to be involved in spindle maintenance given that perturbations at later stages have minimal effects (Kalab et al., 2006). Upon injection of dominant-negative gradient regulators, the major phenotype is a lag in the transition from mono- to bi-polar spindle organization. Thus, although the RanGTP gradient provides a kinetic stimulus, it is not the main driving force for spindle assembly in cells that possess centrosomes. Nevertheless, chromosome-mediated organization of microtubules is clearly a significant contributor to mitotic spindle assembly even in cells containing centrosomes. Intriguingly, although the RanGTP-gradient becomes established around any piece of chromatin in mitotic cytoplasm, several in-situ studies have demonstrated that non-centrosomal microtubules form primarily in the vicinity of the centromere and not around chromosome arms in somatic cells (Witt et al., 1980; Khodjakov et al., 2003; Maiato et al., 2004; Tulu et al., 2006).

The ability of centromeres to nucleate and organize K-MTs was first suggested in the 1960s (Inoue and Sato, 1967) and supported by subsequent work using isolated chromosomes and mitotic cell lysates (Telzer et al., 1975; Snyder and McIntosh, 1975; Gould and Borisy, 1978; Witt et al., 1980). The direct nucleation of microtubules from kinetochores poses a conceptual problem because of the predicted orientation of plus ends, which would be distal to the kinetochore as microtubules grow out. Such a polarity is the opposite of what is observed in mitotic spindles. This issue was resolved through examination of serial-section EM reconstructions of CHO cells after colcemid washout, which revealed short microtubules forming not on but, rather, immediately adjacent to kinetochores (Witt et al., 1980). Thus, microtubules assume the proper orientation through cytoplasmic nucleation followed by attachment of plus ends to the kinetochore. It remains to be determined whether microtubule nucleation is entirely restricted to the vicinity of the kinetochore, given the distribution of RCC1 along the entire length of mitotic chromosomes. Microtubules are more likely to form in association with kinetochore clusters than with individual kinetochores (Witt et al., 1980; Tulu et al., 2006), which suggests that kinetochores/centromeres emit a short-range factor to promote microtubule nucleation that perhaps enhances the effects of RanGTP.

Formation of microtubules in the vicinity of centromeres has important implications for the search-and-capture mechanism. Clearly, these can be easily captured by the kinetochore. Association of microtubule plus ends with the kinetochore induces their polymerization and the growth of a microtubule bundle (K-fiber) from the kinetochore. This type of kinetochore-mediated formation of K-fibers has been directly observed in mammalian (PtK) and *Drosophila* (S2) cells (Khodjakov et al., 2003; Maiato et al., 2004). Thus, the classical search-and-capture mechanism and the chromosome-mediated spindle assembly pathway appear to be interrelated. The search-and-capture principle applies even to those microtubules that are formed by chromosome-mediated mechanisms (see also Wadsworth and Khodjakov, 2004; Gadde and Heald, 2004). In other words, chromosomes do not passively wait to be discovered by centrosomal microtubules. Instead, they actively form their own K-fibers by promoting local microtubule nucleation followed by capture at their kinetochores.

Cooperative interactions of microtubules in spindle assembly

If each individual chromosome can create and organize its own K-fibers, then an average human cell could end up with 46 individual mini-spindles. Since this does not occur, the cell must possess mechanisms for integrating all components into a common structure. Indeed, K-MTs nucleated at kinetochores are ultimately combined with those originating from centrosomes. Several recent studies have indicated that integration can be achieved through capture of astral microtubules by kinetochore-nucleated K-fibers. The distal ends of K-fibers are then transported poleward along astral microtubules by dynein motors (Fig. 1C) (Khodjakov et al., 2003; Maiato et al., 2004). The same mechanism is responsible for capture and poleward transport of other spindle components, such as individual microtubules and non-kinetochore microtubule bundles (Rusan et al., 2002; Tulu et al., 2003). As such, centrosomes in animal cells serve as 'attraction points' to which pre-assembled spindle components are transported and then seamlessly integrated into a common spindle structure (Heald et al., 1997; Wadsworth and Khodjakov, 2004).

Spindles in anastral systems lack the integrative properties of centrosomes; yet they are also able to incorporate chromosomes into a single mitotic or meiotic apparatus. This means additional mechanisms must function to 'corral' the large number of microtubules that form around chromosomes. An elegant recent study of the determinants of spindle geometry has provided insight into how the spindle is organized in the absence of centrosomes. Employing chromatin-coated beads, which support formation of spindles in *Xenopus* extracts (Heald et al., 1996), Gaetz and co-workers used a magnetic field to generate linear bead arrays of various lengths to explore the relationship between chromatin and spindle geometry (Gaetz et al., 2006). The width and overall shape of the spindles were unaffected by the length of the chromatin-coated bead assemblies, even though dynamic microtubules could be visualized probing the entire length. The authors concluded that factors other than mitotic chromosomes constrain spindle morphology. One of these factors was identified as dynein. Upon disruption of dynactin, a dynein accessory protein required for motor function, spindles cannot establish or maintain a standard width and instead spread out along the entire length of the chromatin-coated bead arrays.

Dynactin, as well as an additional dynein accessory protein, NuMA, can bind to microtubules (Waterman-Storer et al., 1995; Merdes et al., 1996) and the entire complex participates in crosslinking of microtubules and generates sliding forces between them. The requirement for dynein/dynactin to restrict spindle width is likely to be due to transport of microtubules by motors at the periphery of a forming spindle. In this model, microtubules capture (or are captured by) other spindle microtubules through dynein and its accessory proteins. At the same time, transport and incorporation of peripheral microtubules into the main body of the spindle prevents their

accumulation (Gaetz et al., 2006). This phenomenon constitutes yet another variation of search and capture. However, in this case, microtubules perform two functions cooperatively: incorporation of peripheral microtubules adds to the spindle framework, which captures and transports additional peripheral microtubules. Although the underlying molecular mechanisms remain obscure, the coordinated activity of molecular motors that dynamically focus microtubule minus ends and the crosslinking action of NuMA, a protein that helps maintain the integrity of spindle poles, are likely to be essential elements (Compton, 1998).

Cooperative mechanisms might not only organize spindle microtubules but also help generate them. The ATPase katanin, a microtubule-severing protein (McNally and Vale, 1993), contributes to the overall microtubule density in *C. elegans* meiotic spindles during the early stages of spindle formation (McNally et al., 2006). Electron tomography of wild-type and mutant *C. elegans* oocytes demonstrates a requirement for katanin to generate a large pool of microtubules around the meiotic chromatin (Srayko et al., 2006). Severing creates more microtubules, which then grow and also get severed. Many iterations of this process should dramatically increase polymer density in a cooperative manner. The activity of katanin may contribute to the distribution of microtubule minus ends observed throughout meiotic spindles (Burbank et al., 2006). It is important to note that the *C. elegans* oocyte is an acentrosomal system, and the contribution of katanin must also be assessed in cells possessing centrosomes. This will address whether katanin-mediated microtubule formation is a universal mechanism contributing to spindle formation or a specialized adaptation to supplement meiosis in the absence of centrosomes. There is also evidence suggesting that microtubules nucleated from the sides of pre-existing microtubules contribute cooperatively to spindle assembly (reviewed by Luders and Stearns, 2007). This has been directly demonstrated in fission yeast (Janson et al., 2005) and plants (Murata et al., 2005). Further, it has been shown that new microtubules form inside the spindle in *Drosophila* S2 cells lacking functional centrosomes (Mahoney et al., 2006). It is noteworthy that microtubule nucleation inside the spindle is also compatible with the observed distribution of microtubule minus ends throughout the spindle (Burbank et al., 2006).

Cooperativity in chromosome positioning

One of the more curious phenomena observed during mitotic spindle formation is the 'centrophilic' behavior of chromosomes (Fig. 2, supplementary material Movie 1). Shortly after nuclear envelope breakdown, many chromosomes engage in lateral interactions with astral microtubules and move rapidly along them toward the pole, presumably transported by dynein (Hayden et al., 1990; Rieder and Alexander, 1990). As spindle assembly proceeds, chromosomes continue to move toward the centrosomes, albeit more slowly (Skibbens et al., 1993). There they reside until suddenly moving back (congressing) to the spindle equator (Fig. 2C-E). The initial translocation toward the spindle pole appears to be counterproductive, given that it is in the opposite direction to the desired destination, the metaphase plate. Yet, this type of behavior is exhibited by many chromosomes during their initial attachment to the spindle as well as during correction of chromosome attachment errors (Lampson et al., 2004).

In the classical view of mitosis, congression was believed to be a consequence of chromosome bi-orientation (reviewed by Rieder and Salmon, 1998). The search-and-capture hypothesis postulated that mono-oriented chromosomes congress after they capture microtubules connected to the distal spindle pole (McEwen et al., 1997). More recent work has demonstrated that, in fact, chromosomal congression can take place through alternative strategies.

First, the unattached kinetochore on mono-oriented chromosomes can develop K-fibers by centrosome-independent mechanisms. These fibers, growing out from the kinetochore, subsequently capture astral microtubules and are transported to the distal spindle pole, as outlined above. As a result, the chromosome slides toward the spindle equator and ultimately becomes incorporated into the metaphase plate (Khodjakov et al., 2003; Maiato et al., 2004). One advantage of such a mechanism is the increased probability that a K-fiber, as opposed to a small (200-nm) kinetochore, will encounter a microtubule extending from the distal pole.

Another mechanism by which kinetochores proactively incorporate into the spindle has come to light as a result of detailed examination of kinetochore-microtubule interactions during chromosome congression in PtK cells (Kapoor et al.,

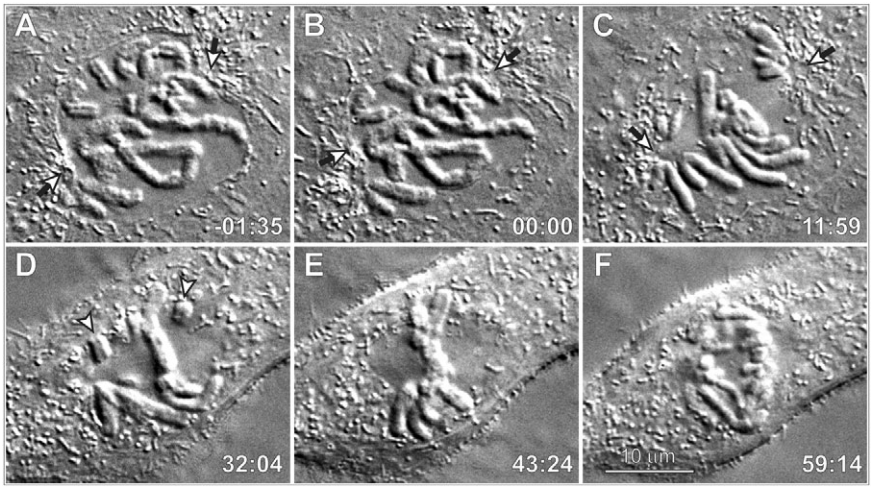

Fig. 2. Typical mitosis in a vertebrate (PtK_1) somatic cell imaged with DIC microscopy. Note the chromosomes that migrate first to the poles (arrows in A-C) before moving to the metaphase plate (D, arrowheads). Time shown is relative to nuclear envelope breakdown (NEB). Anaphase ensues approximately one hour after NEB.

2006). This work revealed that kinetochores driving congression exhibit one of two types of attachment. The first is classical end-on binding of K-MTs from the distal pole, as predicted by the original search-and-capture model. However, this kinetochore is frequently bound to the K-fiber of another, already bi-oriented chromosome [see (4) in Fig. 1C]. In this case, the kinetochore laterally attaches to the walls of microtubules and glides along the K-fiber surface owing to the plus-end-directed motor activity of the kinesin-7 family member CENP-E. At the same time trailing kinetochores on congressing chromosomes consistently possess end-on microtubule attachments to the proximal spindle pole via a mature K-fiber. Thus, at least in some cases, chromosomes can congress before becoming bi-oriented. Congression becomes a mechanism to facilitate bi-orientation by positioning mono-oriented sister chromosomes closer to the distal, unattached pole, where microtubules emanating from it are present at higher density.

Sliding of mono-oriented chromosomes along pre-formed K-fibers is a clear example of spindle cooperativity, creating additional bi-oriented chromosomes that, in turn, can be used to propagate the process. Future work will need to determine the details of microtubule attachments during this process. Currently, it is unknown whether the lateral attachment of the mono-oriented chromosome's leading kinetochore to the K-fiber must be dissolved before stable, end-on attachment of microtubule plus-ends can be established.

One outstanding question concerns how kinetochores that are laterally attached to microtubules make the choice to move toward the minus or plus ends. The rapid poleward movement along a single astral microtubule that occurs when chromosomes initially attach is exclusively minus end directed (Hayden et al., 1990). By contrast, mono-oriented chromosomes congressing to the metaphase plate through lateral attachments to K-fibers are transported to the plus ends (Kapoor et al., 2006). It is not understood how the kinetochores in each case discriminate between these two subsets of microtubules. One possibility is that kinetochores detect differences in the amount of tension applied to different types of microtubule in the spindle. Astral microtubule minus ends are anchored in the centrosome and the plus-ends are free; as a result, tension is absent. The K-fibers of bi-oriented chromosomes, by contrast, have both plus and minus ends anchored in the kinetochore and centrosome, respectively, and they experience forces exerted by the competing activities of motors at either end (Kapoor and Compton, 2002).

Conclusions and perspectives

We are making significant progress in our efforts to understand mitotic spindle assembly. As imaging tools and technology continue to improve, a unified model is emerging that reconciles two, formerly competing, hypotheses of microtubule organization: one based on centrosomes, the other on chromosomes. The two mechanisms are not mutually exclusive and, instead, appear to contribute simultaneously to spindle assembly. By harnessing multiple pathways to nucleate and organize microtubules around chromatin, the cell is able to expedite what would otherwise be a slow process of chromosome capture and congression. The kinetic advantage conferred by a RanGTP gradient is likely to be attributable to a direct role in establishment of the cooperative mechanisms outlined above. By nucleating and/or stabilizing microtubules from multiple sources (i.e. kinetochores and centrosomes), cells increase the probability of an interaction that will result in the incorporation of a chromosome or microtubule into the spindle. For example, consider a K-fiber nucleated at a kinetochore by the local accumulation of RanGTP. This K-fiber grows and subsequently captures an astral microtubule whose proximity is encouraged by the microtubule-stabilizing factors released by the same gradient. The resulting amphitelic attachment of the chromosome to two mature K-fibers can then be used by another, mono-oriented chromosome to congress through lateral interactions and CENP-E motor activity. The spindle poles are the guideposts of the system, providing spatial cues to organize and incorporate the components. Experimental manipulations of the RanGTP gradient, coupled with high-resolution microscopy of microtubules and chromosomes and correlative electron microscopy, will be needed to assess the extent of RanGTP's role in integrating the assembly pathways.

Finally, the centrosome and chromosome spindle-assembly pathways use the same underlying principle of search and capture: microtubule-kinetochore, kinetochore-microtubule, and microtubule-microtubule combinations all contribute. Therefore, the search-and-capture model was conceptually correct; however, its original formulation proved to be too stringent and needs to be expanded with regard to the nature of the interactions it describes. Ultimately, cooperativity results when kinetochores and microtubules use previously existing spindle structures. These synergistic relationships accelerate the speed at which components are incorporated. One significant challenge for the future will be to uncover additional novel interactions among spindle components that contribute to spindle cooperativity.

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