

Mechanisms of plant spindle formation

Han Zhang · R. Kelly Dawe

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Abstract In eukaryotes, the formation of a bipolar spindle is necessary for the equal segregation of chromosomes to daughter cells. Chromosomes, microtubules and kinetochores all contribute to spindle morphogenesis and have important roles during mitosis. A unique property of flowering plant cells is that they entirely lack centrosomes, which in animals have a major role in spindle formation. The absence of these important structures suggests that plants have evolved novel mechanisms to assure chromosome segregation. In this review, we highlight some of the recent studies on plant mitosis and argue that plants utilize a variation of “spindle self-organization” that takes advantage of the early polarity of plant cells and accentuates the role of kinetochores in stabilizing the spindle midzone in prometaphase.

Key words plant · spindle · microtubule · chromosome · kinetochore · centromere · mitosis · meiosis · nuclear envelope · RanGTP · preprophase band · *Hordeum vulgare*

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H. Zhang · R. K. Dawe
Department of Genetics, University of Georgia,
Athens, GA 30602, USA

R. K. Dawe (✉)
Department of Plant Biology, University of Georgia,
Athens, GA 30602, USA
e-mail: kelly@plantbio.uga.edu

Abbreviations

GDP	Guanosine diphosphate
GFP	Green fluorescence protein
GTP	Guanosine triphosphate
INCENP	Inner centromere protein
Mis12	Minichromosome instability 12
Ndc80	Nuclear division cycle 80
Nuf2	Nuclear filament-containing protein 2
NuMA	Nuclear mitotic apparatus protein
PPB	Preprophase band
Rae1	Ribonucleic acid export
Ran	Ras-related nuclear protein
RCC1	Regulator of chromosome condensation 1
Spc24	Spindle pole body component 24
Spc25	Spindle pole body component 25
TPX2	Targeting protein for Xklp2
Xklp2	<i>Xenopus</i> plus end-directed kinesin-like protein 2

Introduction

Chromosome segregation relies entirely on the formation of a stable, bipolar spindle that can direct the separated chromosomes away from each other and into daughter nuclei. Spindles are made primarily of microtubules that have a natural polarity. One end of a polymerized microtubule is known as the plus end, since new tubulin subunits tend to be incorporated there, and the other end is known as the minus end. In animal somatic cells, the minus ends are embedded in

specialized organelles known as centrosomes while the plus ends radiate outwards and undergo rapid cycles of growth and shrinkage (Howard and Hyman 2003). Kinetochores can capture the dynamic plus ends to form stable bundles. As both the spindle poles and the replicated kinetochores occur in pairs, these forces along with hundreds of facilitating proteins can form durable bipolar spindles. This centrosome-based mechanism of spindle formation is referred to as the “search and capture” model (Kirschner and Mitchison 1986).

The search and capture model does not account for all cell types and can be misleading in its reliance on preexisting poles. Many types of animal cells lack centrosomes and appear to form perfectly functional spindles. In such cases, such as animal oocytes that naturally lack centrosomes (Matthies et al. 1996), somatic cells that lack centrosomes due to laser ablation (Khodjakov et al. 2000; Khodjakov and Rieder 2001), or somatic cells that lack centrosomes by genetic manipulation (Bonaccorsi et al. 1998; Megraw et al. 2001), the microtubules are nucleated at or near the chromosomes. In a particularly influential study using *Xenopus* egg extracts lacking centrosomes, bipolar spindles were shown to self-organize around DNA-coated beads (Heald et al. 1996). The *Xenopus* system exemplifies a mechanism of spindle formation known as “spindle self-organization” and is viewed as an alternative to the centrosomal pathway (Heald et al. 1996; Karsenti and Vernos 2001).

Higher plant cells lack centrosomes and apparently have no functional equivalent at the spindle poles. Consequently, it has been argued that plant spindles form in a manner similar to the spindle self-organization pathway in animals (Karsenti and Vernos 2001). In this review, we highlight recent studies on plant mitosis that support and expand this view. We emphasize studies on the Ras-related nuclear protein (Ran) pathway and how the results relate to plant spindle formation, briefly review new data on the role of preprophase bands in conferring early polarity to plant spindles, and discuss the fact that kinetochores play a particularly large role in plant spindle formation in mitosis.

“Ran-regulated spindle self-organization” is a mechanism common to both plants and animals

The spindle self-organization mechanism was first established using an *in vitro* system in which plasmid

DNAs were coupled to magnetic beads in *Xenopus* egg extracts containing chromatin assembly and spindle assembly components (Heald et al. 1996). As this system lacks centromeric DNA or centrosomes, the “search and capture” pathway is inaccessible; nevertheless, morphologically normal bipolar spindles formed around the DNA-coated beads. A follow-up study further demonstrated that spindle shape and orientation is largely defined by the chromatin (Dinarina et al. 2009). These observations raised many questions, such as how microtubules are stabilized by chromatin alone and how microtubules can self-assemble to form a bipolar structure.

Subsequent studies using *Xenopus* egg extracts have shed light on both the mechanism and molecular components involved in this pathway. The guanosine triphosphatase Ran (RanGTP) is believed to be a regulator of chromosome-based microtubule nucleation and spindle formation (Kalab et al. 1999; Ohba et al. 1999; Carazo-Salas et al. 1999; Wilde and Zheng 1999). Ran has two states, the guanosine triphosphate (GTP)-bound form and the guanosine diphosphate (GDP)-bound form. In the nucleus, Ran interacts with chromatin and proteins that maintain it in the GTP-bound state. Ran can bind directly to histone H3 and histone H4 (Bilbao-Cortes et al. 2002) or indirectly to nucleosomes through regulator of chromosome condensation 1 (RCC1), a protein found in animals but not plants. RCC1 is a guanine nucleotide exchange factor of Ran, which efficiently converts Ran from the GDP to GTP state (Makde et al. 2010). In the cytoplasm, the GTPase-activating protein RanGAP together with its cofactor RanBP1 or RanBP2 (Bischoff et al. 1994, 1995) stimulates the reverse reaction and promotes the GDP-bound state. As a result, RanGTP is highly enriched in the nucleus. When the nuclear envelope breaks down (preceding mitosis or meiosis), the compartmentalization is lost, but a gradient is formed: with RanGTP enriched in the vicinity of the chromosomes and RanGDP concentrated at the cell periphery. This gradient is thought to be critical for mitotic spindle assembly around chromosomes (Caudron et al. 2005; Kalab et al. 2006).

Ran activates mitotic spindle assembly by releasing spindle assembly factors from their nuclear transport receptors (importin α and importin β) (Wiese et al. 2001; Nachury et al. 2001; Gruss et al. 2001). Only where the RanGTP concentration is above a threshold,

such as in close proximity to chromatin, can free spindle assembly factors exist (Caudron et al. 2005). A number of spindle assembly factors are thought to be regulated by RanGTP (Kalab and Heald 2008). Three examples are nuclear mitotic apparatus protein (NuMA), targeting protein for Xklp2 (TPX2), and ribonucleic acid export 1 (Rae1). In the presence of these proteins, microtubules tend to be more stable and thus more likely to form the bundled arrays that make up a spindle.

NuMA is localized in the nucleus during interphase and moves to the spindle poles at the onset of mitosis (Merdes et al. 1996, 2000). NuMA binds microtubules directly and plays a critical role in zippering microtubules into focused poles (Merdes et al. 1996, 2000; Silk et al. 2009). Similarly, TPX2 binds to microtubules and tends to concentrate at the spindle poles (Wittmann et al. 1998, 2000). It has been shown that TPX2 is required for microtubule nucleation as well as spindle pole organization (Gruss et al. 2002; Garrett et al. 2002; Schatz et al. 2003). Rae1 likewise binds to microtubules (Blower et al. 2005) as well as the N-terminal end of NuMA (Wong et al. 2006). Previous data suggested that the interaction between Rae1 and NuMA is important for bipolar spindle formation.

The evidence in favor of a similar Ran-based pathway of spindle formation in plants is limited but nevertheless convincing. Ran itself is highly conserved in plants and other species (Ach and Grussem 1994; Merkle et al. 1994; Haizel et al. 1997). As in animals, green fluorescence protein (GFP)-tagged Ran localizes to nuclei in tobacco cells (Yano et al. 2006). Overexpression of Ran in rice increases the proportion of cells in G2 phase (Wang et al. 2006), indicating that Ran is involved in cell cycle progression. *Arabidopsis* RanGAP localizes to spindles as well as the late spindle midzone (the phragmoplast; (Pay et al. 2002), while AtRanBP1 is localized to the cytoplasm where it maintains its conserved function as a co-activator that stimulates the hydrolysis of RanGTP (Kim and Roux 2003). A recent study in *Arabidopsis* (Xu et al. 2008) showed that RanGAP further localizes to preprophase bands (PPBs), which are cortical rings of microtubules found in plant cells during late interphase and prophase (see below for discussion of the PPB). These observations strongly suggest that the Ran cycle is associated with plant spindle assembly.

Spindle assembly factors are also found in plants. Homologs of NuMA, TPX2, and Rae1 have been identified based on sequence homology and their conserved functions during spindle formation. Similar to its vertebrate homolog, *Arabidopsis* TPX2 localizes to nuclei during interphase and associates with spindle microtubules during mitosis (Vos et al. 2008). AtTPX2 binds importin α and promotes spindle assembly in vitro (Vos et al. 2008) and is therefore believed to be involved in the RanGTP-regulated spindle assembly pathway. Microinjection of TPX2 antibodies immediately after nuclear envelope breakdown causes cell cycle arrest, suggesting that TPX2 is required to initiate plant spindle assembly (but, interestingly, is not required to complete mitosis (Vos et al. 2008)). Rae1 has also been identified in plants and appears to have a highly conserved function. Tobacco Rae1 binds microtubules and is required for bipolar spindle formation (Lee et al. 2009). NuMA, although the least characterized of the three, is present in plants as judged by limited molecular homology and immunolocalization with heterologous antibodies (Yu and Moreno Diaz de la Espina 1999).

Plants appear to have further elaborated on the Ran pathway to enrich the population of microtubules around nuclei. It is known that plant nuclei serve as efficient nucleation centers for microtubules (Stoppin et al. 1994) and that Ran is concentrated near the nuclear envelope (Ma et al. 2007). A recent report from tobacco sheds light on the mechanism, showing that histone H1 forms ring-shape complexes with tubulin dimers at the nucleus–cytoplasm interface and promotes the formation of aster-like structures (Hotta et al. 2007; Nakayama et al. 2008). In *Leishmania* (a protozoan; (Smirlis et al. 2009)), Ran and histone H1 co-localize at the nuclear envelope and bind each other in vitro. These data suggest that in plants, the Ran-based pathway nucleates microtubules around the nucleus even before nuclear envelope breakdown.

Taken together, the available data indicate that the spindle self-organization pathway has a major role in plant mitosis. It is noteworthy that spindle self-organization occurs naturally in animals as well, even though the centrosomal pathway is dominant. Recent studies show that microtubules are nucleated around animal chromosomes independent of centrosomes (Khodjakov et al. 2003; Maiato et al. 2004; O'Connell et al. 2009) and, conversely, that when the Ran cycle and its effectors are disrupted, mitotic spindle

formation is impaired despite the presence of centrosomes (Guarguaglini et al. 2000; Gruss et al. 2002; Garrett et al. 2002). Although spindle self-organization is a remarkably robust mechanism, it appears to operate most efficiently when there are other features such as centrosomes available to reinforce and stabilize the resulting polarity. In plants, likely stabilizing factors are the preprophase bands and kinetochores.

Preprophase band: an analog of centrosomes?

Many have observed that plant mitotic spindle poles are not defined points, but instead loose collections of ends that coalesce (Fig. 1). What causes this to happen such that the resulting structure is bipolar and not multipolar? The answer probably lies in the fact that plant cells have an unusual type of microtubule array called the preprophase band (Lloyd and Chan 2006). The preprophase band (PPB) is a ring of cortical microtubules that forms during G2/prophase at the equator of (future) cell division. As such, the PPB encircles the cell and accurately marks where the metaphase plate will form. In principle, an array that marks the outlines of equator can have the same effect as marking the two ends of a bipolar structure: instead of marking the axis of symmetry, the PPB marks the plane perpendicular to the axis of symmetry. Several forms of data support this interpretation.

PPBs are microtubule-based structures that form shortly before the nuclear envelope breaks down and bipolarity is initiated. Recent data reveal that the microtubules organized by the PPB radiate outwards and make contact with the nucleus. This small group

of microtubules is known as bridging microtubules (Ambrose and Cyr 2008). At this stage, generally coinciding with late prophase, the outlines of the future spindle poles can be seen. The abundance of bridging microtubules affects where the nucleus lies within the cell and where the spindle equator will be formed (Ambrose and Cyr 2008). Extreme perturbations of microtubule arrays at prophase can result in double PPBs, and when this happens, the early prometaphase spindles are generally multipolar (Yoneda et al. 2005). These results suggest that the PPB has a causal role in defining spindle polarity. It has also been reported that PPBs define the future division plane through the activity of RanGAP, which remains associated at the division plane throughout mitosis and cytokinesis (Xu et al. 2008). An interpretation of how the Ran pathway and PPB may interact to initiate spindle assembly is shown in Fig. 2.

As with centrosomes in animals, it is not the case that plant spindle formation requires the prior formation of a preprophase band. All plant meiotic cells (Bannigan et al. 2008) and a small proportion of somatic cells (Chan et al. 2005) naturally lack preprophase bands yet still form bipolar spindles. Under these conditions, it is likely that chromatin and the Ran pathway suffice to form functional bipolar spindles. Such data suggest preprophase bands are dispensable for spindle assembly but increase the fidelity of mitosis by providing spatial cues.

Kinetochores: a re-evaluation

There is little doubt that kinetochores are integral components of intact spindles and that they are required

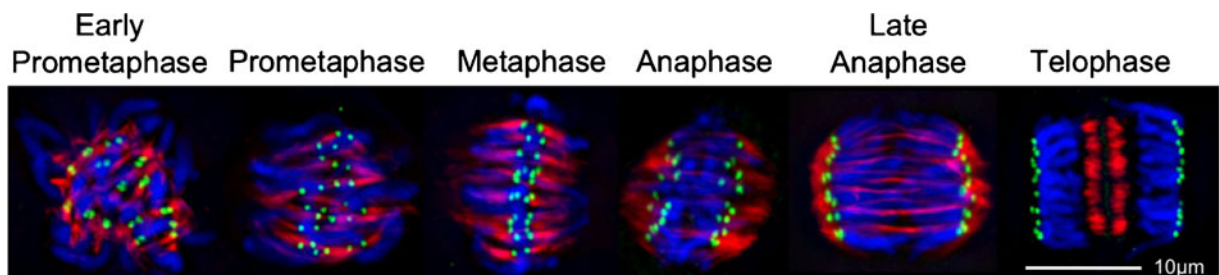


Fig. 1 Immunofluorescence of barley mitosis. Barley root tips were fixed in 4% paraformaldehyde, digested with cellulase and pectinase, and the cells dropped onto polylysine-coated slides. Cells were stained with anti-tubulin (*red*) and anti-CENH3 (an inner kinetochore protein, *green*) (Nagaki et al. 2004). Chromo-

somes are shown in blue with the substages of mitosis indicated. Note that microtubules interact with kinetochores even in the earliest stages of prometaphase immediately following nuclear envelope breakdown

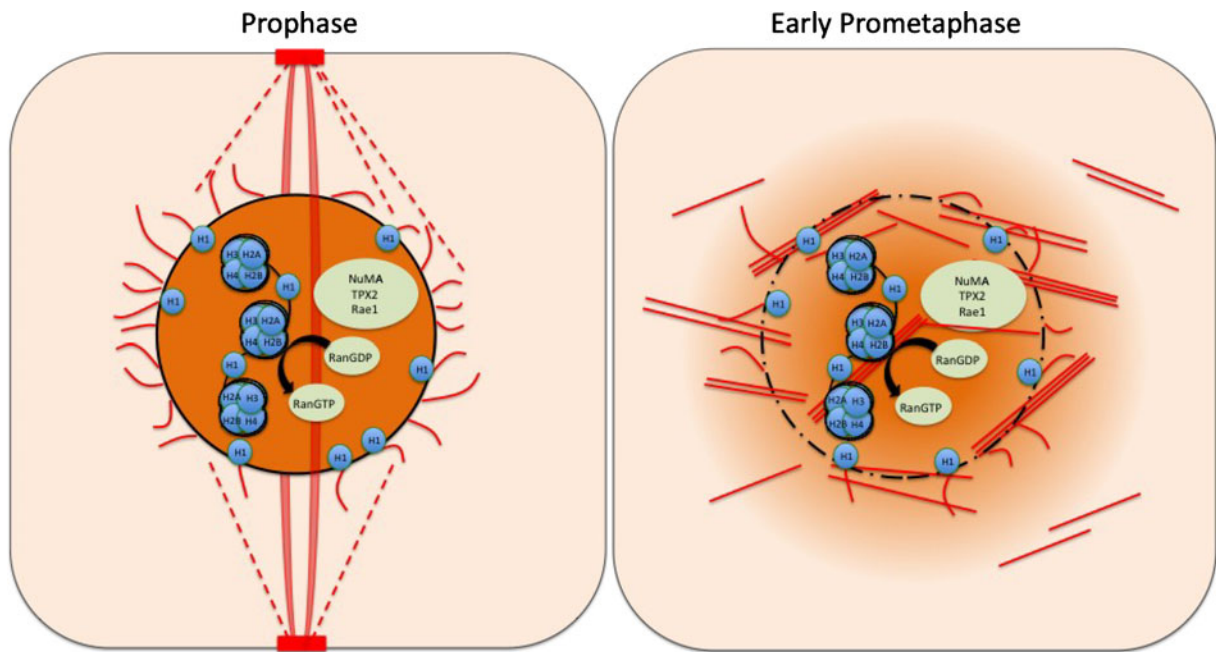


Fig. 2 Illustration of how the Ran pathway and the PPB interact to initiate spindle assembly in plant cells. Microtubules are shown in *red*. The PPB is a ring that encircles the cell in prophase. The majority of microtubules at prophase lie within the PPB itself, although there are a small number of bridging microtubules (*dotted lines*) that connect the PPB to the nucleus. The surface of the nucleus also organizes microtubules by a

mechanism that involves Histone H1. Nucleosomes and histones, representing all the chromatin in the cell, are shown in *blue*. RanGTP, NuMA, TPX2, and Rae1 are all concentrated in the nucleus. This gradient of concentration is illustrated in *orange*. When the nuclear envelope breaks down, spindle subunits are preferentially assembled where the concentration of RanGTP is high

for chromosome movement. In both animals and plants, chromosome fragments without kinetochores fail to segregate (Dawe and Cande 1996; Wigge and Kilmartin 2001; DeLuca et al. 2005). There are also extensive prior data from animal cells suggesting that kinetochores participate in organizing their own kinetochore fibers (Rieder 2005). Particularly in plants, it is difficult to envision a model for plant spindle formation that does not involve the strong stabilizing effects of kinetochores. As can be seen in a series of images from barley root tips (collected by the primary author, Fig. 1), plant kinetochores appear to initiate their own kinetochore fibers early in prometaphase, either by directly nucleating microtubules or promoting microtubule growth in their vicinity. Similar images and interpretations can also be found in the animal literature (Tulu et al. 2006; Torosantucci et al. 2008).

In animals, the recruitment of microtubules to kinetochores can be facilitated by a set of proteins known as passenger proteins that promote microtubule assembly in the absence of RanGTP (Maresca et al. 2009). Since

passenger complex proteins are preferentially concentrated at inner centromeres, they are thought to be important signals in kinetochore-mediated spindle formation (O'Connell et al. 2009). In plants, however, the key components of the chromosome passenger complex (such as INCENP) appear to be absent. Recent data nonetheless suggest that kinetochores alone may be sufficient to initiate and retain microtubule attachment (Akiyoshi et al. 2010) and that the four-protein nuclear division cycle 80 (Ndc80) complex is indispensable for this function (Cheeseman and Desai 2008). The Ndc80 complex, which is among the most conserved features of all kinetochores, forms a rod-like structure with globular regions at either end (Ciferri et al. 2005; Wei et al. 2005, 2006, 2007). One end of the rod is composed of Ndc80 and nuclear filament-containing protein 2 (Nuf2). The Ndc80/Nuf2 complex binds directly to tubulin monomers by electrostatic interactions with the negatively charged C-terminal tails of tubulins (Ciferri et al. 2008). The other end of the rod is composed of spindle pole body component 24 (SpC24) and spindle pole body component 25

(Spc25), which face the inner kinetochore and interact with minichromosome instability 12 (Mis12) complex (Petrovic et al. 2010; Maskell et al. 2010). Recent studies showed that an oligomeric array of Ndc80 complexes move along the microtubules through biased diffusion (Powers et al. 2009; Alushin et al. 2010). In this way, microtubules are able to attach to kinetochores while at the same time sustaining the loss or gain of tubulin subunits at the microtubule plus ends.

The interaction between the Ndc80 complex and microtubules is regulated by Aurora B kinase, which phosphorylates Ndc80 and reduces its binding affinity *in vitro* (Cheeseman et al. 2006). A recent study reported that the physical distance between Aurora B kinase and Ndc80 determines whether Ndc80 is phosphorylated or not (Liu et al. 2009). Since Aurora kinase tends to be localized in the inner kinetochore area, correctly attached, stretched, and bi-oriented outer-kinetochores are separated from the kinase and retain their tight association with microtubules (Liu et al. 2009). On improperly aligned kinetochores, Aurora B kinase and Ndc80 are in close proximity, causing the

microtubule attachments to be unstable until proper attachments can be made. This mechanism is entirely consistent with the available limited literature on plants. Aurora kinases are present in plants and bind to kinetochores (Demidov et al. 2005), and homologs of Ndc80 and Mis12 have been identified in maize and localize outside the inner kinetochores (Du and Dawe 2007; Li and Dawe 2009). Other components of the Ndc80 complex can also be detected in plants by sequence similarity (Meraldi et al. 2006) but have not yet been shown to be kinetochore-localized or to interact directly with Ndc80. The emerging view is that Ndc80, as regulated by selective phosphorylation of key outer residues, is sufficient to organize and maintain stable contacts with spindle microtubules (Akiyoshi et al. 2010).

The primary arguments against the idea that kinetochores are involved in spindle formation derive from fact that spindles can form in *Xenopus* extracts that lack centromeres. However, it is important to note that while bipolar spindles do self-assemble in such extracts, it has never been

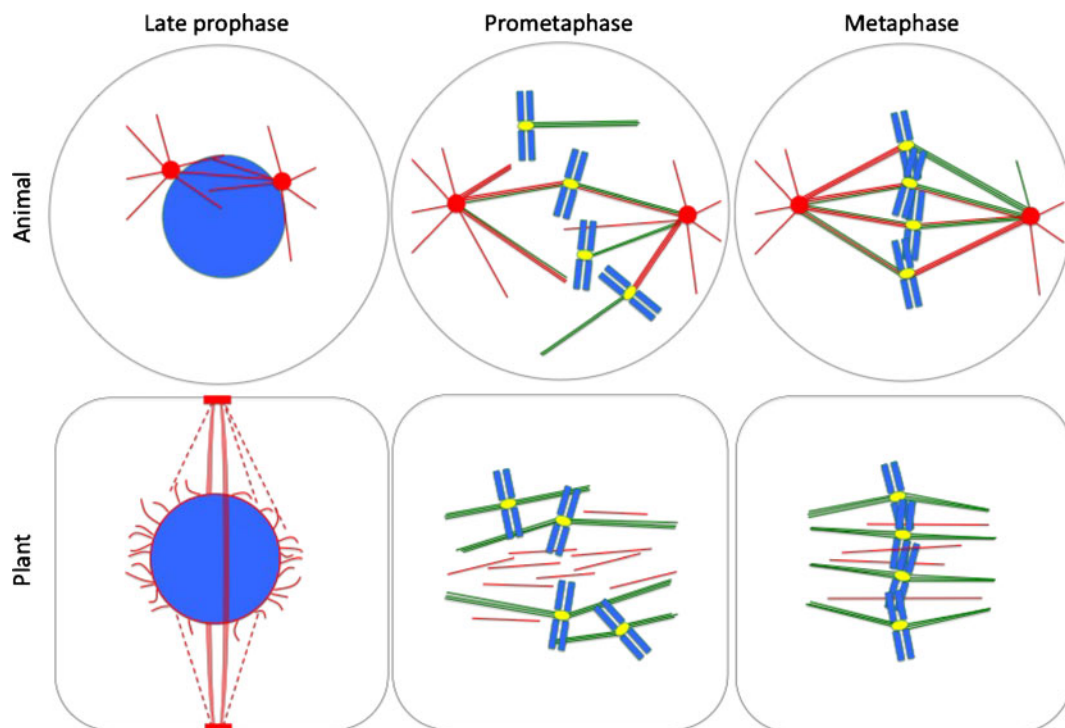


Fig. 3 A comparison of spindle formation in animals and plants. Centrosomal and PPB-associated microtubules are shown in *red*, while kinetochore-associated microtubules are shown in *green*. Chromatin is shown in *blue* and kinetochores

in *yellow*. The PPB is a ring that encircles the cell and the great majority of microtubules lie within the band. A small minority of microtubules (bridging microtubules, shown with *dotted lines*) extend from the band to the nucleus

shown that the DNA-coated beads align to a metaphase plate or move in an anaphase fashion. We believe that the *Xenopus* results may be more reflective of the role of chromosome arms, which in both animals and plants are known to bind microtubules and facilitate chromosome bi-orientation (Khodjakov et al. 1996; Antonio et al. 2000; Funabiki and Murray 2000; Brouhard and Hunt 2005). This view is supported by a recent study of human cells where the authors showed that when the (Ndc80 complex) protein Nuf2 was knocked down, the balance of microtubules shifted towards chromosome arms (O'Connell et al. 2009). One interpretation is that the Ran cycle naturally promotes the formation of microtubules around chromatin early in the cell cycle when the kinetochores are underdeveloped. As the cell cycle proceeds and kinetochores mature, the kinetochores become the strongly preferred sites of microtubule attachment.

Summary and discussion

The elegance of mitosis has caught the imagination of scientists for over a century and spawned many models to explain it (Mitchison and Salmon 2001). These have ranged from an early kinetochore-centric perspective, to the centrosome-heavy “search and capture” model, and most recently the chromatin-is-sufficient self-organization pathway. The current view is more of a hybrid among these concepts, presuming that multiple forces operate at different times and with varying importance depending on the species (Gadde and Heald 2004; Wadsworth and Khodjakov 2004). Microtubules form around chromosomes, kinetochores, and spindle poles such that they interact and converge into the mature spindle.

Here, we argue that the same basic principles of spindle formation apply to plants but with the addition of the preprophase band that facilitates the orientation of spindles (Lloyd and Chan 2006; Bannigan et al. 2008) and a heavier reliance on kinetochores as organizing centers (O'Connell and Khodjakov 2007; Mogilner and Craig 2010). We emphasize that this perspective is necessarily based on a very small plant literature and relies heavily on data from animals. Given these caveats, we can infer that plant spindle formation is initiated by a RanGTP gradient that favors the formation of microtubules around chromosomes following nuclear envelope breakdown. We presume that

the placement and orientation of the spindle is influenced by the presence of bridging microtubules derived from the preprophase band. The newly stabilized microtubules, already adopting a bipolar orientation, are then further oriented by the paired kinetochores, which take the primary role in stabilizing and reinforcing a strictly bipolar arrangement. Kinetochores promote microtubule nucleation and form kinetochore fibers by stabilizing the plus ends while the minus ends aggregate into loose poles by natural bundling factors such as kinesins (Bannigan et al. 2008). A comparison of spindle formation in animals and plants can be viewed in Fig. 3.

Unfortunately, the molecular mechanism of these processes is still largely unknown. There are multiple basic observations still missing from the plant literature. These include the facts that the RanGTP gradient has not been visualized in plants; that there is no known plant equivalent of animal RCC1, the chromatin bound protein that maintains Ran in its GTP-bound form; that we do not know whether plant spindle assembly factors are regulated by RanGTP or how they stabilize microtubules *in vivo*; that it is not known how preprophase bands are initiated or how bridging microtubules enhance spindle bipolarity; and that we do not know if the Ndc80 complex is sufficient to organize microtubules at kinetochores, or if other “passenger proteins” similar to those found in animals are required (Tseng et al. 2010). These studies are difficult and can be viewed as confirmatory but are nevertheless required for further progress. Other more fundamental limitations are that an *in vitro* plant-based model of the same power as *Xenopus* has yet to emerge and that the chromosomes and spindles of *Arabidopsis* are nearly too small to be useful for mitosis research. Efforts to address these key weaknesses are likely to be met with great enthusiasm and provide the necessary impetus to move the field forward.

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