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Two distinct regions of Mto1 are required for normal microtubule nucleation and efficient association with the γ-tubulin complex in vivo

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SUMMARY

Cytoplasmic microtubule nucleation in the fission yeast *Schizosaccharomyces pombe* involves the interacting proteins Mto1 and Mto2, which are thought to recruit the γ-tubulin complex (γ-TuC) to prospective microtubule organizing centers. Mto1 contains a short amino-terminal region (CM1) that is conserved in higher eukaryotic proteins implicated in microtubule organization, centrosome function and/or brain development. Here we show that mutations in the Mto1 CM1 region generate mutant proteins that are functionally null for cytoplasmic microtubule nucleation and interaction with the γ-TuC (phenocopying *mto1*Δ), even though the Mto1-mutant proteins localize normally in cells and can bind Mto2. Interestingly, the CM1 region is not sufficient for efficient interaction with the γ-TuC. Mutation within a different region of Mto1, outside CM1, abrogates Mto2 binding and also impairs cytoplasmic microtubule nucleation and Mto1 association with the γ-TuC. However, this mutation allows limited microtubule nucleation in vivo, phenocopying *mto2*Δ rather than *mto1*Δ. Further experiments suggest that Mto1 and Mto2 form a complex (Mto1/2 complex) independent of the γ-TuC and that Mto1 and Mto2 can each associate with the γ-TuC in the absence of the other, albeit extremely weakly compared to when both Mto1 and Mto2 are present. We propose that Mto2 acts cooperatively with Mto1 to promote association of Mto1/2 complex with the γ-TuC.

Keywords

fission yeast; γ-tubulin complex; centrosomin-related proteins

INTRODUCTION

Microtubule (MT) nucleation in eukaryotic cells involves the γ-tubulin complex (γ-TuC), a multi-protein complex containing gamma-tubulin and several additional proteins (for reviews, see Luders and Stearns, 2007; Raynaud-Messina and Merdes, 2007; Wiese and Zheng, 2006)). In higher eukaryotes, the γ-TuC exists primarily as a large (~2 MDa) complex, the γ-tubulin ring complex (γ-TuRC). The γ-TuRC consists of several copies of a smaller complex (γ-tubulin small complex; γ-TuSC) containing γ-tubulin and the proteins GCP2 and GCP3, plus other proteins that are γ-TuRC-specific. Obvious homologs of γ-TuSC proteins are found in all eukaryotes, while homologs of γ-TuRC specific proteins may not be ubiquituous--for example, they have not been found in budding yeast, which has a relatively simple mode of microtubule organization. However, they have been identified and characterized in the fission yeast *Schizosaccharomyces pombe*, although they are

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signficantly diverged from their higher eukaryotic counterparts (Anders et al., 2006; Fujita et al., 2002; Venkatram et al., 2004).

Fission yeast serves as a useful model organism for understanding microtubule nucleation by the γ-TuC not only because of its conservation of γ-TuC components but also because of its diversity of cytoplasmic microtubule organizing centers (MTOCs; for review, see Sawin and Tran, 2006)). Only a small number of cytoplasmic MTs are present at any given time in fission yeast (Hoog et al., 2007), and these can be imaged by expressing GFP-tubulin at physiological concentrations. During interphase, MTs are nucleated not only from the spindle pole body (SPB; the yeast centrosome equivalent) but also from several interphase microtubule organizing center "satellite" particles (iMTOCs) present on the nuclear envelope, in the cytoplasm, and on MTs themselves. During late mitosis, cytoplasmic astral MTs analogous to those seen in budding yeast are nucleated from the duplicated SPBs. At the end of cell division, a post-anaphase array (PAA) of MTs is nucleated from the equatorial MTOC (eMTOC) at the site of septation. Because fission yeast MTOCs change throughout the cell cycle, they may provide a useful model for understanding the changes in MT organization that accompany differentiation of many higher eukaryotic cell types, for which relatively little is known, especially in relation to non-centrosomal MT nucleation (reviewed in Luders and Stearns, 2007; Raynaud-Messina and Merdes, 2007; Wiese and Zheng, 2006).

Two key regulators of cytoplasmic MT nucleation in fission yeast are the non-essential proteins Mto1 (initially called Mbo1/Mod20) and Mto2 (Janson et al., 2005; Samejima et al., 2005; Sawin et al., 2004; Venkatram et al., 2004; Zimmerman and Chang, 2005). In a variety of assays *mto1*Δ mutants are completely defective in nucleation of MTs from all types of cytoplasmic MTOCs (Sawin et al., 2004; Venkatram et al., 2004). However, *mto1*Δ mutants are viable, because intranuclear mitotic spindle assembly and function is independent of Mto1; intranuclear MT nucleation is most likely driven by an Mto1 paralog, Pcp1, which is present on the nucleoplasmic face of the SPB (Flory et al., 2002). Mto1 localizes to cytoplasmic MTOCs and co-immunoprecipitates the γ-TuC, and collectively these results have led to a model in which Mto1 functions by recruiting the γ-TuC to prospective cytoplasmic MTOCs (Sawin et al., 2004; Venkatram et al., 2004; Zimmerman and Chang, 2005).

Mto2 colocalizes with and physically interacts with Mto1, almost certainly directly (Samejima et al., 2005; Venkatram et al., 2005). In addition, Mto2 can co-immunoprecipitate the γ-TuC (Janson et al., 2005), and in *mto2*Δ mutants, Mto1 fails to co-immunoprecipitate the γ-TuC (Samejima et al., 2005). This could suggest a simple model in which Mto2 provides a "bridge" between Mto1 and the γ-TuC. However, unlike *mto1*Δ mutants, *mto2*Δ mutants are defective in nucleation from only a subset of cytoplasmic MTOCs; they can nucleate MTs from the SPB in both interphase and mitosis, and they can also nucleate a small number of MTs from the eMTOC at the end of mitosis. Thus, an alternative interpretation, based primarily on mutant phenotypes, might be that Mto1 retains some ability to recruit and/or activate the γ-TuC in the absence of Mto2 (e.g. at the SPB), even if to date this has not been detected biochemically.

Mto1 is a large coiled-coil protein with a ~60-amino acid amino-terminal region (centrosomin motif 1; CM1) that is conserved in fungal and animal cells (Sawin et al., 2004; Zhang and Megraw, 2007). Proteins containing CM1 are generally similar in structure: they are all large proteins (100-300 kDa); they contain several predicted coiled-coil regions; and CM1 is typically found towards the amino-terminus of the coding region. Most of these proteins have not been extensively studied, but those that have been characterized, including the *Drosophila* protein centrosomin (Cnn) and the human proteins myomegalin and

CDK5RAP2, have been implicated in microtubule organization, centrosome function and/or brain development (Sawin et al., 2004; Bond et al., 2005; Ching et al., 2000; Fong et al., 2008; Megraw et al., 1999; Terada et al., 2003; Veith et al., 2005; Verde et al., 2001; Zhang and Megraw, 2007). To address the question of how Mto1 and Mto2 may function in MT nucleation in more detail, we constructed site-directed mutations in Mto1 and examined the effects of the mutations on protein localization, interaction and function using a range of cytological and biochemical assays. Our data suggest not only that the conserved CM1 region is critical for Mto1 interaction with the γ-TuC, but also that binding of Mto2 to Mto1 is equally important for interaction with the γ-TuC. Further experiments suggest that Mto1 and Mto2 act cooperatively to promote interaction of an Mto1/2 complex with the γ-TuC, and thereby efficient MT nucleation.

RESULTS

Mutation of Mto1 within CM1 creates an *mto1*-null mutant

We targeted the CM1 region of the single chromosomal copy of fission yeast *mto1*$^+$ to construct three different site-directed mutants: *mto1-9A1*, *mto1-9A2* and *mto1-9A3* (Fig. 1A, B). Each mutation converts nine consecutive amino acids of CM1 to alanine. In a morphology assay, *mto1-9A3* cells were slightly curved relative to wild-type cells, while *mto1-9A1* and *mto1-9A2* cells were strongly curved, like *mto1*Δ cells (Fig. 1C; Sawin et al., 2004)). This suggests that *mto1-9A1* and *mto1-9A2* mutations strongly impair Mto1 function, while the *mto1-9A3* mutation has only a mild effect.

Upon cold-induced MT depolymerization in wild-type cells, Mto1 and associated γ-TuC proteins redistribute to sites on the surface of the nuclear envelope, and when cells are re-warmed, MTs are rapidly nucleated from these sites (Fig. 1D; supplementary material Fig. S1; Sawin et al., 2004). In contrast, in the same MT-regrowth assay *mto1*Δ mutants are completely defective in cytoplasmic MT nucleation and instead slowly nucleate intranuclear MTs (most likely via Pcp1). These intranuclear MTs eventually escape into the cytoplasm , acting as progenitors of essentially all cytoplasmic MTs in the mutant cells and forming abnormal MT bundles that often curve around the cell tips (Fig. 1D; during steady-state growth of *mto1*Δ mutants, intranuclear spindle MTs escaping into the cytoplasm at the end of mitosis perform an equivalent function; Sawin et al., 2004; Zimmerman and Chang, 2005). In the MT-regrowth assay we found that *mto1-9A1* and *mto1-9A2* mutants were indistinguishable from *mto1*Δ cells, both before cold-induced MT depolymerization and during recovery (Fig. 1D). *mto1-9A3* mutants behaved like wild-type cells, except that both before depolymerization and after repolymerization, some cells in *mto1-9A3* mutants had curved microtubule bundles. However, this was not as extreme as in *mto1*Δ, *mto1-9A1* or *mto1-9A2* mutants (Fig. 1D).

Analysis of movies of live cells co-expressing GFP-tubulin (GFP-Atb2; Sawin et al., 2004) and the SPB marker Sad1-dsRed (Chikashige et al., 2004) also showed *mto1-9A1* and *mto1-9A2* mutants to be indistinguishable from *mto1*Δ cells. All three of these mutants showed essentially no new nucleation of interphase cytoplasmic MTs at steady state, while *mto1-9A3* mutants showed approximately half the nucleation frequency of wild-type cells (Fig. 1E, supplementary material Movies 1-5, see Materials and Methods). Consistent with this, *mto1-9A1* and *mto1-9A2* mutants also showed the unusual interphase MT behaviors characteristic of *mto1*Δ mutants, such as bend-breakage when MT bundles curve around cell tips (Sawin et al., 2004), and a strong reduction in SPB oscillations, which likely reflects reduced interactions between cytoplasmic MTs and the SPB (Fig. 1E, supplementary material Movies 2-4). In dividing cells, *mto1-9A1* and *mto1-9A2* mutants, like *mto1*Δ cells, did not nucleate either cytoplasmic astral MTs or PAA MTs (Sawin and Tran, 2006), while *mto1-9A3* mutants behaved like wild-type cells (Fig. 1E, supplementary material Movies

1-5). Taken together, these results show that *mto1-9A1* and *mto1-9A2* mutants are null for Mto1-dependent MT nucleation, while *mto1-9A3* mutants are partially functional.

Mutation of Mto1 within CM1 specifically disrupts γ-TuC binding

We investigated the ability of the Mto1-mutant proteins to physically interact with Mto2 and the γ-TuC (Samejima et al., 2005; Vardy and Toda, 2000; Venkatram et al., 2005). In immunoprecipitation experiments with Myc-tagged Mto1 proteins expressed from the endogenous *mto1*$^{+}$ promoter, we found that all of the mutant proteins were present at normal wild-type levels, and all co-immunoprecipitated Mto2 to the same extent as wild-type cells (Fig. 2A). However, while both wild-type Mto1-Myc and Mto1-9A3-Myc co-immunoprecipitated γ-tubulin (Gtb1) and HA-tagged Alp4 (GCP2 homolog), a well-characterized component of the fission yeast γ-TuSC (Vardy and Toda, 2000), Mto1-9A1-Myc and Mto1-9A2-Myc did not co-immunoprecipitate these γ-TuC components (Fig. 2A).

We next examined the localization of the Mto1-mutant proteins. In the MT regrowth assay described above, all Mto1-mutant proteins redistributed to the nuclear envelope upon cold-induced MT depolymerization, as in wild-type cells (supplementary material Fig. S1; Sawin et al., 2004). To investigate localization more closely in live cells, we tagged the Mto1-mutant proteins with GFP and also tagged Alp4 with the RFP tandem-dimer Tomato (tdT; Shaner et al., 2004; Snaith et al., 2005). In wild-type cells we observed colocalization of Mto1-GFP with Alp4-tdT at SPBs, eMTOCs, and at iMTOC satellites (Fig. 2B, C; Zimmerman and Chang, 2005). Interestingly, while most Mto1-GFP interphase satellites contained Alp4-tdT, some satellites did not, suggesting that the γ-TuC is not always stably associated with Mto1 at prospective MTOCs. This result differs slightly from a previous study using CFP- and YFP-tagged proteins (Zimmerman and Chang, 2005) and may be attributable to our improved imaging conditions.

In *mto1* mutants, we found that Mto1-9A1-GFP, Mto1-9A2-GFP and Mto1-9A3-GFP localized to SPBs, to the eMTOC, and to interphase satellites that appeared to track the cytoplasmic MTs formed in *mto1* mutants by the nuclear escape mechanism (Fig. 2D-F). However, we did not observe any Alp4-tdT associated with either equatorial or interphase-satellite Mto1-9A1-GFP or Mto1-9A2-GFP. We did observe Alp4-tdT at SPBs, consistent with previous observations of Alp4-GFP and other γ-TuC proteins at SPBs in *mto1*Δ mutants; this most likely represents γ-TuC interacting with Pcp1 on the nucleoplasmic face of the SPB (Flory et al., 2002; Sawin et al., 2004; Venkatram et al., 2004; Zimmerman and Chang, 2005).

Collectively, these results indicate that the failure of Mto1-9A1 and Mto1-9A2 to associate with γ-TuC is not due to a mislocalization of the Mto1-mutant proteins, and thus that CM1 is important for γ-TuC binding *per se*. In addition, the demonstration that Mto1 can localize to prospective MTOCs independent of its ability to bind the γ-TuC supports our previous hypothesis that Mto1 is “upstream” of the γ-TuC in determining sites of cytoplasmic MT nucleation (Samejima et al., 2005; Sawin et al., 2004).

The Mto1 CM1 region is not sufficient for interaction with the γ-TuC

In order to test whether the CM1 region of Mto1 is sufficient for interaction with the γ-TuC, we created several different Myc-tagged carboxyl-terminal truncations of Mto1, expressed from the endogenous *mto1* promoter, and performed immunoprecipitation experiments using strains also expressing HA-tagged Alp4; (Fig. 3, see also Fig. 1A). All of the truncated Mto1 proteins were efficiently immunoprecipitated, some much better than full-length Mto1. Interestingly, although all but the shortest truncation contained the entire CM1 region, we found that truncations up to and including Mto1-(1-500)-Myc did not co-

immunoprecipitate Alp4-HA, while Mto1-(1-800)-Myc and larger proteins did (Fig. 3). Thus, in spite of the clear importance of CM1 for the interaction of Mto1 with the γ-TuC, additional sequences beyond CM1 are also important for this interaction.

Disrupting the Mto1-Mto2 interaction phenocopies *mto2*Δ

Previously we showed that amino acids 476-549 of Mto1 are important for binding to Mto2 in vitro (Samejima et al., 2005). This suggested the possibility that in addition to the CM1 region, the Mto2-binding region of Mto1 might also be required for the interaction of Mto1 with the γ-TuC. We therefore wanted a means to test directly whether binding of Mto2 to Mto1 is required for Mto1 association with the γ-TuC, and/or whether Mto2 has additional roles in cytoplasmic microtubule nucleation independent of its association with Mto1.

We constructed several small deletions within the putative Mto2-binding region of Mto1 and targeted these to the chromosomal copy of *mto1*$^+$(Fig. 1A; additional data not shown). An allele deleting amino acids 523 through 537, *mto1-334*, produced a protein that failed to interact with Mto2 in immunoprecipitation experiments (Fig. 4A) as well as in vitro (data not shown). The *mto1-334* mutant also showed cell-shape defects like other *mto1* and *mto2* mutants (Figs. 4B, 1C). In addition, we found that Myc-tagged Mto1-334 did not co-immunoprecipitate the γ-TuC (Fig. 4C).

Thus far, these results would be consistent with *mto1-334* being a null allele of *mto1*. However, in MT regrowth assays we found that *mto1-334* mutants, like *mto2*Δ mutants, were able to nucleate cytoplasmic MTs from the SPB but not from non-SPB sites (Fig. 4D; Samejima et al., 2005). By contrast, *mto1*Δ mutants cannot nucleate MTs from any cytoplasmic sites (Fig. 1D; Sawin et al., 2004). In addition, live *mto1-334* mutant cells expressing GFP-Atb2 and Sad1-dsRed displayed a profile of cytoplasmic MT-nucleation behaviors identical to *mto2*Δ mutants but distinct from *mto1*Δ mutants. This included: nucleation of cytoplasmic MTs during interphase, but only from the SPB (often accompanied by strong SPB oscillations); nucleation of cytoplasmic astral MTs in mitosis; and very limited MT nucleation from the eMTOC at the end of mitosis (Fig. 4E, supplementary material Movies 6 and 7).

Upon cold-induced depolymerization of MTs in MT-regrowth assays, Mto1 in *mto2*Δ mutants does not redistribute to the surface of the nuclear envelope, although the biological significance of this is not known (Samejima et al., 2005; see Discussion). In this assay, Mto1-334 localization in *mto2*+ (wild-type) cells was identical to Mto1 (wild-type) localization in *mto2*Δ cells (supplementary material Fig. S1). In addition, when we tagged Mto1-334 with GFP, we found that the localization of Mto1-334-GFP in *mto2+* (wild-type) cells was identical to Mto1-GFP (wild-type) in *mto2*Δ cells, as was the localization of Alp4-tdT in the two strains (Fig. 4F). Collectively these results suggest that *mto1-334* is not a null allele but rather an interaction-specific allele of *mto1* that disrupts binding of Mto1 to Mto2. Because the *mto1-334* phenotypes (i.e., in an *mto2*+ background) are indistinguishable from those of *mto2*Δ, we conclude that the function of Mto2 in MT nucleation is mediated primarily, if not exclusively, via its binding to Mto1. Moreover, the failure of Mto1-334 to immunoprecipitate the γ-TuC indicates that the Mto1-Mto2 interaction is required for an efficient association of Mto1 with the γ-TuC, as detected in cytoplasmic extracts.

Evidence for cooperative binding of Mto1 and Mto2 to γ-TuC

In experiments in which anti-Mto2 immunoprecipitates from cell extracts were washed with different salt concentrations, we found that Mto1 is much more stably associated with Mto2 than are γ-TuC proteins, suggesting that Mto1 and Mto2 may be part of a distinct protein complex or subcomplex that can interact with the γ-TuC (Fig. 5A). To investigate this

further we constructed trans-heterozygous diploid strains, in order to determine whether both the CM1 region and the Mto2-binding region of Mto1 need to be present on the same polypeptide for proper Mto1 function. This was the case, as all *mto1* mutants were complemented by wild-type *mto1*+, while we did not observe any complementation in the trans-heterozygous mutant strains (Fig. 5B, C). This suggests that CM1 and the Mto2-binding region work closely together in a common function to promote interaction of Mto1 with the γ-TuC.

We therefore considered the possibility that Mto1 and Mto2 might each bind the γ-TuC independently, albeit very weakly, achieving stronger, cooperative binding when both species are present in an Mto1/2 complex. We TAP-tagged Mto1 and Mto2 (Gould et al., 2004; Venkatram et al., 2005)and partially purified TAP-tagged Mto1 from wild-type and *mto2*Δ cells, as well as TAP-tagged Mto2 from wild-type and *mto1*Δ cells. The TAP-tagged proteins were expressed at physiological levels (Fig. 6A), and these partial purifications gave higher yield and purity than conventional immunoprecipitation methods used in this and previous work (data not shown). We probed Western blots of the purifications with antibodies to γ-tubulin as well as with newly-raised antibodies to Alp4 and Alp6 (GCP2 and GCP3 homologs; Fig. 6 B,C; see Materials and Methods for technical details). As expected from previous studies (Janson et al., 2005; Sawin et al., 2004; Venkatram et al., 2004), the γ-TuC proteins co-purified both with Mto1-TAP and with Mto2-TAP (Fig. 6B, lanes 1, 2). Under normal exposure conditions, we did not detect an association between Mto1 and γ-TuC in *mto2*Δ strains (Fig. 6B, lane 3), consistent with previous immunoprecipitation experiments (Samejima et al., 2005). Moreover, we did not observe an association between Mto2 and the γ-TuC in *mto1*Δ strains (Fig. 6B, lane 4). Thus not only does Mto1 association with γ-TuC depend on Mto2, but also Mto2 association with γ-TuC depends on Mto1. Interestingly, however, with more sensitive detection and longer exposures (see Materials and Methods) we found evidence for a physical interaction between Mto1 and the γ-TuC in the absence of Mto2, and also a possible weaker interaction between Mto2 and the γ-TuC in the absence of Mto1 (Fig. 6C, lanes 3,4). We did not detect the γ-TuC in purifications from several negative-control strains, including both untagged strains and unrelated Mod5-TAP strains (Snaith and Sawin, 2003) (also H. Snaith, University of Edinburgh UK, personal communication) used to assess non-specific binding of γ-TuC to TAP-tagged proteins as well as possible unforeseen aberrant properties (e.g. “stickiness”) of γ-TuC subunits in *mto1*Δ and/or *mto2*Δ backgrounds (Fig. 6 B,C, lanes 5-7). These results suggest that Mto1 and Mto2 may bind cooperatively to the γ-TuC.

DISCUSSION

The work presented here provides novel insights into how fission yeast proteins Mto1 and Mto2 act to promote cytoplasmic MT nucleation via interactions with the γ-TuC. Our cytological and biochemical analysis of *mto1-9A1*, *mto1-9A2*, and *mto1-334* mutants demonstrates that two distinct regions of Mto1—the CM1 region and the Mto2-binding region--are required for an efficient association of Mto1 with the γ-TuC. *mto1-9A1* and *mto1-9A2* mutants completely fail to nucleate cytoplasmic MTs (Table 1), and Mto1-9A1 and Mto1-9A2 mutant proteins do not interact with the γ-TuC, although the mutant proteins still interact with Mto2 and localize to all prospective MTOC sites in vivo. Together with the conservation of CM1 and work in other organisms (see below) these results implicate the CM1 region of Mto1 as a likely γ-TuC-binding region. At the same time, however, our characterization of *mto1-334* mutants, which phenocopy *mto2*Δ mutants (Table 1), demonstrates that binding of Mto1 to Mto2 is of comparable importance in promoting the interaction of Mto1 with the γ-TuC.

The stability of the Mto1-Mto2 interaction, together with our observation that the CM1 region and the Mto2-binding region of Mto1 must be present on the same polypeptide in order to achieve proper Mto1 function, suggested that Mto1 and Mto2 might form a complex in which Mto1 and Mto2 bind independently but cooperatively to the γ-TuC. Accordingly, using TAP-purifications and sensitive detection methods we obtained evidence for an association of Mto1 with the γ-TuC in *mto2*Δ mutants, and an association of Mto2 with the γ-TuC in *mto1*Δ mutants. In both cases, however, these associations were significantly weaker than those observed in wild-type cells. A model of cooperative binding of an Mto1/2 complex to the γ-TuC explains these results, as well as several aspects of both *mto1*Δ and *mto2*Δ phenotypes and the differences between them (Janson et al., 2005; Samejima et al., 2005; Sawin et al., 2004; Venkatram et al., 2004; Venkatram et al., 2005; Zimmerman and Chang, 2005). In *mto2*Δ mutants, the limited amount of cytoplasmic MT nucleation observed, primarily at the SPB, can be attributed to a high local concentration of Mto1 weakly interacting with the γ-TuC, likely via CM1, and possibly supplemented by additional mechanisms that may be specific to the SPB. By contrast, the absence of cytoplasmic MT nucleation in *mto1*Δ mutants can be understood if Mto2 were able to bind the γ-TuC weakly but not "activate" it for nucleation; in any case, because the localization of the Mto1/2 complex is governed primarily by Mto1 and not by Mto2 (Samejima et al., 2005); see also below), the γ-TuC would not be recruited and/or concentrated to specific subcellular sites in *mto1*Δ cells. In the future it will be of interest to investigate the nature of the interactions between Mto1, Mto2 and the γ-TuC at a deeper molecular level. Thus far we have not been able to detect interaction of either Mto1 or Mto2 with individual components of the γ-TuC in directed yeast-two hybrid assays or in vitro binding experiments (our unpublished data). Given the weakness of the interactions detected in the Mto1- and Mto2-TAP purifications from *mto2*Δ and *mto1*Δ strains, addressing this question may require additional and/or novel methods.

One aspect of our work suggests that Mto2 could have additional roles in promoting Mto1 function beyond that suggested here, although such potential roles remain ill-defined. Specifically, compared to wild-type cells, Mto1 (or Mto1-334) in *mto2*Δ and *mto1-334* mutants does not accumulate on the nuclear envelope upon cold-induced MT depolymerization (supplementary material Fig. S1; (Samejima et al., 2005). While this is admittedly an "artifactual" situation, and nearly all other aspects of Mto1 localization remain unchanged in *mto2*Δ or *mto1-334* mutants, in these mutants there also appear to be fewer Mto1-GFP (or Mto1-334-GFP) particles associated with interphase MTs during steady-state growth ((Fig. 2C, 4F) see also (Samejima et al., 2005)). The molecular basis for these differences is unknown. We note, however, that comparable differences in Mto1 localization are not observed with Mto1-9A1 and Mto1-9A2 proteins (Fig. 2D,E; supplementary material Fig. S1); it is therefore unlikely that the effects of disrupting Mto1-Mto2 interaction on these more subtle aspects of Mto1 localization are directly linked to aberrant MT distribution and/or reduced interaction of Mto1 with the γ-TuC. Further work on the biochemistry of the Mto1/2 complex may help to illuminate whether and how Mto2 may further contribute to Mto1 function.

While this paper was in preparation, two studies were published investigating the role of the CM1 region in higher eukaryotic proteins. While there are some general similarities between these studies and our own, there are also some important differences. A cytological study in *Drosophila* showed that a deletion within the CM1 region of the Cnn protein leads to reduced and/or altered recruitment of γ-tubulin and two other proteins, D-TACC and Msps, to mitotic spindle poles, with a concomitant impairment of some microtubule-dependent processes (Zhang and Megraw, 2007). Allowing for the different nature of the fly and yeast systems, these results are consistent with our own. However, in the *Drosophila* work, there was no biochemical investigation of protein-protein interactions. In addition, the single

deletion mutation within CM1 did not indicate whether all of CM1 or only a particular sub-region was important for function. By contrast, we have shown that *mto1-9A1* and *mto1-9A2* mutants are functionally null, while *mto1-9A3* mutants, which are altered in an immediately adjacent part of CM1, have only slightly impaired function. Moreover, in immunoprecipitation experiments we have shown a specific defect in the physical interaction of Mto1-9A1 and Mto1-9A2 with the γ-TuC, with no effects on the interaction with Mto2. Thus our findings significantly extend the mutational analysis of CM1 in *Drosophila*.

Another recent study, in human cells, has provided evidence that the CM1 region of one of the two human Mto1/Cnn-related proteins, CDK5RAP2, is itself sufficient to interact with the γ-TuRC (Fong et al., 2008), at least when overexpressed well above probable physiological levels. These findings are also consistent with our own, although to date we have not been able to demonstrate convincingly an interaction between the fission yeast γ-TuC and bacterially-expressed Mto1 fragments containing CM1 (our unpublished data). Beyond this, however, we have further shown that in fission yeast, an additional region of Mto1, which binds the interacting partner Mto2, is also required for efficient association of Mto1 with the γ-TuC, at least when Mto1 is present at physiological levels. It will be interesting to determine whether the proposed mechanism of cooperative interaction of the Mto1/2 complex with the γ-TuC in fission yeast also applies in some form to higher eukaryotic proteins. Presently there are no recognizable homologs of Mto2 in higher eukaryotes, or even in fungi (our unpublished data). However, it is possible that these organisms possess functional equivalents of Mto2 that are highly diverged in sequence, as several fission yeast proteins involved in MT nucleation are only poorly related in primary sequence to their higher-eukaryotic counterparts. Examples of this include the non-essential fission yeast proteins Gfh1, Mod21 and Alp16, which have been shown by combined biochemical, cytological, and genetic analyses to be orthologs of mammalian γ-TuC proteins GCP4, GCP5, and GCP6, respectively, even though the actual amino-acid sequence identity between the fission yeast and mammalian proteins is too low to be convincing in its own right (Anders et al., 2006; Fujita et al., 2002; Venkatram et al., 2004). Another example is Mto1 itself, in which only the CM1 region is conserved from yeast to higher eukaryotes, even though all of the CM1-containing proteins are generally similar in their overall predicted structural features. Indeed, CDK5RAP2 has recently been identified as a particularly rapidly-evolving protein in primates, which may relate to its role in regulating brain size (Bond et al., 2005; Evans et al., 2006). Future biochemical analyses of the higher eukaryotic homologs of Mto1 should shed light on this question.

MATERIALS AND METHODS

Strain construction

Standard yeast genetic and physiological methods were used throughout (Moreno et al., 1991). Strains and genotypes are listed in supplementary material Table S1. The *mto1-9A1*, *-9A2*, and *-9A3* mutants were constructed by a two-step gene-replacement at the *mto1* locus. In the first step, the CM1 region was deleted and replaced with the *ura4+* gene, using PCR-based tagging. The *ura4+* gene was then replaced by mutated CM1 sequences (Fig. 1B) that had been generated by mega-primer PCR. The resulting strains were confirmed by genomic sequencing. The *mto1-334* mutant was constructed in a similar manner. In the first step, amino acids 494-600 of Mto1 were deleted and replaced with the *ura4+* gene. In the second step, the *ura4+* gene was replaced with a linearised plasmid fragment from which the coding sequence for amino acids 523-537 (LMRMEQQWREDVDQL) had been deleted. The resulting strains were confirmed by colony PCR and Western blotting. Diploid strains were made by *ade6-210*/*ade6-216* transcomplementation.

PCR-based tagging and truncations of genes were performed using methods and template plasmids as described (Bahler et al., 1998; Brune et al., 2005; Snaith et al., 2005). Apart from the *nmt81:*GFP-tubulin (Sawin et al., 2004), all tagged proteins were tagged at their C termini and expressed from endogenous promoters. Tagging of the endogenous (essential) Alp4 with the tandem-dimer-Tomato (tdT) RFP tag (Shaner et al., 2004) did not lead to significant deleterious effects on MT behavior. However, when crossing the Alp4-tdT strain with various mutants we sometimes noticed subtle effects on mutant cell morphology. This suggests that Alp4-tdT may have minor effects on MT function, as has been observed with other tagged versions of Alp4 (Anders et al., 2006).

Cell morphology assays and DIC imaging in return-to-growth experiments were performed as described (Anders et al., 2006; Snaith and Sawin, 2003); cells were grown to stationary phase in rich medium for 2-3 days and then refed with fresh medium, and fixed and imaged 3 hours after refeeding.

Biochemical methods

For Myc- and HA-tagged proteins, monoclonal antibodies 9E10 and 12CA5 were used. For Mto1 and Mto2, serum or affinity-purified polyclonal antibodies were used as described previously (Samejima et al., 2005; Sawin et al., 2004). For γ-tubulin (Gtb1), monoclonal antibody GTU-88 (Sigma) was used. Antibodies to Alp4 and Alp6 were raised by immunization of sheep with SDS-PAGE-purified GST-Alp4 and GST-Alp6 fusion proteins (from purified inclusion bodies), and either serum (anti-Alp4) or blot-affinity-purified antibody (anti-Alp6) was used for Western blots.

For immunoprecipitations, extracts were made from liquid-nitrogen frozen cells as described previously (Anders et al., 2006; Samejima et al., 2005; Sawin et al., 2004). Protein A Sepharose, Protein G Sepharose, or Protein G Dynabeads were used for immunoprecipitations, depending on the primary antibody. For salt-washes of immunoprecipitations, two intermediate wash steps were supplemented with KCl to the final salt concentration indicated.

A single-step purification of TAP-tagged proteins, up to and including release of proteins bound to IgG beads by cleavage with TEV protease, was performed following the steps described by Gould et al. (Gould et al., 2004), with only minor modifications. In the experiments shown in Fig. 6 we note that different amounts of Mto1 and Mto2 are seen in the lanes 1-4 under “TEV elution, short exposures”. These differences are likely to have a simple technical explanation. Comparing lanes 2 and 4, it can be seen that more Mto2(-TAP) is purified from *mto1*Δ cells than from wild-type cells, even though equal levels of Mto2(-TAP) are present in the cell extracts. This suggests that the TAP tag on Mto2 may not be easily accessible when Mto2-TAP is part of the Mto1/2 complex, consistent with previously published work that did not successfully detect γ-TuC proteins by mass spectrometry after Mto2-TAP purification (Venkatram et al., 2005). At the same time, comparing lanes 1 and 2, it can be seen that comparable amounts of Mto2 are purified from Mto1-TAP and Mto2-TAP purifications in wild-type cells, while in the same cells, significantly less Mto1 is purified from Mto2-TAP purification as compared to Mto1-TAP purification. We interpret this as follows: In the Mto1-TAP purification from wild-type cells, all of the Mto2 purified is derived from the Mto1/2 complex. In the Mto2-TAP purification from wild-type cells, most of the Mto2(-TAP) purified may be derived from “free”, uncomplexed Mto2, because much of the Mto2(-TAP) that is present as Mto1/2 complex in these cell extracts is poorly accessible and is therefore not purified. Therefore, only a small proportion of Mto2(-TAP) that is purified here is derived from the Mto1/2 complex, and that is why there is less Mto1 in lane 2 than in lane 1. The increased Mto2 in lane 4 compared to lane 2 is, accordingly, the result of a higher proportion of total Mto2 in

cell extracts being accessible to purification. According to this view, the different amounts of γ-TuC purified in lanes 1 and 2 would be correlated with the different amounts of total Mto1/2 complex that were purified. We also note that exposures were chosen to illustrate these intensity differences, and therefore intensities can be compared within panels but not between panels.

Immunofluorescence

Microtubule cold-shock and regrowth experiments were performed exactly as described previously, using methanol fixation (Samejima et al., 2005; Sawin et al., 2004). For anti-tubulin immunofluorescence, monoclonal antibody TAT-1 was used (Woods et al., 1989). For anti-Mto1 immunofluorescence, affinity-purified polyclonal antibody to Mto1 was used (Sawin et al., 2004). TAT1 anti-tubulin-stained cells were further stained with Alexa-Fluor-488-labelled secondary antibody (Invitrogen) and observed by spinning-disk confocal microscopy, using a Nikon 100×/1.45 NA PlanApo objective on a Nikon TE2000 inverted microscope, connected to a Yokogawa CSU-10 spinning disk confocal head (Visitech) and a Coolsnap HQ CCD camera (Photometrics). Images were acquired using Metamorph software (Molecular Devices). Anti-Mto1-stained cells were further stained with Alexa-Fluor-568-labelled secondary antibody, counterstained with DAPI and observed by wide-field microscopy, using a Nikon 100×/1.40 NA Plan Apo objective on a Nikon TE300 inverted microscope, connected to a Coolsnap HQ CCD camera. Images were acquired using Metamorph software. Note that methanol fixation leads to “shrunken” cells relative to live-cell imaging; this can be seen comparing cell lengths to scale bars.

Live-cell imaging and videomicroscopy

Live-cell imaging used a system and methods described previously (Anders et al., 2006). Cells were grown in minimal medium at room temperature (23-25°C). For videomicroscopy of cells expressing GFP-labelled tubulin (*nmt81*:GFP-Atb2, with the promoter induced) and RFP-labelled spindle pole bodies (Sad1-dsRed), 8 Z-sections, 0.6 μm apart, were acquired every 20 seconds, using Metamorph software (Molecular Devices). Video sequences were deconvolved using Softworx (Applied Precision), and maximum projections were used for movies at 10 frames per second (i.e. 200X time compression relative to real-time), using Quicktime 7.4.1 (Apple) and H.264 video compression. Maximum projections are also shown in the image stills of Figs. 1E and 4E. Still images of cells expressing Mto1-GFP or mutant Mto1-GFP with Alp4-tdT were acquired as above but not deconvolved. Maximum projections are shown, except for Fig. 2B. Apart from deconvolution for movies, all microscope images were adjusted only by linear contrast enhancement using Photoshop (Adobe), and all relevant panels within a given figure were adjusted identically, allowing direct comparison of intensities.

Quantification of new cytoplasmic microtubule nucleation frequency

For measurements of new MT nucleation frequency in wild-type cells and *mto1* mutants expressing GFP-Atb2 and Sad1-dsRed, 20 cells of each genotype were analyzed from movies (Fig. 1E, supplementary Movies 1-5). The appearance of new MT bundles was measured in each cell for 26 minutes (80 time-points); values shown are the mean nucleation frequency per minute per cell, ± SD. Obvious intranuclear MT nucleation, which is sometimes apparent in *mto1*Δ, *mto1-9A1* and *mto1-9A2* mutants, was ignored. The low levels of apparent cytoplasmic nucleation measured in *mto1*Δ, *mto1-9A1* and *mto1-9A2* mutants may reflect occasional mis-scored intranuclear MT nucleation (note that SD is greater than the mean in these mutants).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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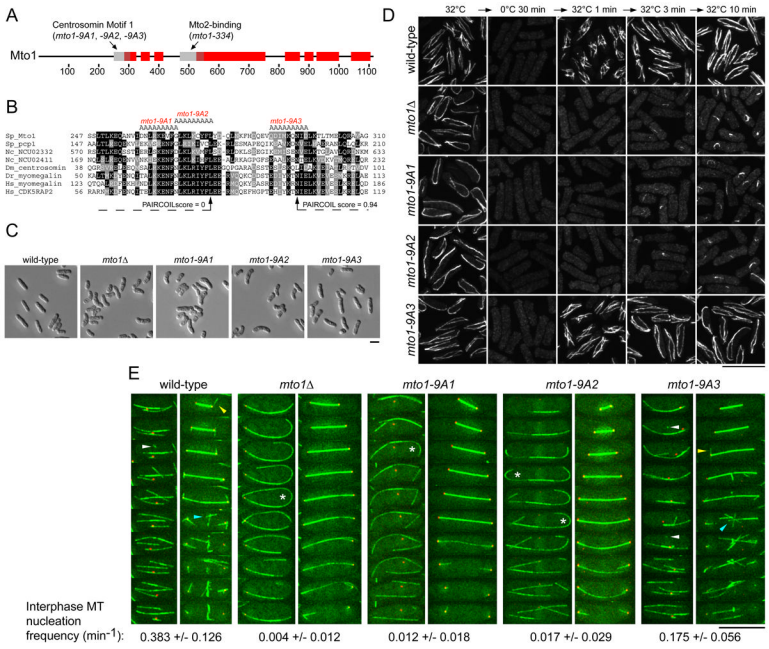

Fig. 1.

Two of three mutations in the Centrosomin motif 1 (CM1) region of Mto1 abolish cytoplasmic MT nucleation. (A) Schematic of Mto1 protein, showing CM1 and Mto2-binding region in grey. Predicted coiled-coils (PAIRCOIL score > 0.5) are indicated in red (Berger et al., 1995). Amino acid residue numbers are indicated below. (B) Alignment of Mto1 CM1 region with selected related proteins in fission yeast, *Neurospora*, *Drosophila*, zebrafish and human, with the amino acids mutated to alanine in the *mto1-9A1*, *mto1-9A2*, and *mto1-9A3* mutants indicated above. The *mto1-9A3* mutation lies at the beginning of the first predicted coiled-coil of Mto1. (C) Wild-type and *mto1* mutant cell morphology (strains KS515, KS1017, KS2007, KS2010, KS2013) after growth to stationary phase and refeeding with fresh medium. (D) Anti-tubulin immunofluorescence time-course of MT regrowth after cold-induced depolymerization in wild-type and mutant strains (strains KS515, KS1017, KS2007, KS2010, KS2013). (E) Stills from movies of strains expressing GFP-tubulin and the SPB component Sad1-dsRed (strains KS2863, KS3609, KS3604, KS3605, KS3607; see supplementary material Movies 1-5). Interphase cells are shown on the left of each pair (40 sec intervals) and mitotic cells on the right (100 sec intervals). White arrowheads indicate examples of interphase MT nucleation. Yellow arrowheads indicate examples of mitotic astral MTs. Blue arrowheads indicate example of PAA MTs. Asterisks indicate bend-breakage events in which a single MT or MT bundle breaks into two. Values underneath images indicate average number of interphase nucleation events per cell per minute (see Materials and Methods). Bars, 10 μm.

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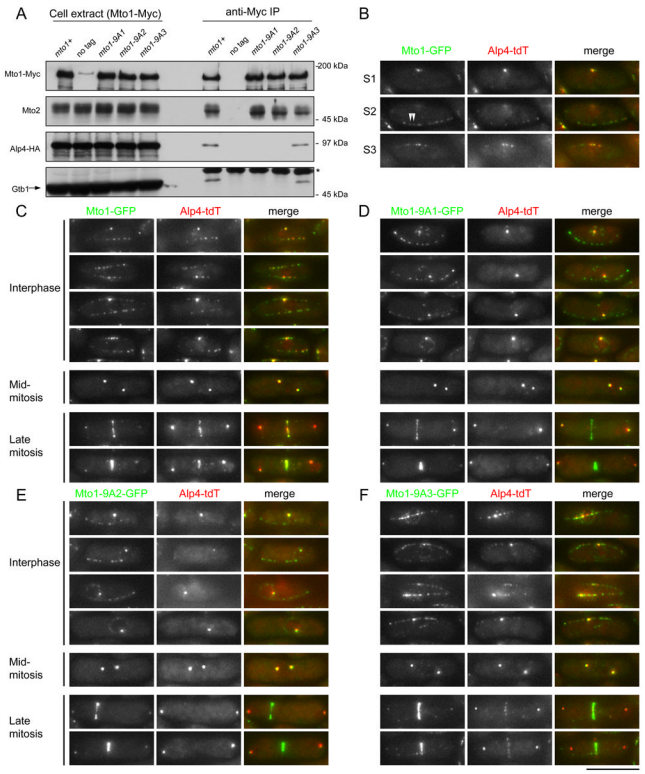

Fig. 2.

Mto1-9A1 and Mto1-9A2 mutant proteins do not interact with the γ-tubulin complex (γ-TuC). (A) Anti-Myc immunoprecipitates of Myc-tagged wild-type and mutant Mto1 proteins (strains KS1517, KS1370, KS2290, KS2292, KS2294), probed with antibodies to the indicated proteins. The band migrating near Mto1-Myc on the anti-Myc blot of cell extract, “no tag” lane is an unrelated protein that is not precipitated by the anti-Myc antibody. IgG on the anti-γ-tubulin blot is marked by asterisk. (B) Individual sections (S1, S2, S3) of a single interphase cell expressing Mto1-GFP and Alp4-tdTomato from endogenous promoters (strain KS3957). The SPB is apparent in S1. Note in S2 that some Mto1-GFP satellites do not contain visible Alp4-tdT (middle section, arrowheads). (C-F) Maximum projections of cells expressing Mto1-GFP, Mto1-9A1-GFP, Mto1-9A2-GFP, or Mto1-9A3-GFP, together with Alp4-tdT (strains KS3957, KS3961, KS3962, KS3965) in interphase and mitosis. Bar, 10 μm.

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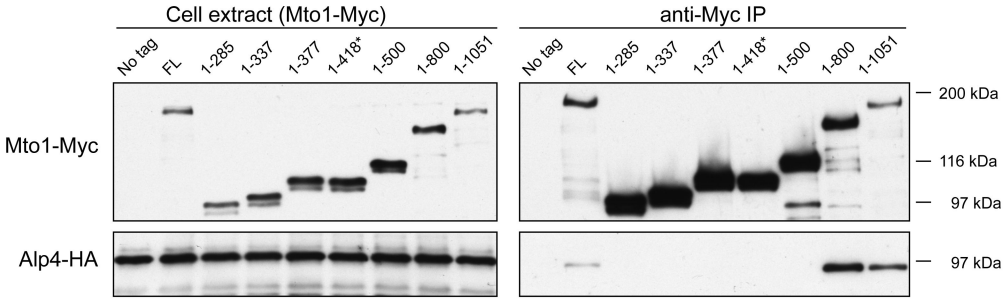

Fig. 3.

The CM1 region of Mto1 is not sufficient for interaction with the γ-TuC. Anti-Myc immunoprecipitates of Myc-tagged carboxy-terminal truncations of Mto1 (strains KS1366, KS1517, KS1910, KS1822, KS1824, KS1825, KS1914, KS1916, KS1933), probed with antibodies to Mto1-Myc and γ-TuC protein Alp4-HA. “FL” indicates full-length Mto1 (1-1115). Abnormally fast migration of Mto1-(1-418)-Myc (marked with asterisk) is due to a reduced number of Myc tags.

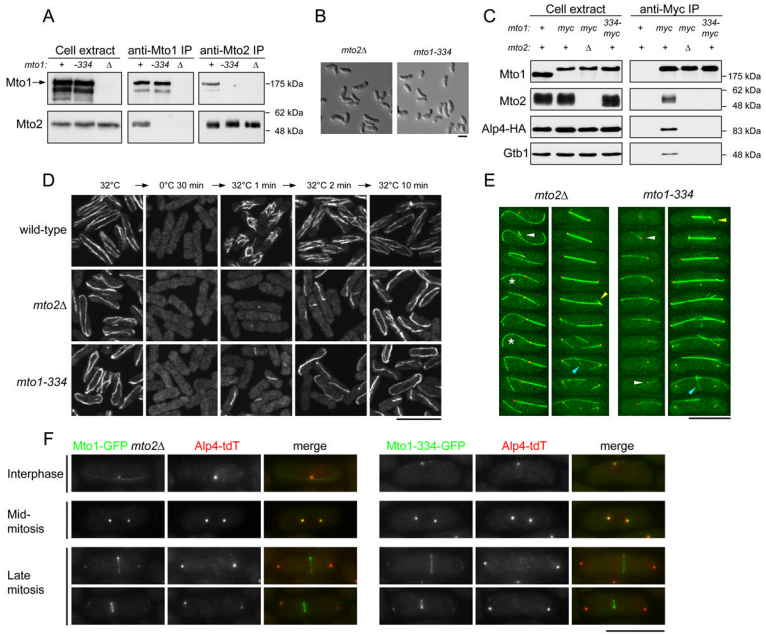

Fig. 4.

Mto2 binding to Mto1 is required for efficient interaction of Mto1 with the γ-TuC. (A) anti-Mto1 and anti-Mto2 immunoprecipitates from wild-type, *mto1-334* and *mto1*Δ cells (strains KS516, KS2272, KS1017), probed with anti-Mto1 and anti-Mto2 antibodies. (B) *mto2*Δ and *mto1-334* mutant cell morphology (strains KS977, KS3734) after growth to stationary phase and refeeding with fresh medium. (C) Anti-Myc immunoprecipitates of Mto1, Mto1-Myc or Mto1-334-Myc, in the *mto2* backgrounds shown (strains KS1370, KS1517, KS2169, KS3742), probed with antibodies to the indicated proteins. (D) Anti-tubulin immunofluorescence time-course of MT regrowth after cold-induced depolymerization in wild-type, *mto2*Δ and *mto1-334* cells (strains KS516, KS976, KS2272). (E) Stills from movies of *mto2*Δ and *mto1-334* cells expressing GFP-tubulin and Sad1-dsRed (strains KS2785, KS3765; see supplementary material Movies 6 and 7). Interphase cells are shown on the left of each pair (40 sec intervals) and mitotic cells on the right (100 sec intervals). White arrowheads indicate interphase MT nucleation, restricted to the SPB. Yellow arrowheads indicate mitotic astral MTs. Blue arrowheads indicate examples of weak nucleation of MTs from eMTOC. Asterisks indicate bend-breakage events in which a single MT or MT bundle breaks into two. (F) Maximum projections of cells expressing Mto1-GFP in *mto2*Δ background or Mto1-334-GFP in wild-type *mto2+* background, together with Alp4-tdT (KS4074, KS4039). In some but not all *mto2*Δ and *mto1-334* mutants, very faint Alp4-tdT could be observed at the equator, which may represent sub-detection amounts of γ-TuC that are likely responsible for the very small amount of equatorial MT nucleation observed in these mutants (see Fig. 4E). Bars, 10 μm.

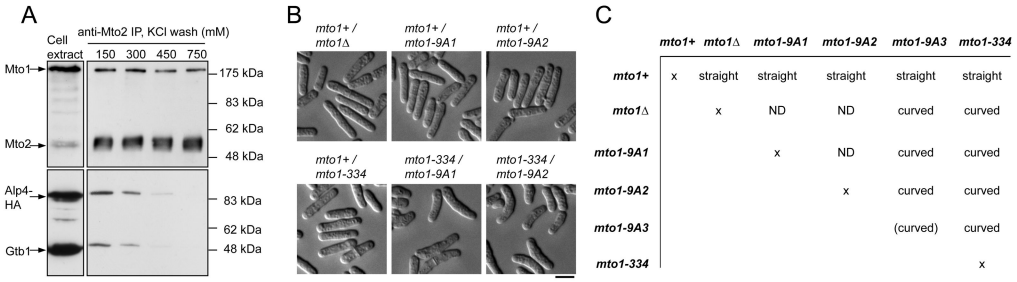

Fig. 5.

Mto1 and Mto2 form a stable complex in which Mto1 must contain both an intact CM1 region and an Mto2-binding region for normal function. (A) Anti-Mto2 immunoprecipitates from *alp4-HA* cells (strain KS1370), washed with the indicated concentrations of KCl and probed with antibodies to the proteins shown. (B) DIC images of representative diploid strains of the indicated genotypes described in (C) after growth to stationary phase and refeeding with fresh medium. Bar, 10 μm. (C) Cell morphology of diploids combining the different *mto1* alleles shown in the rows and columns in *trans* (strains KS3786 × KS3780, KS3785 × KS3775, KS3786 × KS3789, KS3786 × KS3777, KS3795 × KS3784, KS3781 × KS3776, KS3780 × KS3784, KS3774 × KS3777, KS3775 × KS3784, KS3788 × KS3776, KS3789 × KS3784, KS3776 × KS3778, KS3777 × KS3784, reading from left to right). *mto1-9A3* did not trans-complement any of the other *mto1* mutant alleles, probably because *mto1-9A3* is itself a partial loss-of-function allele, as shown in the morphology of *mto1-9A3*/*mto1-9A3* homozygous diploids (see also Figs. 1 and 2; supplementary material Movie 4). Other homozygous diploids were not constructed; ND, not determined.

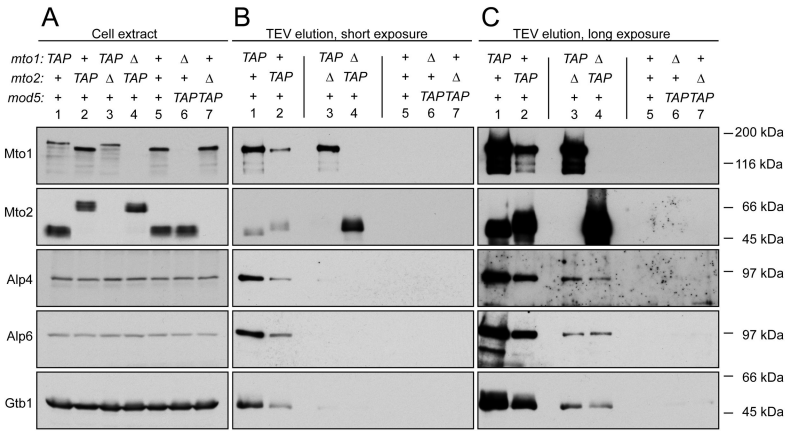

Fig. 6.

Cooperative binding of the Mto1/2 complex to γ-TuC. Purification of TAP-tagged Mto1 and Mto2 in wild-type and mutant backgrounds (strains KS3524, KS3956, KS4323, KS4326, KS516, KS4267, KS4270), probed with antibodies to the indicated proteins. Differently-migrating forms of Mto1 and Mto2 represent TAP-tagged, cleaved TAP-tagged, and untagged forms of the proteins. Vertical lines indicate blank lanes.

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Table 1

Summary of *mto1 and mto2* mutant phenotypes

strain	interphase nucleation from:	cytoplasmic astral MTs in mitosis?	PAA nucleation from eMTOC?
wild-type	SPB and iMTOCs	yes	yes
*mto1*Δ	none	no	no
mto1-9A1	none	no	no
mto1-9A2	none	no	no
mto1-9A3	SPB and iMTOCs (reduced)	yes	yes
*mto2*Δ	SPB only	yes	limited
mto1-334	SPB only	yes	limited