

## Dynamics of the mitotic spindle - potential therapeutic targets

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**Inhibition of mitosis is a useful strategy for treating diseases involving excessive cell proliferation. Anti-mitotic drugs currently in clinical use perturb microtubule dynamics and thereby disrupt the function of the mitotic spindle. Protein regulators of microtubule dynamics and microtubule motors are also essential for mitotic spindle function. In this chapter, we evaluate the potential of these proteins as candidate targets for anti-mitotic drugs. We review in depth a number of proteins of particular interest, highlighting their known functions in mitosis and the effects of their inhibition on cell cycle progression.**

The dynamic nature of microtubules underlies many of the most basic activities of the cell. The establishment of cell polarity, cell differentiation, and cell division all require microtubules and make use of their intrinsic capacity for rapid structural rearrangement. In interphase cells, microtubules radiate outward from the MTOC (Microtubule Organizing Center) and act as relatively static "railroad tracks" for vesicles and protein cargoes. Upon entering mitosis, the cell dismantles and rebuilds this radial array into a bipolar mitotic spindle that carries out the segregation of sister chromatids. Precise regulation of microtubule dynamics underpins the elaborate series of structural rearrangements necessary for this dramatic transformation to occur properly (1).

These same dynamic rearrangements make the mitotic spindle a vulnerable target for drugs that interfere with cell cycle progression. Inhibition of this cell division machinery underlies the clinical treatment of many human diseases characterized by excessive cell proliferation. As discussed in Chapter 12, small molecules that affect microtubule dynamics are one of the most effective classes of anti-mitotic therapeutics, possibly because perturbation of microtubule dynamics in the spindle leads to a prolonged mitotic arrest, followed by cell death.

One problem with direct inhibitors of tubulin is their lack of specificity for dividing cells. Microtubules provide important structural and transport functions in neurons and other non-mitotic cells. Drugs that target the microtubule cytoskeleton without discriminating between dividing and non-dividing cells may thus cause undesired toxicity in living organisms. For example, the microtubule stabilizing drug paclitaxel causes peripheral neuropathy, which is thought to result from perturbation of the microtubule network in post-mitotic neurons (2). It is natural to ask whether we can target mitotic cells more specifically, perhaps by inhibiting proteins that play a large role in mitosis but not interphase.

In this chapter, we ask from a cell biology perspective whether proteins that regulate microtubule dynamics and organization in the spindle might make

good targets for inhibiting cell division. We begin by reviewing the fundamentals of microtubule dynamics and the stereotyped physical rearrangements essential to the function of the mitotic spindle. The bulk of this chapter is then devoted to reviewing microtubule regulating proteins that may be of particular interest in screens for cell cycle inhibitors.

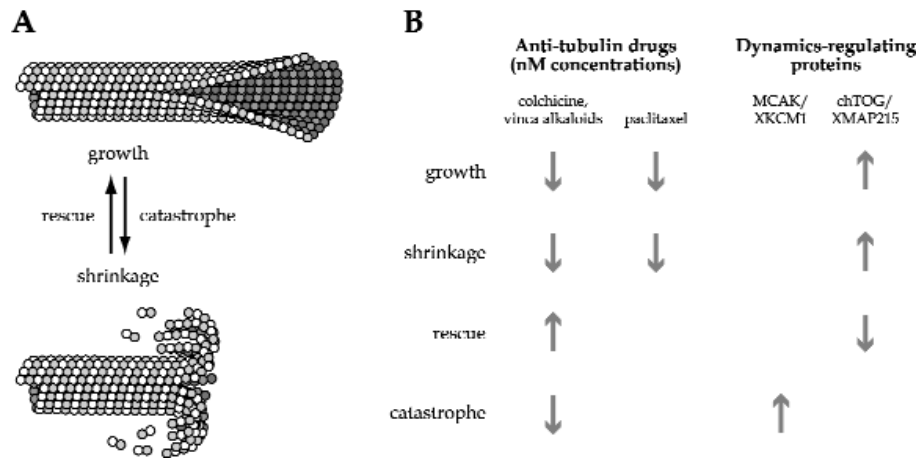
### MICROTUBULE DYNAMICS AND SPINDLE ASSEMBLY

**The polymerization dynamics of microtubules are essential to their function.**

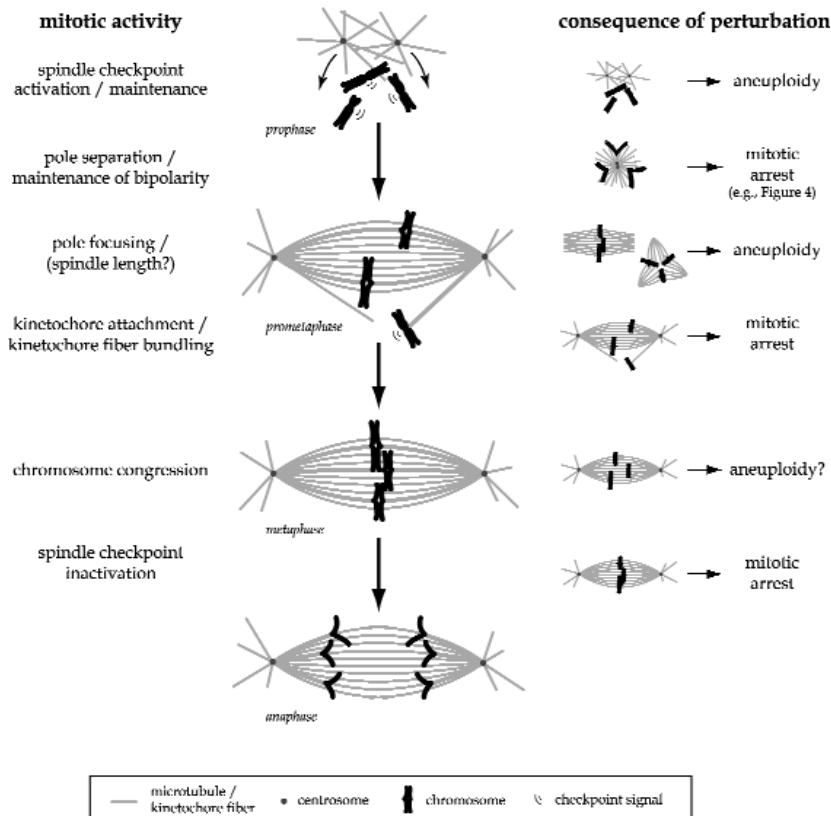
For a comprehensive review of microtubule dynamics, see (3). Briefly, microtubules are composed of  $\alpha$ -/ $\beta$ -tubulin heterodimers, which hydrolyze GTP during polymerization. Unlike typical equilibrium polymers, microtubules never reach steady-state length and instead exhibit a behavior termed "dynamic instability" – that is, stochastic transitions between phases of growth and shrinkage. The four classic parameters of microtubule dynamics are growth rate, shrinkage rate, catastrophe frequency, and rescue frequency (Figure 1A). As depicted, polymerization is correlated with the formation of protofilament sheets at the growing end of the microtubule and depolymerization with a "ram's horn" conformation of curled protofilaments. The two ends of microtubules have different dynamic characteristics. The "plus end" polymerizes more rapidly than the "minus end." It is not clear how or whether protein regulators of microtubule dynamics affect microtubule minus ends, which *in vivo* are usually embedded in the MTOC or pericentrosomal material. In this review, we focus on plus end dynamic behavior.

**Protein regulators of microtubule dynamics are potential targets for cell cycle inhibitors.**

*In vivo*, protein regulators of microtubule dynamics play a key role in transforming the long, stable microtubules of interphase into the short, dynamic microtubules of the mitotic spindle. Direct measurement of microtubule dynamics in different systems have demonstrated that the most significant change in dynamics is either an increase in catastrophe frequen-



**Figure 1.** A. Four parameters are conventionally measured to describe microtubule dynamics. Microtubules typically undergo stochastic transitions (rescue, catastrophe) between phases of polymerization and depolymerization (growth, shrinkage). B. Tubulin inhibitors and microtubule-associated proteins affect the dynamics of microtubules. In contrast to the high (>1  $\mu$ M) concentrations of anti-tubulin drug typically used in cell biological studies, nanomolar concentrations of anti-tubulin drugs inhibit microtubule dynamics without changing total microtubule polymer mass (7, 122). These nanomolar concentrations approximately correspond to the effective plasma concentrations in patients during chemotherapy (123). Two microtubule dynamics regulating proteins that have been well characterized *in vitro* are shown for comparison.



**Figure 2.** Many distinct activities are required between chromosome condensation and sister chromatid separation. The central column depicts the morphological changes of the spindle during mitotic progression, the left column describes the sequence of some of the biochemical activities underlying these rearrangements, and the right column describes the consequences reported for inhibition of some of the involved proteins.

cy (4), a decrease in rescue frequency (5), or, in the case of mammalian tissue culture cells, both (6). Direct anti-tubulin drugs like paclitaxel or vinca alkaloids can inhibit cell cycle progression by altering microtubule dynamics, even at concentrations too low to affect total polymer mass (7) (Figure 1B). Accordingly, inhibiting some of the proteins that regulate microtubule dynamics *in vivo* (3) might be expected to result in mitotic arrest. As will be discussed, such inhibitors might also act in synergy with tubulin inhibitors.

**Many microtubule-regulating proteins are essential for mitotic progression.**

In addition to regulators of microtubule dynamics, many other proteins have been shown to be involved in spindle assembly and function. These include motor proteins, enzymes which use the energy of ATP hydrolysis to move along microtubules; nucleating factors, which stimulate the polymerization of new microtubules; and structural proteins, which do not appear to have any enzymatic activity but which are necessary for spindle structure (8).

In Figure 2, we outline the complex microtubule rearrangements that occur in mitosis and depict the predicted consequence of inhibiting some proteins involved in these events. Prior to nuclear envelope breakdown, the pair of centrosomes at the center of the MTOC begins to separate until the metaphase inter-polar distance is achieved. The upregulation of dynamic instability at mitosis allows microtubule plus ends to explore the volume of the cell. A microtubule that comes in contact with a kinetochore will be stabilized and will be incorporated into a bundle of microtubules termed the kinetochore fiber. Kinetochore fibers transmit force to move chromosomes and allow the trafficking of structural and signaling proteins between kinetochores and poles. Minus ends are held together at the poles, giving rise to the characteristic fusiform shape of the mitotic spindle. The intrinsic bipolarity of the mitotic spindle enables it to push and pull on sister chromatids in two opposite directions, resulting in the migration of bioriented chromosomes to the metaphase plate at the center of the spindle and allowing equal partitioning of chromosomes to the two daughter cells. A spindle checkpoint exists to ensure that this sister chromatid separation does not occur until all chromosomes are properly oriented at the metaphase plate. Some of the proteins we will discuss are involved in this error checking mechanism and communicate with the signaling proteins discussed in Chapter 43. Upon sister chromatid separation, the chromosomes are moved towards the poles, the poles are moved further apart, and a cleavage furrow forms to separate the cytoplasm and give two daughter cells.

**Many of the proteins responsible for microtubule rearrangements during mitosis may be interesting potential targets for cell cycle inhibitors.**

Some of the proteins that regulate microtubule rearrangements in mitosis are listed in Table 1. Since the action of anti-microtubule drugs seems to involve prolonged activation of the spindle checkpoint, our

discussion will highlight those proteins that have been shown to cause mitotic arrest when perturbed and therefore might be potential anti-mitotic drug targets. It should be kept in mind that most of the cell biology data upon which we rely are based on depletion or deletion studies and that small molecule inhibition of a protein may be more likely to phenocopy a dominant negative mutation.

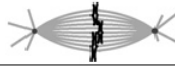









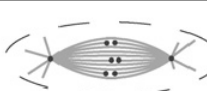
We will also review some proteins for which inhibition does not trigger the spindle checkpoint, but which are essential for function of the mitotic apparatus. There may be some risk associated with the inhibition of some of these proteins, since their perturbation may give rise to aneuploidy, which has been proposed to be an oncogenic mechanism (9). For example, as will be discussed, inhibition or depletion of some microtubule dynamics regulators and some mitotic kinesins leads to defective chromosome segregation. This is perhaps because many of these proteins are important both for spindle structure and spindle checkpoint function.

The proteins in this chapter are divided into microtubule destabilizers, microtubule stabilizers, and mitotic motors. In discussing each protein, we attempt to highlight the effects of its inhibition on cell cycle progression, provide information on its *in vitro* activity, present evidence for its function in mitotic and interphase cells, and discuss potential strategies that may be used to screen for small molecule inhibitors. In general, assays that already exist for dynamics regulators or motor proteins can often be scaled up for high-throughput screening with relative ease. A number of simple assays are well-established for the measurement of nucleotide hydrolysis rates under equilibrium or nonequilibrium conditions; these assays might be used to measure ATPase motor activity (10) or the GTPase activity of microtubule polymerization. Alternatively, cell-based screens for the phenotypic outcome of mitotic arrest may yield inhibitors that would be difficult to obtain *in vitro* - for example, in the case of proteins with relatively low ATPase activity. Some proteins might also be subject to cell cycle-specific regulation or require cellular co-factors difficult to supply in an *in vitro* assay.

**MICROTUBULE DYNAMICS REGULATORS: DESTABILIZERS**

Microtubule destabilizers constitute an important class of potential anti-mitotic targets. Paclitaxel, by analogy, blocks microtubule destabilization, resulting in excessive stabilization and mitotic block (Chapter 33). Inhibiting a catastrophe-inducing protein that is upregulated during mitosis might target mitotic cells more specifically. The importance of the precise regulation of microtubule dynamics in mitosis is underscored by the observation that at least one destabilizing protein, Op18, is upregulated in cancer cells.

However, genetic and functional depletion data present a pessimistic forecast for microtubule destabilizers as targets for cell cycle inhibitors. Inhibition of microtubule destabilization leads more often to abnormal chromosome segregation than to mitotic arrest.

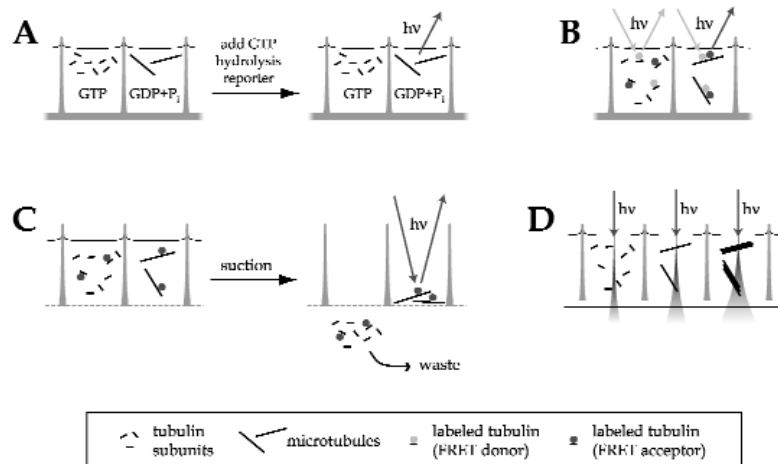
	Key Homologues	Metaphase Localization	Metaphase Function	Inhibition gives Mitotic Arrest?
<i>destabilizers</i>				
MCAK [KNSL6] (80 kDa)	KIF2A/2C ( <i>Mm</i> ); XKCM1[XKIF2] ( <i>Xl</i> )		MT catastrophe	N
Op18 [stathmin,p18, p19, prosolin, metablastin] (18 kDa)	Op18 ( <i>Mm, Xl</i> )		?	N
Katanin (60 kDa/80 kDa)	katanin ( <i>Mm, Dm, Xl, Spu</i> ); Mei1/2 ( <i>Ce</i> ); Erh3/Fra2[Atktn] ( <i>At</i> )		MT severing at poles?	N
<i>Stabilizers</i>				
ChTOG (218 kDa)	XMAP215 ( <i>Xl</i> ); Msps( <i>Dm</i> ); Zyg9 ( <i>Ce</i> ); Stu2 ( <i>Sc</i> ); Dis1/Mtc1[Alp14] ( <i>Spo</i> )		MT growth MT rescue	Y?
EB1 (35 kDa)	EB1 ( <i>Mm, Xl</i> ); Bim1 ( <i>Sc</i> )		MT growth ? MT targeting ?	N
<i>plus end motors</i>				
Eg5 [KNSL1,KSP] (120 kDa) [BimC family]	KIF11 ( <i>Mm</i> ); Eg5[KNSL1] ( <i>Xl</i> ) KLP61F/KRP130 ( <i>Dm</i> ) Cin8/Kip1 ( <i>Sc</i> ); Cut7 ( <i>Spo</i> )		pole separation	Y
Hklp2 (160 kDa)	Xklp2 ( <i>Xl</i> ); KRP180 ( <i>Spu</i> )		pole separation ?	N
MKLP1 [CHO1] (100 kDa)	KIF23 ( <i>Mm</i> ); CHO1 ( <i>Cg, Xl</i> ); Pavarotti ( <i>Dm</i> ); Zen4 ( <i>Ce</i> )		?	Y?
CENP-E (312 kDa)	CENP-E ( <i>Xl</i> ); CENP-meta ( <i>Dm</i> )		kinetochore attachment	Y
<i>minus end motors</i>				
HSET [KNSL2] (75/80 kDa) [Kar3 family]	KIFC1/C4/C5A ( <i>Mm</i> ) CHO2 ( <i>Cg</i> ); XCTK2 ( <i>Xl</i> ); Ncd ( <i>Dm</i> ); Kar3 ( <i>Sc</i> ); Klp2/Pkl1 ( <i>Spo</i> ); KLP4 ( <i>An</i> )		pole focusing spindle length? K-fiber bundling? intraspindle trafficking?	N
Cytoplasmic Dynein (~2 MDa)	dynein ( <i>eukaryotes...</i> )		pole focusing kinetochore attachment cortical attachment intraspindle trafficking checkpoint inactivation	Y

**Table 1. Some potential drug targets in the spindle.** Square brackets denote alternate naming conventions, slashes denote homologues within same organism. Localization and Function columns reflect observations in mammalian cells if known, and *Xenopus* egg extract otherwise. The Mitotic Arrest column gives the end result of inhibition. *Mm*=*Mus musculus*; *Cg*=*Cricetulus griseus*; *Xl*=*Xenopus laevis*; *Dm*=*Drosophila melanogaster*; *Ce*=*Caenorhabditis elegans*; *Spu*=*Strongylocentrotus purpuratus*; *Sc*=*Saccharomyces cerevisiae*; *Spo*=*Schizosaccharomyces pombe*; *An*=*Aspergillus nidulans*; *At*=*Arabidopsis thalianus*.

We might speculate that this is because endogenous microtubule destabilizers may influence microtubules only at specific times or places. Another possibility is that many of these dynamics regulators may have a direct role in the spindle checkpoint mechanism.

Although inhibitors of a microtubule regulating protein may not cause mitotic arrest, they might act synergistically with anti-tubulin drugs. For example, inhibition of Op18, a destabilizer that will be discussed in this section, does not cause mitotic arrest but does appear to make cells more sensitive to the anti-mitotic effects of low doses of paclitaxel (11).

We discuss three destabilizers in depth: XKCM1, katanin, and Op18. Destabilizers that we do not discuss in detail include EF-1 $\alpha$ , for which a reported microtubule severing activity is now widely believed to be an artifactual consequence of bundling activity (12); ELP70, reported to decrease polymerization rate and increase catastrophe frequency (13); and tubulin folding co-factor D, a reported tubulin-sequestering protein whose mitotic implications remain unclear (14). For each of the proteins we discuss below, *in vivo* or *in vitro* screens assaying the polymerization state of microtubules are reasonable options, although anecdotal



**Figure 3.** Several assays that measure the activity of microtubule regulators can be adapted to a high throughput format in multi-well plates. Many assays make use of the well established protocols for fluorescent labeling of purified tubulin (for protocols, see <http://mitchison.med.harvard.edu/Protocols.htm>). Depicted here are cross-sections of two or more wells in which microtubule polymerization state is reported by: **A.** GTP hydrolysis; **B.** fluorescence resonance energy transfer (FRET); **C.** microtubules separated from tubulin subunits by filtration; or **D.** light scattering. As depicted, the light scattering can also be used to assay for microtubule bundling activity.

evidence suggests that the overwhelming majority of positives will end up being drugs that directly stabilize microtubules. *In vitro* assays amenable to high-throughput screening include those relying on GTP hydrolysis, FRET (fluorescence resonance energy transfer) between tubulin monomers, light scattering, and determination of polymer concentration by filtration assays (Figure 3). Where possible, we suggest alternative screens that exploit characteristics specific to each protein.

**MCAK (XKCM1) is a catastrophe factor involved in spindle assembly, but its inhibition does not lead to mitotic arrest.**

The mitotic Kin I kinesins have central motor domains and induce microtubule catastrophe. The best-studied members of this family are MCAK (*mammalian centromere-associated kinesin*) and its homologue XKCM1 (*Xenopus kinesin central motor 1*) (15, 16). In most systems, these proteins localize prominently to centromeres (17), though XKCM1 also shows general spindle microtubule localization (18). XKCM1 is believed to be responsible for high mitotic catastrophe rates in *Xenopus* egg extracts, and immunodepletion from that system results in long stable microtubules and total inhibition of spindle assembly (18).

However, inhibition of MCAK in cultured hamster ovary cells does not result in mitotic arrest. Instead, these cells assemble normal-looking spindles and undergo defective chromosome segregation (19). The lack of obvious changes in microtubule polymerization and distribution may be due to MCAK's specific localization to the centromere, where microtubule depolymerization appears to be important for chromosome movement, either in congression to the metaphase plate or during anaphase segregation. Small-molecule inhibitors of

MCAK might also affect non-mitotic cells, since MCAK appears to be active throughout the cell cycle (20).

With all these caveats, MCAK remains one of the more important microtubule destabilizers discovered to date and would probably be one of the simplest to screen *in vitro*. As will be discussed, kinesins represent a particularly tractable class of screening targets. Practical advantages of MCAK include the fact that baculovirus-expressed, active protein can easily be purified from Sf9 cells (16). The protein has high specific activity and can induce catastrophe in taxol-stabilized microtubules, which are easier to work with than dynamic microtubules.

**Katanin is a well-characterized microtubule severing protein complex with no clear mitotic role.**

The name of this p60/p80 protein complex as "katanin" is derived from - Japanese for "sword" - because it severs microtubules along their lengths (21). The complex localizes to centrosomes throughout the cell cycle (22) and current evidence suggests that katanin severs microtubules at the centrosome to allow microtubule redistribution or disassembly.

A katanin inhibitor would not be likely to produce mitotic arrest. In mammalian tissue culture cells, inhibition of katanin's severing activity does not affect spindle assembly and function (23). Katanin inhibitors might also have adverse effects on postmitotic cells, particularly neurons (24), where the complex appears to have a role in microtubule distribution. Mutations in a katanin-related human protein (spastin) are associated with a neuronal disease, hereditary spastic paraplegia (25).

For screening purposes, the *in vitro* microtubule severing assay used to purify katanin would not be easily adapted to a high-throughput screening format and katanin inhibition does not have a phenotype

strong enough for screening in tissue culture cells. However, the ATPase activity of katanin might be exploited in a high-throughput assay. Another assay is suggested by the observation that mutation of katanin homologues inhibits proper development and cell wall biosynthesis in plants (26, 27) and disrupts meiosis in *C. elegans* (28). If one were interested in inhibiting cell development in plants or fertility in worms (*i.e.*, for anti-helminthic drugs), phenotypic screens using those organisms might potentially uncover a katanin inhibitor.

**Op18 is a catastrophe factor that also sequesters tubulin monomers, and its inactivation is required for cell cycle progression.**

This oncoprotein of 18 kD is upregulated in several types of cancer (29) and undergoes complex phosphoregulation at multiple sites, both in response to extracellular signals (30) and during the cell cycle (29). Op18 is mostly cytoplasmic throughout the cell cycle, although it is enriched at spindle poles during mitosis (31). The protein has two distinct microtubule-destabilizing domains, one that promotes catastrophe and one that sequesters tubulin monomers (32). Op18 is expressed in most dividing cells as well as neurons, where it appears to be involved in differentiation (33).

While it is tempting to view Op18 as a link between upregulated microtubule destabilization and the increased cell proliferation seen in tumorigenesis, the majority of work to date implicates a role for Op18 activity in interphase, not mitosis (20). Inhibition of Op18 using either RNAi (11) or antibody microinjection (34) does not result in mitotic arrest. Intriguingly, however, cells subjected to both Op18 RNAi and paclitaxel treatment were arrested at lower doses of paclitaxel than control cells, indicating that inhibition of Op18 may sensitize cells to mitotic arrest by paclitaxel (32).

It is possible that activators of Op18 or inhibitors of Op18 inactivation might be effective anti-mitotics. Phosphorylation and inactivation of Op18 have been shown to be required for proper cell cycle progression (29). The reasons for this are not clear, although they are presumed to be due to effects on microtubule dynamics. As an example of the latter, Op18 inactivation has been hypothesized to have a role in microtubule stabilization near chromatin (35, 36). A recent report further suggests that Op18 dephosphorylation and activation may be necessary for spindle disassembly and exit from mitosis (37). Though it is currently difficult to predict whether or not inhibitors or activators of Op18 would constitute effective anti-mitotics, the complex regulation and behavior of this protein in cells is clearly interesting and potentially relevant to anti-cancer research.

The co-crystal structure of Op18 and tubulin suggests that it might be difficult to disrupt their interaction with a small molecule, since the two proteins interact through a very large interface (38). Alternatively, one could screen for drugs that cause changes in the phosphorylation state of Op18.

**MICROTUBULE DYNAMICS REGULATORS: STABILIZERS**

Most stabilizing MAPs (*microtubule-associated proteins*) would not be expected to be ideal targets for anti-mitotic drugs. During mitosis, MAPs are generally phosphorylated, which is associated with both decreased microtubule binding affinity and decreased stabilizing activity (39). Inhibition of these proteins would therefore probably not affect mitotic cell cycle progression. However, drugs that activate MAPs or inhibit MAP phosphorylation might inappropriately stabilize microtubules during mitosis and induce mitotic arrest themselves or enhance the effects of microtubule stabilizing drugs like paclitaxel. In human tissue culture cells, transfection of the microtubule stabilizer MAP4 with mutated putative cdc2 phosphorylation sites leads to slowed cell cycle progression and increased microtubule stability (40). Cdc2 is a general cell cycle regulator, but the existence of the microtubule-specific kinase MARK (MAP/microtubule affinity-regulating kinase, also called par-1) suggests that more specific inhibition may be possible (41). Inhibition of a kinase like MARK might have the further advantage of simultaneously activating many MAPs of potentially redundant function.

Though we recognize the potential for microtubule stabilizing proteins as drug targets, we discuss only one, ch-TOG, in detail. Of the known microtubule-stabilizing proteins, ch-TOG has the best-characterized mitotic phenotype. Ch-TOG/XMAP215 appears to play a complex and important role in mitosis, counterbalancing the microtubule destabilizer MCAK/XKCM1 (42). In fact, it was recently demonstrated that combining appropriate concentrations of XMAP215 and XKCM1 alone is sufficient to produce *in vitro* microtubule dynamics quantitatively similar to those seen *in vivo* (43).

Other stabilizers that may play a role in mitosis include EB1, a plus end-binding protein that affects mitotic dynamics and spindle positioning in yeast (44); APC (*adenomatous polyposis coli*), a plus end-binding protein often mutated in colon cancer that binds EB1 and enhances its polymerization activity (45); and orbit, mutation of which in *Drosophila* results in spindle assembly defects and polyploidy (46). The human homologues of orbit, the CLASPS (Clip-170 Associated Proteins), have not been examined for a role in mitosis. These are all potentially interesting proteins and we eagerly await further information on their role in mitosis.

**ch-TOG increases microtubule growth and dynamics and its inhibition may result in mitotic arrest.**

ch-TOG (colonic hepatic tumor overexpressed gene) and its well-characterized homologue XMAP215 (*Xenopus microtubule-associated protein* of 215 kD) appear to play key roles in mitosis by stabilizing microtubules. ch-TOG differs from simple stabilizers in making microtubules not only longer but also more dynamic (47). ch-TOG localizes to centrosomes during mitosis in most model organisms. In some organisms, like *Xenopus* and *Drosophila*, the protein also localizes

more generally to spindle microtubules (48, 49) and in others, like fission and budding yeast, appears to be enriched on microtubules near the kinetochores (50-52). It has been hypothesized that ch-TOG stabilizes short nascent microtubules at the centrosome, thus spatially restricting microtubule growth to the poles and possibly playing a role in spindle pole focusing.

Ch-TOG may be an attractive target for anti-mitotic drugs for several reasons. First, from genetic and functional depletion data available in frog, fly, yeast, and worm, we know that ch-TOG plays an important and complex role in spindle assembly. The two most common phenotypes of ch-TOG inhibition are short, destabilized microtubules and defects in spindle pole formation (53). Its localization to poles is apparently important for function—mutations in proteins that localize ch-TOG such as D-TACC (54) or *ncd* (55) result in phenotypes consistent with microtubule destabilization. Second, mutations in at least one homologue, mini-spindles in *Drosophila*, lead to mitotic arrest (49). A small-molecule inhibitor of this ch-TOG might further be valuable as a tool for understanding the etiology of the colonic and hepatic cancers in which the protein is overexpressed (56).

Like most MAPs, XMAP215 is phosphorylated in mitosis and more active in interphase (39). However, although interphase microtubule defects occur in plant and yeast ch-TOG mutations (57, 58), interphase defects have not been reported upon inhibition in animal cells. It is possible that inhibition of the interphase activity of ch-TOG results in centrosome duplication defects or abnormalities in centrosome structure that become apparent only in mitosis.

High-throughput screens for inhibitors of ch-TOG *in vitro* might make use of the same assays of microtubule polymerization described earlier for destabilizers. *In vivo* assays are also possible (screening for short microtubules or small spindles) but would undoubtedly yield a large number of drugs that target tubulin.

#### MICROTUBULE MOTOR PROTEINS

Control of microtubule dynamics alone is not sufficient to organize microtubules into a bipolar mitotic spindle. Microtubule motor proteins, structural proteins, and microtubule nucleating proteins are also necessary for the complex rearrangements underlying spindle assembly and function. For a number of reasons, we believe that motors are particularly amenable to inhibition by small molecules. First, inhibitors of enzymatic activity have historically been easier to find than inhibitors of protein-protein interactions (for a rare counterexample, see (59).) It may thus be easier to block the ATPase activity of motor proteins than the protein-protein interactions involved in structural protein function and microtubule nucleation. Second, the global conformational changes intrinsic to motor function may increase the probability of finding allosteric inhibitors — that is, small molecules that act by stabilizing a particular conformation and thereby prevent motion necessary for the function of the motor (for

examples of this principle in a variety of proteins, see (60-63)). Finally, as will be discussed, small molecules have already been found that prevent the motor activity of the kinesin Eg5 without affecting the activity of other kinesins. This demonstrates that although the motor domain is highly conserved, it can be specifically inhibited.

The inhibition of many mitotic motors results in mitotic arrest (Table 1 and Figure 2). Of particular interest are motors responsible for spindle pole separation and maintenance of spindle bipolarity. When these motors are inhibited, spindle poles collapse together, resulting in a monoaster, a radial array of microtubules. This phenotype is often accompanied by mitotic arrest. Two motors implicated in spindle bipolarity, Eg5 and Xklp2, are reviewed below. Inhibition of the motors responsible for kinetochore attachment, kinetochore fiber formation, or spindle checkpoint inactivation can also lead to mitotic arrest. We discuss in detail two such motors, CENP-E and cytoplasmic dynein.

As mentioned in the introduction, there is some risk associated with targeting the activities of the mitotic spindle. Disruption of some mitotic motors involved in spindle assembly may give rise to aneuploidy without mitotic arrest (Figure 2). For example, we discuss below the motor protein HSET, a kinesin involved in spindle pole focusing. Inhibition of this protein results in sparse bipolar spindles or multipolar microtubule arrays, both of which can result in chromosome missegregation.

The motors of this section are grouped according to the direction in which they appear to walk along microtubules *in vitro*. Eg5, Xklp2, MKLP1 and CENP-E are all plus end-directed kinesins. In addition to their other functions, each appears to mediate the interaction of antiparallel microtubules in metaphase or anaphase. HSET and cytoplasmic dynein are both minus end-directed motors help to focus spindle poles. In assessing these motors as potential anti-mitotic drug targets, we review their roles in mitosis and interphase and highlight the effects of their inhibition on mitotic progression.

#### **Eg5 is required for spindle bipolarity and its inhibition leads to mitotic arrest.**

Eg5, a member of the BimC (blocked in *mitosis*) family of plus end-directed kinesins, is a key motor in the establishment of spindle bipolarity (64). These BimC proteins form bipolar homotetramers with motor domains positioned on opposite ends of a central rod, suggesting an ability to crosslink and slide apart antiparallel microtubules (65). Eg5 localizes to spindle microtubules and is involved in spindle pole separation and production of the bipolar spindle in many organisms including humans (66). Microinjection of inhibitory antibodies into HeLa cells results in monoasters and mitotic arrest (67, 68).

Based on our current understanding, we would not expect an inhibitor of Eg5 to affect interphase cells. The presence of Eg5 in post-mitotic neurons has led to speculation that Eg5 and other mitotic motor proteins may play roles in axonal elongation and retraction (69,

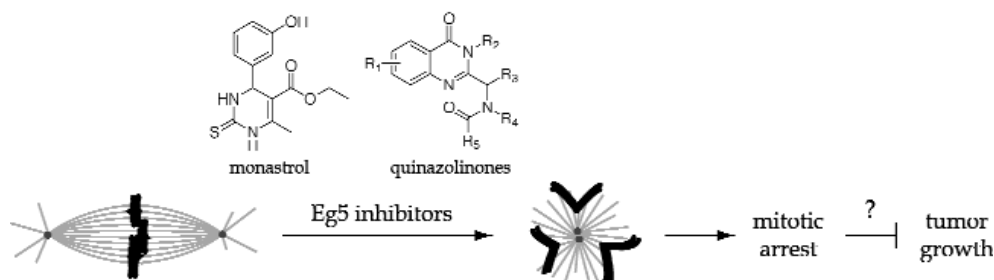


Figure 4. Small molecule inhibitors of Eg5 result in monoastral mitotic figures and mitotic arrest.

70). However, no functional evidence has yet been published to support this hypothesis. Eg5 itself appears to be tightly regulated through the cell cycle. In interphase cells, Eg5 is diffusely cytoplasmic and a conserved putative site of phosphorylation by the mitotic kinase cdc2 appears to be necessary for localization to the mitotic spindle (66, 67).

A number of specific small molecule inhibitors of Eg5 have been identified using high-throughput screens (71, 72). In some mammalian cells, these inhibitors cause monoasters very similar to those seen upon antibody inhibition. The first of these compounds was found in a three step screen (71). A high throughput assay detecting cell cycle-dependent nucleolin phosphorylation was initially used to identify compounds that increase the mitotic index of mammalian cells. Visual assays were then performed on the hits from this primary assay to eliminate compounds that perturb tubulin polymerization *in vitro*. Finally, fluorescence microscopy was used to visualize the effects of the remaining compounds on spindle morphology in mammalian cells. The signature phenotype of Eg5 inhibition was observed for a 1,4-dihydropyrimidine-based compound (Figure 4) which was confirmed to inhibit Eg5 in an *in vitro* microtubule gliding assay. This compound, monastrol, does not inhibit the *in vitro* motility of conventional kinesin and does not disrupt the cellular localization of lysosomes or the Golgi in interphase cells, indicating that the kinesins involved in the localization of these organelles are not perturbed.

Recent meeting abstracts report the identification of a broad class of quinazolinone Eg5 inhibitors (Figure 4) (72). One of these, a 2-(aminomethyl) quinazolinone, appears to have broad spectrum activity against murine solid tumors and human colon tumor xenografts without causing peripheral neuropathy in mice, and the researchers plan to bring this compound to clinical trials (73). These results suggest that an Eg5 inhibitor can inhibit mitosis without causing undesired side effects in interphase cells even at the organismal level.

#### Hklp2 may be required for bipolarity, but its inhibition may not lead to mitotic arrest.

The human homologue, Hklp2, of the plus end-directed *Xenopus* kinesin-like protein Xklp2 (74) remains poorly characterized (75). Xklp2 is concentrated on centrosomes throughout the cell cycle, while the

sea urchin homologue KRP180 localizes along astral microtubules in interphase and to the spindle midzone during mitosis (74, 76).

Initial data suggested that Xklp2 is involved in spindle pole separation and the establishment of spindle bipolarity. A putative dominant negative Xklp2 truncation construct prevented spindle pole separation in *Xenopus* egg extracts (74). Both this construct and inhibitory antibodies led to dramatic inward collapse of the spindle poles in sea urchin embryos (76). However, immunodepletion of Xklp2 from *Xenopus* egg extracts does not perturb cycled spindle assembly (77) and allows normal bipolar spindles to form around chromatin beads (78). Given these contradictory results, it is difficult to assess the role of Xklp2 in the establishment or maintenance of spindle bipolarity.

The majority of sea urchin embryos in the above inhibition studies proceed into anaphase with normal timing, suggesting that Xklp2 may not be a good anti-mitotic drug target even if it does play a role in spindle bipolarity (76).

#### MKLP1 has a poorly understood role in mitosis, but its inhibition has been reported to cause mitotic arrest.

Early work on this family of plus end-directed kinesins suggested a role in anaphase spindle elongation. However, the bulk of genetic evidence points to an important role in cytokinesis. Human MKLP1 (*mammalian kinesin-like protein 1*) is localized to centrosomes and nuclei during interphase, to the midzone microtubules during anaphase, and eventually at the midbody during telophase. *In vitro*, it can bundle and slide apart antiparallel microtubules in an ATP-dependent fashion (79).

Inhibition of MKLP1 in mammalian cells and sea urchin embryos by antibody microinjection causes mitotic arrest with apparently normal metaphase spindles (80, 81). However, in subsequent genetic studies, disruption or deletion of the *C. elegans* and *Drosophila* homologues did not cause mitotic arrest and instead led to failures in cytokinesis, giving rise to multinucleate cells (82, 83). This discrepancy has not been resolved, although an intriguing recent report shows that at least two alternative splice products exist in the mitotic cell and may have different inhibition phenotypes (84).

Inhibitors of MKLP1 may disrupt the structure and function of some specialized postmitotic cells. Several interphase functions have been identified for MKLP1, including the formation of major processes in podocytes and the establishment of dendritic identity in neurons (85, 86). In both cases, MKLP1 helps to establish an antiparallel microtubule array in these specialized cellular processes. If MKLP1 is pursued as an anti-mitotic drug target, the phenotypes of metaphase arrest or multinucleate cells observed with its inhibition might readily lend themselves to image-based phenotypic screening. The *in vitro* bundling activity of MKLP1 might also be exploited for high-throughput screening using a light scattering assay (Figure 3).

**CENP-E is necessary for chromosome alignment and spindle checkpoint signaling, and its inhibition leads to mitotic arrest.**

This kinesin-like centromere-associated protein is a plus end-directed kinesin with a second microtubule-binding site in addition to that of the motor domain (87-89). CENP-E associates with kinetochores upon nuclear envelope breakdown and remains there until anaphase, when it rapidly relocates to the midzone (87, 90).

We believe that CENP-E is a particularly attractive candidate drug target. Inhibition of CENP-E (in mammalian cells) results in a strong mitotic arrest, as demonstrated by antibody microinjection or antisense experiments (91, 92). CENP-E has been implicated in a number of important spindle events. It is necessary for bioriented attachment of chromosomes and subsequent alignment of chromosomes at the metaphase plate (88, 91, 93, 94). A direct functional role for CENP-E in these processes is further supported by *in vitro* studies demonstrating that CENP-E can couple chromosome movement to microtubule depolymerization (90). As discussed in Chapter 43, CENP-E also interacts with spindle checkpoint proteins at the kinetochore and appears to be an essential component of the checkpoint, although the detailed mechanism of its function remains unclear (91, 95). Finally, additional anaphase function is suggested by its paired microtubule binding sites and the changes in its phosphorylation and localization at anaphase onset (89, 90). Adding to its attractiveness as a potential drug target, CENP-E does not appear to have any role in interphase or postmitotic cells, and it is degraded at the end of mitosis (87).

In addition to the conventional screens for motor activity, an interesting possible screening strategy may be suggested by the observation that CENP-E has a CAAX box and is farnesylated. Since farnesyl transferase inhibitors often arrest cells in mitosis with misaligned chromosomes, it is possible that CENP-E is one of the true targets of this actively pursued class of anti-tumor agents (96, 97).

**HSET is necessary for spindle pole focusing, and its inhibition does not result in mitotic arrest.**

HSET (*human spleen, embryonic tissue and testes*) is a member of the Kar3p (*karyogamy defects during mating*) family of minus end-directed motors. This

family appears to play a highly conserved role in spindle pole focusing and spindle length determination. In eukaryotes, this protein localizes to spindle poles and spindle microtubules (98-101) and electron microscopy suggests that HSET localizes between microtubules in parallel bundles (101).

Inhibition of HSET does not induce mitotic arrest or any obvious spindle defects in mammalian cells (101). This differs from the spindle pole defects and chromosome segregation problems resulting from inhibition of the best-characterized metazoan homologue, the *Drosophila* protein Ncd (*Non-claret disjunction*, after its role in chromosome disjunction and proximity to the claret locus.) Interestingly, inhibition of Kar3p family members in human, fruit fly and fungus rescues the loss of spindle bipolarity that accompanies inhibition of Eg5 (101-103). No interphase role has been shown for members of this family, although they remain present throughout the cell cycle (104, 105).

HSET may have a from a cross-linking function or may play a role in trafficking of spindle components. Ncd has recently been shown to be necessary for the polar localization of the *Drosophila* ch-TOG homologue Msps (55), which might suggest that HSET inhibition might synergize with inhibition of ch-TOG.

**Cytoplasmic dynein has multiple roles in mitosis, and its inhibition leads to mitotic arrest.**

Cytoplasmic dynein in mammalian cells is a complex of two heavy chains, three intermediate chains, and four light chains (106), with multiple isoforms of each chain present in most model organisms (107). The complex is so named to distinguish it from flagellar dynein. The genetic or biochemical differences distinguishing cytoplasmic and flagellar dynein remain poorly understood. The functional cytoplasmic dynein complex appears to require association with another large complex, the *dynein activator* dynactin (108).

Cytoplasmic dynein deserves attention as a potential drug target because its inhibition results in mitotic arrest (109). Dynein is essential in higher eukaryotic mitosis and its inhibition results in mitotic arrest. The complex localizes at the nuclear envelope in early prophase in mammalian cells, and later at the cortex and on spindle poles, microtubules, and kinetochores in a wide range of organisms (110). In mammalian cells, dynein has been shown to be necessary for multiple events in mitosis, including spindle positioning, pole focusing, proper localization of pole and kinetochore components, and the delocalization of spindle checkpoint proteins after proper attachment of kinetochores to microtubules. It is also implicated in the poleward movement of chromosomes during spindle assembly and anaphase (111-116).

Cytoplasmic dynein is also important in interphase cells, playing major roles in ER and Golgi trafficking and assembly (111). However, dynein null yeast are viable, showing only mild spindle positioning and chromosome separation defects (117). At the organismal level, dynein is likely to play a number of

important developmental roles tied to spindle positioning (118) and vesicle transport (111). Another risk of targeting cytoplasmic dynein is that inhibition of flagellar dynein results in infertility, respiratory disorders, and *situs inversus* (119). The development of dynein as a therapeutic target may require better understanding of its differential regulation in the cell.

Since recombinant expression of a functional dynein-dynactin complex may prove difficult, *in vitro* screens will likely require biochemically purified complex. The ATPase activity of dynein purified from bovine brain was reported to have a  $V_{max}$  an order of magnitude lower than that of conventional kinesin (120), so an *in vitro* ATPase assay will require sensitive detection. Cell-based phenotypic assays may prove necessary in the search for dynein inhibitors.

### CONCLUSION

The mitotic spindle is a complex, dynamic structure. The morphogenesis and organization of which we are just beginning to understand at the molecular level. The success of paclitaxel and the vinca alkaloids as anti-mitotic drugs highlights the vulnerability of the spindle as a drug target. The possibility that inhibitors of proteins that regulate microtubule dynamics and organization may also be effective anti-mitotics remains largely unexplored.

Predicting the clinical efficacy or toxicity of targeting these proteins is impossible from basic cell biology, but we can point to certain properties that suggest their potential as viable drug targets. Many of the proteins we have discussed in this chapter are regulated or degraded by the cell cycle machinery, suggesting that these may allow specific targeting of dividing cells. Inhibition of many of these proteins in a number of model systems results in a mitotic arrest promisingly similar to that obtained by anti-mitotic drugs that directly target microtubules.

Inhibition of other proteins discussed in this chapter does not lead to mitotic arrest. However, as has been suggested in the case of Op18, inhibitors of these proteins may act in synergy with anti-tubulin drugs. Such a synergy might both produce more effective mitotic arrest and lower the individual doses required for therapeutic efficacy, minimizing adverse effects. The strategy of combination therapy has been successfully applied to the treatment of bacterial infection, HIV, and many types of cancer (121).

Throughout this chapter, we have attempted to assess the potential of microtubule regulators as candidate drug targets by examining their known functions in mitosis and highlighting the effects of their inhibition on cell cycle progression. As the example of Eg5 shows, improvements in our understanding of the cell biology of mitosis continue to suggest new targets for anti-mitotic drugs.

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