

Regulated Assembly of the Mitotic Spindle: A Perspective from Two Ends

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Abstract

Chromosome segregation and cell division requires the regulated assembly of the mitotic spindle apparatus. This mitotic spindle is composed of condensed chromosomes attached to a dynamic array of microtubules. The microtubule array is nucleated by centrosomes and organized by associated structural and motor proteins. Mechanical linkages between sister chromatids and microtubules are critical for spindle assembly and chromosome segregation. Defects in either chromosome or centrosome segregation can lead to aneuploidy and are correlated with cancer progression. In this review, we discuss current models of how centrosomes and chromosomes organize the spindle for their equal distribution to each daughter cell.

Introduction

A fundamental property of all cells is their ability to multiply and reproduce. The replication and segregation of the genome must be performed with high fidelity, ensuring that each daughter cell receives a full complement of DNA. For all eukaryotic cells, separation of the replicated genome is accomplished by the mitotic spindle during the M-phase of the cell cycle. For a rapidly dividing mammalian cell, M-phase lasts for about an hour out of each 24 hour cell cycle. The spindle is assembled at M-phase and is a transient structure, built to accurately move chromosomes and then disassemble. In this review we discuss mechanisms responsible for assembling the mitotic spindle and attaching replicated chromosomes and centrosomes to this biological machine. To provide a reference point, we begin by describing the structure of the spindle at metaphase - the midpoint of mitosis. We then describe the roles of centrosomes, microtubule associated proteins (MAPs), motor proteins and chromosomes in assembling and maintaining the mitotic spindle and anchoring chromosomes and centrosomes to this structure.

The Spindle at Metaphase

The major structural components of the spindle are the microtubules, polymers of α and β tubulin subunits. The

microtubule lattice has an intrinsic structural polarity due to the polarized arrangement of tubulin dimers within the lattice. The two ends of the microtubule differ in growth rate, with the faster growing (plus) end localized away from the centrosome. Spindle microtubules are organized into two overlapping arrays that form the bipolar metaphase spindle (see Figure 1). Here the fast growing plus ends originating at each centrosome meet in the spindle midzone (for more extensive reviews see Inoue and Salmon, 1995; Wittmann *et al.*, 2001).

Chromosomes attach to the spindle by binding the plus ends of microtubules. Before anaphase begins, each chromosome is actually a pair of replicated sister chromatids (reviewed in Salmon, 1989). These chromatids are held together along the lengths of their arms by a complex of proteins called cohesins (below). At metaphase, chromosomes are aligned in the center of the spindle, with sister chromatids facing the opposite poles. Chromosome attachment is mediated by the kinetochore, a complex of DNA and proteins (shown in red in Figure 1B,C). The kinetochores of sister chromatids are organized on opposite sides of the chromosome complex. Based on the organization of the sister kinetochores, the attachment of a kinetochore to microtubules from one half spindle positions the opposite kinetochore toward the opposite pole. This organization ensures that replicated chromatids are attached to opposite poles and that each daughter cell will receive one, and only one, copy of each chromosome as cells divide (reviewed in Salmon, 1989).

The opposite ends of the microtubules, the minus ends, are focused *near* the centrosome, but not directly attached to it. The spindle microtubules are nucleated at the centrosome, but then released from the nucleation site. The minus ends of these free microtubules are then focused and anchored by several structural and motor proteins (discussed below). The centrosome is likely held to the minus ends of microtubules by additional proteins. This centrosome anchoring mechanism would serve to link the centrosome to the microtubules of the spindle and allow each daughter cell to inherit one, and only one, centrosome.

The majority of spindle microtubules do not form attachments to chromosomes. These non-kinetochore microtubules turn over rapidly by dynamic instability, a novel behavior where individual microtubules exist in persistent phases of elongation or rapid shortening, with infrequent and abrupt transitions between these phases. The transition from elongation to rapid shortening has been termed catastrophe, while the transition from rapid shortening to elongation has been termed rescue (Walker *et al.*, 1988).

Setting the stage: Microtubule reorganization at the G2/M transition

As the cell enters mitosis, a radial array of long interphase microtubules is disassembled to make way for the spindle.

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Dissolution of the interphase microtubule array occurs at the G2/M transition and coincides with the brief period of time when the nuclear envelope is broken apart (Zhai *et al.*, 1996). Changes in the dynamic turnover of microtubules likely play a major role in disassembly of the interphase microtubules. During interphase, the frequency of rescue (the switch from a shortening state to a growth state) is relatively high. Frequent rescues allow microtubules to grow to the longer lengths typical of interphase cells (~ 100 μm in a typical mammalian epithelial cell; Gliksman *et al.*, 1993). At the G2/M transition, rescue frequency drops about 8-fold (Rusan *et al.*, 2002). This drop in rescue, combined with a reduction in the amount of time microtubules spend neither growing nor shortening (paused), results in an overall loss of microtubule polymer (Rusan *et al.*, 2002).

The removal of microtubules from the peripheral regions of cells is also partially accomplished by microtubule bundling and dynein-driven transport of these bundles (Figure 2). Bundles are moved to the minus ends of microtubules and these bundles can be incorporated into the forming spindle (Rusan *et al.*, 2002). This dynein-dependent clearing of microtubules from the cytoplasm continues through prometaphase and metaphase stages of mitosis (Rusan *et al.*, 2002). Dynein-dependent transport of free microtubule bundles has also been observed recently within mitotic spindles (Khodjakov *et al.*, 2003).

By late prophase, growing microtubule ends are concentrated in the area surrounding the nuclear envelope. These microtubules are stabilized by dynein/dynactin associated with the nuclear envelope (Piehl and Cassimeris, 2003). Since dynein/dynactin specifically associates with the nuclear envelope at prophase (Busson *et al.*, 1998), the motor complex provides local microtubule stabilization during a brief window of time. The interaction between nuclear envelope-bound dynein and microtubules allows dynein-dependent tearing of the nuclear envelope to facilitate breakup of the envelope (Salina *et al.* 2002; Beaudouin *et al.* 2002; see Figure 2).

By the processes outlined above, both the interphase microtubule array and the nuclear envelope are broken down. The stage is now set for spindle assembly. Microtubules rapidly assemble into the nuclear area once the nuclear envelope is broken apart. Since microtubule assembly begins at the centrosome, in the next section we discuss how centrosomes contribute to spindle assembly.

The Centrosome: Microtubule nucleation and perhaps a bit more

The centrosome contributes to spindle assembly and mitotic fidelity through several functions. The major function of the centrosome is to nucleate microtubule assembly. Centrosomes can also serve as a scaffold to anchor regulatory proteins and they are necessary for cell cycle progression, as discussed below.

Centrosomes and microtubule nucleation

Centrosomes are microtubule-organizing centers capable of nucleating microtubules - they stimulate microtubule

assembly at tubulin concentrations insufficient to allow spontaneous microtubule assembly. Within the centrosome, microtubules are nucleated by ring complexes composed of γ tubulin and associated proteins (Oakley and Oakley, 1989; Zheng *et al.*, 1995). The precise mechanism by which these γ tubulin ring complexes nucleate microtubule assembly remains a controversial issue (Job *et al.*, 2003).

Each centrosome nucleates a starburst array of microtubules, such that each interphase cell typically has a single microtubule array, and each mitotic cell, with its replicated and separated centrosomes, forms a bipolar spindle composed of two such arrays (Figure 1). Thus, centrosomes determine where microtubule assembly begins and can organize those microtubules into simple patterns depending on the placement of the centrosomes relative to each other. When separation of replicated centrosomes fails at entry into mitosis, the resulting monopolar spindle contains a single radial array of microtubules, which cannot support chromosome segregation.

The centrosome's capacity to nucleate microtubules increases approximately 5-fold at mitosis (Synder and McIntosh, 1975; Kuriyama and Borisy, 1981; Telzer and Rosenbaum, 1979), beginning at the G2/M transition (Piehl *et al.*, 2002). This increase in nucleation rate correlates with an increase in the concentrations of γ tubulin and pericentrin associated with the centrosome (Khodjakov and Rieder, 1999; Dictenberg *et al.*, 1998). It is not yet known whether the recruitment of γ tubulin, or other proteins necessary for nucleation, is sufficient to generate the higher mitotic rate of microtubule nucleation, or whether other regulatory mechanisms, such as phosphorylation, contribute to centrosome maturation. A second pathway independent of γ tubulin, may also contribute to microtubule nucleation during mitosis (Hannak *et al.*, 2002). The higher rate of nucleation during mitosis contributes significantly to the approximately 4 to 10-fold increase in the number of microtubules per cell.

Although microtubules are nucleated at the centrosome, spindle microtubules do not remain tethered to the centrosome. Instead, they are released, and the release rate is increased at mitosis (Belmont *et al.* 1990; reviewed in Compton, 2000; Bornens, 2002). It is not yet clear whether katanin, a microtubule severing protein localized to centrosomes, contributes to this microtubule release (Buster *et al.*, 2002).

Centrosomes as depots for regulatory proteins

Studies over the last several years have pointed to an additional function of centrosomes as a depot for regulatory kinases and other potential upstream regulators of cell cycle progression. Multiple kinases and phosphatases have been localized to centrosomes, and these are often associated with scaffold proteins such as AKAP (reviewed by Zimmerman *et al.*, 1999; Diviani and Scott, 2001; Doxsey, 2001). Centrosome localization may contribute to the substrate specificity of some kinases and phosphatases (see Diviani and Scott, 2001; Doxsey, 2001).

The centrosomal protein pericentrin, which is required

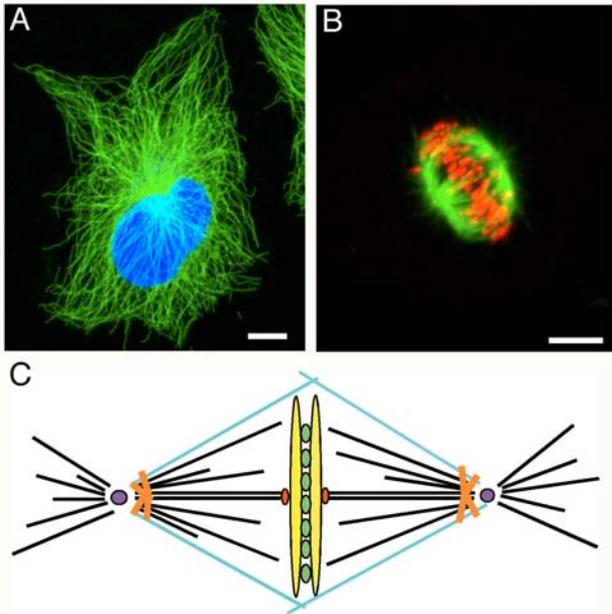


Figure 1. Microtubule organization in interphase (A) and mitotic (B) HeLa cells. (A) Microtubule (green) and chromosomes (blue) in an interphase cell. A single radial array of microtubules emanates from the single centrosome located near the nucleus (shown here only as the brightest green spot). (B) Microtubules (green) of the metaphase spindle are organized into two overlapping arrays. Kinetochores of chromosomes are shown in red. (C) Diagram of spindle organization at metaphase. Centrosomes are shown as violet circles, microtubules as black or blue lines and chromosomes as yellow ellipses. Microtubule minus ends are anchored to spindle poles by structural and motor proteins (shown as thick orange lines), while some plus ends make connections to the kinetochores of chromosomes (red). Overlapping anti-parallel microtubules are highlighted in blue. Cohesion factors, anchoring the chromatids together are shown in green. Each of these components is discussed in detail in the text.

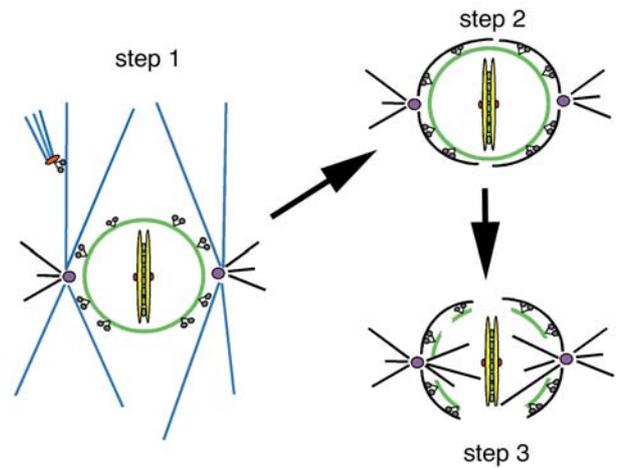


Figure 2. Illustration of proposed steps of microtubule reorganization at the G2/M transition. Step 1, the long microtubules of the G2 interphase array (blue) are disassembled or bundled (shown as a red complex at the minus ends of the bundled microtubules) and moved towards the centrosome by dynein/dynactin. At this stage, dynein/dynactin also transiently associates with the nuclear envelope. Step 2, new microtubules are stabilized at the nuclear envelope by binding to dynein/dynactin. Step 3, dynein-dependent pulling forces tear the nuclear envelope apart and microtubules now have access to the chromosomes. See text for citations to relevant experiments.

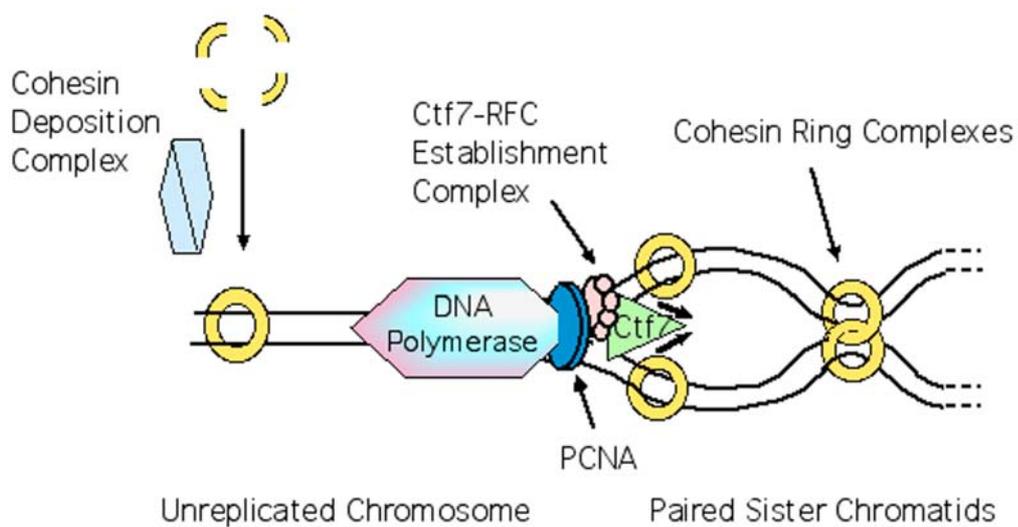


Figure 3. Illustration of one model for chromatid pairing. Cohesin association with chromatin requires the deposition complex (shown on the left). A subset of cohesins appears associated with chromatin prior to S-phase, suggesting stepwise assembly (not shown). To establish pairing, cohesin rings must either become catenated (yellow rings, shown on the right) or a single cohesin ring encircles both sister chromatids (not shown). The role of the establishment complex is unknown, but may facilitate ring catenation during DNA replication (shown in the center).

for microtubule nucleation and forms a structural lattice with gamma tubulin at the centrosome (DICTENBURG *et al.*, 1998), is a member of the AKAP scaffold family (DIVIANI and SCOTT, 2001). The sequence similarity between AKAPs and pericentrin suggests that a dual scaffold is possible: one that can both participate in microtubule nucleation and anchor signaling molecules.

Several other regulatory proteins are also localized to centrosomes including regulatory proteins of the ubiquitin degradation pathway and the tumor suppressor, p53 (CICIARELLO *et al.*, 2001; reviewed by ZIMMERMAN *et al.*, 1999). The localization of p53 to centrosomes may be related to its function in cell cycle arrest (CICIARELLO *et al.*, 2001), but p53 also appears necessary for maintenance of normal centrosome numbers (reviewed by TARAPORE and FUKASAWA, 2002).

Centrosomes: spindle integrity and cell cycle progression

Centrosomes are essential for spindle assembly in most, but not all, cells (SLUDER and RIEDER, 1985; ZHANG and NICKLAS, 1995). For example, spindles do not assemble in insect spermatocytes if centrosomes are removed at prophase or early stages of mitosis (ZHANG and NICKLAS 1995). Surprisingly, laser ablation of centrosomes suggests that some cells can assemble a spindle in the absence of centrosomes (KHODJAKOV *et al.*, 1999). When centrosomes are present, as is usually the case, they dominate spindle assembly by nucleating microtubules (HEALD *et al.*, 1997). Once spindle assembly has progressed to metaphase/anaphase, centrosomes are no longer necessary for spindle maintenance (MITCHISON and SALMON, 1992; NICKLAS *et al.*, 1989).

Given that centrosomes do not appear necessary for maintenance of microtubules and bipolar spindle structure once cells progress to metaphase or anaphase, it is surprising that centrosomes are required for mitotic exit, completion of cytokinesis and entry into the next S phase (KHODJAKOV and RIEDER 2001; PIEL *et al.*, 2001; reviewed by DOXSEY, 2001). These results suggest that centrosome inheritance is important for cell cycle progression. We suggest that during mitosis, it is necessary to attach centrosomes to spindle poles to ensure their delivery to daughter cells. Centrosome inheritance may be necessary not only to provide a microtubule organizing center, but also to ensure the proper localization of regulatory proteins, such as those discussed above.

Defects in the number of centrosomes have been observed in a number of human tumors, including cells from the early stages of tumor development (PIHAN *et al.*, 2001; LINGLE *et al.*, 2002; D'ASSORO *et al.*, 2002). In one case, expression of human papillomavirus E6 and E7 oncoproteins was sufficient to upset the normal coupling between centrosome replication and the cell cycle, resulting in extra centrosomes and aneuploidy (DUENSING *et al.*, 2000). While the experiments with papillomavirus protein expression suggest a causative role for increased centrosome number in causing aneuploidy, it is not yet known whether aberrant numbers of centrosomes causes the aneuploidy associated with cancers, or results from that aneuploidy.

Microtubule Dynamics during mitosis

The above discussion focused on several possible functions for centrosomes, but their role as microtubule nucleators is clearly the most important for spindle assembly. Once nucleated, these microtubules turn over at a fast rate, primarily by dynamic instability. Below we discuss regulation of this dynamic turnover by the antagonistic activities of proteins able to stabilize or destabilize microtubules. This dynamic turnover is necessary for spindle assembly by allowing rapid attachments to form between microtubules and chromosomes (HILL *et al.*, 1985; KIRSCHNER and MITCHISON, 1986).

Microtubule assembly dynamics are regulated by the cell cycle machinery, resulting in much more dynamic microtubules during mitosis. The ~ 10 fold faster turnover of microtubules was initially observed using photobleaching methods almost 20 years ago (SAXTON *et al.*, 1984). Observations of individual microtubules in a number of different experimental systems has shown that microtubule growth rate is faster in mitosis, although increased rates vary from a small 1.1 - 1.3 fold increase (RUSAN *et al.*, 2001; BELMONT *et al.*, 1990) to a near doubling in velocity (PIEHL and CASSIMERIS, 2003; HAYDEN *et al.*, 1990). Experiments in *Xenopus* egg extracts (BELMONT *et al.*, 1990) suggested that the major change in microtubule dynamics at mitosis is a large increase in catastrophe frequency. In other systems, such as sea urchin egg extracts and mammalian epithelial cells, rescue is regulated to a larger degree (GLICKSMAN *et al.*, 1992; RUSAN *et al.*, 2002). These changes in catastrophe or rescue rates could each generate short microtubules with more rapid turnover rates.

Regulation of microtubule assembly dynamics by associated proteins

The changes in microtubule assembly dynamics throughout the cell cycle are regulated by the activation or inactivation of microtubule associated proteins (MAPs; reviewed in CASSIMERIS, 1999). The cell cycle regulation of oncoprotein 18 (Op18), a microtubule destabilizer, is the best-studied example. Op18 is phosphorylated at entry into mitosis and all 4 serine residues must be phosphorylated to allow mitotic progression (LARSSON *et al.*, 1997). Phosphorylation precedes sequentially: ser 25 and 38 are first phosphorylated by CDK1, allowing subsequent phosphorylation of ser 16 and 63 by an unknown kinase(s) (LARSSON *et al.*, 1997; MARKLUND *et al.*, 1996). Expression of non-phosphorylatable Op18 mutants prevents assembly of the mitotic spindle, presumably because microtubule assembly is severely compromised by the presence of excess microtubule destabilizing activity.

The idea that microtubule dynamics are regulated by a balance between stabilizing and destabilizing MAPs was first proposed for XMAP215 (a stabilizer) and XKCM1 (a destabilizer). Depletion of one protein from *Xenopus* egg extracts resulted in very long or very short microtubules, depending on the protein removed. Depletion of both proteins returned microtubule lengths and catastrophe frequency to near normal levels (TOURNEBIZE *et al.*, 2000).

The counteracting activities of these associated proteins has also been demonstrated for their *S. cerevisiae* homologs (Severin *et al.*, 2001), but whether the mammalian homologs function to antagonize each other is not yet clear.

XMAP215 is phosphorylated by CDK1, which significantly reduces the ability of XMAP215 to stimulate microtubule growth rate (Vasquez *et al.*, 1999). CDK1-phosphorylated XMAP215 still binds microtubules (Vasquez *et al.*, 1999) and protects microtubules from the destabilizing activity of XKCM1 (Tournebize *et al.* 2000; Popov *et al.*, 2001). Therefore, mitotic phosphorylations can modify a protein's activity without simply turning the protein "off". In contrast to XMAP215, XKCM1 does not appear regulated by phosphorylation.

The antagonistic activities of XMAP215 and XKCM1 are not the only balancing act able to regulate microtubule assembly. The microtubule stabilizer MAP4 counteracts the microtubule destabilizing activities of both XKCM1 and Op18 (Holmfeldt *et al.* 2002). It is not yet clear whether the counter-acting activities of these proteins is important during mitosis. Op18 is turned off by mitotic phosphorylation (Larsson *et al.*, 1997), as is the rescue-promoting activity of MAP4 (Ookata *et al.*, 1995). Since XKCM1 (MCAK in mammalian cells) counteracts the stabilizing activities of both XMAP215 and MAP4, mitotic phosphorylation of both stabilizers may allow XKCM1/MCAK to gain the upper hand and generate the more dynamic microtubules present during mitosis.

Dissecting the functions of specific MAPs in mitotic spindle assembly has not been straightforward since many proteins show complex binding patterns. The XMAP215 family of MAPs is predominately localized to centrosomes (Cullen *et al.*, 1999; 2001; Charrasse *et al.*, 1998; Wang and Huffaker, 1997), where they may contribute to microtubule nucleation (Lee *et al.*, 2001; Popov *et al.*, 2002). Genetic evidence also points to a function for this protein family at microtubule plus ends or kinetochores (Nabeshima *et al.*, 1998; Garcia *et al.*, 2001). Likewise, XKCM1 is likely necessary for the high rate of mitotic microtubule turnover since this kinesin stimulates catastrophes (Walczak *et al.*, 1996), but XKCM1 is also necessary for microtubule attachments to kinetochores (Walczak *et al.*, 2002). Disruption of the mammalian homolog, MCAK, also delays onset of anaphase (Maney *et al.*, 1998).

While dynamic microtubule turnover is required for spindle assembly, subsets of microtubules become differentially stabilized during mitosis. The most obvious example of local stabilization occurs when microtubule plus ends attach to kinetochores. By attaching to microtubule plus ends, kinetochores reduce the dynamic turnover of these microtubules by approximately 7 fold (Cassimeris *et al.*, 1990; Zhai *et al.*, 1995). It is important to note that kinetochore binding to microtubule plus ends still allows tubulin subunit addition and subtraction from these ends (Mitchison *et al.*, 1986), but the rates of addition and subtraction are greatly reduced.

Microtubules also turn over by flux, where the microtubule polymer is pulled toward the microtubule minus end (the poles), with concomitant assembly at the plus end

and disassembly at the minus end. Flux is likely driven by minus end directed motors, but the motor responsible has not been identified. The rate of flux differs considerably in different systems, and thus the contribution of flux to microtubule turnover rates can vary significantly (Maddox *et al.*, 2002; Mitchison, 1989; Waterman-Storer *et al.*, 1998).

Dynamic microtubules and motor proteins can self-organize into a bipolar spindle

Dynamic microtubules nucleated by the centrosome are sculpted into a fusiform spindle in large part by molecular motors. This is clearly observed for the focusing of microtubule minus ends at the spindle pole. Microtubules released from the centrosome are focused by two types of motors, cytoplasmic dynein and the Kin C class of kinesins (Gaglio *et al.*, 1996; Mountain *et al.*, 1999; Walczak *et al.*, 1998). These motors are all minus end-directed motors and would move along microtubules toward the spindle pole. By binding and crosslinking adjacent microtubules, these motors pull the minus ends together. For cytoplasmic dynein, crosslinking requires the associated complex dynactin and the structural protein NuMA (Merdes *et al.*, 2000). The plus end directed kinesin Eg5 (bimC kinesin family) is also required for spindle pole organization, although the precise function of this motor in spindle pole formation is not known (Gaglio *et al.*, 1996; Sawin *et al.*, 1992; Heck *et al.*, 1993; Blangy *et al.*, 1995; Wilson *et al.*, 1997).

The crosslinking of focused minus ends is critical for spindle function. The presence of free minus ends at the spindle poles is thought necessary for microtubule flux, while these minus ends also must be anchored to generate tension on chromosomes and allow chromosome movement. In the absence of proper anchorage, the chromosomes would remain stationary and the poles and microtubules would be pulled toward them (Nicklas, 1989), as shown experimentally after inhibition of both NuMA and HSET, a kin C kinesin (Gordon *et al.*, 2001).

Plus and minus end directed kinesins also act antagonistically to each other to generate a bipolar spindle. These antagonistic functions were first demonstrated in *S. cerevisiae* (Saunders and Hoyt, 1992) and have been shown subsequently in a wide range of experimental systems (Sharp *et al.*, 1999; Mountain *et al.*, 1999). In the absence of Eg5 or related plus end kinesins (bimC family), centrosome separation fails and a monopolar spindle forms (Saunders and Hoyt, 1992; Sawin *et al.*, 1992; Gaglio *et al.*, 1996; Sharp *et al.*, 1999; Mountain *et al.*, 1999; Kapoor *et al.*, 2000). These results suggest that the plus end directed bimC kinesins push anti-parallel microtubules apart. Each bimC kinesin is a bipolar, tetrameric molecule making it capable of sliding antiparallel microtubules past each other (Kashina *et al.*, 1996). The plus end directed sliding of antiparallel microtubules would push the spindle poles apart and would antagonize the tendency of minus end directed motors to pull the centrosomes together. For all experimental systems tested, simultaneous inactivation of the bim C (plus end directed) and Kin C (minus end directed) kinesins allows formation of a bipolar spindle. Neither class of motors is absolutely required for spindle

assembly since bipolar spindles will assemble, provided both classes of motors are absent.

Structural proteins, such as TPX2 and the XMAP215 family, also contribute to spindle bipolarity (Gruss *et al.*, 2002; Garrett *et al.*, 2002; Gergely *et al.*, 2003). Whether these proteins function to cross-bridge anti-parallel microtubules or act in conjunction with motor proteins is not yet known.

Chromosomes and spindle integrity

Microtubule dynamics, spindle assembly and maintenance of the mitotic apparatus until anaphase onset are critically dependent not only on centrosomes, MAPs and motors, but also on the chromosomes which bridge the two half-spindles. In this context, chromosomes represent both a chemical depot that generates a microtubule plus-end stabilizing environment and a mechanical structure that can generate forces and link two half-spindles together. The importance of chromosomes in spindle stability was elegantly demonstrated by Zhang and Nicklas (1995). Using micromanipulation to detach chromosomes and move them out into the cytoplasm, they showed that spindles disassembled when the chromosomes were taken away.

Chromosomes as chemical depots of microtubule regulatory factors.

Although most cells rely on centrosomes to organize a bipolar spindle, some meiotic oocytes and plant cells are able to assemble a spindle in the absence of centrosomes. In meiotic oocytes, or cell extracts derived from them, chromosomes play the central organizing role. By binding factors that act upstream to regulate microtubule stability, chromosomes mark the area where microtubule assembly will occur (Kalab *et al.*, 1999; Carazo-Salas *et al.*, 1999; Ohba *et al.*, 1999; Wilde and Zheng, 1999; Zhang *et al.*, 1999). Motor proteins, such as dynein/dynactin and Eg5, along with structural proteins such as NuMA, and TPX2, then organize and gather these microtubules into a bipolar spindle shape (Walczak *et al.*, 1998; Carazo-Salas *et al.*, 2001; Gruss *et al.*, 2001; Wiese *et al.*, 2001; Nachury *et al.*, 2001).

Chromosomes are thought to create a local microtubule stabilizing environment in their vicinity because several regulatory proteins have been localized to mitotic chromatin, including RCC1, a guanine nucleotide exchange factor for Ran (Carazo-Salas *et al.*, 1999), and a Polo-like kinase (Budde *et al.*, 2001). Recent experiments have demonstrated a local high concentration of Ran-GTP around chromatin in meiotic *Xenopus* egg extracts (Kalab *et al.*, 2002), suggesting that downstream effectors may be regulated in this local environment. Each of these regulators is thought to act upstream to regulate specific MAPs. For example, local inactivation of a microtubule destabilizing protein (oncoprotein 18) near chromatin would favor microtubule assembly in this region of the cell (Anderson *et al.*, 1997; Budde *et al.*, 2001).

Potential downstream targets of the Ran pathway include TPX2, (Gruss *et al.*, 2001), NuMA (Wiese *et al.*,

2001; Nachury *et al.*, 2001), and the kinesin Eg5 (Wilde *et al.*, 2001). Both TPX2 and NuMA are structural components necessary to organize spindle poles (reviewed in Compton, 2000). TPX2 and NuMA are nuclear proteins during interphase and therefore both are cargo for the nuclear import pathway, and have the sequences necessary to form a complex with the importins. Ran-GTP can release these proteins from importin β , freeing them to participate in spindle assembly (Gruss *et al.*, 2001; Wiese *et al.*, 2001; Nachury *et al.*, 2001).

In tissue cells containing centrosomes, it is not known whether a local environment favoring microtubule stabilization is present during assembly of the mitotic spindle, or whether such an environment is necessary. In *C. elegans* embryos, Ran is required for spindle assembly, but RCC1 is not (Askjaer *et al.*, 2002). It is likely that centrosomes dominate spindle assembly when they are present and can override any defects in a Ran-dependent stabilization pathway (*e.g.*, Faruki *et al.*, 2002).

For a local stabilizing gradient to function, the gradient would have to decay with dimensions approximating that of the spindle. Odde has developed a model to estimate the hypothetical shape of a gradient of a phosphorylated protein. This model incorporates a localized kinase, a uniform concentration of phosphatase, and free diffusion of the substrate. Based on assumptions of enzyme phosphorylation and dephosphorylation rates, and protein diffusion, he predicts that a gradient of phosphorylated substrate would decay exponentially over approximately 10 μm (Odde, 2001). This 10 μm dimension is approximately the length of a typical mammalian spindle, suggesting that "microtubule chemotaxis" toward chromosomes is feasible.

Chromosomes as mechanical bridges and force producing organelles

A centrosome and associated microtubule radial array comprise a half-spindle, an organelle capable not only of force generation but motility. In large vertebrate cells, premature centrosome separation during early prophase can result in two independent and free moving half-spindles that "swim" throughout the cytoplasm (Bajer, 1982; Waters *et al.*, 1993). Proper spindle assembly or fusion of the two half-spindles into a stable mitotic apparatus requires that opposing centrosomes become mechanically tethered through microtubule interactions. Three possible linkages that can tether half-spindles together involve 1) anti-parallel microtubule interactions (described above), 2) kinetochore - microtubule attachments and 3) microtubule interactions with kinesins localized to chromosome arms (termed chromokinesins).

One component of the mechanical linkage between two half-spindles involves interdigitation of opposing polar microtubules. As described above, crosslinking of microtubules from opposing spindle poles are in part produced by a variety of kinesin-like motor proteins. While sufficient to initiate the association between two half spindles, polar microtubule cross-linkages may not be sufficient to maintain a spindle structure. For instance, separated asters devoid of chromosomes initially form a

bipolar spindle-like array, but this structure decays over time (Faruki *et al.*, 2002). Thus, chromosomes remain an important contender in both spindle assembly and maintenance.

A second component linking two half spindles together involves chromosome interactions with microtubules via the kinetochore. Kinetochores are unique protein complexes assembled onto the single centromere DNA region of each sister chromatid (Cheeseman *et al.*, 2002; Kitigawa and Hieter, 2001). Kinetochore-microtubule interactions provide, on average, a poleward pulling force (Skibbens *et al.*, 1993; Khodjakov and Rieder, 1996; Waters *et al.*, 1996). This net pulling force occurs through coupled microtubule plus-end depolymerization and motor protein activities (such as CENP-E, MCAK/XKCM1, and cytoplasmic dynein) located at the kinetochore (Pfarr *et al.*, 1990; Steuer *et al.*, 1990; Yen *et al.*, 1992; Wordeman and Mitchison, 1995; Walczak *et al.*, 1996). These poleward-directed pulling forces act to counterbalance the centrosome separation forces supplied by opposing polar microtubule-associated motors (Kapoor and Compton, 2002). In the presence of chromosomes, half-spindles remain independent up until microtubule plus-ends of one half-spindle are captured and stabilized by kinetochores of chromosomes associated with the other half spindle. Images obtained using high resolution video microscopy and electron microscopy indicate that microtubule capture by a single opposing kinetochore is sufficient for two half-spindles to initiate fusion, effectively producing a mitotic spindle apparatus that is stable over time (Bajer, 1982; Nicklas and Kubai, 1985; Rieder *et al.*, 1990). These observations (and below) reveal that chromosomes ultimately tether centrosomes together.

Amazingly, the kinetochore-microtubule attachment site continues to tether chromosomes to spindle poles during tubulin addition and loss - allowing for chromosome motion away and toward the centrosome while mechanically linked to spindle poles (Rieder and Salmon, 1998). The role of the kinetochore-microtubule mechanical linkage is evident in the characterization of yeast kinetochore proteins such as the Duo1p, Dam1p, Dad1p complex or Slk19p that associate with centromere DNA and microtubules. Loss of any one of these kinetochore proteins not only results in high rates of chromosome missegregation, but also in a diversity of spindle defects. For mutations in either Duo1p, Dam1p, Dad1p, cells contain aberrant spindle structures including bent spindles and partially elongated spindles that contain discontinuous or decreased microtubule staining in the spindle center (Cheeseman-Barnes *et al.*, 2001; Enquist-Newman *et al.*, 2001; Jones *et al.*, 2001). It is likely that these cells contain two half spindles that are no longer joined together, similar to the two separate prometaphase half spindles occasionally observed in newt lung cells (Bayer, 1982; Waters *et al.*, 1993). In contrast, loss of Slk19p function resulted in cells that contained abnormally short, but still bipolar, spindles (Zeng *et al.*, 1999). Cells harboring mutations in both Slk19p and Kar3p (a kinesin-like microtubule minus-end destabilizing factor) were unable to assemble a bipolar structure and instead contained monopolar spindles. If allowed to pre-form, bipolar spindles

quickly collapsed (Zeng *et al.*, 1999; Meluh and Rose, 1990; Endow *et al.*, 1994). While the above observations reveal that opposing kinetochore microtubule attachments provide a mechanical link that tethers together centrosomes of opposing half-spindles, not all kinetochore defects result in overt spindle defects but instead cause cell cycle arrest. This observation may be due to the intimate involvement of the kinetochore-spindle assembly checkpoint system or in partial abrogation of kinetochore structure. The reader is directed to reviews on kinetochore structure and mitotic checkpoints (Cheeseman *et al.*, 2002; Hoyt, 2001; Kitigawa and Hieter, 2001; Amon, 1999).

Several recent findings reveal that a third component of spindle assembly and maintenance involves chromosome-microtubule interactions via chromokinesins (XKLP1, KLP38B and KID) - kinesins associated along chromosome arms. The chromokinesins KLP1 and KLP38B are critical for establishing and maintaining spindle integrity. For instance, XKlp1 depletion by antisense oligonucleotides resulted in unstable spindles. Addition of XKlp1 antibodies to mitotic extracts containing preformed spindles resulted in spindle collapse and dissociation of spindle microtubules. Likewise, defects in KLP38B function or KLP38B depletion resulted in aberrant circular mitotic structures and abnormal anaphase (Vernos *et al.*, 1995; Wang and Adler, 1995; Molina *et al.*, 1997; Ruden *et al.*, 1997; Walczak *et al.*, 1998). Thus, microtubule association with one class of chromokinesins associated along the chromosome arms appears central to spindle assembly and maintenance.

In contrast, KID-like kinesin family members are not required for spindle assembly/maintenance but instead appear to generate pushing or polar ejection forces that promote chromosome movement away from the spindle pole. Typically, chromosomes reside at the half-spindle periphery near microtubule plus-ends. Depletion of Kid from chromosomes within either monopolar or bipolar spindles resulted in chromosome positioning close to the centrosomes and defects in chromosome congression to the metaphase (or meiosis II metaphase) plate (Antonio *et al.*, 2000; Funabiki and Murray, 2000; Levesque and Compton, 2001; Perez *et al.*, 2002). Intriguingly, spindles remained predominantly intact under these conditions, suggesting that the kid class of chromokinesins contribute to chromosome positioning rather than spindle maintenance.

Sister chromatid cohesion - gluing together kinetochore and chromokinesin functions

Given the central role of chromosomes in both assembling and maintaining spindle integrity, it is not surprising that the stable association of two half spindles in part relies on the glue that holds sister chromatids together. During S-phase, each chromosome is replicated to produce two identical chromosomes - termed sister chromatids. Sister chromatids are paired along their entire length by an ill-defined interaction of three different cohesion complexes: the structural cohesins Smc1p, Smc3p, Mcd1p/Scclp, Scclp/Irr1p, and Pds5p; the deposition complex comprised of Scclp and Scclp; and the establishment factors Ctf7p/

Eco1p, Ctf18p/Chl12p, Ctf8p and Dcc1p (Figure 3) (Strunnikov *et al.* 1993; Kurlandzka *et al.* 1995; Guacci *et al.* 1997; Michaelis *et al.* 1997; Toth *et al.* 1999; Hartman *et al.* 2000; Panizza *et al.* 2000; Ciosk *et al.*, 2000; Furuya *et al.*, 1998; Skibbens *et al.*, 1999; Hanna *et al.*, 2001; Mayer *et al.*, 2001). Structural cohesion factors assemble as rings structures that either encompass both sisters or one ring encircling each sister with the rings catenated together (Carson and Christman; 2001, Melby *et al.*, 1998; Akhmedov *et al.*, 1998; Campbell and Cohen-Fix, 2002; Ivanov *et al.*, 2002). This pairing, or cohesion, sterically constrains sister chromatids so that sister kinetochores face away from each other, promoting proper chromosome bi-orientation. Cohesion also resists the poleward forces generated at the kinetochore-microtubule interface and spindle poles that act to pull sister chromatids apart. Tension resulting from poleward pulling forces and sister chromatid cohesion is monitored by eukaryotic cells to ensure that each sister chromatid is properly associated with spindle microtubules before initiating anaphase onset.

Loss of cohesion is thus predicted to result both in an imbalance of forces, favoring chromosome-to-pole motion, and disruption of the mechanical coupling that links two half-spindles together. In both cases, the predictions are borne out by experimentation. Mechanical or laser-induced severing between sister chromatids or ablating the kinetochore itself results in movement of the intact sister chromatid(s) toward its associated spindle pole (Nicklas and Staehly, 1967; McNeill and Berns, 1981; Rieder *et al.*, 1986; Hays and Salmon, 1990; Skibbens *et al.*, 1995). Similarly, yeast cells harboring mutations in the cohesion factors such as Ctf7p/Eco1p, Mcd1p/Scc1p and Pds5p have partially elongated and often bent spindles with separated but unequal masses of DNA (Guacci *et al.*, 1997; Skibbens *et al.*, 1999; Hartman *et al.*, 2000). Cohesion-deficient cells delay in mitosis by activating the kinetochore/spindle checkpoint. Coupled with the observation that these spindles are only partially elongated (*i.e.*, not true anaphase) with broken spindles or spindles with decreased microtubule intensity in the mid-region (*i.e.*, half-spindle separation), we suggest that defects in sister chromatid cohesion result in the inability of kinetochore poleward pulling forces to counteract the forces produced by microtubule motors generating centrosome separation forces. Thus, integrity of the spindle apparatus must in part rely on maintaining cohesion between sister chromatids.

In the beginning: links between centrosome duplication and establishment of sister chromatid cohesion

The geometry of two separated centrosomes, each nucleating a radial array of microtubules, is central to bipolar spindle assembly and proper chromosome segregation during mitosis. Typically, a single centrosome is passed along to each daughter cell and replicated prior to division. Under experimental conditions, centrosomes can form *de novo* if the original centrosomes are first experimentally destroyed. Cells apparently have a mechanism to sense the absence of centrosomes and assemble new ones only if necessary (Khodjakov *et al.*, 2002). Cells lacking a

centrosome will not enter S phase (Khodjakov and Rieder, 2001), suggesting that DNA replication requires that a cell have a centrosome.

In normal circumstances, however, centrosomes are not produced *de novo* but duplicated in a highly regulated fashion during S-phase under the control of CDK2 complexed with cyclin E (Hinchcliffe *et al.*, 1998; Lacey *et al.*, 1999). For centrosome duplication, one target of active CDK2 is nucleophosmin, a component of the nucleolus. Phosphorylation likely regulates the activity or localization of nucleophosmin, since expression of a non-phosphorylatable mutant prevented centrosome duplication by blocking centriole separation (Okuda *et al.*, 2000).

Similarly, sister chromatid pairing is intimately coupled to the DNA replication machinery. For instance, numerous Replication Factor C (RFC) complex subunits (Rfc2p, Rfc4p and Rfc5p) and at least one DNA polymerase (Pol α or Trf4p) are now known to play additional roles in cohesion (Wang *et al.*, 2000; Mayer *et al.*, 2001; Hanna *et al.*, 2001; Kenna and Skibbens, 2003). Ctf7p appears to be a critical link between sister chromatid cohesion and DNA replication (Figure 3). Ctf7p is required only during S-phase and associates with each of three Replication Factor C (RFC) complexes containing Rfc2p-Rfc5 and one of three larger subunits: Rfc1p, Rad24p, or Ctf18p (Skibbens *et al.*, 1999; Kenna and Skibbens, 2003). RFC complexes load sliding clamps onto DNA and thus promote processive DNA replication (Kelman, 1997). Two such sliding clamps have been identified. The homotrimeric PCNA sliding clamp functions during the bulk of DNA replication while the heterotrimeric Mec3p, Rad17p and Ddc1p sliding clamp is a component of the DNA damage checkpoint mechanism (Green *et al.*, 2000; Kondo *et al.*, 1999; Longhese *et al.*, 1997; Naiki *et al.*, 2000; Paciotti *et al.*, 1998). The association of the cohesion establishment factor Ctf7p with all three RFC complexes suggests that each is capable of participating in cohesion establishment.

It is likely that cross-talk exists between the processes required to activate centrosome duplication during S-phase and those that couple cohesion establishment to DNA replication. Toward this end, recent experiments in yeast cells reveal a direct physical interaction between regulators of chromatid cohesion and the spindle pole body duplication machinery (L. Antoniaci, M. Kenna, P. Uetz, and R. V. Skibbens, manuscript in preparation). Our understanding of the mechanisms coupling DNA replication, cohesion establishment and centrosome replication are likely to expand in the next few years. It will be interesting to identify the feedback controls that monitor each replication process, and how it communicates to the other system when problems arise. Such a feedback loop would ensure that DNA and centrosome replication remain in synchrony, and contribute to mechanisms to prevent aneuploidy.

Concluding remarks

Numerous studies have detailed the critical roles centrosomes and chromosomes play in spindle assembly (while a few have suggested that each structure may not be necessary at all times!). Given the importance of centrosomes for subsequent cell cycle progression and

for the localization of various signaling molecules (*e.g.* p53), we suggest that centrosomes attach themselves to the minus ends of microtubules to ensure their transport to daughter cells, while chromosomes attach themselves to the plus ends of microtubules for the same goal. Centrosomes have staked themselves at homeplate, the separated chromatids run to join them, giving each daughter the right stuff to continue on and on.

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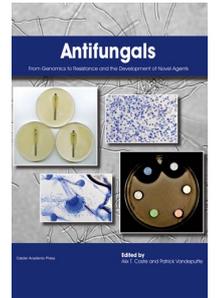
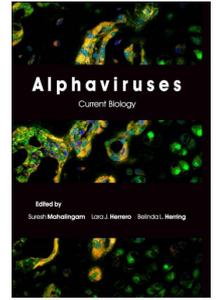
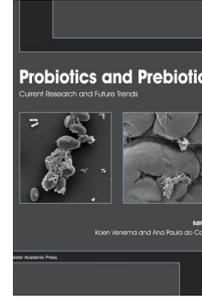
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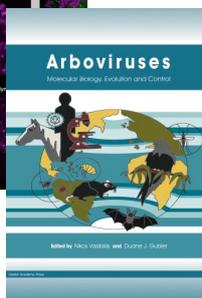
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