

Aurora A activates D-TACC–Msp complexes exclusively at centrosomes to stabilize centrosomal microtubules

Teresa P. Barros,¹ Kazuhisa Kinoshita,² Anthony A. Hyman,² and Jordan W. Raff¹

¹The Wellcome Trust/Cancer Research UK Gurdon Institute, Department of Genetics, Cambridge CB2 1QN, England, UK

²Max Planck Institute of Molecular Cell Biology and Genetics, D-01307 Dresden, Germany

Centrosomes are the dominant sites of microtubule (MT) assembly during mitosis in animal cells, but it is unclear how this is achieved. Transforming acidic coiled coil (TACC) proteins stabilize MTs during mitosis by recruiting Minispindles (Msps)/XMAP215 proteins to centrosomes. TACC proteins can be phosphorylated *in vitro* by Aurora A kinases, but the significance of this remains unclear. We show that *Drosophila melanogaster* TACC (D-TACC) is phosphorylated on Ser863 exclusively at centrosomes during mitosis in an Aurora A-dependent manner. In embryos expressing only a mutant

form of D-TACC that cannot be phosphorylated on Ser863 (GFP-S863L), spindle MTs are partially destabilized, whereas astral MTs are dramatically destabilized. GFP-S863L is concentrated at centrosomes and recruits Msps there but cannot associate with the minus ends of MTs. We propose that the centrosomal phosphorylation of D-TACC on Ser863 allows D-TACC–Msps complexes to stabilize the minus ends of centrosome-associated MTs. This may explain why centrosomes are such dominant sites of MT assembly during mitosis.

Introduction

The centrosome is the main microtubule (MT) organizing center in animal cells, and it plays an important part in organizing many processes in the cell, including cell polarity, intracellular transport, and cell division (Glover et al., 1993). Aurora A protein kinases are centrosomal proteins that are essential for mitosis and have been widely implicated in human cancer (Meraldi et al., 2004; Marumoto et al., 2005). They have several functions in mitosis, and they appear to play a particularly important part in regulating centrosome behavior. They are, for example, required for the dramatic recruitment of pericentriolar material to the centrosome, which occurs as cells enter mitosis (Hannak et al., 2001; Berdnik and Knoblich, 2002). This centrosome “maturation” is thought to ensure that centrosomes are the dominant sites of MT assembly during mitosis.

It was recently shown that Aurora A can phosphorylate the transforming acidic coiled coil (TACC) family of centrosomal proteins *in vitro* (Giet et al., 2002; Pascreau et al., 2005). TACC proteins stabilize spindle MTs in flies (Gergely et al., 2000a; Lee

et al., 2001), humans (Gergely et al., 2003), worms (Bellanger and Gonczy, 2003; Le Bot et al., 2003; Srayko et al., 2003), and frogs (O’Brien et al., 2005) apparently by recruiting the MT-stabilizing protein Minispindles (Msps)/XMAP215/ch-TOG (colonic and hepatic tumor overexpressing gene; hereafter referred to as Msps) to the centrosome. Msps proteins bind directly to MTs and regulate MT dynamics primarily by influencing events at MT plus ends (for review see Cassimeris, 1999; Ohkura et al., 2001; Kinoshita et al., 2002). In *Xenopus laevis* egg extracts, for example, the balance of activity between XMAP215 and the MT-destabilizing protein XKCM1/MCAK (mitotic centromere-associated kinesin) at MT plus ends seems to be the main parameter that determines the overall stability of MTs (Tournebize et al., 2000; Kinoshita et al., 2001).

These findings present something of a paradox; Msps proteins act mainly on MT plus ends, yet, *in vivo*, they are most strongly concentrated at centrosomes, where the minus ends of MTs are clustered. To explain this paradox, it has been proposed that TACC proteins recruit Msps to centrosomes to ensure either that Msps is efficiently “loaded” onto MT plus ends as they grow out from centrosomal nucleation sites or that Msps can stabilize the minus ends of centrosomal MTs after they have been released from their nucleating sites (Lee et al., 2001). The finding that a GFP–*D. melanogaster* TACC (D-TACC)

Correspondence to Jordan W. Raff: j.raff@gurdon.cam.ac.uk

Abbreviations used in this paper: D-TACC, *Drosophila melanogaster* TACC; Msps, minispindles; MT, microtubule; P-D-TACC, phospho-D-TACC; TACC, transforming acidic coiled coil; X-TACC, *Xenopus laevis* TACC.

The online version of this article contains supplemental material.

fusion protein appears to associate with both the plus and minus ends of MTs in living *D. melanogaster* embryos is consistent with both possibilities (Lee et al., 2001).

Ser626 of *X. laevis* TACC3/maskin has recently been identified as a major site of Aurora A phosphorylation in vitro (Kinoshita et al., 2005; Pascreau et al., 2005), and this site is conserved in humans (Ser558) and flies (Ser863). We have investigated the potential significance of the phosphorylation of this site in D-TACC in regulating MT behavior in *D. melanogaster* embryos. Our findings suggest that D-TACC–Msp complexes can stabilize MTs in two ways: (1) when not phosphorylated on Ser863, they can stabilize MTs throughout the embryo, presumably through interactions with MT plus ends; (2) when D-TACC is phosphorylated on Ser863, the complexes can stabilize MTs by interactions with MT minus ends. This second mechanism appears to be activated by Aurora A specifically at centrosomes, which perhaps explains why centrosomes are such dominant sites of MT assembly during mitosis.

Results

D-TACC is phosphorylated at Ser863 in vivo in an Aurora A-dependent manner

Aurora A can phosphorylate TACC proteins in vitro (Giet et al., 2002; Pascreau et al., 2005), and Ser626 of *X. laevis* TACC3/maskin is a major site of Aurora A phosphorylation in vitro (Kinoshita et al., 2005; Pascreau et al., 2005). This phosphorylation site is conserved in humans (Ser558) and flies (Ser863). To begin to investigate the functional significance of the phosphorylation of TACC by Aurora A, antibodies were raised in rabbits against a phosphopeptide from this region of D-TACC. Sera from these rabbits were first depleted of antibodies that recognize nonphosphorylated D-TACC by passing the sera over a column of nonphosphorylated peptide until no further immunoreactivity against the nonphosphopeptide could be detected in the sera in ELISA assays. Antibodies were then purified from this depleted sera with a column containing the phosphopeptide.

In immunofluorescence experiments, both anti-D-TACC and antiphospho-D-TACC (P-D-TACC) antibodies strongly stained centrosomes during mitosis; anti-D-TACC antibodies also stained spindle MTs, but this staining was essentially undetectable with anti-P-D-TACC antibodies. This demonstrates that these antibodies did not recognize all of the D-TACC in the embryo, presumably because not all of the D-TACC was phosphorylated on Ser863 (Fig. 1 A). In early embryos, in which rapid mitotic cycles lack G1 and G2 phases, both antibodies stained centrosomes throughout the cell cycle (not depicted), whereas in cellularized embryos (Fig. 1 B), they stained centrosomes only during mitosis. Thus, in somatic cells, D-TACC and P-D-TACC are only detectable at centrosomes during mitosis.

To confirm that anti-P-D-TACC antibodies recognized only P-D-TACC, we generated flies that expressed a fusion protein in which GFP is coupled to either wild-type D-TACC (GFP-D-TACC) or a mutant D-TACC in which Ser863 was replaced by a leucine residue (GFP-S863L). In *d-tacc* mutant

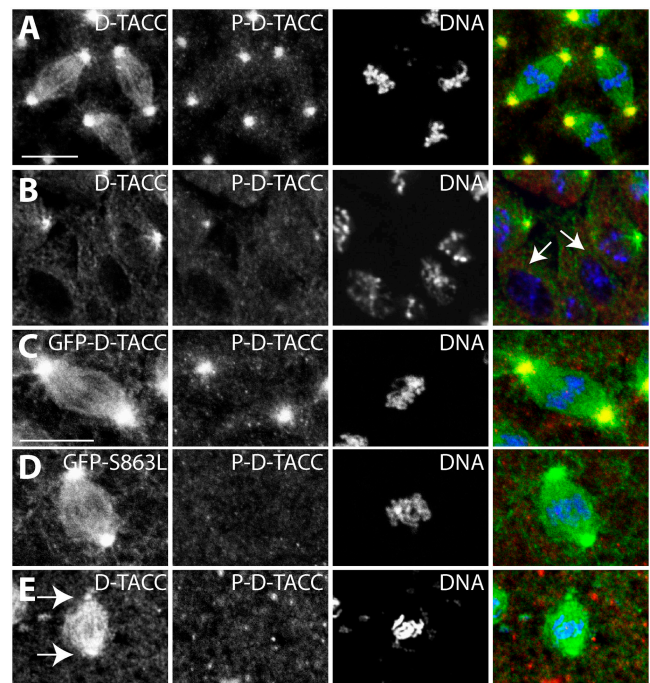


Figure 1. P-D-TACC is concentrated at centrosomes during mitosis. (A and B) The distribution of D-TACC, P-D-TACC, and DNA (green, red, and blue in merged image, respectively) in a wild-type syncytial embryo (A) and a wild-type cellularized embryo (B). D-TACC and P-D-TACC are not detectable at centrosomes in interphase cells in cellularized embryos (interphase cells are indicated by arrows). Note that only a single centrosome from each mitotic cell is present in the focal planes shown. (C) The distribution of GFP-D-TACC, P-D-TACC, and DNA in a *d-tacc* mutant embryo that has no endogenous D-TACC. GFP-D-TACC is concentrated at centrosomes and spindles, whereas P-D-TACC is concentrated at centrosomes. (D) The distribution of GFP-S863L, P-D-TACC, and DNA in a *d-tacc* mutant embryo. P-D-TACC is no longer detectable at centrosomes. (E) The distribution of D-TACC, P-D-TACC, and DNA in *aur¹* mutant embryos. D-TACC is weakly detectable at centrosomes but P-D-TACC is not. Bar, 10 μ m.

embryos, which lacked detectable endogenous D-TACC protein (Fig. 2 A), anti-P-D-TACC antibodies no longer stained centrosomes (not depicted). Centrosome staining was restored in *d-tacc* mutant embryos that expressed GFP-D-TACC (hereafter referred to as GFP-D-TACC embryos; Figs. 1 C and 2 A) but not in *d-tacc* mutant embryos that expressed GFP-S863L (hereafter referred to as GFP-S863L embryos; Fig. 2 A) even though GFP-S863L was present on centrosomes and spindle MTs (Fig. 1 D). Thus, P-D-TACC antibodies appear to specifically recognize D-TACC that has been phosphorylated on Ser863.

To test whether the phosphorylation of D-TACC on Ser863 was dependent on Aurora A, we examined embryos that were laid by homozygous mutant *aur¹* females. These embryos have \sim 10% of wild-type Aurora A levels, and they invariably die as a result of mitotic defects (Glover et al., 1995; unpublished data). Aurora A is required for the efficient recruitment of several proteins to centrosomes during mitosis (Hannak et al., 2001; Berdnik and Knoblich, 2002; Giet et al., 2002). In *aur¹* mutant embryos, D-TACC was distributed normally on mitotic spindle MTs, but greatly reduced levels were present at centrosomes (Fig. 1 E, arrows). In such embryos, however, P-D-TACC was essentially undetectable at cen-

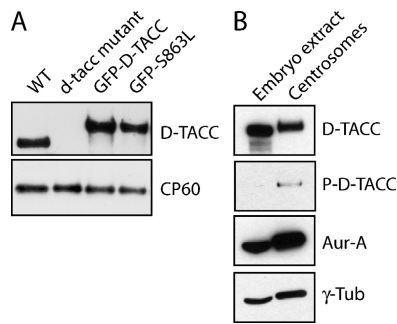


Figure 2. Western blot analysis of GFP-D-TACC and GFP-S863L expression and of the association of P-D-TACC with centrosomes. (A) A Western blot probed with anti-D-TACC antibodies. First lane, wild-type (WT) embryos; second lane, *d-tacc* mutant embryos; third lane, *d-tacc* mutant embryos expressing GFP-D-TACC; fourth lane, *d-tacc* mutant embryos expressing GFP-S863L. The same blot was reprobed with antibodies against the centrosomal protein CP60 as a loading control. (B) Western blots of whole embryo extracts (first lane) and purified centrosomes (second lane) probed with anti-D-TACC, -P-D-TACC, -Aurora A, and - γ -tubulin antibodies.

trosomes (Fig. 1 E). Thus, Aurora A is required to generate P-D-TACC at centrosomes.

P-D-TACC can only be detected at centrosomes

These immunofluorescence studies suggested that P-D-TACC was more concentrated at centrosomes than the bulk of the D-TACC protein. To confirm this, we performed Western blotting experiments on whole embryo extracts and on purified centrosome fractions. Anti-D-TACC antibodies recognized D-TACC in both preparations, and we calculated that $\sim 1\%$ of total embryonic D-TACC is present in purified centrosome fractions (Fig. 2 B; see Centrosome purification). We obtained similar results with antibodies against other centrosomal proteins, including Aurora A, γ -tubulin (Fig. 2 B), Msps, CP190, and CP60 (not depicted). In contrast, anti-P-D-TACC antibodies did not recognize D-TACC in the embryo extract, again demonstrating that these antibodies do not recognize the majority of D-TACC in the embryo, presumably because it is not phosphorylated on Ser863 (Fig. 2 B, left lane). These antibodies did, however, recognize a protein of exactly the same size as D-TACC in purified centrosome fractions (Fig. 2 B, right lane). Together with our immunofluorescence studies (Fig. 1), these data indicate that our anti-P-D-TACC antibodies recognize only P-D-TACC, that only a small fraction of total D-TACC in the embryo is phosphorylated at Ser863, and that this fraction of P-D-TACC appears to be exclusively found at centrosomes.

Ser863 is essential for the function of D-TACC but not for its localization to centrosomes

To test the functional significance of the phosphorylation of D-TACC on Ser863, we examined the behavior of GFP-D-TACC and GFP-S863L embryos. We first tested the viability of these embryos. Most *d-tacc* mutant embryos die early in embryonic development as a result of either a failure in pronuclear fusion or an accumulation of mitotic defects; both phenotypes appear

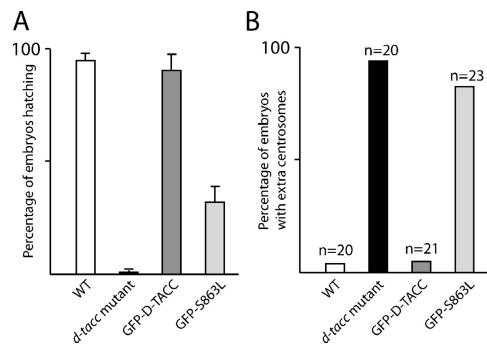


Figure 3. Quantitation of viability and mitotic defects in GFP-D-TACC and GFP-S863L embryos. (A) A bar chart showing the percentage of embryos that hatch from the following genotypes: wild-type (WT), *d-tacc* mutant, *d-tacc* mutant expressing GFP-D-TACC, and *d-tacc* mutant expressing GFP-S863L. Error bars represent SD. (B) A bar chart showing the percentage of embryos with extra centrosomes at nuclear cycle 14 (indicative of mitotic errors during earlier rounds of nuclear division; Raff, 2003). Numbers at the top of each bar are the number of embryos that were counted.

to be caused by a destabilization of MTs (Gergely et al., 2000b; Lee et al., 2001). Less than 1% of *d-tacc* mutant embryos were viable (Fig. 3 A), and those that did develop had a high incidence of mitotic defects (Fig. 1 B), which were quantified in this study as the percentage of embryos with “extra centrosomes” at the cortex during nuclear cycle 14 (these extra centrosomes are indicative of earlier failures in mitosis; Fig. 3 B; for review see Raff, 2003). In contrast, $\sim 90\%$ of GFP-D-TACC embryos were viable (Fig. 3 A), and these embryos had very few mitotic defects (Fig. 3 B), suggesting that GFP-D-TACC was almost fully functional. The viability of GFP-S863L embryos, however, was only $\sim 30\%$ (Fig. 3 A), and those embryos that did develop had a high incidence of mitotic defects (Fig. 3 B), suggesting that GFP-S863L was only partially functional.

To investigate why GFP-S863L was only partially functional, we examined the behavior of GFP-D-TACC and GFP-S863L in living *d-tacc* mutant embryos by using time-lapse confocal microscopy. Like D-TACC in normal embryos, GFP-D-TACC was strongly localized to centrosomes (Fig. 4 A and Video 1, available at <http://jcb.org/cgi/content/full/jcb.200504097/DC1>). GFP-S863L was also strongly concentrated on centrosomes (Fig. 4 B and Video 2, <http://jcb.org/cgi/content/full/jcb.200504097/DC1>), although this concentration was consistently slightly weaker than that seen with GFP-D-TACC (Fig. S1). In embryos that were treated with colchicine to depolymerize MTs, GFP-D-TACC (Fig. 5 A) and GFP-S863L (Fig. 5 B) remained concentrated at centrosomes, demonstrating that both proteins behave as integral centrosomal proteins (although the centrosomal localization of GFP-S863L was again slightly weaker than that seen with GFP-D-TACC; unpublished data). Moreover, the recruitment of Msps to centrosomes was essentially indistinguishable in GFP-D-TACC and GFP-S863L embryos (Fig. 5). Thus, although GFP-S863L is only partially functional, its ability to localize to centrosomes and recruit Msps there appears largely unperturbed. In support of this conclusion, we found that GFP-D-TACC and GFP-S863L interacted equally well with Msps in immunoprecipitation experiments (Fig. S2).

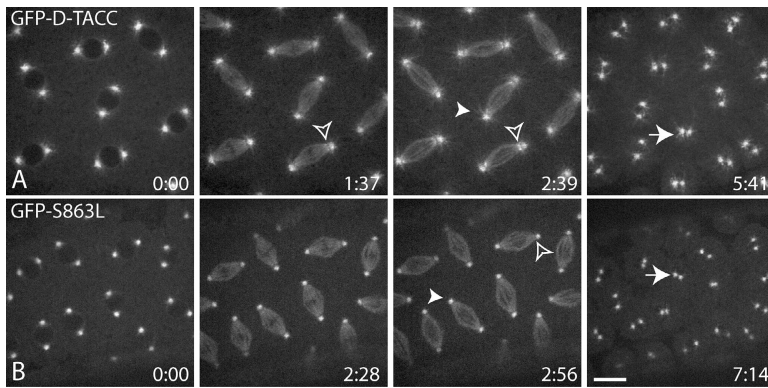


Figure 4. Analysis of living GFP-D-TACC and GFP-S863L embryos. Selected images from videos of living GFP-D-TACC (A) and GFP-S863L (B) embryos (see Videos 1 and 2, respectively, available at <http://www.jcb.org/cgi/content/full/jcb.200504097/DC1>). Time (minutes/seconds) is shown at the bottom right of each image. Images show the embryos just before entry into mitosis, in metaphase, early anaphase, and early interphase of the following nuclear cycle. Arrows indicate the position of centrosomal flares; closed arrowheads indicate the long arrays of astral MTs in anaphase; open arrowheads indicate the concentration of the GFP fusion protein on the minus ends of spindle MTs. These features are readily detectable in GFP-D-TACC embryos but not in GFP-S863L embryos (see text for details). Bar, 10 μ m.

Astral MTs are destabilized in GFP-S863L embryos

Although GFP-S863L was concentrated on centrosomes and spindles, there were significant differences in its behavior when compared with GFP-D-TACC. GFP-D-TACC exhibited prominent “flaring” activity (Lee et al., 2001; Megraw et al., 2002) as the protein moved back and forth from the centrosome in association with the astral MTs (Fig. 4 A, arrow; and Video 3, available at <http://jcb.org/cgi/content/full/jcb.200504097/DC1>). Because this movement is also exhibited by Msp-GFP and is dependent on MTs, we have speculated that it reflects the movement of D-TACC–Msp complexes on the growing and shrinking plus ends of MTs (Lee et al., 2001). GFP-S863L also exhibited some flaring, but it was consistently much less than that seen with GFP-D-TACC (Fig. 4 B, arrow; compare Videos 1 with 2 and Videos 3 with 4, available at <http://jcb.org/cgi/content/full/jcb.200504097/DC1>). Moreover, during anaphase, when centrosomes normally organize very long astral MTs, astral MTs were easily visible in GFP-D-TACC embryos (Fig. 4 A, arrowheads) but were much less prominent in GFP-S863L embryos (Fig. 4 B, arrowheads). These observations suggested that either there were fewer astral MTs in GFP-S863L embryos or GFP-S863L was specifically unable to bind to astral MTs.

To distinguish between these possibilities, we fixed GFP-D-TACC and GFP-S863L embryos and stained them with antitubulin antibodies to examine the distribution of MTs (Fig. 6). In GFP-D-TACC embryos, astral MTs were readily detectable at all stages of the cell cycle and were particularly prominent during anaphase (Fig. 6 A). In GFP-S863L embryos, few astral MTs were detectable at any stage of the cell cycle, including anaphase, although spindle MTs appeared to be relatively normal (Fig. 6 B). To confirm that spindle MTs were relatively unaffected in GFP-S863L embryos, we compared the lengths of mitotic spindles in living GFP-D-TACC and GFP-S863L embryos at the same stage of mitosis (10 s before the initiation of anaphase) during nuclear cycle 10. The mean spindle length was $14.7 \pm 0.35 \mu\text{m}$ (mean \pm SD) in GFP-D-TACC embryos (22 spindles were measured from three different embryos) and $13.2 \pm 0.5 \mu\text{m}$ in GFP-S863L embryos (20 spindles were measured from three different embryos), which is a difference of only $\sim 10\%$. In contrast, when we injected anti-D-TACC antibodies into wild-type embryos to

perturb global D-TACC function, the mitotic spindles shortened by $\sim 25\%$ (Gergely et al., 2000b; unpublished data). Thus, although GFP-S863L is apparently unable to stabilize astral MTs, it can at least partially stabilize spindle MTs. It is presumably this lack of astral MTs that explains the high frequency of mitotic defects in GFP-S863L embryos (de Saint Phalle and Sullivan, 1998).

GFP-S863L appears unable to interact with MT minus ends

We previously observed that a GFP-D-TACC fusion protein was concentrated at the minus ends of spindle MTs, which are slightly separated from the centrosome (Gergely et al., 2000b). We observed a similar localization of GFP-D-TACC in this study when we observed mitosis in either live or fixed GFP-D-TACC embryos (Fig. 4 A, open arrowheads; and Fig. 6 A, arrowheads). We failed, however, to observe this localization in any live or fixed GFP-S863L embryos that we observed in mitosis (Fig. 4 B, open arrowheads; and Fig. 6 B, arrowheads). Thus, GFP-S863L appears unable to interact with the minus ends of spindle MTs.

Discussion

We have investigated the function of phosphorylation on Ser863 of D-TACC, which is a major site of Aurora A phos-

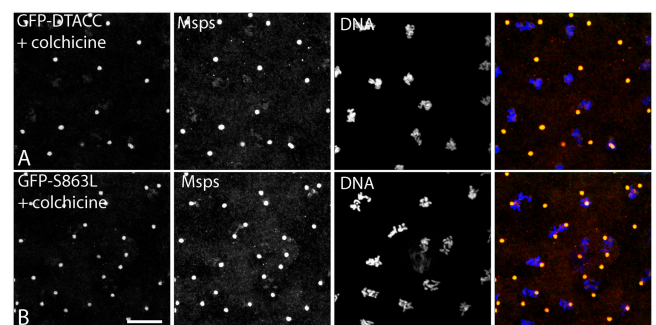


Figure 5. The distribution of Msp and GFP-D-TACC or GFP-S863L in embryos treated with colchicine. (A) A GFP-D-TACC (green in merged image) embryo treated with colchicine to depolymerize MTs. The distribution of Msp (red in merged image) and DNA (blue in merged image) are also shown. (B) A GFP-S863L embryo treated with colchicine, labeled as in A. Bar, 10 μ m.

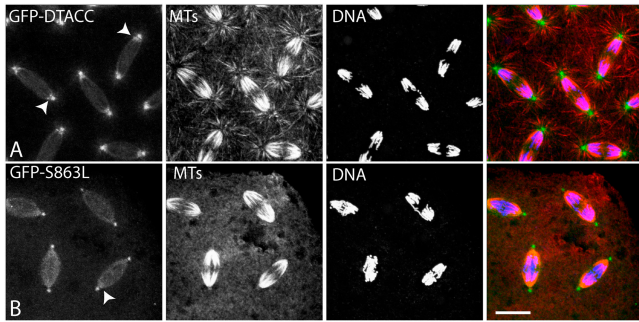


Figure 6. The distribution of MTs in GFP-D-TACC and GFP-S863L embryos. The distribution of MTs (red in merged images) and GFP-D-TACC or GFP-S863L (green in merged images) is shown in GFP-D-TACC (A) and GFP-S863L (B) embryos during anaphase. Arrowheads indicate the position of GFP-D-TACC binding to the minus ends of spindle MTs. This is not detectable in GFP-S863L embryos. DNA is shown in blue in the merged images. Bar, 10 μ m.

phorylation in vitro (Kinoshita et al., 2005; Pascreau et al., 2005). We find that D-TACC phosphorylated on this site (P-D-TACC) is only detectable at centrosomes, whereas nonphosphorylated D-TACC, like most other centrosomal proteins (including γ -tubulin, Aurora A, Msp, CP190, CP60, and centrosomin), has large pools of protein that are present in the cytoplasm of *D. melanogaster* embryos. We conclude that Aurora A only stimulates the phosphorylation of D-TACC at centrosomes and that, once phosphorylated, P-D-TACC is either unable to exchange with the soluble pool of D-TACC or is rapidly dephosphorylated when it leaves the centrosome. As Ser863 is a conserved site of Aurora A phosphorylation in vitro, it seems likely that Aurora A directly phosphorylates Ser863 in vivo, although we cannot exclude the possibility that Aurora A indirectly stimulates the phosphorylation of Ser863 at centrosomes by activating another kinase. It is unclear why Aurora A only stimulates the phosphorylation of D-TACC at centrosomes, but we note that two activators of Aurora A kinase, TPX2 and Ajuba, are themselves concentrated at centrosomes (Hirota et al., 2003; Tsai et al., 2003).

It has previously been reported that Aurora A is required to recruit D-TACC to centrosomes (Giet et al., 2002). We find, however, that GFP-S863L still concentrates at centrosomes, although this concentration is somewhat weaker than that seen with GFP-D-TACC, demonstrating that phosphorylation on Ser863 plays some part in recruiting D-TACC to centrosomes but is not absolutely essential. In the accompanying paper (Kinoshita et al., 2005), we show that the *X. laevis* TACC (X-TACC) protein X-TACC3 is phosphorylated by Aurora A in vitro on three sites that are conserved between frogs and humans, only one of which (Ser863) is conserved in flies. A form of X-TACC3 that mutated at all three serines localizes to centrosomes very weakly. It is possible, therefore, that there are other, nonconserved, Aurora A phosphorylation sites in D-TACC that have a more important role in recruiting the protein to centrosomes. Importantly, GFP-D-TACC and GFP-S863L interact equally well with Msp in immunoprecipitation experiments, and the localization of Msp to centrosomes appears largely unperturbed in GFP-S863L embryos. Thus, we con-

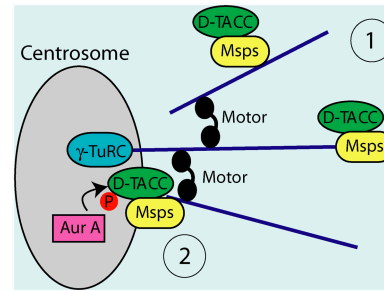


Figure 7. A schematic model of how the D-TACC-Msp complex stabilizes MTs in *D. melanogaster* embryos. MTs are nucleated at centrosomes by the γ -tubulin ring complex (γ -TuRC). These MTs are often released from γ -TuRC but are held in the vicinity of the centrosome by the action of MT motors. The bulk of D-TACC-Msp complexes (that are present at centrosomes, along MTs, and throughout the cytoplasm) can bind these MTs either laterally or at plus ends and stabilize them (mechanism 1). Aurora A can specifically activate a small fraction of D-TACC-Msp complexes that are at the centrosome. This allows the phosphorylated complexes (P, red) to interact with and stabilize MT minus ends (mechanism 2). Importantly, this mechanism is only active at centrosomes, and any MTs that form in the cytoplasm will not be stabilized in this way. This may explain, at least in part, why centrosomes are such dominant sites of MT assembly in mitosis.

clude that the defects in centrosome/MT behavior that we observe in GFP-S863L embryos are unlikely to arise simply from a failure to recruit D-TACC-Msp complexes to centrosomes.

Although GFP-S863L concentrates at centrosomes, it is only partially functional. Whereas spindle MTs are relatively unperturbed in GFP-S863L embryos, astral MTs are dramatically destabilized. In addition, unlike GFP-D-TACC, GFP-S863L appears unable to interact with the minus ends of spindle MTs, suggesting that this interaction requires the Aurora A-dependent phosphorylation of D-TACC. If this were so, we might expect to detect P-D-TACC on the minus ends of spindle MTs. Although this is not usually the case (Fig. 1 A), we can detect such a staining with anti-P-D-TACC antibodies in favorable preparations of fixed embryos (Fig. S3, available at <http://www.jcb.org/cgi/content/full/jcb.200504097/DC1>). We suspect, therefore, that P-D-TACC generated at the centrosome can interact with the minus ends of spindle MTs, but this is difficult to visualize in fixed preparations. In addition, we speculate that P-D-TACC can bind to the minus ends of all centrosomal MTs (not just those in the spindle), but this interaction can only be visualized in the spindle, where large numbers of minus ends are tightly clustered in a region that is slightly separated from the centrosome.

Altogether, our observations suggest a model for how Aurora A, D-TACC, and Msp may cooperate to stabilize MTs during mitosis in *D. melanogaster* embryos (Fig. 7). We propose that D-TACC-Msp complexes normally stabilize MTs in two ways. First, when D-TACC is not phosphorylated on Ser863, the complexes are present throughout the embryo and can potentially stabilize all MTs through either lateral interactions with MTs or interactions with MT plus ends (Fig. 7, mechanism 1). We favor the latter possibility because both D-TACC and Msp appear to concentrate at MT plus ends (Lee et al., 2001), and Msp family members primarily influence MT dynamics through interactions with plus ends. As

this stabilization is independent of phosphorylation on Ser863, GFP-S863L can fulfill this function, which would explain why the expression of GFP-S863L significantly rescues the viability of *d-tacc* mutant embryos (from <1% to ~30%). In support of this possibility, we show in an accompanying paper that nonphosphorylated X-TACC3 can enhance the ability of XMAP215 to stabilize MTs in vitro (Kinoshita et al., 2005).

The Aurora A–dependent phosphorylation of D-TACC on Ser863 at centrosomes, however, activates a second MT stabilization mechanism that acts exclusively on MTs associated with the centrosome. This mechanism cannot operate in GFP-S863L embryos, and, as a result, astral MTs are dramatically destabilized. The lack of this stabilization mechanism in GFP-S863L embryos, however, appears to have only a limited effect on spindle MTs. We speculate that this is because there is a chromatin-based acentrosomal pathway of spindle assembly that can compensate for the instability of centrosomal MTs. Such a pathway exists in many cell types (Heald et al., 1996; de Saint Phalle and Sullivan, 1998; Khodjakov et al., 2000; Giansanti et al., 2001; Megraw et al., 2001) and is especially robust in *D. melanogaster* (Raff, 2001). Because centrosomes still nucleate many MTs in GFP-S863L embryos (centrosomal MTs are simply less stable than normal), these centrosomal MTs can interact with the MTs that assembled around the chromatin to form relatively normal spindles (although they are clearly error prone; Fig. 3 B). In contrast, astral MTs, which are exclusively nucleated by centrosomes and do not interact with MTs nucleated around the chromosomes, are dramatically destabilized in GFP-S863L embryos. In an accompanying paper (Kinoshita et al., 2005), we show that the Aurora A–dependent phosphorylation of X-TACC3 is also required to stabilize centrosomal (but not spindle) MTs in *X. laevis* egg extracts, suggesting that this mechanism is conserved at least in frogs and flies.

Although it is unclear how the phosphorylation of D-TACC on Ser863 leads to MT stabilization at centrosomes, we propose that phosphorylation allows D-TACC to interact with MT minus ends and stabilize them (Fig. 7, mechanism 2). This proposal will be controversial, as Msps proteins appear to stabilize MTs mainly through interactions with MT plus ends (Cassimeris, 1999; Ohkura et al., 2001; Kinoshita et al., 2002). Msps proteins are thought to have such a dramatic effect on MT plus end stability because they specifically counteract the MT destabilizing activity of Kin I kinesins at plus ends (Tournebise et al., 2000; Kinoshita et al., 2001, 2002; Ohkura et al., 2001; Popov et al., 2001; Usui et al., 2003; van Breugel et al., 2003). Several Kin I kinesins, however, are also concentrated at centrosomes (for review see Moore and Wordeman, 2004). In *D. melanogaster* embryos, the Kin I kinesin Klp10A has been reported to destabilize the minus ends of centrosomal MTs (Rogers et al., 2004). Like D-TACC, Klp10A is concentrated both at centrosomes and on the minus ends of spindle MTs that are clustered close to centrosomes (Rogers et al., 2004), and we find that Klp10A remains clustered at these MT minus ends in GFP-S863L embryos (Fig. S4, available at <http://www.jcb.org/cgi/content/full/jcb.200504097/DC1>). Perhaps the phosphorylation of D-TACC on Ser863 allows D-TACC–Msps complexes to counteract the destabilizing activity of Klp10A at MT

minus ends. If so, then a balance between the activities of Msps/XMAP215 and a Kin I kinesin seems to regulate the stability of MTs at both plus and minus ends.

Finally, our findings provide important insight into why centrosomes are the dominant sites of MT assembly during mitosis. As cells enter mitosis, centrioles recruit pericentriolar material in the Aurora A–dependent process of centrosome maturation, which increases the MT nucleating capacity of centrosomes (Hannak et al., 2001; Berdnik and Knoblich, 2002). Our results suggest that this increase in nucleating capacity is insufficient on its own to generate large centrosomal arrays of MTs during mitosis; Aurora A must also phosphorylate D-TACC to activate D-TACC–Msps complexes at centrosomes, which can then stabilize these centrosomal MTs. In this new model, Aurora A ensures that centrosomes are the major site of MT assembly during mitosis both by increasing the MT nucleating capacity of centrosomes and by stabilizing centrosomal MTs. As Aurora A, TACC, and ch-TOG (the human homologue of Msps) have all been implicated in human cancer (Raff, 2002; Meraldi et al., 2004), it will be interesting to determine whether their common role in stabilizing centrosomal MTs is linked to their roles in oncogenesis.

Materials and methods

Antibodies, immunofluorescence, and Western blotting

Anti-P–D-TACC antibodies were raised in rabbits by Eurogentec against the phosphopeptide IDRSSLKFD^P (where the underlined S represents phosphorylated Ser863). The sera from these rabbits were first depleted from any antibodies that might cross react with the nonphosphorylated form of D-TACC by repeated passage over a column of the nonphosphopeptide until no immunoreactivity of the sera could be detected against the nonphosphopeptide in ELISA assays. The antibodies were then affinity purified with a column containing the phosphopeptide. Affinity-purified rabbit anti-D-TACC, anti-Msps, and anti-CP60 antibodies have been described previously (Raff et al., 1993; Gergely et al., 2000b; Lee et al., 2001). Antibodies were raised in rabbits by Eurogentec against a maltose-binding protein fusion to the first 104 amino acids of *D. melanogaster* Aurora A and to the first 228 amino acids of Klp10A; these were purified as described previously (Huang and Raff, 1999). All antibodies were used at 1–2 µg/ml in both immunofluorescence and Western blotting experiments. The mouse mAbs DM1a and GTU88 (Sigma-Aldrich) were used at a 1:500 dilution to detect α - and γ -tubulin, respectively. Embryos were fixed with either methanol or formaldehyde and were processed for immunofluorescence as described previously (Huang and Raff, 1999). Embryos were treated with colchicine (to depolymerize MTs) before fixation as described previously (Gergely et al., 2000b). Methanol-fixed embryos were suspended in protein sample buffer, subjected to SDS-PAGE, and processed for Western blotting using either Supersignal West Pico or Supersignal West Dura chemiluminescent reagents (Pierce Chemical Co.) as described previously (Huang and Raff, 1999).

Centrosome purification

Centrosomes were purified from embryo extracts as described previously (Moritz and Alberts, 1999). On SDS gels, 0.1% of the total embryo extract fraction and 10% of the total centrosome fraction (which equated to approximately equal amounts of protein as judged from Coomassie-stained gels) were loaded in each lane, and these were processed for Western blotting as described above. We estimated the percentage of proteins that was present in the centrosome fraction by comparing the total signal in each lane. A 1:1 ratio of signal would mean that ~1% of the total protein that was present in the embryo extract was present in the purified centrosome fraction.

Image acquisition and processing

Fixed embryos were examined with a microscope (Eclipse 800; Nikon) and a 60 \times NA 1.4 plan Apo objective by using a confocal system (Radi-

ance; BioRad Laboratories) that was equipped with LaserSharp 2000 software (Bio-Rad Laboratories). Live embryos were aligned on coverslips as described previously (Huang and Raff, 1999) and were examined on a microscope (Axiovert 200M; Carl Zeiss MicroImaging, Inc.) with either a 63× NA 1.25 or 100× NA 1.3 plan Neofluor objective on a spinning disc confocal system (UltraView RS; PerkinElmer) that was equipped with UltraView RS software (PerkinElmer). Images of fixed embryos are all maximum intensity projections of five to eight image stacks that were taken at 0.5-μm intervals and made with the LaserSharp 2000 software. All images were imported into Adobe Photoshop 7.0 and adjusted to use the whole range of pixel intensities. An unsharp mask filter was applied to some images. In these images, the filter was applied to the whole image, and control and experimental images were treated in exactly the same way. Videos were made using Velocity 3.0 software (Improvision), and spindle length was measured with this software.

Quantitation of the centrosomal fluorescence of GFP-D-TACC and GFP-S863L in living embryos was performed by making maximum intensity projections of 10–12 image stacks that were taken at 0.5-μm intervals from cycle 10–11 embryos in midinterphase. The projections were imported into Metamorph software (Universal Imaging Corp.), and mean fluorescence intensities were measured in a small area that was manually positioned around 30 centrosomes in each embryo. The mean pixel intensity per centrosome for each embryo was calculated.

Generation of GFP-D-TACC- and GFP-S863L-expressing flies

A full-length D-TACC cDNA in pBluescript-SK (Stratagene) was modified by PCR to allow GFP to be inserted in frame upstream of the initiating ATG codon (generating pBS-GFP-D-TACC). This plasmid was modified by using PCR to replace Ser863 with a leucine (generating pBS-GFP-S863L). All PCR products were sequenced to confirm that they contained no sequence errors. GFP-D-TACC and GFP-S863L were then subcloned into the pWR-pUbq transformation vector (Huang and Raff, 1999), and *w,f* flies were transformed using standard techniques (Roberts, 1986). Transformed flies containing the GFP-D-TACC or GFP-S863L transgene were mated with *d-tacc^{stella592}* flies to generate stocks that were homozygous for the *d-tacc* mutation and contained one copy of the transgene. All experiments were performed with two independent transformed lines for each construct, and results from both lines were pooled.

Online supplemental material

Videos of the embryos shown in Fig. 4 are included, as are additional videos showing the flaring behavior of GFP-D-TACC and GFP-S863L. Four additional figures are also included that show (1) the quantitation of centrosomal levels of GFP-D-TACC and GFP-S863L; (2) the interaction between Msps and GFP-D-TACC or GFP-S863L in immunoprecipitation experiments; (3) the staining of P-D-TACC on the minus ends of spindle MTs; and (4) the localization of Klp10A in GFP-S863L embryos. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200504097/DC1>.

We thank members of the Raff laboratory for critical comments on the manuscript and Alex Sossick for help with imaging and image quantitation.

This work was funded by a Senior Research Fellowship from Cancer Research UK (to J.W. Raff) with earlier support from a Wellcome Trust Senior Research Fellowship (to J.W. Raff), Fundação para a Ciência e a Tecnologia of Portugal (to T.P. Barros), and a Uehara Memorial Foundation Fellowship (to K. Kinoshita).

Submitted: 18 April 2005

Accepted: 4 August 2005

References

Bellanger, J.M., and P. Gonczy. 2003. TAC-1 and ZYG-9 form a complex that promotes microtubule assembly in *C. elegans* embryos. *Curr. Biol.* 13:1488–1498.

Berdnik, D., and J.A. Knoblich. 2002. *Drosophila* Aurora-A is required for centrosome maturation and actin-dependent asymmetric protein localization during mitosis. *Curr. Biol.* 12:640–647.

Cassimeris, L. 1999. Accessory protein regulation of microtubule dynamics throughout the cell cycle. *Curr. Opin. Cell Biol.* 11:134–141.

de Saint Phalle, B., and W. Sullivan. 1998. Spindle assembly and mitosis without centrosomes in parthenogenetic *Sciara* embryos. *J. Cell Biol.* 141:1383–1391.

Gergely, F., C. Karlsson, I. Still, J. Cowell, J. Kilmartin, and J.W. Raff. 2000a.

The TACC domain identifies a family of centrosomal proteins that can interact with microtubules. *Proc. Natl. Acad. Sci. USA.* 97:14352–14357.

Gergely, F., D. Kidd, K. Jeffers, J.G. Wakefield, and J.W. Raff. 2000b. D-TACC: a novel centrosomal protein required for normal spindle function in the early *Drosophila* embryo. *EMBO J.* 19:241–252.

Gergely, F., V.M. Draviam, and J.W. Raff. 2003. The ch-TOG/XMAP215 protein is essential for spindle pole organization in human somatic cells. *Genes Dev.* 17:336–341.

Giansanti, M.G., M. Gatti, and S. Bonaccorsi. 2001. The role of centrosomes and astral microtubules during asymmetric division of *Drosophila* neuroblasts. *Development.* 128:1137–1145.

Giet, R., D. McLean, S. Descamps, M.J. Lee, J.W. Raff, C. Prigent, and D.M. Glover. 2002. *Drosophila* Aurora A kinase is required to localize D-TACC to centrosomes and to regulate astral microtubules. *J. Cell Biol.* 156:437–451.

Glover, D.M., C. Gonzalez, and J.W. Raff. 1993. The centrosome. *Sci. Am.* 268:62–68.

Glover, D.M., M.H. Leibowitz, D.A. McLean, and H. Parry. 1995. Mutations in aurora prevent centrosome separation leading to the formation of monopolar spindles. *Cell.* 81:95–105.

Hannak, E., M. Kirkham, A.A. Hyman, and K. Oegema. 2001. Aurora-A kinase is required for centrosome maturation in *Caenorhabditis elegans*. *J. Cell Biol.* 155:1109–1116.

Heald, R., R. Tournebize, T. Blank, R. Sandaltzopoulos, P. Becker, A. Hyman, and E. Karsenti. 1996. Self-organization of microtubules into bipolar spindles around artificial chromosomes in *Xenopus* egg extracts. *Nature.* 382:420–425.

Hirota, T., N. Kunitoku, T. Sasayama, T. Marumoto, D. Zhang, M. Nitta, K. Hatakeyama, and H. Saya. 2003. Aurora-A and an interacting activator, the LIM protein Ajuba, are required for mitotic commitment in human cells. *Cell.* 114:585–598.

Huang, J., and J.W. Raff. 1999. The disappearance of cyclin B at the end of mitosis is regulated spatially in *Drosophila* cells. *EMBO J.* 18:2184–2195.

Khodjakov, A., R.W. Cole, B.R. Oakley, and C.L. Rieder. 2000. Centrosome-independent mitotic spindle formation in vertebrates. *Curr. Biol.* 10:59–67.

Kinoshita, K., I. Arnal, A. Desai, D.N. Drechsel, and A.A. Hyman. 2001. Reconstitution of physiological microtubule dynamics using purified components. *Science.* 294:1340–1343.

Kinoshita, K., B. Habermann, and A.A. Hyman. 2002. XMAP215: a key component of the dynamic microtubule cytoskeleton. *Trends Cell Biol.* 12:267–273.

Kinoshita, K., T.L. Noetzel, L. Pelletier, K. Mechtler, D.N. Drechsel, A. Schwager, M. Lee, J.W. Raff, and A.A. Hyman. 2005. Aurora A phosphorylation of TACC3/maskin is required for centrosome-dependent microtubule assembly in mitosis. *J. Cell Biol.* 170:1047–1055.

Le Bot, N., M.C. Tsai, R.K. Andrews, and J. Ahringer. 2003. TAC-1, a regulator of microtubule length in the *C. elegans* embryo. *Curr. Biol.* 13:1499–1505.

Lee, M.J., F. Gergely, K. Jeffers, S.Y. Peak-Chew, and J.W. Raff. 2001. Msps/XMAP215 interacts with the centrosomal protein D-TACC to regulate microtubule behaviour. *Nat. Cell Biol.* 3:643–649.

Marumoto, T., D. Zhang, and H. Saya. 2005. Aurora-A - a guardian of poles. *Nat. Rev. Cancer.* 5:42–50.

Megraw, T.L., L.-R. Kao, and T. Kaufman. 2001. Zygotic development without functional mitotic centrosomes. *Curr. Biol.* 11:116–120.

Megraw, T.L., S. Kilaru, F.R. Turner, and T.C. Kaufman. 2002. The centrosome is a dynamic structure that ejects PCM flares. *J. Cell Sci.* 115:4707–4718.

Meraldi, P., R. Honda, and E.A. Nigg. 2004. Aurora kinases link chromosome segregation and cell division to cancer susceptibility. *Curr. Opin. Genet. Dev.* 14:29–36.

Moore, A., and L. Wordeman. 2004. The mechanism, function and regulation of depolymerizing kinesins during mitosis. *Trends Cell Biol.* 14:537–546.

Moritz, M., and B.M. Alberts. 1999. Isolation of centrosomes from *Drosophila* embryos. *Methods Cell Biol.* 61:1–12.

O'Brien, L., A.J. Albee, L. Liu, W. Tao, P. Dobrzyn, S.B. Lizarraga, and C. Wiese. 2005. The *Xenopus* TACC homologue, maskin, functions in mitotic spindle assembly. *Mol. Biol. Cell.* 16:2836–2847.

Ohkura, H., M.A. Garcia, and T. Toda. 2001. Dis1/TOG universal microtubule adaptors - one MAP for all? *J. Cell Sci.* 114:3805–3812.

Pascreau, G., J.G. Delcros, J.Y. Cremet, C. Prigent, and Y. Arlot-Bonnemains. 2005. Phosphorylation of maskin by aurora-A participates to the control of sequential protein synthesis during *Xenopus laevis* oocyte maturation. *J. Biol. Chem.* 280:13415–13423.

Popov, A.V., A. Pozniakovsky, I. Arnal, C. Antony, A.J. Ashford, K. Kinoshita, R. Tournebize, A.A. Hyman, and E. Karsenti. 2001. XMAP215 regulates microtubule dynamics through two distinct domains. *EMBO J.* 20:397–410.

- Raff, J.W. 2001. Centrosomes: central no more? *Curr. Biol.* 11:R159–R161.
- Raff, J.W. 2002. Centrosomes and cancer: lessons from a TACC. *Trends Cell Biol.* 12:222–225.
- Raff, J.W. 2003. Cell division: genome maintenance. *Nature.* 423:493, 495.
- Raff, J.W., D.R. Kellogg, and B.M. Alberts. 1993. *Drosophila* γ -tubulin is part of a complex containing two previously identified centrosomal MAPs. *J. Cell Biol.* 121:823–835.
- Roberts, D. 1986. *Drosophila: A Practical Approach*. IRL Press, Oxford. 295 pp.
- Rogers, G.C., S.L. Rogers, T.A. Schwimmer, S.C. Ems-McClung, C.E. Walczak, R.D. Vale, J.M. Scholey, and D.J. Sharp. 2004. Two mitotic kinesins cooperate to drive sister chromatid separation during anaphase. *Nature.* 427:364–370.
- Srayko, M., S. Quintin, A. Schwager, and A.A. Hyman. 2003. *Caenorhabditis elegans* TAC-1 and ZYG-9 form a complex that is essential for long astral and spindle microtubules. *Curr. Biol.* 13:1506–1511.
- Tournebise, R., A. Popov, K. Kinoshita, A.J. Ashford, S. Rybina, A. Pozniakovsky, T.U. Mayer, C.E. Walczak, E. Karsenti, and A.A. Hyman. 2000. Control of microtubule dynamics by the antagonistic activities of XMAP215 and XKCM1 in *Xenopus* egg extracts. *Nat. Cell Biol.* 2:13–19.
- Tsai, M.Y., C. Wiese, K. Cao, O. Martin, P. Donovan, J. Ruderman, C. Prigent, and Y. Zheng. 2003. A Ran signalling pathway mediated by the mitotic kinase Aurora A in spindle assembly. *Nat. Cell Biol.* 5:242–248.
- Usui, T., H. Maekawa, G. Pereira, and E. Schiebel. 2003. The XMAP215 homologue Stu2 at yeast spindle pole bodies regulates microtubule dynamics and anchorage. *EMBO J.* 22:4779–4793.
- van Breugel, M., D. Drechsel, and A. Hyman. 2003. Stu2p, the budding yeast member of the conserved Dis1/XMAP215 family of microtubule-associated proteins is a plus end-binding microtubule destabilizer. *J. Cell Biol.* 161:359–369.