Katanin Contributes to Interspecies Spindle Length Scaling in Xenopus

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SUMMARY

Bipolar spindles must separate chromosomes by the appropriate distance during cell division, but mechanisms determining spindle length are poorly understood. Based on a 2D model of meiotic spindle assembly, we predicted that higher localized microtubule (MT) depolymerization rates could generate the shorter spindles observed in egg extracts of X. tropicalis compared to X. laevis. We found that katanin-dependent MT severing was increased in X. tropicalis, which, unlike X. laevis, lacks an inhibitory phosphorylation site in the katanin p60 catalytic subunit. Katanin inhibition lengthened spindles in both species. In X. tropicalis, k-fiber MT bundles that connect to chromosomes at their kinetochores extended through spindle poles, disrupting them. In both X. tropicalis extracts and the spindle simulation, a balance between k-fiber number and MT depolymerization is required to maintain spindle morphology. Thus, mechanisms have evolved in different species to scale spindle size and coordinate regulation of multiple MT populations in order to generate a robust steady-state structure.

INTRODUCTION

The metaphase spindle is a bipolar and dynamic steady-state structure composed of microtubule (MT) polymers and hundreds of other factors. Spindle length varies several fold among organisms and cell types to enable chromosome segregation over different length scales. Although many manipulations can alter the size and shape of the spindle in a variety of systems (Bird and Hyman, 2008; Brust-Mascher et al., 2009; Dumont and Mitchison, 2009), the physiologically relevant mechanisms underlying spindle scaling are poorly understood. In some mitotic divisions, the dimensions of the dividing mother cell may determine spindle size (Hara and Kimura, 2009; Wühr et al., 2008). However, in the egg and large cells of the embryo, cytoplasmic mechanisms are expected to regulate the size of the spindle, which occupies a small volume relative to the cell (Hara and Kimura, 2009; Wühr et al., 2008). How cytoplasmic factors coordinate spindle length with cell size is unknown.

Cytoplasmic Xenopus egg extracts reconstitute many cell cycle events in vitro including meiotic spindle assembly (Maresca and Heald, 2006) and provide a powerful approach to investigate intrinsic mechanisms of organelle sizing in the absence of the cell. We previously compared egg extracts prepared from Xenopus laevis to those of the closely related, smaller frog Xenopus tropicalis and found that spindle size was reduced in X. tropicalis by dose-dependent cytoplasmic activities and only weakly influenced by the amount of DNA (Brown et al., 2007). This established a system to investigate mechanisms of spindle scaling by identifying differences in spindle MT behavior in the two extracts and then determining whether the proteins responsible function as regulatory factors.

Here, we have combined computational modeling with egg extract experiments to elucidate a mechanism of scaling of spindle length in Xenopus by MT severing. Previous experimental (Budde et al., 2001; Gaetz and Kapoor, 2004; Goshima et al., 2005; Houghtaling et al., 2009; Oh et al., 2007) and in silico studies (Loughlin et al., 2010) have shown that MT depolymerization activities regulate spindle length, leading us to examine this parameter in the two egg extracts. We demonstrate that the MT severing protein katanin has lower activity in X. laevis than in X. tropicalis due to inhibitory phosphorylation. A hexameric AAA ATPase, katanin stimulates the production of MT seeds and limits spindle length during meiosis in C. elegans, although its physiological role in acentrosomal vertebrate spindles is unknown (McNally et al., 2006; Srayko et al., 2006). In Drosophila S2 cells, katanin functions at the kinetochore, destabilizing kinetochore-MT plus-ends and contributing to chromatid separation during anaphase A, while the related severing proteins spastin and fidgetin destabilize MTs at the spindle pole during metaphase (Zhang et al., 2007a).

In addition to altering spindle length, we found that inhibition of katanin in X. tropicalis egg extract uncoupled the dynamics of interpoolar spindle MTs from the MT bundles connected to chromosomes at their kinetochores (k-fibers), revealing their greater stability. We utilized our computational model of the meiotic Xenopus spindle to simulate this effect by adding k-fibers as bundled MTs with specific plus-end properties. Our model predicted that decreasing the number of k-fibers would allow...
We confirmed this prediction experimentally, demonstrating that katanin regulates spindle length in part by coordinating the stability of different MT populations.

RESULTS

Katanin-Dependent MT Severing Is Elevated in *X. tropicalis*

Our computational simulation of a 2D spindle showed that spindle length varies dramatically with plus-end catastrophe and minus-end depolymerization rates, mechanisms that could potentially generate the spindle length scaling observed in egg extracts of *X. laevis* and *X. tropicalis* (Loughlin et al., 2010). Since measured MT plus-end catastrophe frequencies were not significantly different between the two species (Brown et al., 2007), we investigated the MT destabilization rates utilizing a flow cell assay in which taxol-stabilized, rhodamine-labeled MTs were incubated with either *X. laevis* (left panels) or *X. tropicalis* (right panels) egg extract. Movie S1 shows that MTs disappear much more rapidly in *X. tropicalis* extract. The scale bar represents 10 μm.

A steady-state spindle structure to form. We confirmed this prediction experimentally, demonstrating that katanin regulates spindle length in part by coordinating the stability of different MT populations.

Katanin Phosphorylation Underlies Activity Differences between the Two Species

To elucidate the molecular basis of differential katanin activity, we first compared its properties in egg extracts of the two species using an antibody raised against the human katanin p60, which is 84% identical to both *X. laevis* and *X. tropicalis* (Loughlin et al., 2010). Since measured MT plus-end catastrophe frequencies were not significantly different between the two species (Brown et al., 2007), we investigated the MT destabilization rates utilizing a flow cell assay in which taxol-stabilized, rhodamine-labeled MTs were incubated on a coverslip and monitored by time-lapse fluorescence microscopy upon introduction of crude egg extracts. Whereas MTs persisted with a half-life of 18.8 ± 4.2 min in *X. laevis* extract, they disappeared with a half-life of 0.92 ± 0.52 min in *X. tropicalis* extract, a ~20-fold decrease in MT stability (mean ± SD, n = 3, Figure 1A, Movie S1 available online). Like spindle length (Brown et al., 2007), the MT destabilizing activity was dose dependent, since mixed extracts gave intermediate rates of MT disappearance (Figure 1B). To identify the responsible factor, antibodies were added to *X. tropicalis* extracts to inhibit candidate molecules including the two major kinesin-13 proteins found in egg extracts, MCAK and Kif2a, and the catalytic subunit p60 of the MT severing protein katanin (Figure 1C, Movie S2). Only katanin antibodies inhibited MT disassembly, consistent with its original purification and characterization by this assay (McNally and Thomas, 1998; McNally and Vale, 1993; Vale, 1991). Careful examination of MTs as they disappeared revealed the formation of gaps indicative of severing activity all along the length of MTs, and this effect was blocked by katanin p60 immunodepletion from the extract (Figure 1D). Thus, katanin-driven severing underlies the higher MT depolymerization activity observed in *X. tropicalis* egg extracts.

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X. tropicalis extract failed to accelerate MT depolymerization in X. laevis extract, severing was rapid in any reaction containing X. tropicalis p60. Addition of X. laevis extract with or without katanin did not slow MT depolymerization in X. tropicalis extract. This experiment indicates that the activity difference derives from the katanin proteins, and not from their extract environments, ruling out one possible scenario in which X. laevis extracts contain factors that protect MTs against katanin.

Probing for intrinsic differences in the proteins, we observed that katanin p60 localized more strongly to X. tropicalis than X. laevis spindle poles by immunofluorescence (Figures 2C and 2D). Since MT binding promotes p60 oligomerization, which is required for severing activity (Hartman and Vale, 1999), we reasoned that an increase in MT affinity could underlie the elevated activity of X. tropicalis katanin. Although katanin p60 protein sequences were 95% identical in the two species, most differences occurred in the putative MT-binding domain (Figures 2E and S1A). Consistent with this hypothesis, significantly greater amounts of X. tropicalis p60 copelleted with taxol-stabilized MTs in the presence of ATPγS, which inhibits katanin-dependent severing (Figures 2F and S1B).

We purified recombinant Maltose Binding Protein (MBP)-tagged X. laevis and X. tropicalis katanin p60 to investigate how minor changes in protein sequence confer differential severing activity. Interestingly, MT affinities were similar for both, in the range of 0.8–1.0 μM (Figures S2A and S2B). Correspondingly, MT severing activities of the pure proteins were indistinguishable, though dependent on ATP (Figures 3A and 3B, Movie S3, and Figure S2C). These results suggest that the two recombinant katanin proteins have very similar intrinsic
activities and that posttranslational modification may underlie activity differences. To test phosphorylation as a potential mechanism, the recombinant p60 proteins were added to the two metaphase-arrested egg extracts in the presence of ATP γ-S, then modified with p-nitrobenzyl-mesylate (PNBM), retrieved, and probed for incorporation of the thio-phosphate with a nitrobenzyl-thioester specific antibody (Figure 3C). X. laevis p60 was more highly phosphorylated than X. tropicalis p60 in both extracts (Figure 3D). Close inspection of the amino acid sequences revealed a serine residue of a potential Aurora kinase consensus site at position 131 in the X. laevis p60 and N-terminal MT binding domains by Aurora B. Phosphorylation of this putative Aurora phosphorylation site.

To test whether X. laevis katanin p60 is inhibited by phosphorylation at Ser 131, we supplemented X. laevis egg extracts with wild-type proteins or X. laevis p60S131A to block phosphorylation, or X. laevis p60S131E to mimic it, and quantified severing activity. Whereas X. laevis p60 and the S131E mutant or the MBP control had no significant effect on severing, both X. tropicalis p60 and X. laevis p60S131A decreased MT half-life approximately 3- to 4-fold (Figures 3F and S2E, Movie S4). Altogether, these results suggest that X. laevis katanin is inhibited by phosphorylation at Ser 131 by Aurora B kinase, while X. tropicalis katanin lacks this regulatory site.

MT Severing Activity Scales Spindle Length in a Meiotic Spindle Simulation

Katanin can directly depolymerize MTs, and also promote kinesin-13 driven depolymerization by exposing new MT ends (Buster et al., 2002; Zhang et al., 2011). To investigate whether its elevated activity in X. tropicalis compared to X. laevis could make shorter spindles, we introduced MT severing into our 2D meiotic spindle simulation (Loughlin et al., 2010).
invokes a small number of factors that organize dynamic MTs into a steady-state bipolar structure, including kin-5-dependent MT antiparallel sliding, and dynein-dependent delivery of NuMA oligomers to minus-ends which recruit kin-13 MT depolymerizing activity (Figure 4A). The balance of MT lifetime and poleward sliding rate sets the length of the spindle (Loughlin et al., 2010). Based on the spindle pole localization of katanin in egg extracts (Figure 2C), MT severing was coupled to NuMA, similar to how kin-13 activity was implemented in the model (Loughlin et al., 2010). Severing events generated new dynamic plus-ends and new minus-ends subject to depolymerization by kin-13 activity. The probability of a severing event increased with NuMA density and could occur along the length of a MT, but the polar localization of NuMA biased the severing toward the minus-ends of MTs reaching the poles. Spindle length scaled linearly with the severing rate (Figures 4B and 4C). Thus, the net effect of increased katanin activity in the model is increased polar MT depolymerization and decreased spindle length.

**Inhibition of Katanin Causes Spindle Elongation**

Because the model lacked centrosome- and kinetochore-associated MTs, we first examined the effect of katanin inhibition on spindles assembled around chromatin-coated beads, the simplest Xenopus spindle type most closely related to the simulation. Addition of inhibitory katanin antibodies caused on average a 4.6 μm (20%) and 5.5 μm (24%) increase in chromatin bead spindle length in X. laevis and X. tropicalis, respectively (Figure 5A). This confirms that katanin contributes to spindle length scaling in Xenopus by accelerating MT depolymerization, and indicates that the contribution of katanin to the total MT destabilization activity is greater in X. tropicalis than in X. laevis.

We next examined the effects of katanin inhibition in more complex spindles formed around X. laevis sperm that had progressed through interphase in the extract, with each sperm nucleus contributing a duplicated centrosome and paired sister kinetochores on 18 replicated chromosomes. Whereas sperm spindles formed in X. laevis extracts showed a similar length increase as chromatin bead spindles upon katanin inhibition, sperm spindles in X. tropicalis extracts became much longer, as thin and curly MT bundles protruded through the spindle poles (Figures 5B and 5D, Movie S5). The spindle midzone retained the antiparallel MT organization observed in control spindles. But whereas MTs in control spindles moved to the pole and depolymerized, MT bundles in katanin-inhibited spindles were stable, as revealed by speckle microscopy (Movie S6). Since chromatin bead spindles did not exhibit this phenotype, and centrosomes often fell off the ends of sperm spindles (unpublished data), we reasoned that these wispy spindle extensions originated from k-fibers. If k-fiber formation was blocked by addition of antibodies recognizing Nuf2, a component of the Ndc80 complex crucial for K-fiber formation (DeLuca et al., 2002; McCleland et al., 2003), the extensions did not appear upon katanin inhibition (Figures 5C and 5D). Thus, whereas chromatin bead spindles and X. laevis sperm spindles formed in X. laevis extracts lengthen proportionally upon katanin inhibition, X. laevis sperm in X. tropicalis egg extracts generate k-fibers that appear to overwhelm the MT depolymerization activity, disrupting the spindle poles (Figure 5E).
Depolymerization Coordinates Spindle and K-Fiber MTs

The wispy spindle extensions observed upon katanin inhibition in *X. tropicalis* extracts likely indicate a failure to adjust the minus-ends of the k-fibers to terminate precisely at the spindle poles. To study how such coordination is achieved, we added a new class of fibers to the model representing the bundles of MTs formed in each k-fiber. The plus-ends of k-fibers grew at the speed of flux but were tethered to the midplane of the spindle, thereby forcing k-fibers to move poleward. The plus-ends of k-fibers never underwent catastrophe, and minus-ends were indistinguishable from other MT minus-ends (Figure 6A, k-fibers in yellow). Since MT depolymerization and severing activity function similarly in the model, for simplicity we utilized MT depolymerization in simulations with k-fibers to minimize the number of parameters. Introduction of 5 k-fibers did not disrupt simulated spindle morphology (Figure 6B). However, if minus-end MT depolymerization activity associated with NuMA was decreased, mimicking katanin inhibition at spindle poles, k-fibers grew past non-k-fiber MTs and disrupted the poles (Figure 6C). The response was non-linear: spindles maintained pole integrity only above a certain threshold of depolymerization activity (Figure 6C, green circles).

When k-fibers protruded through the spindle pole, they continued to grow for the duration of the simulation without reaching a steady state (up to 80 min real-time, Figure 6C, red x’s), resembling the phenotype upon katanin inhibition in *X. tropicalis* spindles. A similar threshold phenomenon was observed if depolymerization was held constant and the number of k-fibers varied. Simulated spindles could reach a steady state within 45 min with up to 10 k-fibers, and spindle length scaled with the number of k-fibers (Figures 6D and 6E, Movie S7). Additional k-fibers disrupted poles, and spindles did not reach steady state (Figure 6D, red x’s). By explicitly including MT severing as described in Figure 4 and changing the number of k-fibers, the same threshold behavior could be observed (Figures 6F and 6G).

The simulation indicated that a balance between k-fiber number and MT depolymerization is required to maintain a steady-state spindle structure, and predicted that decreasing the number of k-fibers could compensate for lower katanin activity. To test whether this mechanism operates in *X. tropicalis* spindles, we added *X. tropicalis* sperm nuclei (10 chromosomes) instead of *X. laevis* sperm nuclei (18 chromosomes) and observed that katanin inhibition lengthened *X. tropicalis* sperm spindles 4.4 ± 2.0 μm (mean ± SE, n = 4 extracts), approximately...
the same amount as chromatin bead spindles in X. tropicalis, but that the integrity of the poles was preserved (Figure 6H). Occasionally, two X. tropicalis sperm fused together, yielding spindles with twice the number of chromosomes and kinetochores. Such spindles again displayed the long, wispy MT phenotype with disrupted poles upon katanin inhibition (Figure 6H). Katanin therefore not only regulates spindle length, but also plays a role in adjusting the length of the k-fibers, a feature that becomes apparent above a threshold number of k-fibers or below a threshold level of MT destabilization.

**P60 Serine 131 Phosphorylation by Aurora B Is a MajorDeterminant of Spindle Length**

To more definitively link spindle length regulation in Xenopus with phosphorylation of katanin p60 at Serine 131, we performed depletion and add-back experiments in X. tropicalis. Consistent
with antibody addition experiments (Figure 5B), depletion of p60 increased spindle length and disrupted spindle poles, effects that could be counteracted by adding back either X. tropicalis p60 or X. laevis p60S131A, but not MBP, X. laevis p60 or X. laevis p60S131E. Pairwise t test between MBP and other rescue conditions p value < 0.0001 except X. laevis p60 S131E (p value = 0.48). (mean ± SE. n = 240-500 spindles from three extracts per condition).

(B) Quantification of X. tropicalis spindle pole morphology in depletion/add-back experiments. P60 depletion leads to a high percentage of spindles with wispy MT extensions protruding through the spindle pole (see Figure 5B and C), which can be rescued by adding back X. tropicalis p60 or X. laevis p60S131A, but not MBP, X. laevis p60 or X. laevis p60S131E. (mean ± SD; n = 3 extracts, at least 150 spindles evaluated per condition).

(C and D) Aurora B inhibition decreases spindle length in X. laevis and is rescued by inhibiting p60 (D) but does not decrease spindle length in X. tropicalis (E). Quantification of spindle length in the presence of 5 μm ZM447439 with and without p60 katanin inhibition in the two extracts. (mean ± SD; N > 70 spindles from two extracts). The scale bar represents 10 μm.

(E) Model of spindle length scaling through phosphorylation of katanin p60 by Aurora B. P60 (red) is shown in its hexameric ring form.

DISCUSSION

By combining computational modeling with experiments in egg extracts, we have elucidated a MT destabilization mechanism underlying meiotic spindle length scaling between two different sized species of Xenopus frog. Although many proteins altering MT dynamics and spindle length have previously been characterized (Budde et al., 2001; Gaetz and Kapoor, 2004; Goshima et al., 2005; Houghtaling et al., 2009; Ohi et al., 2007), our study identifies katanin as a source of the physiological difference between related species. Katanin-mediated MT severing activity was originally characterized in Xenopus egg extracts (Vale, 1991), and has been shown to be an important regulator of meiotic spindle length in C. elegans (McNally et al., 2006). We now show that katanin activity is differentially regulated to scale the meiotic spindle through a phosphorylation mechanism. A single phosphorylation site missing from the X. tropicalis protein leads to higher activity compared to X. laevis and is largely responsible for the observed difference in spindle length.
The threshold effect observed when k-fibers were added to spindles in the simulation reflects a fundamental property of the model that appears to hold true in the *X. tropicalis* spindle. In the model, spindle length is determined by the distance that antiparallel MTs slide poleward during their lifetime. K-fiber MTs were implemented to reflect their documented properties of increased stability, resistance to MT depolymerization drugs, and slightly slower rates of poleward flux (Maddox et al., 2003). As the ratio of their number to the rate of MT minus end depolymerization was increased, the spindle lengthened due to increased MT lifetime, and at some threshold of stable k-fibers there was so little depolymerization per MT that their growth became unbounded. Thus, normal MT depolymerization not only affects spindle length, but also regulates k-fibers so that they terminate at the poles. This is a simple mechanism to coordinate the spindle MTs and the MTs composing the k-fibers, and is functionally robust to the fact that the two classes of MTs have different characteristics. Thus, the complementary MT depolymerization activities of kin-13 and katanin can be seen as a buffer necessary to ensure that k-fibers reliably terminate at the spindle poles. Experiments altering the ratio of k-fiber number/MT depolymerization activity in *X. laevis*, however, have thus far not given the same phenotype (unpublished data), suggesting that the k-fiber stability threshold is further from the steady-state situation in *X. laevis*. This variation likely reflects fundamental differences in spindle architecture between the two species, such as the ratio of k-fiber to spindle MTs, and we predict that other systems will have different sensitivities to k-fiber number, stability and MT depolymerization, which may correlate with spindle size or other functional demands. Characterizing these parameters in a wide variety of systems will provide important insight into spindle function and evolution.

Our experiments identify inhibitory phosphorylation as a regulatory mechanism of *X. laevis*, but not *X. tropicalis* katanin that lacks Serine 131, a consensus Aurora kinase site. A corresponding residue is found in katanin of most species including human, suggesting that this regulation is generally conserved. Aurora B phosphorylates katanin more efficiently in vitro, and downregulates other MT stabilizers including kinesin-13 (Knowlton et al., 2006; Knowlton et al., 2009; Zhang et al., 2007b) and Op18 (Gadea and Ruderman, 2006), supporting the overall MT-stabilizing role of this mitotic kinase family. While most katanin in *Xenopus* spindles colocalizes with Aurora A, its phospho-regulation is clearly complex since mutation of Ser 131 does not abolish all Aurora B phosphorylation of *X. laevis* katanin. *X. tropicalis* can be phosphorylated given long enough reaction times with high Aurora B activity despite missing the equivalent Ser 131 residue (unpublished data), and the Polo like kinase, Pxl-1, has been implicated in regulating katanin (McNally et al., 2002). The phosphorylation by Aurora B suggests that in *X. laevis* spindles katanin is downregulated near the chromatin and kinetochores while *X. tropicalis* katanin may be active in these locations, and plus-end stabilization of kinetochore MTs may contribute to the wispy k-fiber phenotype observed upon p60 depletion. The difference in katanin-mediated severing activity between the two species’ extracts (approximately 20-fold) is more dramatic than the difference in spindle length, which is less than 2-fold. This discrepancy is perhaps not surprising, since many MT stabilizing activities in the spindle may counteract and control MT depolymerization, and severing proteins including spastin and katanin are known to be more active toward polyglutamylated brain MTs used in the severing assay (Lacroix et al., 2010). Furthermore, the contribution of p80, the targeting/regulatory subunit of katanin, has not yet been determined (McNally et al., 2000).

The model of a meiotic spindle steady state helped us identify potential scaling activities, and we tested some of these mechanisms based on our ability to experimentally inhibit katanin. However, multiple spindle sizing mechanisms must operate, since inhibition of katanin in extracts does not increase *X. tropicalis* spindle length to the length of *X. laevis* spindles without disrupting spindle poles. During *Xenopus* embryonic development, spindles shorten approximately five-fold (Wühr et al., 2008), and the size of the cell becomes a major factor in setting the dimensions of the spindle. In addition to differential phosphorylation, other mechanisms including alternative splicing, expression level, or titration of regulatory factors could operate. In *C. elegans*, severing by the katanin homolog MEI-1/MEI-2 regulates meiotic spindle length before polar body extrusion (McNally et al., 2006; Srayko et al., 2006). This spindle sizing activity is normally inactivated at the end of meiosis by protein degradation but can also shrink *C. elegans* mitotic spindles (Srayko et al., 2006). We are currently testing whether severing or other mechanisms contribute to spindle scaling during *Xenopus* development. Plus-end catastrophe frequency, which robustly altered spindle length in the simulation, is one candidate mechanism that could operate directly through destabilizing proteins or indirectly through the activity of MT binding proteins, as proposed for centrosome-associated TPXL during *C. elegans* development (Greenan et al., 2010). Combined with theory, experimental approaches in a variety of organisms will reveal other functional mechanisms of spindle morphogenesis and scaling across species.

**EXPERIMENTAL PROCEDURES**

**Xenopus Egg Extracts and Spindle Assembly Reactions**

*X. laevis* and *X. tropicalis* egg extracts were prepared and induced to progress through the cell cycle as described (Brown et al., 2007; Hannak and Heald, 2006; Maresca and Heald, 2006). Chromatin-coated beads were prepared as described (Heald et al., 1996). For antibody inhibition experiments, purified rabbit IgG (Covance), Rabbit anti-p60 and rabbit anti-HuD (gift from the Stukenberg lab) in PBS or XB were added at a 1:10 dilution. Extracts were immunodepleted at room temperature for 15 min following (Hannak and Heald, 2006), 2 μM recombinant p60 or p60 mutants were added for rescue experiments. For inhibition of Aurora B, 5 μM 2M447439 (Tocris biosciences) was added to cyclc spindle reactions with or without inhibition of p60 with an anti-p60 antibody. Live movies of spindle reactions were imaged in the presence of 50 μg/ml X-rhodamine- or Cy3-labeled porcine brain tubulin (Hyman et al., 1991) and an oxygen scavenging mix (Hartman et al., 1998). Images were obtained at 30 s intervals on an Olympus BX51 fluorescence microscope with TRITC and Cy3 filters (Chroma Technology) and a 40x objective (UPlanFl N, Olympus; NA 0.75) controlled by μManager (http://www.micro-manager.org/) with a Hamamatsu Orca-ER cooled CCD camera. Skepted tubulin movies were obtained using a 60x objective (PlanApo N, Olympus; NA 1.42) at 5 s intervals. Immuno-fluorescence staining of fixed spindles was performed according to (Maresca and Heald, 2006). Rabbit anti-p60 and donkey anti-rabbit Alexa 488 (Invitrogen) were used at 1:500. Spindle length and fluorescence intensity were quantified using MATLAB.
MT Depolymerization Assays
MTs were polymerized from bovine or porcine brain tubulin (Cytoskeleton) and our own X-rhodamine-labeled or Alexa-488 labeled porcine brain tubulin (Hyman et al., 1991) or rhodamine-labeled bovine brain tubulin (Cytoskeleton) at a ratio of 20:1 using taxol (Paclitaxel, Sigma). Flow cells were constructed with an 18 x 18 mm glass coverslip and Scotch double-stick tape for a volume of ~10 µl. A mutant rigor kinase (McNally and Thomas, 1998) in KAB (20 mM HEPES pH 7.5, 25 mM K-glutamate, 2 mM MgCl₂, 1 mg/ml BSA, 10% glycerol, 0.02% Triton X-100) with 1 mM ATP (Hartman et al., 1998) was incubated in the flow cell. The cell was washed with KAB, incubated with MTs in KAB with ATP, and washed with BRB80 (80 mM PIPES [pH 6.8], 1 mM MgCl₂, 1 mM EGTA). Finally, 18–20 µl of crude egg extract, extract supplemented with recombinant MBP-p60s or 40 µl of purified recombinant MBP-p60 in KAB+ (KAB supplemented with 20 mg/ml BSA, 5 mM phospho- enol pyruvate, pyruvate kinase and 2 mM ATP), with an oxygen scavenging mix (Hartman et al., 1998) was flowed in and immediately imaged at 10 or 15 s intervals with a 60x objective. The total length of polymerized tubulin in images was quantified using ImageJ.

MT Binding Assays and Protein Concentration
MTs for katanin binding experiments were polymerized as described above with the addition of bovine brain biotinylated tubulin (Cytoskeleton) at a 30:1 ratio to MTs. Extract was incubated with 10 mM ATP, diluted 3x into KAB, and incubated with either BRB80 or BRB80 containing biotin-MTs. Streptavidin MyOne Dynabeads (Invitrogen) were incubated in the solution before being retrieved with a magnet and washed twice with BRB80. The relative concentrations of p60 and tubulin in the egg extract and in MT pulldown experiments were quantified by western blot with rabbit anti-p60 and mouse anti-tubulin (Developmental Studies Hybridoma Bank, E7) antibodies and an Odyssey Infrared Imaging System (LI-COR Biosciences). The ratio of katanin to biotin-tubulin was corrected for the polymerization of endogenous tubulin.

In vitro MT binding assays were performed with E306Q or E310Q ATPase deficiency mutants in X. laevis or X. tropicalis p60, respectively. 10 µM p60 was added to increasing amounts of taxol stabilized MTs in KAB buffer with 2 mM ATP, incubated for 15 min and pelleted by centrifugation at 40,000 x g. Pellets were separated by SDS-PAGE and Coomassie stained, scanned and quantified using ImageJ.

Purification of Recombinant p60
X. laevis or X. tropicalis p60 sequence was PCR amplified and cloned in pMAL-C5X expression vector (New England Bio Labs). ATPase and phospho null mutant proteins were generated by quickchange mutagenesis (Agilent). Protein was induced using 1mM IPTG in BL21-RIPL (Agilent) for ~16 hr at 16°C. Purification was following the manufacturer’s protocol using the following buffer for all steps: 20 mM HEPES [pH 7.7], 250 mM NaCl, 1 mM MgCl₂, 1 mM 2-mercaptoethanol, 10% glycerol, 2 mM ATP.

Katanin Phosphorylation Assays
2–5 µM recombinant p60 was added to extracts supplemented with 2 µM okadaic acid, 1 µM microcystin, 1.25 mM ATP, γ-S, and 1.3 mM GTP, and incubated at room temperature for 30 min. P60 was retrieved by diluting extracts with 400 µl of XB buffer containing 2.5 µM p-nitrobenzyloxymesitylate (Eptepicon) and binding to amylase resin. Incorporation of thio-phosphate into recombinant p60 katanin was determined by western blotting using a nitrobenzyl thio-phosphate ester specific antibody (Epitomics) via the manufacturer’s protocol, imaged with an Odyssey Infrared Imaging System (LI-COR Biosciences) and corrected for total MBP-p60 loaded, as determined by western blotting using an anti-MBP monoclonal antibody (New England Bio Labs) and normalized to the X. laevis p60 signal for each extract. Signals for thio-ester phosphate and MBP were found to be within the linear range of detection (data not shown).

Phosphorylation of purified katanin was performed as previously described utilizing recombinant MBP-p60 or a GST-tagged version of p60 lacking the AAA ATPase domain and GST-Aurora B/INCENP (Sessa et al., 2005) except that ATP concentrations were 250 µM. Reactions were separated by SDS-PAGE and stained with Pro-Q Diamond phosphoprotein gel stain (Invitrogen), scanned with a Typhoon Trio imager (GE Healthcare Lifesciences) then stained for total protein using Sypro Ruby protein gel stain (Invitrogen).

Simulation of MT Severing and Kinetochore MTs in Cytosim
The model is as described in Loughlin et al., 2010, except for the addition of severing. An arbitrary baseline probability for severing was set as a simulation parameter. The effective severing rate for each MT segment was the baseline rate multiplied by the NuMA-recruited MT destabilization activity in the 0.5 µm by 0.5 µm gridbox containing the MT segment (Loughlin et al., 2010). Thus, the effective severing rate increased with NuMA density and decreased with adjacent MT density. Starting at the MT minus-end and proceeding along the MT segments toward the plus-end, a severing event could occur at the severing rate calculated at each segment. Because severing is sufficiently rare, only one severing event per MT per timestep was allowed. When a severing event occurred, a new plus-end and a new minus-end were generated at the severing location on the MT, resulting in two independent MTs. MT plus-ends created by severing were in a shrinking state while new minus-ends were stable. Severing was a stochastic process while depolymerization was modeled continuously.

Kinetochore fibers were modeled as opposing pairs of fibers with their plus-ends anchored at X = 0, corresponding to the center of the RanGTP zone. MT plus-ends constantly grew at the speed of flux without catastrophe and the entire k-fibers were pushed poleward at this same speed. Minus-ends were identical to spindle MT minus-ends.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Extended Experimental Procedures, two figures, and seven movies and can be found with this article online at doi:10.1016/j.cell.2011.11.014.

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EXTENDED EXPERIMENTAL PROCEDURES

Computational Modeling of MT Severing
The simulation and statistical modeling are described in Loughlin et al. (Loughlin et al., 2010). Severing only occurred in the vicinity of NuMA. The probability of an event was additive with respect to NuMA and was distributed to adjacent MTs. Each NuMA radiated its associated severing rate, 90% to MTs within 0.5 μm and 10% to MTs within 0.5–1.5 μm. The severing rate is expressed per unit length of MT (0–0.04 s⁻¹/μm). In the simulations, MTs are represented with segments of 0.5 μm. For simplicity, we thus only tested the severing rate of these segments based on the local NuMA and MT distribution; from minus to plus ends and only one severing event was considered per MT in one time-step. This is not a limitation considering the probability of severing a segment in one time step (0.02 s⁻¹ × 0.005 s = 10⁻⁴).

Xenopus Egg Extracts and Spindle Assembly Reactions
In meiotic (cytostatic factor-arrested) reactions, p60 antibodies were added before or after spindle assembly with similar results. In mitotic reactions, p60 and Nuf2 antibodies were added at the transition from interphase to mitosis. Live imaging of X. tropicalis spindles required coverslips that had been cleaned with NaOH, and treated with PEG-silane (Gelest) in a mixture of ethanol and acetic acid.

For MT destabilization assays, a mutant rigor kinesin was incubated in the flow cell in KAB (see Experimental Procedures) with 1 mM ATP for 2 min. The cell was washed with 100 μl KAB, and then incubated with 10 μl of rhodamine-labeled Taxol-stabilized MTs in KAB with ATP for 2 min. Flow cells were washed with 100 μl of BRB80 before addition of crude egg extract.

For katanin binding experiments extract was incubated with 10 mM ATP₃S for 10 min. After 3x dilution into KAB, the extract was incubated for 10 min with either buffer or buffer with biotin-MTs. Dynabeads were incubated in the solution for 2 min, retrieved with a magnet, and quickly washed twice with BRB80. In MT binding assays, the polymerization of endogenous tubulin was not equivalent in the two extracts. To correct, the ratio of katanin to biotin-tubulin was calculated:

\[
\frac{p_{\text{MTs}}^{60} - p_{\text{buffer}}^{60}}{\text{tubulin}_{\text{MTs}} - \text{tubulin}_{\text{buffer}}}
\]

For immunodepletion experiments, 80 μg of rabbit anti-p60 antibody (SDIX) was necessary to deplete 110 μl of egg extract.

In vitro MT binding shows the loading and pellets only of binding reactions and MBP alone as a control showed no binding (data not shown). Data was fit to the following scaled binding quadratic equation similar to NDC80 complex and the Dam1 complex (Ciferri et al., 2008; Ramey et al.):%

\[ \% \text{bound} = \frac{B_m + K_a + x}{\sqrt{(B_m + K_a + x)^2 - (4B_mx)}} \]

were Bₘₐₓ is the maximal percent bound and x is the tubulin concentration. The binding p60 to MTs saturated at low percent bound necessitating the Bₘₐₓ scaling factor and indicating either limited non-uniformly distributed binding sites such as lattice defects as previously suggested (Davis et al., 2002), or a subset of p60 protein incompetent to bind MTs. In either case, the difference in binding affinity for MTs between the X. laevis and X. tropicalis p60 was not significant enough to be considered different, within the error of the experiment, though small differences in binding affinity cannot be completely excluded. There was little to no difference in MT affinity of the p60 MT binding domains expressed without the ATPase domains (data not shown).

Image Analysis
Image analysis was conducted in MATLAB with the Image Analysis Toolbox. Spindle length was measured by thresholding images and projecting spindles along the long axis. In experiments involving dynein inhibition, thresholded spindles were skeletonized to a single line of pixels to account for curvature. For fluorescence intensity profiles, mitotic X. laevis and X. tropicalis spindles were spun down onto the same coverslip. Fluorescence intensity in arbitrary units was summed in a 20 pixel-wide line along the spindle axis, and the ratio of katanin to tubulin intensity was taken along this line and averaged over all spindles for each species (n = 20 minimum).

The total amount of polymerized tubulin was quantified in flow cells by dynamically thresholding images. Exponential curves (A·e⁻ᵗ/λ) were fit to data by minimizing the sum of squared errors. Half life measurements were then calculated from the decay rate, \( τ_{1/2} = \log(2)/λ \), and represent the time at which half of the polymerized tubulin has been depolymerized.

SUPPLEMENTAL REFERENCES


Figure S1. Comparison of *X. laevis* and *X. Tropicalis* Katanin, related to Figure 2

(A) Protein sequence comparison of *X. tropicalis* and *X. laevis* katanin to the human sequence. Regions marked in yellow, blue, red and green are p80 binding, MT binding, AAA ATPase and oligomerization domains, respectively. Phosphorylation site Serine 131 is present in the *X. laevis* sequence but missing from the *X. tropicalis* sequence.

(B) Western blot of MT copelleting assays in three *X. laevis* and *X. tropicalis* egg extracts probed for katanin and tubulin. Overlay shows tubulin in red and katanin in green.
Figure S2. MT Binding Affinity is Similar for X. Tropicalis and X. laevis p60, related to Figure 3

(A) Purified recombinant X. laevis and X. tropicalis p60 was added to increasing concentrations of taxol-stabilized MTs, which were pelleted and analyzed by SDS-PAGE.

(B) MT Binding curve of X. laevis and X. tropicalis p60.

(C) Severeing of MTs by 800 nM recombinant X. tropicalis p60 in the presence of ADP.

(D) Sequence alignment comparing the amino acids surrounding X. laevis serine 161 in X. tropicalis and 7 other species. The predicted Aurora phosphorylation site is conserved in vertebrates with the exception of X. tropicalis.

(E) Half-life of MTs in flow cell destabilization assays in X. laevis extracts supplemented with indicated proteins. Mean ± SEM, n = 3 extracts.