CHAPTER FOUR

Cell and Molecular Biology of the Spindle Matrix

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Contents

Abstract

The concept of a spindle matrix has long been proposed to account for incompletely understood features of microtubule spindle dynamics and force production during mitosis. In its simplest formulation the spindle matrix is hypothesized to provide a stationary or elastic molecular matrix that can provide a substrate for motor molecules to interact with during microtubule sliding and which can stabilize the spindle during force production. Although this is an attractive concept with the potential to greatly simplify current models of microtubule spindle behavior, definitive evidence for the molecular nature of a spindle matrix or for its direct role in microtubule spindle function has been lagging. However, as reviewed here multiple studies spanning the evolutionary

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spectrum from lower eukaryotes to vertebrates have provided new and intriguing evidence that a spindle matrix may be a general feature of mitosis.

Key Words: Spindle matrix, Spindle formation, Microtubules, Mitosis, Chromosome segregation.

1. Introduction

A mitotic spindle is present in all known eukaryotic cells and its function is essential for chromosomal segregation and cell division to occur (Mitchison and Salmon, 2001). The spindle apparatus is a complex molecular machine known to be made up of polymerized tubulin and various associated motor proteins (Gadde and Heald, 2004; Karsenti and Vernos, 2001; Sharp *et al.*, 2000a). Although much work has been directed toward understanding mitotic spindle apparatus structure and function, it is still unclear what directs and stabilizes the assembly of the spindle. Furthermore, although numerous models have been proposed for how the spindle apparatus may transmit forces, none of these models have been able to account for all the experimentally observed properties of spindle behavior (Bloom, 2002; Gadde and Heald, 2004; Kapoor and Compton, 2002; Mitchison and Salmon, 2001; Scholey *et al.*, 2001; Wittmann *et al.*, 2001), especially, the discovery of microtubule flux and the constant treadmilling of tubulin dimers toward the poles (Cassimeris *et al.*, 1988; Mitchison, 1989; Mitchison and Salmon, 2001; Rogers *et al.*, 2005; Sawin and Mitchison, 1991, 1994) have made it difficult to model how forces are generated to actually move chromosomes on the basis of a metastable structure not anchored in place. For these reasons and based on theoretical considerations of the requirement for force production at the spindle, the concept of a spindle matrix has long been proposed (Johansen and Johansen, 2002; Pickett-Heaps *et al.*, 1982, 1997; Wells, 2001). The spindle matrix is hypothesized to provide a stationary or elastic molecular matrix that can provide a substrate for motor molecules to interact with during microtubule sliding and which can stabilize the spindle during force production (Pickett-Heaps *et al.*, 1997) (Fig. 4.1). Molecules forming a spindle matrix complex would be expected to exhibit several characteristics: (1) they should associate together to form a true fusiform structure coaligned with the microtubule spindle; (2) they should remain associated, forming a polymerized complex in the absence of microtubules; (3) perturbation of one or more of the components should affect spindle assembly and/or function; and (4) one or more members of the complex should interact with microtubules or microtubule-associated molecules such as motor proteins. Such a matrix could also be envisioned to have the added properties of helping to organize

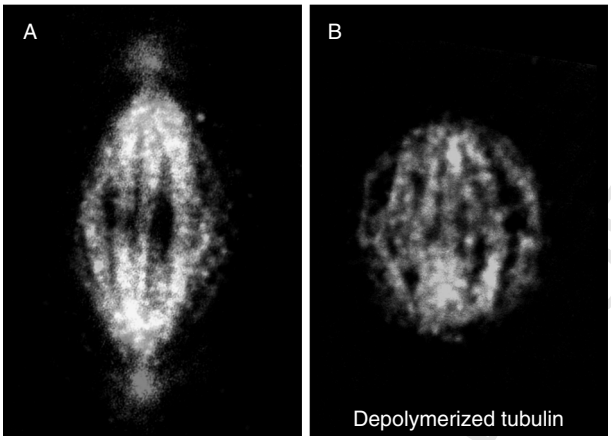

Figure 4.1 Properties of the spindle matrix. The spindle matrix hypothesis proposes the existence of a stationary or elastic molecular matrix that contributes to microtubule spindle function and/or assembly. Molecules forming a spindle matrix would be expected to exhibit several characteristics: they should associate together to form a true fusiform structure coaligned with the microtubule spindle and they should remain associated forming a polymerized complex in the absence of microtubules as illustrated in the micrographs. (A) and (B) Confocal images of *Drosophila* syncytial embryo nuclei at metaphase double labeled with antibodies to tubulin (red) and the spindle matrix protein Megator (green). After depolymerization of microtubules by cold treatment the Megator antibody-labeled matrix is still intact maintaining a fusiform structure (B). (See also color insert.)

and stabilize the microtubule spindle as well as the midbody, an electron dense body implicated in cytokinesis. Whereas the spindle matrix is an attractive concept, for many years there has been little direct experimental evidence for such a structure and its molecular nature has remained enigmatic. However, a number of studies in *Drosophila* (Qi *et al.*, 2004, 2005; Rath *et al.*, 2004; Walker *et al.*, 2000) as well as in vertebrates (Chang *et al.*, 2004; Mitchison *et al.*, 2005; Tsai *et al.*, 2006) have revived interest in the spindle matrix. Here we review evidence for the existence of a spindle matrix and its possible molecular composition in the context of microtubule spindle dynamics and force production.

2. Microtubule Spindle Dynamics and Force Production

The mitotic spindle is a dynamic, complex macromolecular machine constantly being remodeled during the progression through the stages of mitosis (prophase, prometaphase, metaphase, anaphase, and telophase). Most research to date has focused on two classes of molecules that play

critical roles in transducing the forces necessary to align and separate chromosomes precisely to two daughter nuclei, namely microtubules and motor proteins (Kapoor and Compton, 2002; Karsenti and Vernos, 2001; Mitchison and Salmon, 2001; Sharp *et al.*, 2000a,b; Wittmann *et al.*, 2001). Microtubules contribute to these forces in two ways: they are a rigid, polarized cytoskeletal element on which dynein- and kinesin-family motor proteins can move cargo in a directional manner and they also can directly promote movement within the spindle via ''treadmilling'' in which tubulin subunits are dissembled from the minus end while being added to the plus end (Margolis and Wilson, 1978; Margolis *et al.*, 1978; Mitchison *et al.*, 1986). Thus the biophysical properties of microtubules make them an ideal cytoskeletal structural element to confer many of the essential functional properties required of a mitotic apparatus.

Microtubules are hollow cylindrical tubes of 13 parallel protofilaments, each composed of $\alpha\beta$-tubulin heterodimers arranged end-on-end with the α-tubulin subunit exposed at the minus end and the β-tubulin subunit exposed at the plus end (Fan *et al.*, 1996; Hirose *et al.*, 1995; Nogales *et al.*, 1999). This tubular organization of protofilaments is stabilized by lateral interactions forming a ''B lattice'' in which contacts are made between homologous subunits, $\alpha-\alpha$ and $\beta-\beta$ (Song and Mandelkow, 1993), but closing of the cylinder leaves a ''seam'' in which these lateral contacts are slightly altered (Chrétien *et al.*, 1996; Kikkawa *et al.*, 1994; Sosa and Milligan, 1996; Wade and Hyman, 1997). Studies suggest this seam may be an important site of action for the regulation of microtubule dynamics (Sandblad *et al.*, 2006). However, better understood is how MT dynamics are affected by the status of the guanine nucleotide bound to the β-tubulin subunit. Each tubulin subunit binds a GTP nucleotide with hydrolysis of the GTP to GDP occurring only on the β-tubulin subunit (David-Pfeuty *et al.*, 1977; Spiegelman *et al.*, 1977). When the β-tubulin subunits at the end of a MT contain GTP (a ''GTP-cap''; Carlier and Pantaloni, 1981), the MT tends to be stable and growing, but loss of this GTP cap by hydrolysis to GDP favors ''catastrophe'' (Davis *et al.*, 1994), a situation in which the MT rapidly depolymerizes until a ''rescue'' event may reverse the process (Hyman *et al.*, 1992; Mitchison, 1993; Nogales *et al.*, 1999). The rapid switching between a depolymerizing state and a growing state is the hallmark of ''dynamic instability'' for which MTs are known and is a key feature allowing for remodeling of MTs (Cassimeris *et al.*, 1987; Kristofferson *et al.*, 1986; Mitchison and Kirschner, 1984).

The metaphase spindle is a fusiform-shaped structure anchored by two poles with microtubules organized with their plus-ends pointing away from the pole (Euteneuer and McIntosh, 1981; Haimo, 1985; Telzer and Haimo, 1981). Within the mitotic spindle there are different classes of MTs: (1) *astral fibers* which radiate toward the cortex of the cell and are believed to assist in spindle orientation and cleavage plane specification; (2) *kinetochore*

microtubules (*kMTs*), which typically are bundled into *k-fibers*, extend from the pole and attach to the chromosomes at specialized attachment sites called kinetochores; and (3) *interpolar microtubules* (*ipMTs*) which extend across the spindle midzone, interdigitating with microtubules originating from the opposite half of the spindle without interacting with chromosomes (Compton, 2000). This latter class of MTs can be of diverse lengths, as their minus-ends do not always originate at a pole but can be spread out over half of the spindle length (Mastronarde *et al.*, 1993). This observation was unexpected because previously it had been thought that all MTs initiate from a "microtubule organizing center" (MTOC) that for most spindles is provided by one of the duplicated and separated centrosomes found at each half-spindle pole. However, experiments have shown that microtubule spindles can form and function in the complete absence of centrioles and centrosomes (Basto *et al.*, 2006; Bonaccorsi *et al.*, 1998; Hinchcliffe *et al.*, 2001; Khodjakov *et al.*, 2000; Megraw *et al.*, 2001). Furthermore, γ-tubulin, which is involved in MT nucleation, has been observed to be distributed throughout the spindle fibers and not strictly localized to the centrosomes (O'Brien *et al.*, 2005; Raynaud-Messina and Merdes, 2007; Wilde and Zheng, 1999). Thus, based on these findings several different models have been developed to account for the multiple mechanisms that combine to generate the microtubule spindle (Gadde and Heald, 2004; Wadsworth and Khodjakov, 2004).

In the "search and capture" model, MTs that emanate from the poles are highly dynamic, undergoing multiple rounds of growth and shrinkage until they become stabilized by "capture" of a chromosome kinetochore (Kirschner and Mitchison, 1986; Mitchison and Kirschner, 1985; Nicklas and Kubai, 1985). Once the bivalent kinetochore has been captured by MTs emanating from opposite poles, the chromosome will congress to the metaphase plate to form a bipolar metaphase spindle (Nicklas and Kubai, 1985). But a different mechanism for spindle assembly must occur in acentrosomal cells such as certain oocytes and higher plant cells. It has been proposed that such cells build their spindles by a "self-organization" mechanism in which MTs are nucleated from the chromosomes and from within the spindle itself instead of from a MTOC; once formed the MTs are progressively focused by the actions of motor proteins and other scaffolding proteins to form a bipolar spindle (Albertson and Thomson, 1993; Matthies *et al.*, 1996; McKim and Hawley, 1995; Smirnova and Bajer, 1992; Steffen *et al.*, 1986). However, the observation that cells that would normally utilize a centrosomal-mediated mechanism could build spindles by a chromosome-mediated pathway if an essential centrosome component was mutant (Bonaccorsi *et al.*, 1998; Megraw *et al.*, 2001) or if the centrosome was removed or inactivated by chromophore-assisted laser inactivation (Khodjakov *et al.*, 2000), suggested that cells could be induced to use alternate pathways. High-resolution imaging studies revealed that although the centrosome-mediated pathway dominates in *Drosophila* S2

cells, both pathways are operational (Maiato *et al.*, 2004). Thus, the evidence points toward a ''combined model'' in which multiple redundant mechanisms such as centrosome-mediated nucleation of MTs, chromosome-based assembly of MTs, and recruitment, sliding, and bundling of MTs created at other sites interact to give rise to spindle assembly (Gadde and Heald, 2004; Janson *et al.*, 2007; Mahoney *et al.*, 2006; Wadsworth and Khodjakov, 2004).

Whereas microtubule dynamics of assembly and disassembly during spindle formation is regulated through a complex balance between different MT-stabilizing and MT-destabilizing activities (Andersen, 2000; Kline-Smith and Walczak, 2004), less well understood is how spindle MT fluxes are generated or how flux may be linked to actual force generation capable of moving chromosomes during anaphase (Kwok and Kapoor, 2007; Rogers *et al.*, 2005; Sharp *et al.*, 2000a). In addition to microtubules a large number of MT-based motors participate in spindle action via a number of different mechanisms, including cross-bridging and sliding adjacent MTs, transporting chromosomes and other mitotic cargoes along the MTs, and by influencing MT growth and shrinkage (Goshima and Vale, 2003; Goshima *et al.*, 2005a; Kwon *et al.*, 2004; Rogers *et al.*, 2004; Tao *et al.*, 2006). Furthermore, a balance of plus-end–directed and minus-end–directed motors regulate many aspects of spindle morphogenesis and dynamic function (Fuller and Wilson, 1992; Heald and Walczak, 1999; Sharp *et al.*, 2000a,b). Thus the mitotic apparatus is under constant tension, with both microtubule dynamics and opposing motor proteins generating inward and outward forces. Because the metaphase spindle does not collapse, these forces must balance each other out or alternatively the motor proteins must be anchored to a stabilizing structural element that acts as a scaffold or strut (Johansen and Johansen, 2002; Sharp *et al.*, 2000a). Theoretical calculations have been derived in support of the hypothesis that observed spindle dynamics (i.e., centrosome separation, spindle assembly, spindle elongation, and spindle disassembly) can be satisfactorily accounted for based on a structure comprised solely of microtubules and motors (Cytrynbaum *et al.*, 2003; Nedelec, 2002; Scholey *et al.*, 2001). However, even if the forces in the model can be ''balanced'' such that it is not necessary to invoke an additional scaffolding element to assist in the dynamics of spindle assembly or mitotic motility, certain biophysical constraints argue for the existence of a matrix to strengthen the spindle apparatus. Force calculations predict 1- to 10-pN force is generated per motor (Block, 1995; Howard, 1995; Scholey *et al.*, 2003; Svoboda and Block, 1994) and with cooperation of hundreds to thousands of force generators (motors), the actual spindle force measured is in the range of a 1000 pN (Nicklas, 1983). The force to buckle a microtubule is only in the pN range and although bundling of microtubules increases the buckling threshold to an ~100 pN range (Elbaum *et al.*, 1996; Freitas, Jr., 1999; Fygenson *et al.*, 1997), this is still well below the measured forces exerted on the spindle (Nicklas, 1983). It is also difficult to account for why most spindle microtubules are curved. Whether they are

envisioned to exert pulling forces or pushing forces on the chromosomes, microtubules would be expected to be ''straight'' without some kind of tensile element to act against. Thus, current models to explain spindle structural dynamics based solely on the activities of microtubules and motors are likely to be incomplete. Furthermore, there may be many missing elements because the molecular composition of the spindle is still not fully defined: a proteomic analysis of the human mitotic spindle identified 795 proteins, only 151 of which had been previously known to associate with the spindle apparatus (Sauer *et al.*, 2005). Consequently, our understanding of the molecular composition of the mitotic spindle apparatus and how it generate forces to segregate the chromosomes is likely to be only rudimentary. The demonstration of the existence of a spindle matrix would have the potential to clarify many of the outstanding issues and to greatly simplify current models for force generation.

3. Evidence for a Spindle Matrix

Some of the first experimental observations that hinted at the existence of a spindle matrix were from the early experiments of Goldman and Rebhun (1969) and Forer (1969) who found that the volume of the nonmicrotubule portion of the spindle was much greater than that of microtubules. That this could correspond to a ''spindle matrix'' was suggested by data indicating that birefringence in the spindle originates from nonmicrotubule, as well as from microtubule, components. Similar conclusions were made from EM evidence demonstrating linear arrays of particles in the absence of microtubules (Behnke and Forer, 1966; Goldman and Rebhun, 1969). When spindles are treated with nocodazole, a ''spindle remnant'' can be isolated that retains spindle-like morphology despite the absence of microtubules (Leslie *et al.*, 1987; Pickett-Heaps *et al.*, 1984; Wein *et al.*, 1998). Tektin-like antigens associate with spindles as well as spindle remnants generated by cold treatment, implying other structural elements exist in the spindle (Steffen and Linck, 1992). Chromosomes are still pulled to spindle poles in UV-microbeam experiments during mitosis despite the microtubules having been severed (Sillers and Forer, 1983; Spurck *et al.*, 1997) (Fig. 4.2). At least four proteins from two different nuclear compartments that interact with each other and that redistribute during prophase forming a fusiform spindle structure that persists in the absence of polymerized tubulin have been identified in *Drosophila* (Johansen *et al.*, 1996; Qi *et al.*, 2004, 2005; Rath *et al.*, 2004; Walker *et al.*, 2000). The coiled-coil protein NuMA forms a pericentriolar matrix that has been shown to be necessary for proper spindle formation and function (Dionne *et al.*, 1999; Merdes et al, 1996). In addition, a nonmicrotubule

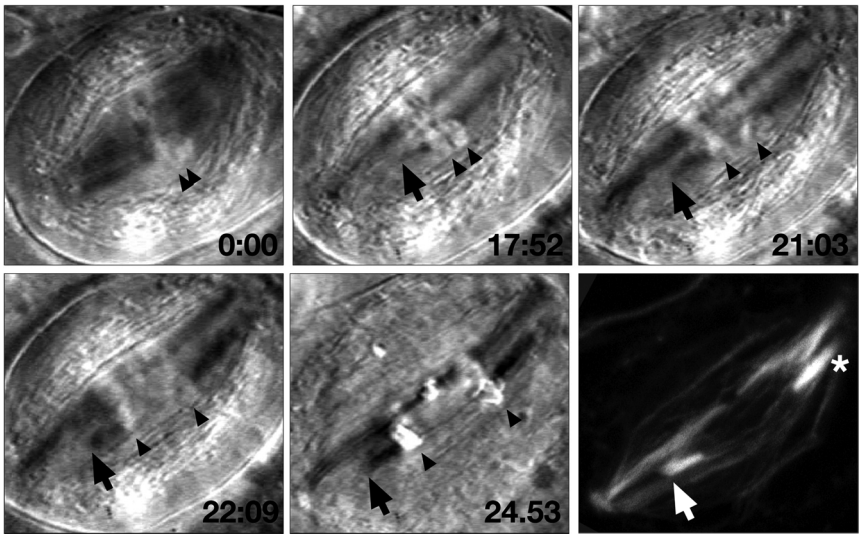

Figure 4.2 Chromosome movement after k-fibers are severed in an irradiated crane-fly spermatocyte spindle. 0.00: Cell before treatment. Chromosomes are aligned at the metaphase plate (arrowheads). 17:52: UV irradiation creates a k-stub (arrow) and an ARB (area of reduced birefringence) indicating loss of microtubules. 21:03: Both k-stub and chromosome move toward the pole in anaphase, despite lack of k-fiber connection to the pole. 22:09: Chromosomal movement during anaphase continues. Note the increased separation between partners (arrowheads) and continued presence of a k-stub (arrow). 24:53: Cell after lysis reveals k-stub (arrow) and separated chromosomes (arrowheads). Final panel: confocal of cell in previous panel stained for tubulin shows clearly that the UV-severed k-fiber remained a stub and was not in contact with the pole. Asterisk denotes a k-stub from a previously irradiated region. Note that both chromosome partners moved toward poles despite severed k-fibers. (Micrographs were generously provided by Drs. A. Forer, T. Spurck, and J. D. Pickett-Heaps).

filamentous structure that colocalizes with spindle microtubules has been observed in yeast nuclei (van Hemert *et al.*, 2002). Studies indicating a nonmicrotubule matrix contributes to force generation in the spindle have also been reported, for example, severing all of the microtubules in a metaphase half-spindle resulted in spindle shortening, suggesting the existence of compressive forces on the central spindle at metaphase (Spurck *et al.*, 1990). Similarly, based on responses to microtubule destabilization experiments in *Xenopus* egg extracts, Mitchison *et al.* (2005) suggested an unidentified tensile element acts in parallel with conventional microtubule lattice factors to generate spindle-shortening forces. One molecular candidate for such an internal matrix in this system is poly(ADP-ribose), a nonprotein macromolecule required for bipolar organization of *Xenopus* extract spindles (Chang *et al.*, 2004). Another candidate molecule is lamin B, a component of the interphase nuclear lamina shown to be required for spindle assembly (Tsai *et al.*, 2006).

Thus a wide range of experimental observations in different organisms has been consistent with the existence of a spindle matrix.

3.1. Early indications of abundant nonmicrotubule components of the spindle and spindle remnants

Porter (1955) reported the presence of fibrillar elements in the spindle that were later shown to be composed of microtubules (Ledbetter and Porter, 1963, 1964; Slautterback, 1963) and which became the focus of most studies of spindle function. However, even at that time a number of different lines of investigation were raising questions about whether additional components in the spindle remained to be identified. Mazia and Dan (1952) first took advantage of the synchronized mitotic populations of sea urchin eggs to develop procedures to obtain substantial quantities of isolated mitotic spindles for such analysis and multiple versions of this original spindle isolation protocol have since been developed (e.g., Kane, 1965, 1967; Mazia *et al.*, 1961; Sauer *et al.*, 2005; Zieve and Solomon, 1982). In independent studies by Goldman and Rebhun (1969) and by Forer (1969), it was found that the volume of the nonmicrotubule portion of the spindle was greater than that of microtubules. That this could correspond to a ''spindle matrix'' was suggested by data indicating that birefringence in the spindle originates from nonmicrotubule as well as from microtubule components (Forer, 1966; Goldman and Rebhun, 1969). Similar conclusions were made from EM evidence demonstrating linear arrays of particles in the absence of microtubules (Behnke and Forer, 1966; Goldman and Rebhun, 1969). Thus, very early on it was clear the mitotic apparatus was a complex structure composed of significantly more material than simply microtubules. However, different isolation procedures could give rise to very different results, as shown in the studies of Forer and Goldman (1969). In these studies isolated spindles included protein, RNA, and carbohydrate-containing material but the actual composition varied depending on pH and isolation times. This has prompted some to question whether mitotic spindles act as ''a sponge'' during the isolation procedure and has led to concerns about the physiological relevance of many components that copurify with the mitotic apparatus (Wells, 2001).

Nonetheless, when isolated spindles are extracted with calcium and/or shifted to low temperatures, a ''spindle remnant'' can be isolated that retains spindle-like morphology despite the absence of microtubules (Leslie *et al.*, 1987; Pickett-Heaps, 1986; Rebhun and Palazzo, 1988; Wein *et al.*, 1998). Rebhun and Palazzo (1988) reported that Ca^{2+}-extracted spindles contained a ~55 kDa polypeptide with an amino acid composition similar to intermediate filament proteins. Leslie *et al.* (1987) used antibodies to study the distribution of kinesin in isolated sea urchin spindles. Whereas the kinesin colocalized with MT spindles including asters in unperturbed spindle preparations, following

MT disassembly it remained associated with the spindle remnant in amorphous, spindle-shaped structures with no astral extensions. This localization was not affected by exogenous ATP addition, supporting the interpretation that in addition to its association with microtubules, kinesin likely also associates with a spindle matrix component. These conclusions were strengthened by studies in *Cylindrotheca fusiformis* in which the kinesin-related protein DSK1 was shown to be part of a matrix in the extracted diatom mitotic apparatus that exists independent of the microtubule spindle (Wein *et al.*, 1998).

Other molecules besides motor proteins show this behavior. Affinity purified polyclonal antibodies against tektin C label tubulin-depleted spindle remnants as well as spindles (Steffen and Linck, 1992). Because tektin itself does not appear to localize to the mitotic apparatus but under renaturing transblot conditions this antibody could recognize tektins A and B due to their high degree of structural similarity, it was proposed that tektin-related protein(s) might be present in the spindle and potentially provide additional structural support (Steffen and Linck, 1992). Other potential cytoskeletal proteins have also been described in spindles, including the keratin-related proteins cytocentrin (Paul and Quaroni, 1993) and astrin (Gruber *et al.*, 2002; Mack and Compton, 2001), titin or titin-related proteins (Wernyj *et al.*, 2001; Zastrow *et al.*, 2006), Protein 4.1 (Huang *et al.*, 2004; Krauss *et al.*, 1997, 2004) and lamin B (Beaudouin *et al.*, 2002; Georgatos *et al.*, 1997; Harel *et al.*, 1989; Maison *et al.*, 1997; Paddy *et al.*, 1996; Tsai *et al.*, 2006).

3.2. Chromosome movement after UV microbeam severing of microtubules

An early indication of a functional spindle matrix was reported by Forer (1965) who used ultraviolet (UV) microbeam irradiation to create an ''area of reduced birefringence'' (ARB) in the spindle fiber and found that it moved to the pole with constant velocity and shape until it disappeared. The fiber simultaneously recovered birefringence from the kinetochore end, suggesting a poleward flux of spindle fiber material. In addition, EM analysis showed that lesions associated with ARBs are devoid of microtubules (Snyder *et al.*, 1991; Wilson and Forer, 1988). Thus this technique was ideally suited to test the prevailing ''Pac-man'' model that proposed chromosome segregation to the poles was powered by disassembly of kMTs at the kinetochore. If this were the sole mechanism generating the separation forces severing k-fibers in the middle of the half-spindle by UV-microbeam irradiation should have halted chromosome segregation during anaphase but, remarkably, this was not the case. Even after UV-mediated kMT disassembly the chromosomes continued to be pulled to the spindle poles (Pickett-Heaps *et al.*, 1997; Sillers and Forer, 1983; Spurck *et al.*, 1997)

(Fig. 4.2). These results suggested poleward forces for chromosome motion are not produced *by* the k-fibers but rather act *on* them and that an intact kMT is not required for force production. In addition, the observation that although some chromosomes moved with unchanged speed, other chromosomes showed a transient acceleration, returning to the normal segregation speed only after the kMT-stub contacted the centrosome (Spurck *et al.*, 1997), leading to the proposal that the rate of MT disassembly in the spindle might serve to *limit* the rate of chromosome motion rather than power it (Forer, 1974; Nicklas, 1975; Pickett-Heaps *et al.*, 1982). Taking into consideration an external source of power generating chromosome movement as well as that MTs adjacent to the ARB showed a tendency to buckle, Spurck *et al.* (1997) favored a model in which the force for chromosome segregation is generated by a component associated with a spindle matrix and where the role of spindle MTs is to resist this force thus setting up the spindle as a kind of tensegrity structure (Ingber, 1993, 2003). Although it has become clear there are multiple and perhaps redundant mechanisms at work generating segregation forces in the spindle, these were critical experiments in advancing the idea that forces external from the kMTs are also involved. However, the debate about the existence of a "spindle matrix" continued, with some favoring its role in contributing to these external forces (Pickett-Heaps and Forer, 2001; Pickett-Heaps *et al.*, 1984, 1996, 1997; Spurck *et al.*, 1997), whereas others envisioning that motor proteins could move chromosomes on intact ipMTs outside of the ARB region after cross-bridging with the kMT-stub (Scholey *et al.*, 2001; see also Maiato *et al.*, 2004 regarding k-stub regrowth and reincorporation into the spindle), an issue that has still not been definitively resolved.

3.3. Molecular identification of a multi-protein spindle matrix complex in *Drosophila*

3.3.1. Molecular components

A longstanding reservation regarding the potential existence of a spindle matrix has been a lack of direct molecular information on its biochemical composition. However, some of the most promising molecular candidates, Skeletor, Chromator, Megator, and EAST (Qi *et al.*, 2004, 2005; Rath *et al.*, 2004; Walker *et al.*, 2000), for constituting a bona fide spindle matrix complex presently fulfilling at least three of its defining criteria have been identified in *Drosophila*. Skeletor, the founding member of this complex, is an 81-kDa protein originally identified by use of a mAb with an intriguing dynamic staining pattern during mitosis in *Drosophila* embryos (Walker *et al.*, 2000) (Fig. 4.3). However, Skeletor encodes a low-complexity protein with no obvious motifs, so it was difficult to predict Skeletor's potential role in spindle matrix function. Consequently, in a search for

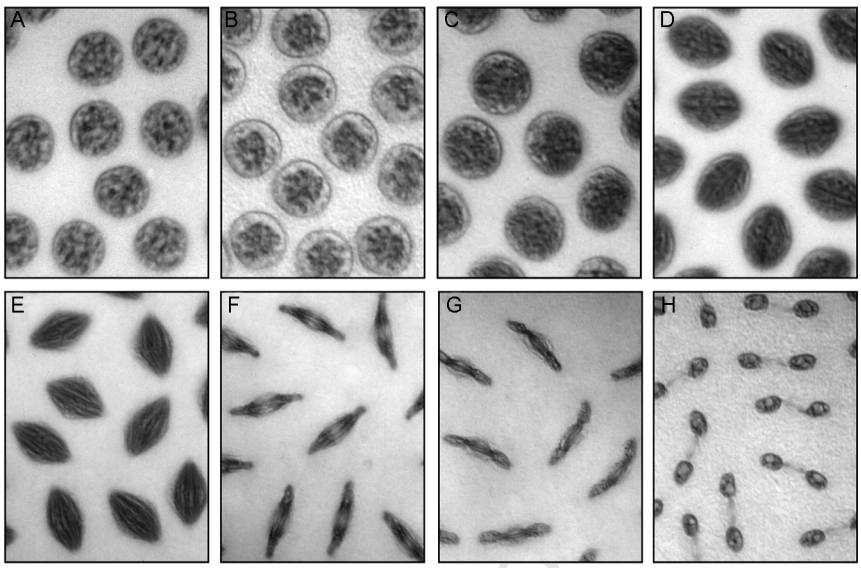

Figure 4.3 Drosophila embryo nuclei labeled by the Skeletor mAb1A1 from various stages of the cell cycle. (A) interphase; (B) prophase; (C) late prophase; (D) prometaphase; (E) metaphase; (F) late anaphase; (G) early telophase; (H) late telophase. At prophase (B), the chromosome localization of the spindle matrix component Skeletor undergoes a redistribution to a structure forming a spindle-like scaffold from late prophase (C) through anaphase (F). During interphase (A) and prophase (B, C) mAb1A1 labeling appears to be also associated with the nuclear envelope. The micrographs are Nomarski images of mAb1A1 labelings visualized using HRP-conjugated secondary antibody. (Modified from *J. Cell Biol.* (2000). 151, 1401–1411. Copyright 2000 Rockefeller University Press.)

other members of a spindle matrix molecular complex, Rath *et al.* (2004) used a yeast two-hybrid screen to identify a novel protein, Chromator, which directly interacts with Skeletor. Chromator is a 101-kDa protein that contains a chromodomain and analysis of P-element mutations has demonstrated that Chromator is an essential protein (Rath *et al.*, 2004). However, for a spindle matrix to form independently or to form a structural scaffold aligned with the microtubule spindle one or more of its molecular components would be predicted to have the ability to form polymers and neither Skeletor nor Chromator appear to contain molecular motifs with such properties. Qi *et al.* (2004, 2005), therefore, used immunocytochemistry and cross-immunoprecipitation experiments to show that two additional proteins, Megator and EAST, also interact with Skeletor and Chromator during mitosis. Megator is a 260-kDa protein with a large NH_2-terminal coiled-coil domain and a shorter COOH-terminal acidic region that originally was referred to as the Bx34 antigen (Zimowska *et al.*, 1997). EAST is another large protein of 153 kDa, which apart from seven potential nuclear

localization sequences and 12 potential PEST sites, does not have any previously characterized motifs or functional domains (Wasser and Chia, 2000). Although Skeletor, Chromator, and EAST appear to have no obvious mammalian homologs, the coiled-coil protein Megator has a striking overall structural and sequence similarity to the mammalian nuclear pore complex Tpr protein (Zimowska *et al.*, 1997) and to the yeast nuclear pore complex-associated proteins Mlp1p and Mlp2p (Kosova *et al.*, 2000; Strambio-de-Castillia *et al.*, 1999). Mlp2p binds directly to core components of the spindle pole body (SPB) and is required for proper SPB function and normal cell division (Niepel *et al.*, 2005). Interestingly, several other nuclear pore complex-associated proteins have also been linked to proper microtubule spindle assembly and function (Orjalo *et al.*, 2006; Schetter *et al.*, 2006; Scott *et al.*, 2005). Furthermore, the presence of a large coiled-coil domain in Megator raises the intriguing possibility that Megator could comprise the structural element of the spindle matrix complex. Interestingly, Megator deletion construct analysis in S2 cells indicates that the NH_2-terminal coiled-coil containing domain has the ability to self assemble into spherical structures in the cytoplasm (Qi *et al.*, 2004). This is in contrast to the acidic COOH-terminal domain, which is targeted to the nucleus implying the presence of a functional nuclear localization signal. Furthermore, the COOH-terminal domain is sufficient for localization to the nuclear rim as well as for spindle localization. Thus, an attractive hypothesis is that the COOH-terminal domain of Megator functions as a targeting and localization domain whereas the NH_2-terminal domain may be responsible for forming polymers that may serve as a structural basis for the putative spindle matrix complex. Supporting this notion is the finding that Megator spindles persist in the absence of microtubules depolymerized by cold or nocodazole treatment (Qi *et al.*, 2004) (Fig. 4.1).

3.3.2. Dynamic redistribution of spindle matrix proteins during mitosis

Coimmunoprecipitation studies, yeast-two hybrid interaction assays as well as immunocytochemistry show that all four proteins interact to form a true fusiform spindle complex during mitosis (Qi *et al.*, 2004, 2005; Rath *et al.*, 2004; Walker *et al.*, 2000). Two of the proteins, Skeletor and Chromator, are localized to chromosomes during interphase (Rath *et al.*, 2004; Walker *et al.*, 2000) whereas the other two, Megator and EAST, occupy the intranuclear space surrounding the chromosomes with Megator additionally being localized to the nuclear rim (Qi *et al.*, 2004; Zimowska *et al.*, 1997) (Fig. 4.4). Thus, the four proteins are derived from two different nuclear compartments. As illustrated in Fig. 4.5A, the establishment of the spindle matrix (as labeled using the Skeletor antibody mAb1A1) appears to precede microtubule spindle formation at prophase. During metaphase the spindle matrix and the microtubule spindles are coaligned (Fig. 4.5B). Importantly,

Spindle matrix proteins in *Drosophila*

Name	Kind	Size	Interphase localization
Skeletor	Novel protein	81 kD	Chromosomes
Chromator	Chromodomain protein	101 kD	Chromosomes
Megator	Coiled-coil protein	260 kD	Intranuclear space
EAST	Novel protein	253 kD	Intranuclear space

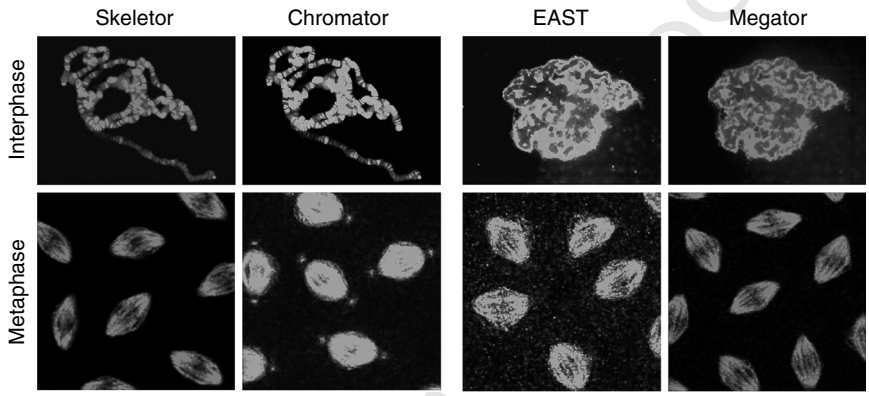

Figure 4.4 Spindle matrix proteins in *Drosophila*. The four nuclear spindle matrix proteins, Skeletor, Chromator, Megator, and EAST redistribute during prophase and interact with each other to form a fusiform spindle structure at metaphase. Two of these proteins, Skeletor and Chromator, are localized to chromosomes during interphase whereas the other two, Megator and EAST, occupy the interchromosomal space surrounding the chromosomes. The micrographs in the upper panel are confocal images from third instar larval salivary gland nuclei double-labeled with antibodies specific to each of the four spindle matrix proteins (red or green) and Hoechst (blue). The lower panel shows confocal images of metaphase nuclei from syncytial embryos labeled with antibodies specific to each of the four spindle matrix proteins (red or green). (See also color insert.)

the mAb1A1-labeled spindle matrix maintains its fusiform spindle structure from end to end across the metaphase plate during anaphase when the chromosomes segregate (Fig. 4.5D). At telophase when the chromosomes start to decondense mAb1A1 labeling still defines a spindle in the midregion (Fig. 4.5C). When embryos are treated with nocodazole to disassemble the microtubules, the mAb1A1-labeled spindle structure persists (Fig. 4.5E). Thus, the mAb1A1-defined spindle exhibits all the properties predicted for the spindle matrix (Walker *et al.*, 2000). It should be emphasized that antibodies to Chromator, EAST, and Megator label the metaphase spindle matrix structure in an identical way to that of the Skeletor antibody (Qi *et al.*, 2004, 2005; Rath *et al.*, 2004). Especially, the finding that the spindle matrix maintains its fusiform spindle structure

during chromosome segregation makes it an ideal candidate for being a scaffold that provides structural support for motor proteins and counterbalancing force production. That this spindle may provide a substrate for the alignment of microtubules is further supported by the finding that microtubules are colocalized with the spindle matrix that remains in the central region during midbody formation at telophase. This alignment of microtubules at the midbody region is largely unaccounted for by most other models but could be explained if the spindle matrix as hypothesized by Walker *et al.* (2000) helps in organizing microtubule fibers. Based on these observations Walker *et al.* (2000) proposed a model in which the spindle matrix constituted by these proteins is assembled during late prophase (Fig. 4.5F) at a time prior to or coinciding with microtubule extension into the nucleus. During mitosis the nuclear lamina in *Drosophila* first breaks down at the poles, thus allowing access of the microtubules into the nuclear interior where they coalign with the spindle matrix (Fig. 4.5G). The spindle matrix remains intact during metaphase and anaphase, thereby providing a stable scaffold to balance the forces and counterforces generated by motor proteins while microtubules shorten and chromosomes are moved to the poles (Fig. 5H). A prediction of this model is that mutations in components of the spindle matrix compromising this scaffold or resulting in its loss will lead to abnormal microtubule spindles and chromosome segregation defects (Fig. 4.5I).

3.3.3. Functional analysis

Unfortunately, a definitive analysis of the role of these proteins in spindle matrix function by mutant and RNAi approaches has been challenging. One of the reasons is that components of the spindle matrix may have essential functions at interphase as well as during mitosis. For example, it is likely Megator plays several important functional roles as a component of multiple subcellular structures that include the nuclear pore complex, the interphase interchromosomal domain, and the spindle matrix (Qi *et al.*, 2004). Thus, although a null allele of the Megator gene has been identified, Megator function in early homozygous embryos could not be tested due to the presence of maternally derived Megator protein that masks any potential phenotypes (Qi *et al.*, 2004). Furthermore, these animals die before hatching precluding larval neuroblast analysis. For these reasons Qi *et al.* (2004) used RNAi methods in S2 cells to deplete Megator protein levels. When Megator levels were knocked down, the number of S2 cells undergoing mitosis was greatly reduced. However, cells with obvious defects in tubulin spindle morphology or chromosome segregation defects were not observed suggesting that depletion of Megator prevents cells from entering metaphase. This could be due to an essential function of Megator in maintaining nuclear structure and/or in maintaining the integrity of the nuclear rim and pore complexes during interphase or a necessary function for nuclear

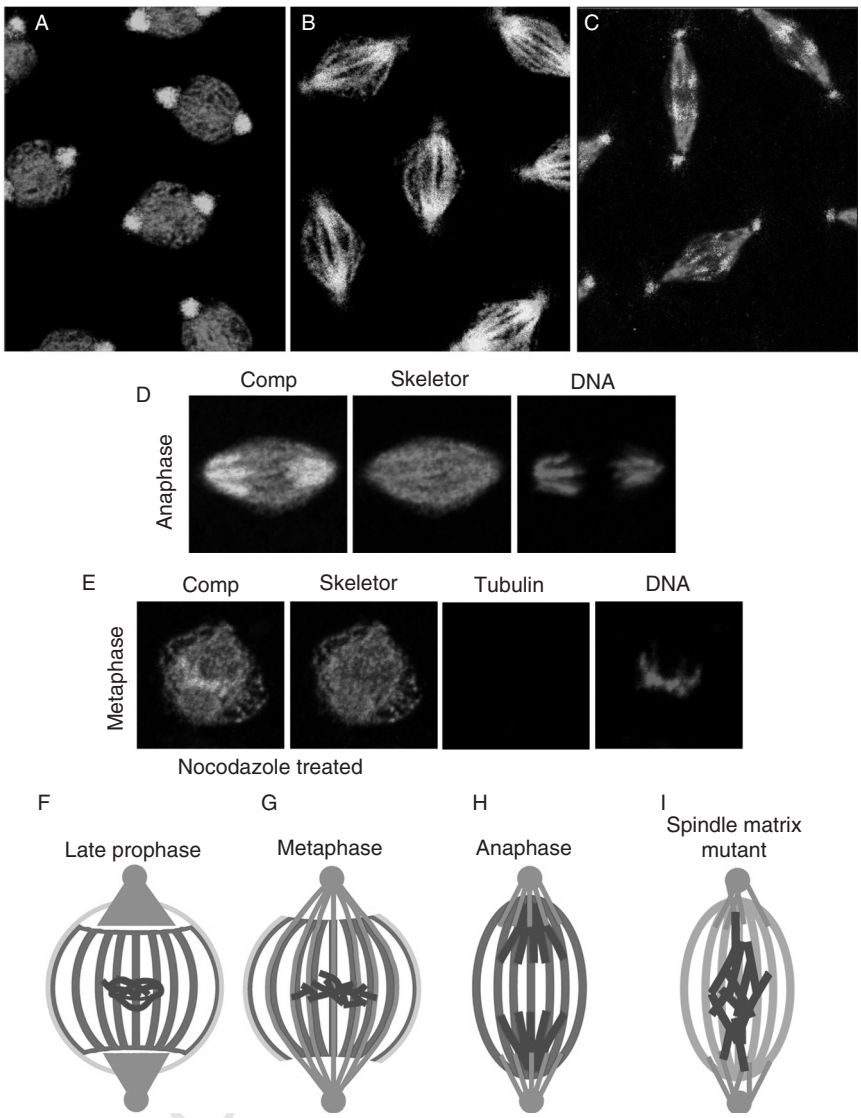

Figure 4.5 Spindle matrix properties in *Drosophila*. (A–C) Spindle matrix formation begins before microtubule entry into the nucleus or nuclear lamina breakdown. Double labelings with mAb1A1 to visualize the spindle matrix protein Skeletor (red) and anti–α-tubulin to visualize the microtubules (green) show that in late prophase, when the microtubules have not yet entered the nuclear space, the Skeletor antibody-labeled spindle is already aligned within the nucleus (A). During metaphase the two spindles are coaligned, although the Skeletor antibody-labeled spindle appears broader than the microtubule spindle (B). During telophase the Skeletor antibody labeled spindle persists in the central region where midbody formation of the microtubules is found to take place (C). QuickTime movies of 3D reconstructions of Skeletor and tubulin labelings

reorganization during prophase. Thus, if Megator plays multiple functional roles as its dynamic localization pattern suggests (Zimowska and Paddy, 2002), it would prevent the analysis of a mitotic function using RNAi approaches. Therefore, the further study of Megator function will require additional methods such as genetic approaches to generate new alleles that behave as conditional lethals or knockout-specific functions of the Megator protein or cell biological/biochemical approaches such as isolation of the matrix.

For the analysis of Chromator function using a mutant approach, a similar situation exists. A null Chromator allele has been isolated but homozygous animals die as embryos or first instar larvae, preventing the study of Chromator function during mitosis (Rath *et al.*, 2004). However, with the report of the generation of hypomorphic alleles of Chromator (Rath *et al.*, 2006), it may soon be possible to study Chromator's effect on cell division in larval neuroblasts. So far the best evidence for a functional role of a spindle matrix protein in mitosis was provided by Rath *et al.* (2004) using RNAi assays in S2 cells demonstrated that depletion of Chromator protein leads to abnormal spindle morphology and chromosomes are scattered in the spindle, indicating defective spindle function in the absence of Chromator (Fig. 4.6A, C). These types of defects would be expected if Chromator functions as a spindle matrix-associated protein that promotes interactions between motor proteins and a stationary scaffold and if these interactions were necessary for chromosome mobility. Interestingly, this phenotype resembles the mitotic chromosome segregation defects observed after RNAi knockdown of some kinesin motor proteins in S2 cells including KLP67A by Goshima and Vale (2003) and KLP59C by Rogers *et al.* (2004) (Fig. 4.6A, B). Thus, these data provide evidence that Chromator is a nuclear-derived protein that plays a role in proper spindle dynamics leading to chromosome separation during mitosis and are compatible with the hypothesis that Chromator may constitute a functional component of a spindle matrix molecular complex.

Although no Skeletor mutants have yet been characterized, studies of chromosome behavior in *east* loss-of-function mutations during mitosis and

can be accessed at http://www.jcb.org/cgi/content/full/151/7/1401/DC1. (D) The Skeletor antibody-labeled spindle (red) persists as an intact spindle extending across the metaphase plate as the chromosomes (blue) segregate to the poles. (E) Nocodazole-treated *Drosophila* embryo at metaphase triple-labeled with mAb1A1 (red), α-tubulin antibody (green), and Hoechst (DNA in blue). The microtubule spindles have completely depolymerized as indicated by the absence of microtubule labeling (green). The mAb1A1-labeled spindle (red) is still intact albeit slightly deformed demonstrating that this structure persists independently of the microtubule spindle. All panels represent confocal images. (F–H) Diagram of spindle matrix protein redistribution during mitosis. The spindle matrix is indicated in red, condensed chromosomes in blue, centrosomes and microtubules in green, and the nuclear lamina in yellow. (I) The spindle matrix hypothesis predicts impaired function of one or more spindle matrix proteins (gray) would lead to abnormal chromosome segregation and/or microtubule spindle defects. (Modified from *J. Cell Biol.* (2000). 151, 1401–1411. Copyright 2000 Rockefeller University Press.) (See also color insert.)

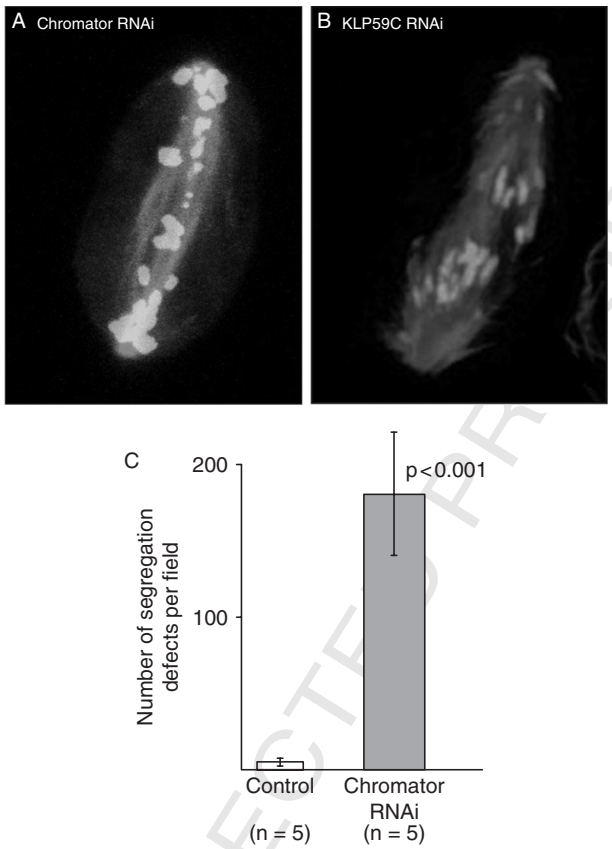

Figure 4.6 RNAi depletion of Chromator leads to spindle and chromosome segregation defects. (A) The most common phenotype in Chromator dsRNA-treated S2 cells of abnormally narrow spindles and mis-segregated chromosomes scattered throughout the spindle-region at anaphase. (B) The phenotype observed in Chromator dsRNAi-treated cultures (A) resembles that observed for dsRNAi depletion of the kinesin motor protein KLP59C (image courtesy of Dr. D. Sharp). Tubulin antibody-labeling is shown in blue and Hoechst labeling of the DNA is in red in (A) and (B). (C) Comparison of chromosome segregation defects in control and Chromator dsRNAi-treated cells. (See also color insert.)

meiosis suggested EAST may play an important role in the congression and alignment of chromosomes during prophase and prometaphase as well as in regulating chromosome movement at metaphase (Wasser and Chia, 2003). Abnormal chromosome localization in *east* mutants can be observed in prophase of male meiosis before nuclear envelope breakdown and before the chromosomes can establish interactions with microtubules (Wasser and Chia, 2003). These findings provide evidence that EAST may function to guide or constrain chromosome congression (Wasser and Chia, 2003). Moreover, the continued colocalization of EAST with Megator during prophase suggests

Megator may interact with EAST to play a role in this process as well and both EAST and Megator have important functions in nuclear reorganization during prophase.

3.4. Spindle length and the spindle matrix

The principle underlying how mitotic spindles maintain a uniform size has been a puzzle, especially when taking into account that their constituent microtubules are constantly undergoing dramatic fluctuations in length due to dynamic instability. This enigma did, however, give rise to the notion that a stabilizing structure such as a spindle matrix would be an elegant solution to account for this phenomenon (Mitchison *et al.*, 2005). Furthermore, differences in spindle matrix architecture could reasonably explain why spindles in closely related species or in different cell types within a single organism can vary significantly in length (Brown *et al.*, 2007). In attempting to identify the principles governing spindle size, it has been found that perturbing a number of different spindle-related functions can result in changes in spindle length. For example, spindles can be elongated or shortened by the action of outward or inward sliding of overlapping antiparallel microtubules mediated by kinesin-5 or kinesin-14 family members, respectively (Ambrose and Cyr, 2007; Kapitein *et al.*, 2005; Mountain *et al.*, 1999; Sharp *et al.*, 1999, 2000c). Alternatively, kinesin-8 and kinesin-13 family members control spindle length by regulating microtubule depolymerization (Gandhi *et al.*, 2004, 2005b; Goshima and Vale, 2003; Maney *et al.*, 2001; Rogers *et al.*, 2004; Savoian *et al.*, 2004; Straight *et al.*, 1998; Walczak *et al.*, 1996) in a length-dependent manner (Varga *et al.*, 2006) as do microtubule-severing proteins such as katanin (McNally *et al.*, 2006). Besides motors and microtubule assembly regulators, chromatid cohesion factors have also been implicated in regulating spindle size (Goshima *et al.*, 1999). A steady-state spindle length has thus been proposed to be the consequence of a balance of forces governing microtubule polymerization dynamics, motor protein activity, and/or cohesion factor functions (Goshima *et al.*, 2005b; Odde, 2005). Alternatively, it has been proposed that a concentration gradient of morphogens diffusing from the chromosomes dictates spindle length (Karsenti and Vernos, 2001). In all of these models a common thread is that regulation of microtubule architecture is ultimately responsible for determining the final spindle length. Au1

However, a very intriguing set of experiments has prompted a reconsideration of the premise that microtubule architecture is solely responsible for the length of the spindle (Mitchison *et al.*, 2005). In this study different components proposed to govern spindle length were experimentally perturbed. To address the role of microtubule polymerization dynamics, Mitchison *et al.* (2005) examined *Xenopus* extract spindles treated with hexylene glycol (2-methypentane-2,4,-diol) or antibodies against MCAK

(a kinesin-13 formerly known as XKCM1), both of which result in microtubules (and spindles) growing in length and volume in a manner consistent with retention of MT treadmilling but loss of MT catastrophe or depolymerization. Interestingly, a large number of MTs appear to curve or buckle as the spindle elongates. In a second set of experiments spindle length became shorter as MTs were rapidly depolymerized using either nocodazole or a caged microtubule-depolymerizing drug (105D) that behaves similarly to nocodazole, albeit with 20-fold less potency. In these experiments the investigators had expected kinetochores would stretch apart due to forces generated as a result of kinetochores pulling the poles inward, but instead the distance between sister kinetochores actually decreased, indicating a loss (not gain) of tension. Furthermore, the kinetochore pairs often twisted laterally away from the spindle axis and k-fibers buckled. Especially with 105D the k-fiber MT bundles were not as efficiently depolymerized and showed major compression effects such as buckling and bending. This result was reminiscent of those reported in Spurck *et al.* (1990) and Snyder *et al.* (1991) in which a UV microbeam was used to sever all of the MTs across a half-spindle in metaphase whereupon there was a movement of the severed pole back toward the chromosomes and buckling of the microtubules. If microtubules were involved in pulling the poles together during spindle shortening, those microtubules would be expected to remain straight under the tension. Instead, in the Mitchison *et al.* (2005) experiments a complete through-focus image scan failed to reveal any straight bundles of microtubules connecting the poles or any sister kinetochore pairs still under tension, suggesting that when overlap microtubules are rapidly removed by 105D the poles are pulled (or pushed) together by something other than microtubules, a notion consistent with earlier "traction fiber" models for a spindle matrix (Forer, 1966; Forer and Wilson, 1994; Pickett-Heaps *et al.*, 1984, 1996, 1997; Sillers and Forer, 1983). Mitchison *et al.* (2005) hypothesized that an unidentified tensile element pulls the poles together and that this element acts to oppose elongation in unperturbed spindles. In considering possible candidates for such an element, Mitchison *et al.* (2005) proposed it might be provided by a membranous sheath surrounding the spindle or alternatively that it might be provided by an internal spindle matrix composed of an as-yet uncharacterized structural element.

3.5. Membranes and the spindle matrix

The potential contribution of membranes to spindle form and function has not been extensively addressed but a number of independent studies have found evidence for membranous structures present in some though not all spindles (Hepler, 1989). In some studies vesicular or tubular membrane elements were found to permeate the spindle and/or ensheathe the chromosomes (Moll and Paweletz, 1980; Paweletz and Fehst, 1984; Rieder and Nowogrodzki, 1983; Waterman-Storer *et al.*, 1993; Wise, 1984), whereas in

other cases membranes formed a "spindle envelope" encasing the spindle (Harel *et al*., 1989; Hepler, 1980; Kramer and Hawley, 2003; Motzko and Ruthmann, 1984; Stafstrom and Staehelin, 1984; Wise and Wolniak, 1984). From these studies it is not clear whether the membrane association with the spindle plays a functional role or is simply a way to apportion membrane components to daughter nuclei. However, the report of a functional requirement for a transmembrane protein associated with the spindle envelope (Kramer and Hawley, 2003) suggests the potential for an operative role in at least some spindle types. The *aberrant X segregation (Axs)* gene encodes a transmembrane protein in *Drosophila* oocytes that reorganizes during germinal vesicle breakdown from the outer nuclear membrane to a fusiform-shaped sheath encapsulating the spindle and remains associated with the central spindle through entry into anaphase (Kramer and Hawley, 2003). A female-specific dominant mutation in this gene, Axs^D, gives rise to shortened spindles as well as defects in chromosome alignment and achiasmate chromosome segregation (Kramer and Hawley, 2003; Whyte *et al*., 1993). Thus this protein, and by extension perhaps the spindle envelope, is required for proper spindle assembly and chromosome segregation. However colchicine treatment that disassembled the microtubules also disrupted Axs localization (Kramer and Hawley, 2003) so evidence that the spindle envelope provides a spindle matrix function distinct from the microtubules is lacking.

Nevertheless, one study on spindle membranes did provide compelling support for the existence if not the identity of a microtubule-independent spindle matrix. Rieder and Nowogrodzki (1983) performed an ultrastructural analysis of *Xenos* oocytes during the first meiotic division and observed that during late prophase the nuclear envelope became convoluted and fenestrated with vesicular and tubular membrane elements permeating the nucleoplasm and ensheathing the condensing tetrads. Remarkably, the tetrads condense and become aligned within the nucleus during late prophase in the complete absence of microtubules. Only after nuclear envelope breakdown initiates do microtubules invade the nuclear space. At that stage the microtubules appear in association with and parallel to the tubular membrane components of the meiotic apparatus. Rieder and Nowogradzki (1983) proposed that membranes associated with the spindle determine the orientation of spindle microtubules and play a role in regulating their formation, roles that would be consistent with that proposed for a spindle matrix.

3.6. Other molecular candidates for an internal spindle matrix structural element

3.6.1. NuMA and the pericentriolar matrix

In considering potential structural elements of a spindle matrix, NuMA was one of the first particularly attractive candidates for such a role. NuMA was originally described as a nuclear matrix protein recognized by human autoantigen antibodies found to redistribute to the spindle poles of the

mitotic apparatus during cell division (Lydersen and Pettijohn, 1980). Microinjection of anti-NuMA antibodies into cells at interphase blocked subsequent mitotic spindle formation, whereas injecting antibodies at metaphase only after spindles were fully assembled resulted in spindle collapse (Yang and Snyder, 1992). In addition to observing similar perturbations on spindle morphology and mitotic progression, another group reported antibody-injected cells often became micronucleated (Kallajoki *et al.*, 1991, 1993). Expression of a truncated NuMA construct lacking its globular head domain resulted in mitotic failure and micronucleation (Compton and Cleveland, 1993). Whereas NuMA had previously been proposed to serve as a nuclear matrix protein at interphase, these results suggested NuMA also plays an essential structural role for mitotic spindle organization and function.

NuMA encodes a large (~230 kD) protein consisting of unique head and tail domains with a large internal coiled-coil domain (Compton *et al.*, 1992; Yang *et al.*, 1992) predicted to oligomerize (Parry, 1994). EM immunogold imaging analysis showed NuMA localized to core filaments of the nuclear matrix (Zeng *et al.*, 1994) but from these studies it was not clear whether NuMA simply decorated the fibers or whether it formed the structural basis of these filaments. However, when NuMA was retained in the cytoplasm by removing its nuclear localization sequence (NLS), it formed networks of interconnected 5-nm filaments of pure NuMA protein (Saredi *et al.*, 1996) indicating that NuMA does indeed have the capacity to independently form a matrix-like structure. Several other studies have confirmed that full-length NuMA can also self-assemble into large matrices (Gueth-Hallonet *et al.*, 1998; Harborth *et al.*, 1999; Saredi *et al.*, 1997) but interestingly, underscoring the "dynamic nature" of the spindle, the majority of NuMA (>80%) in the cell appears to undergo continuous exchange between soluble- and spindle-associated pools as determined by fluorescence recovery after photobleaching (FRAP) analysis (Kisurina-Evgenieva *et al.*, 2004). Because NuMA has been shown to directly bind and bundle microtubules (Haren and Merdes, 2002) and immunodepletion of NuMA from *in vitro* mitotic assembly extracts causes spindles to develop into irregular, unfocused MT arrays, it was proposed that a NuMA-based matrix structure acts to stabilize the mitotic spindle poles (Merdes *et al.*, 1996). In this same study, Merdes *et al.* (1996) observed that NuMA is found in a complex with dynein and dynactin that they proposed localizes NuMA to the poles where it then forms a matrix that promotes and stabilizes the fusiform spindle. This notion is supported by experiments showing that cell free assembly systems require the presence of NuMA to organize MTs into mitotic asters (Gaglio *et al.*, 1995). By immunogold EM techniques NuMA was found to localize to an electron-dense matrix at the spindle pole and in cell fractionation experiments it was retained predominantly in the insoluble fraction, even after nocodazole treatments had shifted tubulin

and conventional MAPs into the soluble fraction (Dionne *et al.*, 1999). Thus, NuMA was proposed to comprise part of the mitotic spindle matrix and it was envisioned that this matrix functioned to organize MT minus ends and counterbalance the forces exerted by microtubule motors at the spindle pole (Dionne *et al.*, 1999). However, NuMA shows a pericentrosomal localization to the spindle at metaphase, and does not form a complete spindle. Thus NuMA may contribute to the spindle matrix, but it is likely there are additional components essential for providing a complete framework.

3.6.2. Fin1p, Ase1p, and the midzone matrix

One candidate for a filamentous molecule that extends throughout the mitotic spindle was reported in a study by van Hemert *et al.* (2002) where they identified a novel *Saccharomyces cerevisiae* 14-3-3-interacting protein that they named Fin1p (for *f*ilaments *i*n between *n*uclei). This protein contains two putative coiled-coil domains suggesting a potential for self-assembly. To test this possibility van Hemert *et al.* (2002) found that 6xHis-tagged Fin1p purified from yeast extract could self-assemble *in vitro* into 10-nm filamentous structures independently of microtubules or other proteins. When a GFP-tagged Fin1p was introduced into yeast cells the distribution of Fin1p dynamically reorganized during the cell cycle from a nonfilamentous nuclear form in nondividing cells to a filamentous structure that colocalized with spindle microtubules extending between the two nuclei of dividing cells during mitosis. More recently, using a 13xmyc-tagged Fin1p construct expressed at endogenous levels Woodbury and Morgan (2007) observed that Fin1p's targeting to spindles occurs specifically at anaphase and only after it is dephosphorylated by the Cdc14 phosphatase. Mutation of Fin1p's consensus phosphorylation sites to alanine ($Fin1^{5A}$) results in a dominant-lethal phenotype but $Fin1^{5A}$-GFP can be expressed in cycling cells using an inducible GALS promoter (Woodbury and Morgan, 2007). Although these cells showed a reduced growth rate and premature association of $Fin1^{5A}$-GFP with the spindle prior to anaphase, there were no obvious spindle defects. In contrast, when $Fin1^{5A}$-GFP was overexpressed in metaphase-arrested cells, the spindles collapsed, yielding unseparated spindle poles and unusually long astral microtubules. Thus, in cycling cells mislocalization of $Fin1^{5A}$ may cause microscopically undetectable spindle defects that impair chromosome segregation ultimately leading to cell lethality, a notion that was supported by finding a sevenfold increase in chromosome loss after a transient, sublethal pulse of $Fin1^{5A}$ was delivered. Although expression of $Fin1^{5A}$ caused metaphase spindle collapse, it appears to play a role in stabilizing anaphase spindles, as shown using an artificial anaphase system in which metaphase-arrested cells can be triggered to enter anaphase by induction of the TEV protease (Higuchi and Uhlmann, 2005; Uhlmann *et al.*, 2000). In this system sister chromatids move to the poles but because cyclins are

stable, spindle elongation at anaphase is abnormal and spindles eventually break. The presence of wild-type Fin1, which due to cyclin stabilization remains phosphorylated and hence does not associate with the spindle, has no effect. However, nonphosphorylatable Fin1^{5A} does target to the spindle and greatly reduces spindle breakage frequency (Woodbury and Morgan, 2007). Interestingly, this requirement for a ''Fin1p matrix'' coincides with the transition of microtubules from a state of high dynamic instability at metaphase to suddenly becoming stable at anaphase (Higuchi and Uhlmann, 2005) and would support previous hypotheses for a role of the spindle matrix in stabilizing the microtubule spindle.

One potential inconsistency with the idea that Fin1p determines a spindle matrix scaffold required for stabilizing microtubules is the observation that deletion of Fin1 does not affect cell viability or cause any obvious spindle defects (van Hemert *et al.*, 2002; Woodbury and Morgan, 2007). However, just as establishment of a proper microtubule spindle is so critical to the cell that redundant pathways have evolved to ensure its successful formation, it is likely redundant pathways to build a spindle matrix have evolved as well. This idea is supported by the finding that a second coiled-coil protein, Ase1p, (*a*naphase *s*pindle *e*longation) has been identified that localizes to the spindle midzone, binds and bundles MTs, and is necessary to maintain anaphase spindle integrity (Loïodice *et al.*, 2005; Pellman *et al.*, 1995; Schuyler *et al.*, 2003). Similarly with FIN1, the null allele of ASE1 is viable (Pellman *et al.*, 1995). However, cells lacking both Fin1p and Ase1p are inviable (Woodbury and Morgan, 2007), consistent with the idea that they may comprise redundant molecular components of a spindle matrix.

3.6.3. Poly(ADP-ribose): A regulatory switch or a spindle matrix "gel"?

The addition of negatively charged poly(ADP-ribose) (PAR) moieties is an unusual and versatile post-translational modification originally linked to DNA damage detection and repair but now associated with an increasing number of biological functions including chromatin modification, transcription, and cell survival/cell death pathways (Kim *et al.*, 2005; Schreiber *et al.*, 2006). The surprising discovery of several different poly(ADP-ribose)polymerases (PARPs) on the mitotic spindle hinted at a potential role for ADP-ribosylation in spindle function as well (Earle *et al.*, 2000; Kickhoefer *et al.*, 1999; Smith, 2001; Smith and de Lange, 1999). This idea received experimental support when Chang *et al.* (2004) discovered that mitotic spindles contain approximately 10-fold higher levels of PAR than the surrounding cytoplasm and PAR levels and/or function are necessary for proper spindle assembly and structure. When PAR polymer levels were decreased enzymatically in preassembled spindles by poly(ADP-ribose)glycohydrolase (PARG), a highly specific processive endoglycosidase and exoglycosidase (Hatakeyama *et al.*, 1986), or functionally blocked with purified anti-PAR antibodies, Chang *et al.* (2004)

observed rapid breakdown of spindle structure with microtubules splaying outward and the two half-spindles becoming disconnected. In spindle assembly assays these treatments resulted in the formation of monopolar microtubule asters, suggesting PAR is not required for microtubule nucleation or dynamics but instead has a specific role in organizing the bipolar spindle, a role consistent with that proposed for a spindle matrix molecule.

The mechanism by which PAR regulates bipolar spindle formation is not known. Given that proteins can be reversibly poly(ADP-ribosyl)ated by the opposing activities of PARPs and PARGs and the significant biochemical consequences that result due to addition or removal of such large, negatively charged groups, one model postulates that PARsylation of proteins acts as a molecular switch regulating protein activity, much in the way phosphorylation does. In this case, the challenge is to identify the potential targets of PARsylation. Using RNAi approaches Chang *et al.* (2005a) identified tankyrase-1 as the PARP responsible for the spindle-associated PAR and one relevant target that has already been identified is NuMA (Chang *et al.*, 2005a,b). However, because PAR was found to extend across the entire spindle (Chang *et al.*, 2004) and is not restricted to the pericentrosomal region where NuMA resides, presumably there are other targets yet to be identified. PARsylation of spindle proteins might regulate their function or binding activities in an analogous manner to how tankyrase-1-mediated PARsylation regulates telomere length by inducing telomeric repeat binding factor 1 (TRF1) displacement to allow telomerase access (Chong *et al.*, 1995; Smith *et al.*, 1998). Au2

An alternative model posits that a PARsylated spindle matrix might be composed of a cross-linked poly(ADP-ribose) gel that stretches and stores elastic energy due to its attachment to spindle poles and plus end-directed motors (Mitchison *et al.*, 2005). The stored elastic energy would provide an explanation for the tensile forces such as were observed in Mitchison *et al.* (2005) and in earlier experiments (Forer, 1966; Forer and Wilson, 1994; Pickett-Heaps *et al.*, 1984, 1996, 1997; Sillers and Forer, 1983) that could not be ascribed to microtubules. Although the actual molecular composition of such a matrix is not yet defined, its regulation by PARsylation would provide a convenient tag to assist in its identification.

3.6.4. Lamin B and a Membranous Spindle Matrix

Early studies in *Drosophila* that had noted a mitotic "spindle envelope" also found that a fraction of the lamin T40 antigen (now known to be the lamin B homolog) remains associated with the mitotic apparatus (Harel *et al.*, 1989). The dynamics of mitotic spindle formation and nuclear lamina breakdown inferred a functional role for the lamina in mitotic spindle formation (Paddy *et al.*, 1996) but because in *Drosophila* a large fraction of the nuclear envelope remains localized to a rim in the nuclear periphery until well into metaphase (Paddy *et al.*, 1996), it was not clear whether this was a consequence of the

so-called ''semi-closed'' mitosis in *Drosophila*. However, the subsequent observation that lamin B also associates with mitotic spindles in mammalian cells (Beaudouin *et al.*, 2002; Georgatos *et al.*, 1997; Maison *et al.*, 1997) argues for a more widely conserved function for lamin B in the spindle. In *Caenorhabditis elegans* reduction of lamin B in early embryos resulted in a range of both nuclear and mitotic defects (Liu *et al.*, 2000). Nonetheless, it Au3 was difficult to differentiate cause and effect in these studies and a functional requirement for lamin in spindle assembly was not explored until more recently.

To assess the potential requirement in spindle assembly and/or function for LB3, the major lamin B isoform in *Xenopus* eggs (Lourim *et al.*, 1996), Tsai *et al.* (2006) took advantage of a powerful spindle assembly system in which spindles can be assembled in vitro from *Xenopus* M-phase extracts with or without depleting factors of interest (Lohka and Maller, 1985; Sawin and Mitchison, 1991; Sawin *et al.*, 1992). Immunostaining of spindles assembled in complete M-phase egg extract revealed the presence of LB3 in the spindle and peripheral region surrounding the spindle. Depleting LB3 from the extract severely disrupted the spindle assembly process with the vast majority of mitotic figures appearing as half spindles or asters indicating a requirement for the presence of lamin B for proper bipolar spindle assembly. To address whether the lamin B spindle localization defined a ''spindle matrix'' independent of microtubules, the authors treated normally assembled spindles with nocodazole to depolymerize the MTs. Despite the complete absence of spindle MTs lamin B3 remained spindle-associated, although it adopted a more granular and vesicular appearance suggesting the presence of membranes. To test whether membranes were associated with the spindle as well as the lamin B matrix, Tsai *et al.* (2006) used CM-diI, a membrane dye that is well retained throughout fixation and permeabilization. Both the complete assembled spindle and the nocodazole-generated lamin B spindle matrix preparations stained with CM-diI indicating the presence of membranes. But when exposed to Triton X-100 prior to fixation, the lamin B matrices were completely disrupted, suggesting this matrix is dependent on a membrane component. Although not examined in this study, it would be interesting to know whether, once assembled, the microtubule spindle is stable through Triton X-100 treatment or whether there is a functional requirement for continued presence of the lamin B matrix. *In vivo* data also supports the indication from the in vitro studies that lamin B plays a role in spindle assembly. Tsai *et al.* (2006) analyzed lamin B distribution in HeLa cells and found by immunostaining that a fraction of both lamin B isoforms (LB1 and LB2) was associated with mitotic spindles. Reducing expression of either lamin B isoform by siRNA resulted in spindle abnormalities including unfocused spindle poles, loss of chromosome congression, and defects in spindle morphology. However, because the lamin B appears to remain associated with membranes, it is not clear

how this mitotic spindle matrix may be organized or how it may relate to force production of the microtubule spindle as originally envisioned for a spindle matrix. It has therefore been suggested the assembly of this lamin B-containing membranous matrix in mitosis may provide a connection between microtubule spindle function and the partitioning of membrane systems during cell division (Zheng and Tsai, 2006).

3.6.5. Actin and myosin

A number of immunolocalization studies in a variety of systems have reported on the presence of actin and myosin in the mitotic spindle (Cande *et al.*, 1977; Czaban and Forer, 1992; Espreafico *et al.*, 1998; Forer and Jackson, 1976, 1979; Fujiwara and Pollard, 1976; Robinson and Snyder, 2005; Sampson, 2004; Sanger, 1975; Schmit and Lambert, 1987; Seagull *et al.*, 1987; Silverman-Gavrila and Forer, 2000a, 2003; Traas *et al.*, 1987; Yasuda *et al.*, 2005). However, other studies have failed to identify significant F-actin in the spindle proper (Clayton and Lloyd, 1985; Derksen *et al.*, 1986; Palevitz and Liu, 1992), yet others suggested that lack of antibody specificity or fixation artifacts may be responsible for apparent actin fibers in the spindle (Aubin *et al.*, 1979; Barak *et al.*, 1981; Schroeder, 1973). It is also possible that actin structures can be lost due to fixation artifacts (Traas *et al.*, 1987). In some cases few actin filaments were observed within the spindle, but an actin network instead appeared as a stretched, ''elastic cage'' encasing the microtubule spindle (Schmit and Lambert, 1987). In an EM study of HeLa cell mitotic spindles, Pollard *et al.* (1984) found no examples of long actin fibers but did detect a large number of short, actin-like filaments. Thus, the presence of actin or myosin in the spindle has historically been controversial.

Nevertheless, a variety of functional studies indicating a requirement for actin and myosin for proper spindle function have been reported and thus raise the prospect that an actin cytoskeletal system may be involved in spindle matrix function in at least some cell types. Inhibition of actin polymerization with cytochalasin D or latrunculin B induces abnormal chromosome alignment and segregation (Forer and Pickett-Heaps, 1998; La Fountain *et al.*, 1992; Sampson *et al.*, 1996). Treatment of cells with the Au4
myosin inhibitor 2,3-butadione monoxine (BDM) has been associated with aberrant chromosome movements on the spindle and a loss of microtubule poleward flux (Sampson, 2001; Silverman-Gavrila and Forer, 2000a,b, 2001, 2003). Although a study has called into question BDM's target specificity (Ostap, 2002), the observation that these same defects are also observed with actin inhibitors (Silverman-Gavrila and Forer, 2000a,b) suggests the relevant target in these studies was myosin. Analysis of the severed ''k-stubs'' created when kinetochore fibers are severed by UV microbeam revealed that in the presence of either actin or myosin inhibitors, subsequent fiber elongation is blocked (Forer *et al.*, 2007). Based on

these results, it was suggested that actin and myosin are involved in generating microtubule flux that results in elongation of kinetochore microtubules (Forer *et al.*, 2007; Silverman-Gavrila and Forer, 2000a). Because microtubule flux has also been attributed to motor activity at the poles (Goshima *et al.*, 2005a; Miyamoto *et al.*, 2004; Rogers *et al.*, 2005; Shirasu-Hiza *et al.*, 2004), there may be multiple mechanisms underlying flux generation or the motors that drive microtubule flux may require interaction with an actinomyosin-based spindle matrix. In this model actin and myosin are a functional component of a spindle matrix, generating a tensegrity structure that can provide the energy necessary to propel kinetochore fibers poleward (Pickett-Heaps and Forer, 2001; Pickett-Heaps *et al.*, 1984, 1996, 1997; Spurck *et al.*, 1997). This force is resisted by the microtubules which in this model are acting as struts (Forer, 1974) and whose rate of disassembly thus determines the rate of chromosome segregation (Forer and Wilson, 1994; Pickett-Heaps *et al.*, 1986, 1997).

One issue that may underlie the difficulty in firmly establishing a connection between an actin spindle matrix component and microtubule function in the spindle is that due to a variety of technical reasons it has been difficult to document interactions between the two systems. These reasons include difficulties fractionating two filamentous, polymeric entities and different fixation conditions required for each to optimize preservation for fluorescent imaging studies, as well as masking of less abundant F-actin structures by the high local concentration of cortical F-actin (discussed in Sider *et al.*, 1999 and references therein). Sider *et al.* (1999) exploited the *Xenopus* oocyte extract system to examine whether microtubules and F-actin could interact and observed a consistent lengthwise colocalization of F-actin with astral microtubules that was correlated with a bending or kinking of microtubules similar to that previously described *in vivo* in lamellipodia by Waterman-Storer and Salmon (1997). Interestingly, the association between microtubules and F-actin was dependent on the presence of one or more microtubule-associated proteins (MAPs), because it was not observed to occur in assays with purified brain tubulin and muscle F-actin unless oocyte microtubule-binding proteins were included in the assay (Sider *et al.*, 1999). Examples of microtubule-actin interactions have now been observed to play an important role in directed cell migration, neuronal growth cone guidance, wound healing, cytokinesis, and cortical flow (Rodriguez *et al.*, 2003). In addition, the MyTH4-FERM domain Au5
cassette present in several unconventional myosins has, in the case of myosin-10, been demonstrated to bind microtubules directly (Weber *et al.*, 2004). Thus, there is precedence for functional interactions between the two cytoskeletal systems.

Furthermore, some cells appear to be dependent on the actinomyosin cytoskeletal system for proper mitotic spindle assembly. Rosenblatt *et al.* (2004) found that perturbation of myosin II by either depleting protein by

RNAi or by inhibiting myosin activity with blebbistatin or inhibiting actin polymerization with latrunculin B led to defects in centrosomal separation and abnormalities in spindle formation. However, in this case they identified cortical myosin as the necessary player and proposed that astral microtubule interaction with the cortex plays a role in triggering centrosomal separation (Rosenblatt *et al.*, 2004). They did not find a requirement for actin or myosin after nuclear envelope breakdown had occurred. In another study in starfish oocytes, nuclear envelope breakdown was found to trigger actin polymerization required for efficient chromosome capture by the spindle during the first meiotic division (Lénárt *et al.*, 2005). Chromosomes or DNA-coated beads were observed to trigger the formation of actin patches connected to each other by a network of actin filaments. Contraction of this network delivered the embedded chromosomes to the animal pole where they could then be captured by spindle microtubules (Lénárt *et al.*, 2005). When the actin cytoskeleton was disrupted by latrunculin B, chromosome congression failed in 75% of the cells resulting in chromosomes loss and aneuploid eggs (Lénárt *et al.*, 2005). Defects in spindle formation have also been observed in *Xenopus* oocytes when the actin cytoskeleton was disrupted with cytochalasin B (Gard *et al.*, 1995; Ryabova *et al.*, 1986). Thus, cells with large nuclear volumes such as oocytes appear to utilize an actin-based system to propel chromosomes toward the centrosomes to increase the efficiency of the microtubule-based "search and capture" mechanism for spindle assembly (Lénárt *et al.*, 2005).

Thus, although the role of actin and myosin in cytokinesis is well established (Glotzer, 2005), their requirement for mitotic spindle assembly or function has been debated over the years. Mutations in myosin II in *Schizosaccharomyces pombe* and *Dictyostelium* did not lead to apparent mitotic defects (Bezanilla *et al.*, 1997; de Hostos *et al.*, 1993) and RNAi depletion of myosin II appeared to affect only cytokinesis (Somma *et al.*, 2002). Perturbing myosin function by injection of anti-myosin II antibodies or myosin II fragments into amphibian eggs did not have any apparent effect on spindle formation or function, though after one cell division subsequent cell cycles were blocked (Kiehart *et al.*, 1982; Meeusen *et al.*, 1980). Furthermore, actin depolymerizing drugs do not prevent spindle formation or function in *Xenopus* egg extracts (Desai *et al.*, 1998, 1999; Sawin and Mitchison, 1991). Interestingly, in some studies where inhibition of actin or myosin blocked anaphase chromosome movements, such blockage was only temporary with chromosomes resuming movement, albeit more slowly, after a delay (Fabian and Forer, 2005; Forer and Pickett-Heaps, 1998; Silverman-Gavrila and Forer, 2001). Thus, contributions of the actinomyosin cytoskeletal system to force production in the spindle may reflect the evolution of multiple, independent mechanisms to create an operational spindle to reliably power chromosome movement despite the diverse array of physiological demands

Au6

placed upon different cell types in the functioning organism (Fabian and Forer, 2005).

3.7. Spindle assembly factors (SAFs) and the spindle matrix

The development of an *in vitro* spindle assembly system from *Xenopus* egg extracts (Lohka and Maller, 1985; Sawin and Mitchison, 1991) has provided a very powerful system in which to dissect out some of the molecular requirements for spindle assembly. Whereas the first models of spindle assembly had been almost exclusively based on the "search and capture" principle in which two opposing centrosomes nucleated microtubules that were stabilized after capturing a kinetochore, thus generating a bipolar spindle (Compton, 2000), subsequent studies employing the *Xenopus in vitro* assembly system revealed that chromatin-coated beads were also highly efficient at promoting formation of bipolar spindles (Heald *et al.*, 1996) and further analysis with this system identified that it was the gradient of RanGTP generated by the chromosomes that was responsible for this activity (Carazo-Salas *et al.*, 2001). Both the spindle localization and MT stabilizing effects of RanGTP were subsequently confirmed *in vivo* in the *Drosophila* embryo system (Trieselmann and Wilde, 2002) and in HeLa cells using a fluorescent biosensor that shows increased fluorescence resonance energy transfer (FRET) signal when liberated from importin-β by RanGTP (Kaláb *et al.*, 2006). The discovery that RanGTP regulates spindle assembly was somewhat unexpected as the focus on Ran had previously uncovered its critical role in regulating transport of RNA and proteins between the nucleus and cytoplasm (Moroianu, 1999). Ran is an abundant, small G-protein that when in the nucleus primarily exists in its GTP-bound form due to the activity of the chromosomally localized GEF (guanine nucleotide exchange factor) RCC1 (the regulator of chromosome condensation) and the high nuclear GTP concentration. RanGTP promotes the dissociation of importin β-like nuclear transport receptors from their cargoes upon reaching the nuclear interior, a critical part of the nuclear import pathway. Among the cargoes imported into the nucleus in this pathway are a number of spindle assembly factors (Goodman and Zheng, 2006). Nuclear localization of these factors sequesters them from the microtubules during interphase. However, upon nuclear envelope breakdown the mixing of nuclear and cytoplasmic compartments results in nuclear SAFs becoming bound and thus inactivated by available nuclear import receptors. RanGTP is able to stimulate MT assembly in the vicinity of the chromosomes as a consequence of the chromosomal RCC1 RanGEF activity that produces a gradient of RanGTP (Carazo-Salas *et al.*, 1999). Just as nuclear RanGTP acts to release cargo from the receptor during the import process, the binding of RanGTP to the nuclear import receptor in the proximity of chromosomes results in release of the SAFs, which are then free to stabilize

MTs and thereby promote spindle assembly (Dasso, 2001; Goodman and Zheng, 2006; Walczak, 2001). One such SAF is NuMA, a protein that had already been proposed to comprise part of the spindle matrix. This raises the question of whether other spindle matrix-associated components may be similarly regulated by RanGTP. A number of RanGTP targets have been identified, although it is not yet known if they mediate their activities in conjunction with the spindle matrix.

3.7.1. TPX2

TPX2 (*T*argeting *P*rotein for *X*klp*2)* was identified as a microtubule-associated protein that directly links the motor protein Xklp2 to microtubules (Wittmann *et al.*, 1998). TPX2 is a basic 82.4-kDa protein with two coiled-coil domains (Wittmann *et al.*, 2000) required for bipolar spindle formation in *in vitro* assembly assays using chromatin beads (Gruss *et al.*, 2001) and in *vivo* studies in HeLa cells (Gruss *et al.*, 2002). TPX2 is inactivated by importin-β via the adaptor protein importin-α. Release of TPX2 from importin-α/β by Ran-GTP frees it to promote spindle assembly (Gruss *et al.*, 2001) Although TPX2 was originally defined as a microtubule associated protein (MAP) (Wittmann *et al.*, 1998) and its colocalization with the lamin B spindle matrix was dependent on the presence of microtubules (Tsai *et al.*, 2006), the report that addition of dominant negative lamin constructs in the assay prevented association of any of the SAFs studied (Tsai *et al.*, 2006) suggests TPX2 predominantly interacts with the microtubule spindle but its association may also be affected by a spindle matrix.

3.7.2. TACC (D-TACC and maskin)

TACC (*T*ransforming *A*cidic *C*oiled *C*oil) proteins were first identified in humans where genomic rearrangements involving the genes encoding these proteins were associated with different types of cancers (Chen *et al.*, 2000; Lauffart *et al.*, 2003; Pu *et al.*, 2001; Still *et al.*, 1999a,b). In addition to being highly acidic, the common feature of these proteins was a highly conserved 200-amino acid coiled-coil domain termed the TACC-domain but the biological function(s) of these proteins was not known. Using a ''reverse genetics'' approach in *Drosophila* in which microtubule-binding proteins were isolated from microtubule affinity columns for subsequent molecular, cellular, and genetic characterization (Kellogg *et al.*, 1989), a protein containing a TACC domain was identified and named D-TACC (Gergely *et al.*, 2000b). Further analysis showed D-TACC is an ~220 kDa centrosomal and mitotic spindle protein required for mitotic spindle function. A hypomorphic mutation in the *d-tacc* gene resulted in many embryos showing mitotic defects with many failing to develop beyond the first mitotic division (Gergely *et al.*, 2000b). Although a D-TACC-GFP fusion protein primarily concentrated to the spindle poles, it also oscillated to and from the centrosomes consistent with an association with microtubule

Au7

plus-ends (Lee *et al.*, 2001). In this same study, Lee *et al.* (2001) found D-TACC associates with Mini-spindles (Msps), the *Drosophila* homolog of XMAP215/ch-TOG (Cullen *et al.*, 1999). XMAP215 plays a critical role in the regulation of MT dynamics by modulating plus-end behavior, both by promoting microtubule assembly (Charrasse *et al.*, 1998; Gard and Kirschner, 1987; Vasquez *et al.*, 1994) and disassembly (Shirasu-Hiza *et al.*, 2003) and its activity is essential for centrosome integrity, spindle pole organization, and bipolar spindle assembly (Cassimeris and Morabito, 2004; Gergely *et al.*, 2003). Thus a model was proposed that centrosomal D-TACC was responsible for loading Msps (*Drosophila* XMAP215) onto the ends of growing microtubules to regulate the microtubule dynamics required for spindle assembly, in particular enhancing MT growth from the centrosome (Lee *et al.*, 2001). D-TACC appears to comprise part of the pericentriolar matrix and maintains its centrosomal localization even after the microtubules have been depolymerized by colchicine (Gergely *et al.*, 2000b), a finding that was also confirmed for the human TACC proteins (Gergely *et al.*, 2000a).

A single TACC homolog had also been previously identified in *Xenopus* oocytes where, due to its activity as an mRNA-binding protein that represses polyadenylation and translation of certain maternally provided stores of mRNA, it had been named Maskin (Stebbins-Boaz *et al.*, 1999). Maskin had been found at the mitotic spindle where it was proposed to regulate localized cyclin B1 mRNA translation during the cell cycle (Groisman *et al.*, 2000) but, more recently, using the *Xenopus* spindle assembly Au8
assay system Maskin has been shown by two independent groups to also play a direct role in mitotic spindle assembly (O'Brien *et al.*, 2005; Peset *et al.*, 2005). Depletion of maskin resulted in small asters, poorly organized spindles with reduced numbers of microtubules, and misaligned chromosomes (O'Brien *et al.*, 2005; Peset *et al.*, 2005). Maskin associates with XMAP215 and its activation and localization to the centrosome is regulated by phosphorylation by the Aurora A kinase (Kinoshita *et al.*, 2005; O'Brien *et al.*, 2005; Peset *et al.*, 2005). Although Maskin lacks a conventional nuclear localization signal it is still able to bind importin-β, maintaining it in an inactive state until the presence of RanGTP promotes its release (Albee *et al.*, 2006) placing it in the family of Ran-regulated SAFs.

3.7.3. NuSAP

Raemaekers *et al.* (2003) identified NuSAP (*Nu*cleolar *S*pindle-*A*ssociated *P*rotein) as a 55 kDa, basic protein found at increased levels in dividing cells that contains an N-terminal SAP domain, a helix-extension-helix motif that is implicated in organizing nuclear architecture by binding MARs (AT-rich nuclear *M*atrix *A*ttachment *R*egion*s* of the DNA) and/or RNA (Aravind and Koonin, 2000), and a C-terminal–charged helical domain. Immunolocalization revealed NuSAP is primarily nucleolar at interphase but

relocalizes to the spindle at metaphase. An *in vitro* sedimentation assay showed purified, recombinant NuSAP is able to directly bind to microtubules via its C-terminal domain suggesting NuSAP may play a role in regulating microtubule dynamics. Indeed, overexpression of NuSAP in COS cells resulted in the appearance of long, curved, highly bundled microtubules that were extremely stable even in the presence of nocodazole, whereas reduction of NuSAP delayed mitotic entry, resulting in defects in chromosome condensation, chromosome alignment, and spindle organization (Raemaekers *et al.*, 2003). NuSAP is regulated by RanGTP in Au9
a complex manner as it must release the blocking of NuSAP's microtubule-stabilizing activity mediated by importin-α and importin-7 as well as the blocking of NuSAP's MT cross-linking activity mediated by importin-β (Ribbeck *et al.*, 2006). The cross-linking activity of NuSAP was observed to be especially dramatic, resulting in the formation of large networks of bundled microtubules that included not only intact microtubules but also other polymerization intermediates including protofilament sheets (Ribbeck *et al.*, 2006). In biochemical reconstitution experiments, NuSAP efficiently adsorbs to chromatin or DNA where it can efficiently promote microtubule formation and retention (Ribbeck *et al.*, 2007). This ability to stabilize such a meshwork of microtubules around the chromosomes may serve a critical role in stabilizing the nascent spindle structure as it encounters forces and counterforces during assembly. It is not currently known whether, in addition to binding microtubules, NuSAP interacts with a spindle matrix. However, the presence of a SAP domain implicated in interacting with nuclear matrix attachment sites during interphase raises the tantalizing prospect that NuSAP may serve as a bridge between the spindle matrix and the microtubule spindle.

3.7.4. HURP

HURP (*H*epatoma *U*p-*R*egulated *P*rotein) had previously been identified as a cancer-related marker for detecting transitional cell carcinoma (Chiu *et al.*, 2002) but its cellular function was not known. Using proteomic, biochemical fractionation, and microarray expression approaches, three different labs independently identified HURP's involvement in mitosis and further characterized it as playing an essential role in spindle organization including mediating k-fiber stabilization and chromosome congression (Koffa *et al.*, 2006; Silljé *et al.*, 2006; Wong and Fang, 2006). HURP was found to be a direct cargo of importin-β released by high concentrations of Ran-GTP whereupon it localizes to kinetochore MTs near the chromosomes. Western blot and mass spectroscopy analysis revealed that HURP migrated at several different sizes including a high molecular weight form that appeared to represent a covalently linked dimeric or oligomeric species, raising the possibility of it forming a meshwork (Koffa *et al.*, 2006). In this latter study, HURP was isolated as part of a complex that includes TPX2,

Aurora A, XMAP215 and Eg5, four Ran-GTP–regulated SAFs that have been suggested to interact with the lamin B spindle matrix (Tsai *et al.*, 2006). The immobility of Eg5 on bipolar spindles treated with a moderate dose of monasterol (an Eg5 inhibitor) relative to the vigorous continued flux of tubulin at this monasterol concentration has been interpreted to indicate a static spindle matrix (Kapoor and Mitchison, 2001). Thus, although HURP's potential association with a spindle matrix has not yet been examined, it appears to show many characteristics consistent with such a role. The demonstration that HURP can polymerize free tubulin *in vitro* into a new configuration composed of antiparallel protofilaments wrapped around the growing end of a normal microtubule could suggest a novel mechanism for regulating MTs, although it is not yet known whether this activity is biologically relevant (Santarella *et al.*, 2007).

3.7.5. Rae1

Rae1 is another direct cargo of importin-β released (activated) by high concentrations of Ran-GTP. A role for the Rae1 RNP complex in regulation of MT dynamics was uncovered by Blower *et al.* (2005) using *in vitro* Au10
assembly assays to identify factors necessary to promote Ran-GTP–induced aster formation. Rae1, which was previously known for its role as an mRNA export factor (Pritchard *et al.*, 1999), was found to associate with spindle microtubules, with the highest levels at the poles, as well as with the aligned chromosomes. Rae1 apparently does not compose a MT-independent structure, as its spindle—but not chromosomal—localization was lost after nocodazole treatment (Blower *et al.*, 2005). Rae1 is found in an RNP Au11
complex with at least 10 other polypeptides and Rae1's aster- and spindle-promoting activity requires the presence of other cofactors including RNA, as RNase treatment blocked assembly. Propidium iodide staining of *in vitro*-assembled spindles revealed the presence of RNA on and adjacent to the mitotic microtubules. Given that Rae1 contains four β-propeller WD-repeats, a domain proposed to serve as a scaffolding platform (Li and Roberts, 2001), Blower *et al.* (2005) speculate that Rae1 may function as a Au12
scaffold that tethers functionally important factors to the mitotic microtubule network.

4. Concluding Remarks

As reviewed here, multiple studies spanning the evolutionary spectrum from lower eukaryotes to vertebrates have provided new and intriguing evidence that a spindle matrix may be a general feature of mitosis. Nonetheless, definitive evidence for its molecular nature and for its role in microtubule spindle function is still lacking. Considering the diversity of

potential and unrelated spindle matrix molecules so far described the possibility exists that different molecules may have attained the same function in different organisms. Alternatively, this could reflect the presence of multiple redundant systems or that different spindle matrices are operational at different stages of the cell cycle or in different cell types. Thus, the challenge remains to directly demonstrate a stationary or elastic molecular matrix independent of microtubule polymerization contributes to microtubule spindle function and/or assembly. However, with the identification of several potential spindle matrix molecules in experimentally tractable systems such experiments may soon be forthcoming. Especially the characterization of a multi-protein spindle matrix complex in *Drosophila* where a wide range of genetic and biochemical approaches are available promises to provide an avenue to directly test the spindle matrix hypothesis and to give insight into its functional dynamics. For example, mutagenesis strategies can be applied to generate a range of mutant alleles that will allow a genetic dissection of the functional requirements of the spindle matrix proteins in different cellular contexts. Such alleles can also be used to explore the potential interaction of spindle matrix proteins with microtubule spindle assembly factors and microtubule-based motors. If spindle matrix proteins form a structural component essential for microtubule spindle function the expectation is that microtubule spindle assembly and alignment will be impaired with abnormal congregation and segregation of chromosomes in dividing cells with spindle matrix protein mutations.

Another interesting issue that can be addressed in live preparations in *Drosophila* by creating transgenic animals with fluorescently tagged spindle matrix proteins is whether nuclear proteins reorganize and begin to form a fusiform spindle structure prior to nuclear envelope breakdown and microtubule invasion of the nuclear space. Immunocytochemical studies of fixed preparations and analysis of cell division in *east* mutants carried out thus far suggest that the spindle matrix in *Drosophila* begins to form independent of microtubule assembly and additionally may play an important role in guiding chromosome congression. However, it is also possible the microtubule spindle apparatus forms independently and the alignment with the spindle matrix complex is a secondary process. If that is the case the spindle matrix may only serve to provide structural support.

Although the studies described here support the spindle matrix hypothesis and indicate it plays an important role in microtubule assembly and function, an alternative hypothesis for spindle matrix function is that it serves as a means to distribute important nuclear components to the forming daughter nuclei. One major strategy to accomplish this goal is exemplified by structural components of the nucleus such as the nucleolus and the nuclear lamina that are completely dismantled and reassembled in the forming daughter nuclei only after chromosome segregation. Many of the proteins making up these structures are either degraded or are recycled through incorporation into vesicles

(Moir *et al.*, 2000; Olson *et al.*, 2000). Other proteins known as ''the chromosomal passengers'' become associated with the condensing chromosomes during prophase, accumulate at the inner centromeres in prometaphase, then at the onset of anaphase leave the chromosomes and transfer to the central spindle before concentrating at the midbody at cytokinesis (Vagnarelli and Earnshaw, 2004). The ''chromosomal passenger protein complex'' (Adams *et al.*, 2001; Terada, 2001) has been functionally implicated in chromosome condensation and segregation as well as in completion of cytokinesis. The spindle matrix complex through interactions with the microtubule spindle may play a similar role in assuring equal distribution to the daughter nuclei of essential proteins that for structural reasons are difficult to degrade or resynthesize and reassemble on a rapid time scale. However, it should be noted that this hypothesis is not mutually exclusive with the ''spindle matrix hypothesis'' in which the spindle matrix proteins play an important role in chromosome congregation and segregation as well as in microtubule spindle function. Thus, the further study of the cellular and molecular biology of the spindle matrix promises to provide important new information on the highly choreographed process for how chromosomes and/or nuclear proteins are segregated during mitosis.

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