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Review

Mechanisms of Centrosome Separation and Bipolar Spindle Assembly

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Accurate segregation of chromosomes during cell division is accomplished through the assembly of a bipolar microtubule-based structure called the mitotic spindle. Work over the past two decades has identified a core regulator of spindle bipolarity, the microtubule motor protein kinesin-5. However, an increasing body of evidence has emerged demonstrating that kinesin-5-independent mechanisms driving bipolar spindle assembly exist as well. Here, we discuss different pathways that promote initial centrosome separation and bipolar spindle assembly.

Introduction

During every cell cycle, the genome of a cell is duplicated and segregated into two daughter cells, such that each daughter cell receives a single, complete complement of the genome. For this to occur, the mitotic spindle, the machine driving chromosome segregation during mitosis, must form a bipolar configuration to partition the chromosomes into two distinct sets. Bipolar spindle assembly can be divided into several steps. First, duplicated centrosomes separate in prophase, before the nuclear envelope has broken down. This initial centrosome separation is accompanied by an increase in microtubule (MT) nucleation at centrosomes. As the nuclear envelope breaks down, microtubules from the two centrosomes not only interact with chromosomes, but also with each other. Furthermore, microtubules growing away from the chromosomes will encounter the cell cortex, where they can be captured by cortical factors. All of these different populations of microtubules, as well as the proteins associated with them, contribute to the forces that control bipolar spindle assembly.

A large amount of work in organisms ranging from yeast to humans has identified the plus-end-directed kinesins of the kinesin-5 family as the key players in driving spindle bipolarity (Ferenz et al., 2010; Scholey, 2009). Kinesin-5 motors are tetrameric kinesins with two motor domains on each side of the tetramer, allowing them to simultaneously bind and walk along two antiparallel MTs, thereby sliding them apart (Kapitein et al., 2005; Kashina et al., 1996). Such antiparallel sliding activity is thought to generate an outward sliding force within the spindle that pushes centrosomes apart, thereby promoting spindle bipolarity. Inhibition of kinesin-5 motors in almost all organisms tested results in the formation of monopolar spindles, demonstrating the importance of this class of motors for spindle bipolarity (Ferenz et al., 2010). Based on the essential role of kinesin-5 in bipolar spindle assembly, inhibitors of this motor have entered clinical trials as anticancer therapeutics.

Over the past years, several strong lines of evidence have emerged that additional, kinesin-5-independent mechanisms must exist as well that promote spindle bipolarity. First, simultaneous inhibition of kinesin-5 and a minus-end-directed motor (dynein or kinesin-14, depending on the experimental system) results in bipolar spindle formation even though kinesin-5 is inactive (Ferenz et al., 2009; Mitchison et al., 2005; Mountain et al., 1999; O'Connell et al., 1993; Saunders and Hoyt, 1992; Sharp et al., 1999; Tanenbaum et al., 2008). Second, inhibition of kinesin-5 in metaphase does not result in spindle collapse in mammalian cells, suggesting an alternative pathway is involved in the maintenance of bipolarity (Cameron et al., 2006; Kapoor et al., 2000; Kollu et al., 2009; Tanenbaum et al., 2009; Vanneste et al., 2009). Third, certain organisms like *C. elegans* and *Dictostelium* do not require kinesin-5 for bipolar spindle assembly (Bishop et al., 2005; Saunders et al., 2007; Tikhonenko et al., 2008). Finally, while kinesin-5 motors seem to be required for initial centrosome separation during prophase in mammalian cells (Tanenbaum et al., 2008; Whitehead and Rattner, 1998; Woodcock et al., 2010), they are not required for prophase centrosome separation in *Drosophila* embryos (Sharp et al., 1999). In this Review, we discuss multiple mechanisms underlying centrosome separation and bipolar spindle assembly, with an emphasis on kinesin-5-independent mechanisms.

Mechanisms of Initial Centrosome Separation

Centrosomes begin to separate in prophase approximately 1 hour before nuclear envelope breakdown (NEB), at which time they migrate along the nuclear envelope (NE) to opposite sides of the nucleus. This mode of centrosome separation is seen in most, if not all, organisms that undergo an "open" mitosis (in which the NE breaks down at the onset of mitosis).

Role of MT Motors

Several studies have found the kinesin-5 motor Eg5 to localize to centrosomes and MT asters in prophase (Blangy et al., 1995; Sawin and Mitchison, 1995; Whitehead et al., 1996) and inhibition studies revealed that Eg5 is needed for centrosome separation during prophase in mammalian cells (Tanenbaum et al., 2008; Whitehead and Rattner, 1998; Woodcock et al., 2010) (Figure 1A). However, in *Drosophila* embryos, inhibition of the kinesin-5 motor Klp61F does not affect centrosome separation in prophase, although it is essential for bipolar spindle assembly after NEB (Sharp et al., 1999). Moreover, if Eg5 is the major force generator that drives prophase centrosome separation, the movement of the two centrosomes would be expected to be coordinated through the antiparallel sliding activity of Eg5.

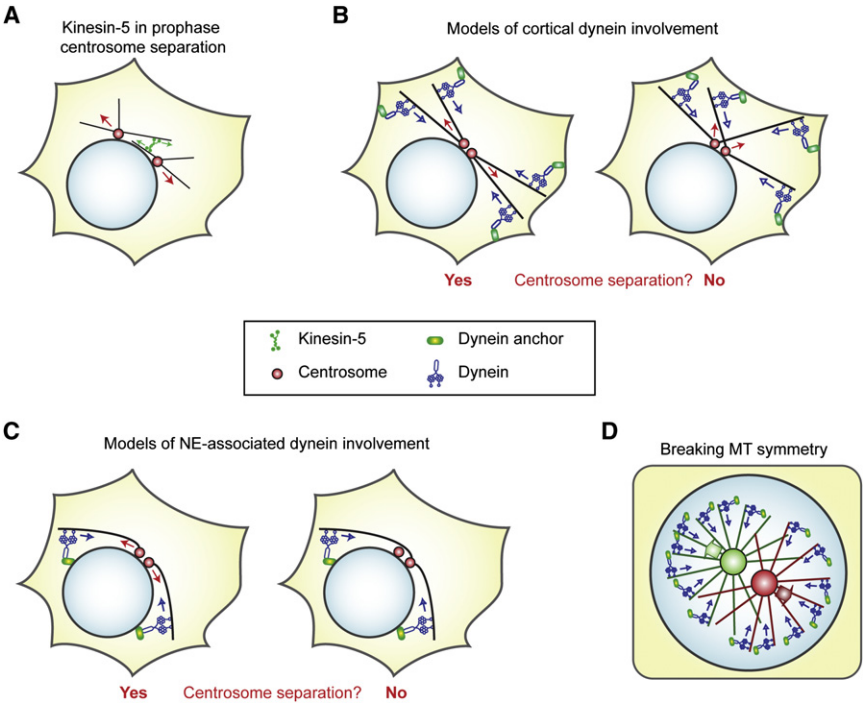

Figure 1. Role of MT Motors in Prophase Centrosomes Separation

Cells in prophase are depicted.

(A) Kinesin-5 motors can crosslink and slide apart antiparallel microtubules (MTs) coming from opposite centrosomes. This MT-sliding activity results in centrosome separation along the nuclear envelope (NE).

(B) Dynein anchored at the cortex can bind centrosomally anchored MTs. The minus-end-directed motility of dynein along these MTs will then pull centrosomes toward the cortex. If microtubules from a centrosome attach to cortical dynein molecules in an asymmetric fashion, with more attachments on one side than on the other side of the cell, while the other centrosomes makes equally asymmetric, but oppositely oriented, attachments, motility of dynein will result in centrosome separation (left). However, if centrosomes attach to cortical dynein in a symmetric fashion, the activity of dynein will only pull centrosomes away from the NE (right).

(C) Similar to (B), but here dynein is anchored at the NE. Centrosome separation will occur if MTs from a single centrosome preferentially attach to dynein molecules on one side of the nucleus, while MTs from the other centrosomes preferentially attach to dynein molecules on the opposing side of the nucleus (left). In contrast, centrosome separation will not occur if MTs from one centrosome attach to dynein on both sides of the nucleus equally (right).

(D) Top view of a prophase nucleus. If centrosomes are located very close together, one centrosome might physically block MT growth from the other centrosome and, thus, break the symmetry in MT outgrowth. This asymmetry in pulling forces would then allow centrosome separation. Small green and blue arrows indicate direction of kinesin-5 and dynein motility, respectively. Red arrows indicate the direction of centrosome movement. Large green and red arrows in (D) indicate direction of movement of centrosomes.

However, direct observations of centrosome movements in mammalian cells using time-lapse microscopy revealed that centrosomes move independently from each other in prophase (Waters et al., 1993), arguing against a role for Eg5 as the sole driver of prophase centrosome separation. Thus, while kinesin-5 motors clearly participate in prophase centrosome separation, at least in some systems, this process must require additional force generators as well.

A second important player in prophase centrosome separation is the minus-end-directed motor dynein. Dynein localizes to many distinct subcellular compartments, including the plus-ends of growing MTs, a plethora of intracellular vesicles, the cell cortex, and the NE (Kardon and Vale, 2009). While a role for dynein in prophase centrosome separation has been established in certain systems (Gonczy et al., 1999; Robinson et al., 1999; Sharp et al., 2000; Vaisberg et al., 1993), the mechanism by which dynein promotes initial centrosome separation is still under debate.

The cortical pool of dynein can pull on astral microtubules, as has been demonstrated during the process of spindle positioning (Dogterom et al., 2005; Galli and van den Heuvel, 2008; Grill et al., 2003; Kozlowski et al., 2007; Moore and Cooper, 2010). Studies performed in yeast have especially provided strong evidence that cortical dynein can pull on microtubules to position the spindle (Carminati and Stearns, 1997; Eshel et al., 1993; Heil-Chapdelaine et al., 2000; Li et al., 1993; Moore and Cooper, 2010). In higher eukaryotes, dynein also localizes to cortical microtubule attachments sites (Busson et al., 1998; Dujardin and Vallee, 2002) and was suggested to pull centrosomes apart through its minus-end-directed motility on astral microtubules, (Figure 1B, left panel) (Cytrynbaum et al., 2005; Sharp et al., 2000). There are, however, several issues with this simple model of cortical dynein as a facilitator of prophase centrosome separation. First, it is unclear how MTs from one centrosome can establish cortical interactions early in the centrosome separation process that are different from the MTs origination from the other centrosome, a prerequisite for migration of centrosomes in different directions (Figure 1B, right panel). Second, each centrosome will only show directed movement when the pulling force from one side is higher than the pulling force from the other side, but it is unclear how such asymmetric pulling forces on a single centrosome could be accomplished (Figure 1B, right panel). Indeed, attempts to model prophase centrosome separation in *Drosophila* embryos using cortical dynein as an outward force generator fail unless an asymmetric distribution of dynein pulling forces is incorporated (Cytrynbaum et al., 2003, 2005). In highly polarized cells in early *Drosophila* embryos, there is evidence for such asymmetric interactions with the cortex (Cytrynbaum et al., 2005), but the molecular basis for such asymmetry is unclear and it remains to be determined if similar asymmetric attachments occur in other cell types. Third, the orientation of the main force vector of cortical pulling forces will often not be in line with the direction of movement of centrosomes along the NE. Rather, this force is expected to pull the centrosomes away from the NE, toward the cell cortex, especially in large nonpolarized cells (Figure 1B, right panel). An additional component is, therefore, required that can tightly tether centrosomes to the nucleus but be sufficiently dynamic to allow

for centrosome movement along the NE. The angle of the force vectors will be highly dependent on the cell geometry and, while such mechanisms may work for certain cells with a well-defined geometry (such as yeast, early *Drosophila* embryos, and mammalian epithelial cells), it is unclear how robust such a mechanism could be in cells with a variable shape (such as the majority of cells in culture).

In addition to its cortical localization, dynein also localizes to the NE, and this pool of dynein has also been implicated in centrosome separation (Gonczy et al., 1999). Interestingly, dynein is specifically recruited to the NE in late G2 (Gonczy et al., 1999; Salina et al., 2002; Splinter et al., 2010; Tanenbaum et al., 2010), just prior to centrosome separation and, thus, suggestive of a function in this process. Furthermore, both in vitro and in vivo data demonstrates that NE-associated dynein can generate a substantial amount of force when pulling on microtubules (Beaudouin et al., 2002; Reinsch and Karsenti, 1997; Salina et al., 2002). From a theoretical point of view, centrosome separation by NE-associated dynein has two advantages over cortical dynein (Figure 1C). First, centrosomes are closer to the NE than to the cortex, greatly facilitating interactions between centrosomal MTs and dynein at the NE. Second, the force vector of NE-associated dynein is directed so that it pulls centrosomes along the NE rather than away from the NE. However, in the NE-associated dynein-pulling model, it is similarly unclear how MTs coming from one centrosome, which grow in all directions, will preferentially interact with dynein molecules on one side of that centrosome to generate asymmetric pulling forces (Figure 1C, right panel). One speculative explanation for this is that MT growth from the two centrosomes may be biased away from each other because MTs growing toward the opposing centrosome will collide with the other centrosome and undergo catastrophe (Janson et al., 2003). This would break the symmetry of MT outgrowth from each centrosome and result in asymmetric pulling forces, promoting centrosome separation (Figure 1D). This mechanism will only work efficiently when centrosomes are close together and could, therefore, function in the initial separation of centrosomes, after which other mechanisms should take over. Interestingly, a recent study suggested that in very large cells, dynein molecules anchored in the cytoplasm (rather than at the cortex or NE) provide pulling forces on astral microtubules. Due to a symmetry breaking event in these radial pulling forces, induced by the opposing microtubule aster (similar to Figure 1D), directed movement of centrosomes is possible (Wuhr et al., 2009, 2010). Careful spatial analysis of microtubule growth during prophase, as has been initiated in *Drosophila* cells (Cytrynbaum et al., 2005), will be required to address whether such biased microtubule growth does indeed occur in mammalian cells. Furthermore, specific perturbations that remove dynein specifically from the NE (Splinter et al., 2010) or cell cortex (Heil-Chapdelaine et al., 2000; Nguyen-Ngoc et al., 2007) will be valuable tools to test which pool of dynein promotes prophase centrosome separation. Finally, analysis in multiple cell types and organisms will provide important information about the generality of a role for dynein in prophase centrosome separation.

The Involvement of Actin

Actin filaments provide important structural support to the cell cortex and, together with their associated myosin motors, can contract, thereby generating a substantial amount of force. Interestingly, the actin cytoskeleton is also involved in centrosome separation in prophase in early *Drosophila* embryos (Cao et al., 2010; Stevenson et al., 2001). Surprisingly, this function of actin does not involve actomyosin contractility (Cao et al., 2010), suggesting that actin plays a structural role in promoting centrosome separation. Loss of actin function phenocopies loss of dynein in *Drosophila* embryos, as both treatments result in a partial inhibition of centrosome migration along the NE (Cao et al., 2010; Robinson et al., 1999; Sharp et al., 2000; Stevenson et al., 2001), suggesting that the major role of actin may be to recruit or activate cortical dynein. Similarly, *Drosophila* embryos lacking a functional version of the protein kinase Akt have reduced cortical MT attachments and decreased centrosome separation (Buttrick et al., 2008). Together, these studies further implicate microtubule attachments to the cell cortex in prophase centrosome separation.

In addition, a study in HeLa cells found centrosomes to be aligned with actin bundles in prophase, suggesting that actin might guide centrosome movement (Whitehead et al., 1996). While disruption of the actin cytoskeleton did not inhibit centrosome movement per se, it did affect the direction of centrosome movement (Whitehead et al., 1996). The conclusions in this study were based, however, on analysis of fixed cells, and it will be important to analyze centrosome movement using live-cell imaging to confirm these findings. A different study in mammalian cells found that actin is needed for centrosome separation specifically after NEB (Rosenblatt et al., 2004). This function of actin in mammalian cells does involve actomyosin contractility, which is different from its proposed role in prophase centrosome separation in *Drosophila* (Cao et al., 2010). In fact, contrary to mammalian cells, actin does *not* appear to be involved in centrosome separation after NEB in *Drosophila* embryos (Cao et al., 2010).

Taken together, three different mechanisms of action of been proposed for actin in centrosome separation: (1) Cortical actin promotes prophase centrosome separation, possibly through recruitment of dynein to the cortex and cortical capture of astral MTs. (2) Actomyosin contractility can position centrosomes after NEB by pulling on astral MTs. (3) Noncortical actin fibers may act to subtly guide prophase centrosome movement. The extent to which these pathways act in distinct organisms appears to be different, and more work is required to fully understand the role of actin and myosin in centrosome movement.

MT Pushing Forces

Both MT assembly and disassembly can generate force, which is coupled to the GTPase activity of tubulin. Assembling MTs can generate pushing forces, while disassembling MTs can generate pulling forces (reviewed in Dogterom et al., 2005). An interesting model that has been proposed is that MT pushing forces may contribute to centrosome separation in prophase (Cytrynbaum et al., 2003). During the process of centrosome separation, MTs growing from one centrosome will encounter the other centrosome and can exert a pushing force on it (Figures 2A and 2B). This force could be especially strong very early during centrosome separation, when centrosomes are close together, since the chance of a MT encountering the opposing centrosome is highest and the force generated by each MT is relatively high because short MTs do not buckle as easily as long MTs

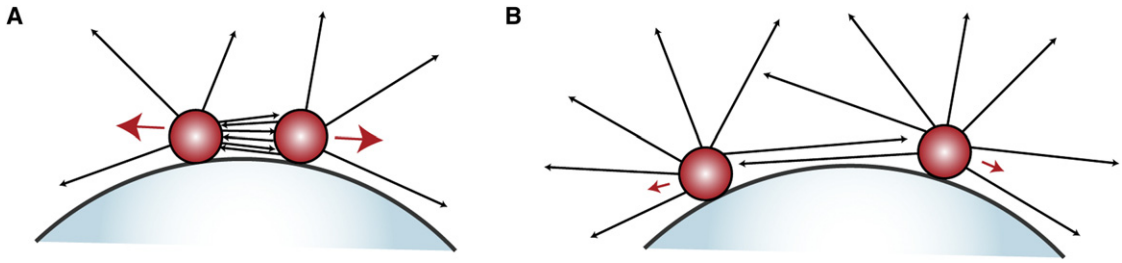

Figure 2. Role of MT Pushing Forces in Prophase Centrosomes Separation
View of centrosomes associated with the NE. MTs are growing from each centrosome in all directions.
(A) When centrosomes are positioned close together, many short MTs will collide with the opposing centrosomes, generating a pushing force that propels centrosomes apart.
(B) When centrosomes are further apart, many fewer MTs will collide with the opposing centrosome and the outward pushing force is much smaller. Size of the red arrows indicates the magnitude of the outward pushing force.

(Dogterom et al., 2005). The fact that growing microtubules can generate substantial pushing forces is supported by the fact that, in vitro, microtubule asters can position themselves solely through MT-pushing forces (Faivre-Moskalenko and Dogterom, 2002; Holy et al., 1997). Centrosome separation based on MT pushing forces is an especially compelling model, as this mechanism would function best when centrosomes are very close together (Figures 2A and 2B), a time at which most other centrosome-separating mechanisms, such as those dependent on antiparallel MT overlap and asymmetric cortical pulling forces, might not function efficiently.

Mechanisms of Bipolar Spindle Assembly

While prophase centrosome separation occurs in almost all cells, the extent to which it occurs is often variable, and cells frequently enter mitosis with only partially separated centrosomes (Rattner and Berns, 1976; Rosenblatt et al., 2004; Toso et al., 2009). Thus, robust mechanisms must exist in prometaphase to drive subsequent centrosome separation and bipolar spindle assembly. While kinesin-5 motors have a clearly defined role in promoting prometaphase spindle bipolarity in almost all systems (Ferenz et al., 2010), recent work has identified several additional pathways important for bipolar spindle assembly.

Kinesin-12 Motors

The kinesin-12 family has clearly been implicated in bipolar spindle formation. This family includes *Xenopus* Xklp2, sea urchin KRP(180), *C. elegans* KLP-18, mouse Kif15, and human Kif15/Hklp2 (Boleti et al., 1996; Rogers et al., 2000; Segbert et al., 2003; Sueishi et al., 2000; Tanenbaum et al., 2009; Vanneste et al., 2009), although somewhat surprisingly, no clear homolog has been identified in *Drosophila*. Kinesin-12 motors have an N-terminal motor domain, a very long central coiled-coil, and a C-terminal leucine zipper (Boleti et al., 1996; Wittmann et al., 1998). The leucine zipper alone localizes to the mitotic spindle, but not to purified MTs, suggesting it requires an adaptor protein to bind MTs (Boleti et al., 1996; Wittmann et al., 1998, 2000). Indeed, TPX2 (Targeting Protein for Xklp2) was identified as a protein that can target the leucine zipper of Xklp2 to MTs both in vitro and in vivo (Wittmann et al., 1998, 2000). TPX2 is also required to target full-length kinesin-12 to the spindle (Tanenbaum et al., 2009; Vanneste et al., 2009; Wittmann et al., 1998, 2000), demonstrating that the leucine zipper is both necessary and sufficient to target kinesin-12 to the spindle. The interaction between TPX2 and the leucine zipper of kinesin-12 is likely direct, as recombinant TPX2 was able to target recombinant kinesin-12 leucine zipper to MTs (Wittmann et al., 2000). In addition to TPX2, the dynein-dynactin complex also appears to regulate kinesin-12 binding to the spindle in *Xenopus* egg extracts. Kinesin-12 accumulates at spindle poles in *Xenopus* extracts, and this depends on dynein-dynactin (Wittmann et al., 1998), although the functional significance of this poleward transport remains unclear. Finally, in mammalian cells, Kif15/Hklp2 was shown to bind to chromosomes through an interaction with the chromosome-associated factor Ki-67 (Sueishi et al., 2000; Vanneste et al., 2009).

The first hint into the function of kinesin-12 came from dominant-negative mutants and inhibitory antibody studies in *Xenopus* egg extracts. Addition of either an antibody to the kinesin-12 C terminus or a recombinant fragment encompassing the kinesin-12 C terminus to *Xenopus* egg extracts potently blocked the formation of bipolar spindles (Boleti et al., 1996). However, further analysis of *Xenopus* kinesin-12 function using protein depletion did not reveal any defects in bipolar spindle assembly (Walczak et al., 1998; Wittmann et al., 2000). This demonstrated that kinesin-12 is not essential for bipolar spindle assembly in *Xenopus* egg extracts and suggested that the dominant-negative approaches used generate a kinesin-12 gain-of-function. A similar study showed that injection of an antibody directed against the kinesin-12 C terminus impaired normal centrosome separation and bipolar spindle assembly in sea urchin embryos (Rogers et al., 2000), but it is unclear if this too is due to a side-effect of the method of inhibition. In this respect, it is interesting to note that the spindle protein RHAMM, which also forms a complex with TPX2 (Groen et al., 2004), has a domain that is highly similar to the TPX2-interaction site in the C terminus of kinesin-12. It is, therefore, possible that this is a general TPX2-binding motif and that the dominant negative kinesin-12 mutants used in these studies also inhibit the interaction between RHAMM and TPX2.

The first solid evidence for a role of kinesin-12 in spindle assembly came from *C. elegans*, where depletion of kinesin-12 results in the formation of monopolar spindles in meiosis (Segbert et al., 2003; Wignall and Villeneuve, 2009). Surprisingly though, kinesin-12 is not needed for bipolar spindle assembly in the subsequent *C. elegans* mitosis (Saunders et al., 2007; Segbert et al., 2003). Similarly, in human cells, kinesin-12 is not

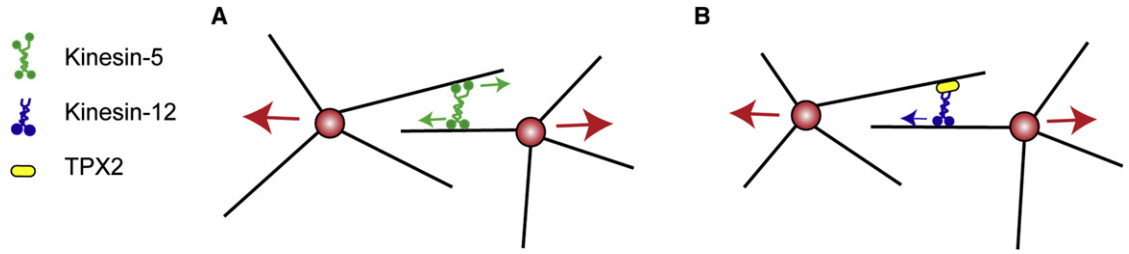

Figure 3. Model for Kinesin-5 and Kinesin-12 Motor Function in Bipolar Spindle Assembly

(A) After NEB, kinesin-5 motors crosslink antiparallel MTs through their bipolar configuration and slide these MTs apart, generating an outward force in the spindle. Green arrows indicate direction of kinesin-5 motility, and red arrows indicate the direction of centrosome movement.

(B) Kinesin-12 can bind one MT directly through its motor domain and another one indirectly, through the targeting protein TPX2. In this way, a complex of kinesin-12 and TPX2 can crosslink two MTs, and MT motility by kinesin-12 would slide anti-parallel MTs apart, generating an outward force similar to kinesin-5. The blue arrow indicates direction of kinesin-12 motility, and red arrows indicate the direction of centrosome movement.

essential for bipolar spindle assembly in mitosis (Tanenbaum et al., 2009; Vanneste et al., 2009; Zhu et al., 2005). However, loss of kinesin-12 does strongly sensitize human cells to kinesin-5 inhibitors (Tanenbaum et al., 2009; Vanneste et al., 2009), suggesting that kinesin-12 acts redundantly with kinesin-5 to promote bipolar spindle assembly. Importantly, in the complete absence of kinesin-5 activity, overexpression of kinesin-12 can fully restore centrosome separation and bipolar spindle assembly (Tanenbaum et al., 2009). This result provides strong evidence that kinesin-12 can produce an outward force within the spindle, similar to kinesin-5, but that in normal cells, sufficient kinesin-5 activity is present within the spindle to mask this function of kinesin-12.

An essential aspect of kinesin-5 function is its ability to form homo-tetramers, allowing it to crosslink anti-parallel MTs and slide them apart (Figure 3A). Kinesin-12 motors likely form dimers, not tetramers. Therefore, these motors must have adopted an alternative mechanism of force generation. Interestingly, kinesin-12 molecules can bind MTs directly using the motor domains, and indirectly through their interaction with TPX2 (Boleti et al., 1996; Heidebrecht et al., 2003; Tanenbaum et al., 2009; Vanneste et al., 2009; Wittmann et al., 2000), suggesting a mechanism by which kinesin-12 might crosslink two microtubules (Figure 3B). This mode of MT crosslinking could drive anti-parallel MT sliding. Indeed, in sea urchin embryos, kinesin-12 localizes prominently to the zone of antiparallel MT overlap during prometaphase and metaphase, further suggesting that it functions at these sites (Rogers et al., 2000). In vitro reconstitution experiments will be required to directly address this intriguing possibility. Kinesin-12 also associates with chromosomes through Ki-67, but specific depletion of kinesin-12 from chromosomes by Ki-67 RNAi actually increases the fraction of bipolar spindles that form in the presence of a low dose of kinesin-5 inhibitors (Vanneste et al., 2009), suggesting that chromosome binding by kinesin-12 might negatively regulate its activity in bipolar spindle formation. Taken together, kinesin-12 motors play an important role in promoting bipolar spindle assembly, and although their mode of action is distinct from kinesin-5 motors, they seem to be able to generate a force in the mitotic spindle that acts redundantly with kinesin-5.

Function of Chromokinesins

Chromokinesins are kinesins that associate with mitotic chromosomes (and in many cases with the spindle as well) and are involved in multiple aspects of cell division. So far, two conserved chromokinesin families have been identified, the kinesin-4 and kinesin-10 families (reviewed in Mazumdar and Misteli, 2005), and both types of chromokinesins have plus-end directed motility (Bieling et al., 2010; Bringmann et al., 2004; Sekine et al., 1994; Yajima et al., 2003). While bound to chromosome arms, chromokinesins can walk along MTs growing from the centrosomes, which generates a force (polar ejection force) that pushes the chromosome arms away from spindle poles (Antonio et al., 2000; Funabiki and Murray, 2000; Goshima and Vale, 2003; Ke et al., 2009; Levesque and Compton, 2001; Mazumdar et al., 2004; Rieder et al., 1986; Vernos et al., 1995). At the same time, the MT that is being used as a track for the chromokinesin should experience an outward force that pushes spindle poles apart, suggesting that chromokinesins might cooperate with kinesin-5 and kinesin-12 motors to promote centrosome separation (Dumont and Mitchison, 2009). Indeed, one study has reported defects in spindle pole separation after inhibition of a chromokinesin (Klp3a, kinesin-4) (Kwon et al., 2004), confirming the notion that chromokinesins promote spindle bipolarity. It should be noted that this latter study also found severe defects in spindle organization, possibly because of the additional activity of kinesin-4 motors in regulating MT dynamics (Bringmann et al., 2004), which could indirectly result in decreased spindle length or centrosome separation. Surprisingly, other loss-of-function studies have not provided strong support for a role of chromokinesins in spindle bipolarity. While many studies have revealed a role for these motors in chromosome alignment/positioning, bipolar spindles form normally in the absence of either kinesin-4 motors, kinesin-10 motors, or both (Antonio et al., 2000; Funabiki and Murray, 2000; Goshima and Vale, 2003; Levesque and Compton, 2001; Mazumdar et al., 2004; Vernos et al., 1995; Walczak et al., 1998). Another study also reported a decrease in spindle length after inhibition of a kinesin-10 motor, but this effect was minor and independent of motor activity and chromosome binding (Tokai-Nishizumi et al., 2005). So why are chromokinesins not essential for spindle bipolarity in the majority of experimental systems? One possibility is that they act redundantly with kinesin-5, similar to kinesin-12 motors. It would, therefore, be very interesting to test whether loss of chromokinesins sensitizes cells to partial inhibition of kinesin-5. Alternatively, the forces generated by chromokinesins might be relatively small

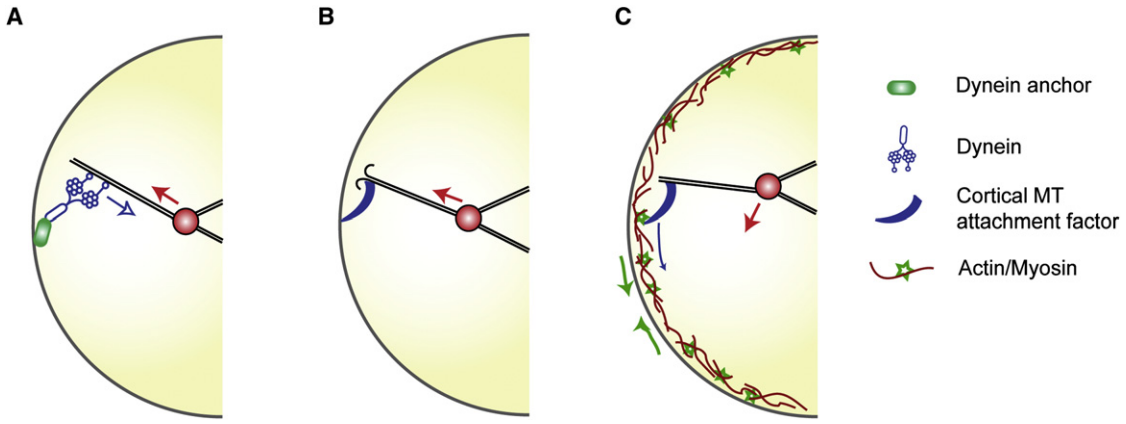

Figure 4. Force Generation through Cortical Pulling Forces

(A) An astral MT is captured by cortical dynein, which generates a pulling force on the centrosome through minus-end-directed motility on the astral MT. The blue arrow indicates direction of dynein motility, and the red arrow indicates the direction of centrosome movement.

(B) An astral MT is captured by a cortically anchored MT binding protein (either motor or nonmotor). When the MT undergoes a catastrophe, the MT binding protein remains attached to the depolymerizing MT, resulting in pulling force on the centrosome toward the cortex. The red arrow indicates direction of centrosome movement.

(C) An astral MT attaches to the cortex through a MT binding protein (motor or nonmotor). Due to the contractility of the actomyosin cortex (green arrows), the MT binding protein moves along the cortex and pulls with it the astral MT and associated centrosome. The blue arrow indicates movement of cortical MT binding protein, and the red arrow indicates movement of the centrosome.

compared to other forces in the spindle (Brouhard and Hunt, 2005; Marshall et al., 2001), and therefore, they would not substantially contribute to an outward sliding force in the spindle. Thus, additional work will be required to determine to what extent chromokinesins contribute to bipolar spindle assembly.

Function of MT-Cortex Interactions

Cortical pulling forces can generate a substantial amount of pulling force on centrosomes, as cortical force generators can position the entire spindle in the cell through interactions with astral MTs (Dogterom et al., 2005; Galli and van den Heuvel, 2008; Grill et al., 2003; Kozlowski et al., 2007). Four types of forces can be generated by MTs interacting with the cortex. First, MTs can grow into the cortex and generate a pushing force (Dogterom et al., 2005; Holy et al., 1997). While this type of force might contribute to positioning of the spindle, it is unlikely to promote centrosome separation. Second, MTs can be captured at the cortex by dynein, which could "pull" on these MTs through its minus-end-directed motility (Figure 4A). Third, similar to the second scenario, astral MTs could be captured at the cortex by either nonmotor MT-associated proteins or minus-end-directed motors (again the prime candidate in higher eukaryotes being dynein). If the cortically attached MT undergoes catastrophe and the cortical attachment site is able to remain attached to the shrinking MT, the cortical MT can generate a pulling force on its associated centrosome toward the cortex (Figure 4B). This mechanism is thought to control spindle positioning in yeast (reviewed in Moore and Cooper, 2010) and is also analogous to kinetochores holding on to shrinking MT and using the energy released from the microtubule lattice during depolymerization for their movement (Asbury et al., 2006; Grishchuk et al., 2005; Powers et al., 2009; Westermann et al., 2006). While this type of force generation seems similar to the dynein-dependent pulling model, it is conceptually distinct because the force is generated by the MT rather than by the motor. These two models are, however, not necessarily mutually exclusive: a cortical dynein motor bound to an astral MT could both generate a pulling force itself through ATP hydrolysis and at the same time utilize the energy from the shrinking MT to indirectly generate a pulling force. Fourth, astral MTs can attach to the cortex stably and experience a force due to movement or flow of the cortical actomyosin network (Figure 4C) (Rosenblatt et al., 2004). In this case, the force generation is independent of MT dynamics. Again, this type of force generation is not mutually exclusive with previous models, as MTs can be pulled on by force-generating cortical sites, while at the same time these cortical sites can move themselves, producing different types of forces simultaneously.

Consistent with a role as an outward force generator at the cortex during spindle assembly, depletion of dynein results in impaired spindle elongation in *Drosophila* embryos (Sharp et al., 2000) and monopolar spindles in *C. elegans* (Gonczy et al., 1999). However, in other systems, studies on the role of cortical dynein in outward force generation during spindle assembly is hampered by two problems. First, inhibition of dynein can influence prophase centrosome separation, as described in the previous section, complicating conclusions about dynein function at later stages. Second, several studies have found that dynein actually pulls centrosomes together during spindle assembly (Ferenz et al., 2009; Mitchison et al., 2005; Tanenbaum et al., 2008). Thus, dynein appears to have two distinct counteracting activities: one that pulls centrosomes apart by generating cortical MT pulling forces and the other that pulls centrosomes together through a function within the spindle. In different systems, the relative strength of these activities could differ, resulting in the opposite outcomes of dynein inhibition. Therefore, it will be especially important to identify specific proteins involved in MT-cortex interactions, based on which new tools can be generated to selectively perturb these interactions, as has been done successfully in yeast (Moore and Cooper, 2010).

Pulling forces have also been elegantly demonstrated to be generated on astral MTs in early prometaphase by contraction of the cortical actomyosin cortex (Rosenblatt et al., 2004). Myosin II activity results in flow of the cortical actin meshwork away from the center of the cell, pulling along astral MTs with their associated centrosomes (Rosenblatt et al., 2004). Importantly, actomyosin-dependent centrosome separation occurs specifically after NEB. While this provocative study clearly demonstrates the importance of actomyosin contractility in mammalian cells in culture, the generality of this mechanism for other systems remains to be determined.

In conclusion, cortical pulling forces can generate a substantial amount of force and appear to promote bipolar spindle assembly in some systems but whether these forces are widely harnessed for centrosome separation and bipolar spindle assembly remains to be determined. Furthermore, the exact mechanism of cortical force generations will be an exciting topic for future research.

Control of Spindle Bipolarity by Kinetochores

Kinetochores are not essential for the formation of a bipolar spindle, as bipolar spindles can form around chromatin-associated beads that lack kinetochores (Heald et al., 1996). Nonetheless, it is likely that kinetochores, when present, do affect the forces that control spindle bipolarity. In a metaphase spindle, bioriented kinetochore pairs are thought to be under tension as the distance between sister-kinetochore is increased compared to unattached kinetochores. The exact nature of the forces that generate this interkinetochore tension are still controversial, but it is reasonable to assume that when sister kinetochores are under tension, they generate an equal inward force on centrosomes through kinetochore-MTs. Consistent with this, spindles are longer in the absence of kinetochore-MT attachments (DeLuca et al., 2002). However, a recent study challenged this idea by showing that inhibiting kinetochore-MT attachments results in delayed centrosome separation and suggested that kinetochores generate an outward pushing force (Toso et al., 2009). It is unclear how this model can be reconciled with the idea of inward force generation by sister kinetochores under tension. Furthermore, earlier studies using laser ablation of oscillating kinetochore pairs did not provide evidence that kinetochores are generating a pushing force (Khodjakov and Rieder, 1996), at least not in metaphase spindles. Perhaps, early in mitosis, when sister kinetochores have monotelic attachments and are, thus, not under tension, kinetochores generate different types of forces than during metaphase, when sister kinetochores are bioriented and under tension. Further studies are clearly required to determine exactly how kinetochores contribute to spindle bipolarity.

Regulators of MT Dynamics and Spindle Bipolarity

Dynamics of MT plus- and minus-ends need to be tightly controlled for proper bipolar spindle assembly. First, kinetochore-bound regulators of MT growth are required for spindle bipolarity by preventing spindle collapse due to continuous kinetochore-MT shortening at the spindle pole (Laycock et al., 2006; Maiato et al., 2002, 2003). In addition, several studies have found an increase in monopolar spindle formation after depletion of MT destabilizing motor proteins (Ganem and Compton, 2004; Holmfeldt et al., 2004; Kline-Smith and Walczak, 2002; Laycock et al., 2006; Rogers et al., 2004). However, in general, these effects are mild and often confined to specific experimental systems (Cassimeris and Morabito, 2004; Tanenbaum et al., 2009). In human cells, it appears that the kinesin-13 motor Kif2a is, in fact, not required for bipolar spindle assembly (Tanenbaum et al., 2009), as suggested previously (Ganem and Compton, 2004). Taken together, these results suggest that regulators of MT dynamics, like several other pathways, contribute to spindle bipolarity in a largely redundant fashion. Support for this idea comes from studies in which the kinesin-13 motor MCAK was depleted in a setting in which kinesin-5 function is compromised (Kollu et al., 2009; Tanenbaum et al., 2009). In this setting, MCAK does become essential for spindle bipolarity, confirming its role as a redundant regulator of spindle bipolarity. Together, these results indicate that overstabilization of the mitotic MT array results in an increased tendency to form monopolar spindles. Interestingly, the opposite also appears to be true: when MT stability is decreased, cells form bipolar spindles, even when kinesin-5 activity is reduced (but not eliminated) (Kollu et al., 2009).

Why does overstabilization of MTs inhibit bipolar spindle assembly? First, it is possible that increased MT stability increases the anti-parallel MT overlap on which minus-end-directed motors can act to pull centrosomes together, as suggested previously (Kollu et al., 2009). While one would expect that outward force generators would also benefit from increased antiparallel overlap, it is possible that in a metaphase spindle (where inward and outward force are likely balanced), outward force is generated by motors both at antiparallel MTs and at other sites (cortical sites, chromosomes, etc.), while inward forces are exclusively generated at antiparallel MTs. Thus, proportionally more inward force would be generated at antiparallel MTs, so increasing this region will pull centrosomes together. Consistent with this, a recent study modeling spindle assembly suggested that antiparallel MTs need to be dynamic to establish a productive force-balance (Civelekoglu-Scholey et al., 2010). Second, it is possible that centrosomes connected to a MT array made of long MTs require more force to move through the cell than small asters (especially if centrosomal MTs extend to the cortex, thereby potentially blocking the movement of the MT network). Although this can explain defects in initial centrosome separation when MT stability is increased, it does not explain why fully formed spindles collapse more readily when MTs are overstabilized (Kollu et al., 2009; Tanenbaum et al., 2009). It is also possible that MT-cortex interactions require MT depolymerization to produce a pulling force on mitotic asters (as described above). Thus, increasing MT stability would inhibit efficient cortical pulling forces, explaining both defects in centrosome separation and bipolar spindle maintenance. Importantly, none of these explanations are mutually exclusive, and it is likely that altered MT dynamics affect spindle bipolarity in multiple ways.

Concluding Remarks

Bipolarity is one of the most apparent and conserved aspects of the mitotic spindle, but we have just started to dissect the different molecular pathways that contribute to spindle bipolarity. The events that lead to bipolar spindle assembly can be separated into two distinct temporal phases: prophase and prometaphase. While some factors, like kinesin-5 and cortical

dynein, may be active during both phases, other factors, like NE-dynein and kinesin-12 function (which requires nuclear release of TPX2), are specific for prophase and prometaphase, respectively. Furthermore, many individual proteins contribute to spindle bipolarity without being absolutely essential for it. In fact, if prophase centrosome separation fails completely, bipolar spindles are still formed in prometaphase, demonstrating that there is substantial redundancy during bipolar spindle assembly. Perhaps redundant pathways are used differentially in different cell types or organisms, for example, depending on the specific geometric properties of the cell. However, it is more likely that such redundancy has evolved to ensure very high level of accuracy of this process, which is absolutely essential for correct chromosome segregation and cell viability. The next challenge will be to fully elucidate the complex layers of redundancy in the system that ensures its robustness. Likely, enhancer/suppressor-type of genetic approaches will help uncover these redundant pathways and give a more complete picture of bipolar spindle assembly.

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