Molecular mechanisms of spindle function
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Abstract

The key molecules involved in regulating the assembly and function of the mitotic spindle are shared by evolutionarily divergent species. Studies in different model systems are leading to convergent conclusions about the central role of microtubule nucleation and dynamics and of kinesin-related motor proteins in spindle function.

The faithful segregation of genetic material to daughter cells is essential for the survival of an organism. The cell has an elegant macromolecular machine, the mitotic spindle, made up of microtubules and associated proteins, to segregate its chromosomes. Microtubules are dynamic, polar polymers composed of α-β tubulin heterodimers that are nucleated from the centrosomes and extend to the spindle equator where they interact with the chromosomes. The chromosomes are attached to the spindle via kinetochores, specialized proteinaceous structures at the centromere that interact with microtubules.

The study of spindle function has evolved dramatically during the last 50 years. Work in the 1950s-1980s focused primarily on cytological observation of spindle function through ever-improving microscopy techniques. These studies provided an observational account of the precise movements achieved by the chromosomes and other partners in the spindle. The 1990s were the molecular years, when many of the key players in spindle assembly were identified and their precise roles began to be elucidated. The next decade should offer great additional insights into the mechanism of mitosis as many key components are achieved by the chromosomes and other partners in the spindle. The 1990s were the molecular years, when many of the key players in spindle assembly were identified and their precise roles began to be elucidated. The next decade should offer great additional insights into the mechanism of mitosis as many key components are achieved by the chromosomes and other partners in the spindle.
Interestingly, klpA is the γ-tubulin gene that requires pkl1 for viability [2]. Several phenotypes were observed in this γ-tubulin mutant. At 19°C bipolar spindles formed and elongated, but chromosome segregation was impaired. Paluh and colleagues also examined the microtubule cytoskeleton in these cells and found that microtubules were hyper-elongated, resulting in long microtubule bundles that wrapped around the cell. The work in S. pombe is consistent with reports from A. nidulans: Oakley’s group isolated point mutations in the γ-tubulin gene and then looked for genetic interactions with other spindle components [3]. They found that a null mutation in the KRP gene klpA caused a dramatic increase in the cold-sensitivity of two γ-tubulin alleles, which could be suppressed by the anti-microtubule drug benomyl. Several phenotypes were observed in this γ-tubulin mutant. At 19°C bipolar spindles formed and elongated, but chromosome segregation was impaired. Paluh and colleagues also examined the microtubule cytoskeleton in these cells and found that microtubules were hyper-elongated, resulting in long microtubule bundles that wrapped around the cell. The work in S. pombe is consistent with reports from A. nidulans: Oakley’s group isolated point mutations in the γ-tubulin gene and then looked for genetic interactions with other spindle components [3]. They found that a null mutation in the KRP gene klpA caused a dramatic increase in the cold-sensitivity of two γ-tubulin alleles, which could be suppressed by the anti-microtubule drug benomyl. Interestingly, klpA is the A. nidulans homolog of the S. pombe pkl1, suggesting conservation of a functional pathway.

In C. elegans, a slightly different approach resulted in similar conclusions about the same process [4]. RNAi was used to deplete γ-tubulin in early embryos. Embryos that had been treated with γ-tubulin RNAi contained bipolar spindles with numerous long astral microtubules but very few microtubules between the poles. These embryos had chromosomes that did not align properly on the spindle and failed to segregate during anaphase. Taken together, all of the results described above suggest that γ-tubulin must play an additional role in the spindle other than nucleation of microtubules from the centrosomes. It appears that this additional role may involve controlling microtubule dynamics, and that the combined effects of a defect in nucleation and a defect in dynamics result in defective chromosome segregation.

**Microtubule dynamics**

In addition to microtubule nucleation, microtubule dynamics must be precisely controlled to allow formation of a spindle. In the self-assembly pathway of spindle formation, microtubule dynamics must be regulated in the region around chromatin to allow for the local nucleation of microtubules that will be sorted and arranged into a bipolar spindle. An in vitro reconstitution of spindle assembly that recapitulates these events can be achieved using cytoplasmic extracts from Xenopus eggs. This system has been very useful in examining protein function during spindle assembly and chromosome segregation. In egg extracts, stathmin/Op18 may be a key component in regulating microtubule dynamics in the region of chromatin [5]. Op18 is a small heat-stable protein that destabilizes microtubules in vitro, in cell extracts and in intact cells (reviewed in [6]). It is regulated by phosphorylation; hyperphosphorylation of Op18 inactivates the protein, making microtubule polymerization more favorable. It has been shown previously that Op18 becomes hyperphosphorylated in the presence of chromatin, suggesting the existence of a chromatin-associated Op18 kinase.

Heald and coworkers described the development of a kinase assay to examine the effects of Op18 phosphorylation and to identify this kinase [7,8]. Xenopus Op18 is normally phosphorylated on three residues: Ser16, Ser25 and Ser39. Two triple mutants were created and then analyzed. Mutation of all three serines to alanine (AAA mutant) should generate a constitutively inactive protein, because the glutamates should mimic phosphorylated residues. Addition of wild-type Op18 to Xenopus egg extracts resulted in small spindles whereas addition of AAA Op18 generated virtually no spindles, and extract with EEE Op18 looked very similar to controls with no Op18. The effects of the different proteins on microtubule polymer levels in extracts when examined by a microtubule sedimentation assay were similar to their respective effects on extracts. By contrast, addition of wild-type Op18 or any of the mutated Op18 derivatives to purified microtubules without the cytoplasmic extract results in microtubule destabilization. These results suggest that extracts contain other factors that can modulate the activity of Op18. The identification of these additional factors, as well as the identification of the chromatin-associated kinase that regulates Op18 activity, will be important areas of future study.

**Motor proteins**

The proper assembly and function of the mitotic spindle would not be possible without the presence of motor proteins,
which couple the energy of ATP hydrolysis to force production. Studies in multiple systems, such as the yeast *Saccharomyces cerevisiae* and HeLa cell extracts have established the concept that antagonistic forces by multiple motor proteins are essential for spindle assembly and function (reviewed in [9]). Studies presented by Sharp and colleagues provide a beautiful illustration of how one can examine the antagonistic forces among different motor proteins in mitosis in vivo using early embryos of the fruitfly *Drosophila melanogaster* [10]. They focused their studies on three motor proteins known to be important for spindle assembly in flies: KLP61F, Ncd, and cytoplasmic dynein. KLP61F is a bipolar KRP proposed to cross-link anti-parallel microtubules to generate sliding forces in the mitotic spindle [11]. Sharp and colleagues examined the function of KLP61F by microinjection of antibodies into early *D. melanogaster* embryos that had previously been injected with fluorescently labeled tubulin so that mitosis could be followed with time-lapse imaging. Inhibition of KLP61F resulted in initial separation of centrosomes that then collapsed back together after nuclear envelope breakdown. These results suggest the presence of an antagonistic force acting against KLP61F and resulting in centrosome collapse. Sharp and coworkers suggested that this opposing force probably comes from the KRP Ncd, which also cross-links microtubules and plays a role in spindle assembly and chromosome segregation [12]. Injection of KLP61F antibodies into ncd null *D. melanogaster* embryos resulted in abnormal spindles, but centrosome collapse was never observed, suggesting that KLP61F and Ncd provide antagonistic forces for centrosome positioning. Another motor important in spindle function is cytoplasmic dynein. In *D. melanogaster*, cytoplasmic dynein is found localized at the cortex. Injection of inhibitory dynein antibodies or of p50 dynamin, which disrupts the dynein-dynactin complex, resulted in inhibition of bipolar spindle formation. Surprisingly, inhibition of both dynein and Ncd resulted in restoration of bipolar spindles. The working model is that dynein acts at the cortex to pull on astral microtubules, resulting in forces that keep centrosomes apart. Taken together, these antibody inhibition experiments reveal the complex integration of motor function that acts to maintain spindle bipolarity in living embryos.

The question of what drives spindle assembly and generates spindle bipolarity remains a mystery. One of the most amazing recent findings is that bipolar spindles can be assembled in the absence of centrosomes, kinetochores or DNA. Activation of the small GTPase Ran induces microtubule aster formation and bipolar spindle assembly (reviewed in [13]). Wilde [14] presented work analyzing the mechanism by which this could occur. Wilde rationalized that Ran must act by inducing microtubule formation, or by inducing microtubule organization, or both. One argument against the idea that Ran acts solely by inducing microtubule formation is that agents that stabilize microtubules, such as dimethylsulfoxide (DMSO) or taxol, induce aster formation but not bipolar spindle formation when added to *Xenopus* egg extracts. But addition of a dominant-active version of Ran to taxol- or DMSO-induced asters assembled in extracts stimulates bipolar spindle formation, favoring the idea that microtubule organization is affected. An analysis of the movements of stabilized microtubule seeds on asters revealed that Ran stimulated plus-end-directed seed motility (microtubule ‘plus’ ends are at the periphery of the aster), suggesting that a plus-end-directed motor might be activated. Wilde hypothesized that Eg5, a bipolar KRP shown previously to be important in establishing spindle bipolarity, might be a downstream target of Ran. Indeed, inhibition of Eg5 reduced the proportion of seeds moving toward the plus ends of microtubules, strengthening the idea that Eg5 might be stimulated after the addition of Ran. The idea that a motor required for spindle bipolarity is stimulated by Ran makes sense in understanding how Ran could induce bipolar spindle formation. Understanding the mechanism of this stimulation and identifying other players in this process will clearly be important areas of future research.

Once a bipolar spindle is assembled, the chromosomes must be aligned at the metaphase plate and then segregated during anaphase to the two daughter cells. Anaphase is functionally subdivided into anaphase A, when the chromosomes separate but the spindle does not change length, followed by anaphase B in which the spindle poles separate from each other. There have been two primary mechanisms proposed for how chromosomes can be moved poleward in anaphase A. In vertebrate somatic cells, microtubule depolymerization at kinetochores accounts for approximately 70-90% of poleward movement, with depolymerization at the poles via poleward microtubule flux accounting for the rest. Recent experiments in spindles assembled in egg extracts had suggested that the majority of anaphase A movement occurs via depolymerization from poles by poleward flux [15]. Is this a unique property of extract-assembled spindles or could it be a general mechanism for chromosome segregation in embryonic systems? Using fluorescent-speckle microscopy [16], Desai described finding that chromosome-to-pole movement in *D. melanogaster* embryos occurred at 2 μm/min, the same rate at which tubulin subunits fluxed toward the poles [17]. This suggests that depolymerization at poles due to poleward flux may be the main driving force for chromosome segregation in embryonic systems. A re-analysis of chromosome movement and flux in spindles assembled in *Xenopus* extracts showed that flux occurred at 75% of the rate of chromosome-to-pole movement, suggesting that some depolymerization at kinetochores must occur in the extract-assembled spindles. Desai and coworkers also began to explore the molecular mechanisms associated with flux. Inhibition of the microtubule-depolymerizing KRP, XKCM1, did not inhibit poleward microtubule flux but did result in an inhibition of microtubule depolymerization at poles, such that microtubules at poles curved around. When XKCM1 was inhibited in combination with the inhibition of the KRP Eg5, microtubule flux...
virtually stopped, suggesting that these two proteins might be important constituents of the flux machinery. Eg5 is the *Xenopus* homolog of the bipolar kinesin KLP61F; thus it would be very interesting to perform similar experiments in *D. melanogaster* embryos and establish whether the two motors behave in the same way in both organisms.

In summary, the field of mitosis is making great progress in establishing the molecular mechanism of mitotic spindle assembly and chromosome segregation. The importance of these processes to the survival of each cell and thus of the organism itself has allowed for the evolution of an intricate network of overlapping players and mechanisms to ensure fidelity. One striking outcome of the studies presented at the meeting is that there are redundant processes in place for each aspect of mitosis and that multiple related proteins are involved at each step. There must be a complete integration of function of the proteins that are involved in mitosis, including those that act directly on the spindle, such as motor proteins, those that control microtubule polymerization dynamics and those that coordinate the function of the spindle with the rest of the cell. Clearly the regulation of this complex machinery will be an important area of future research, as we move beyond the identification of additional protein components and into an era of more mechanistic analysis.

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