

RESEARCH ARTICLE

Microtubule-severing activity of the AAA+ ATPase Katanin is essential for female meiotic spindle assembly

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ABSTRACT

In most animals, female meiotic spindles are assembled in the absence of centrosomes. How microtubules (MTs) are organized into acentrosomal meiotic spindles is poorly understood. In *Caenorhabditis elegans*, assembly of female meiotic spindles requires MEI-1 and MEI-2, which constitute the microtubule-severing AAA+ ATPase Katanin. However, the role of MEI-2 is not known and whether MT severing is required for meiotic spindle assembly is unclear. Here, we show that the essential role of MEI-2 is to confer MT binding to Katanin, which in turn stimulates the ATPase activity of MEI-1, leading to MT severing. To test directly the contribution of MT severing to meiotic spindle assembly, we engineered Katanin variants that retained MT binding and MT bundling activities but that were inactive for MT severing. *In vivo* analysis of these variants showed disorganized microtubules that lacked focused spindle poles reminiscent of the Katanin loss-of-function phenotype, demonstrating that the MT-severing activity is essential for meiotic spindle assembly in *C. elegans*. Overall, our results reveal the essential role of MEI-2 and provide the first direct evidence supporting an essential role of MT severing in meiotic spindle assembly in *C. elegans*.

KEY WORDS: AAA+ ATPase, Microtubule severing, Meiosis, MEI-1, MEI-2, *C. elegans*

INTRODUCTION

Meiosis is a specialized cell division that is essential to produce haploid gametes for sexual reproduction. During oogenesis, meiosis produces a single haploid oocyte, whereas the remaining chromosome sets are discarded into the polar bodies (PBs). This highly asymmetric cell division relies on the assembly and positioning at the cell cortex of a small acentrosomal and anastral meiotic spindle (Dumont and Desai, 2012; Severson et al., 2016). In *Caenorhabditis elegans*, the assembly, function, cortical localization and size of the meiotic spindle requires Katanin, an evolutionarily conserved AAA+ ATPase complex exhibiting microtubule (MT)-severing activity, originally discovered in *Xenopus* (McNally and Vale, 1993).

AAA+ ATPases (ATPases associated with diverse cellular activities) are protein complexes present in all kingdom of life, which share a functional domain of 200–250 amino acids called the AAA+ domain, containing Walker A and B motifs (Walker et al., 1982), which are involved in nucleotide binding and hydrolysis, respectively, and a second region of homology (SRH) involved in subunit communication

(Ogura and Wilkinson, 2001; Vale, 2000; White and Lauring, 2007). They also share a common oligomer organization (usually hexamer) required for the formation of the nucleotide-binding pocket at the interface between two adjacent subunits, allowing them to use ATP hydrolysis to remodel their substrate (Ogura and Wilkinson, 2001; Vale, 2000; White and Lauring, 2007).

Katanin consists of a ‘catalytic’ AAA+ subunit (p60) and an ‘accessory’ subunit (p80), called MEI-1 and MEI-2, respectively, in *C. elegans*. The AAA+ domain of MEI-1 is highly conserved between species and presents extensive homologies with two other MT-severing enzymes, Fidgetin and Spastin (Luke-Glaser et al., 2007) (Fig. S1). The p60 catalytic subunit from the sea urchin is sufficient for MT severing *in vitro* (McNally and Vale, 1993). However, both subunits are required for Katanin activity in *C. elegans* (Mains et al., 1990; McNally et al., 2006), *Tetrahymena* (Sharma et al., 2007) and *Chlamydomonas* (Dymek et al., 2004). These observations indicate that the so-called ‘accessory subunit’ is more than ‘accessory’ in these organisms. However, its contribution is still poorly understood. For instance, while MEI-2 is essential for Katanin activity in *C. elegans* and for MT severing *in vitro*, its precise function within the complex is not known (McNally et al., 2006; Srayko et al., 2000).

Likewise, whereas the MEI-1/MEI-2 complex is essential for meiotic spindle assembly, the biochemical activity of the complex required for this spindle assembly is still unclear. Katanin bundles and severs microtubules (McNally and Vale, 1993; McNally et al., 2014; Srayko et al., 2000, 2006). It has been reported that only the MT-bundling activity is required for meiotic spindle assembly based on the observation that versions of Katanin with disrupted MT-severing activity support the assembly of female meiotic spindles that are longer than those in the wild type (McNally and McNally, 2011). However, this conclusion was based on the analysis of Katanin MEI-1 variants obtained by a forward genetic approach. These variants contain two substitutions, in particular P99L, which disrupt MEI-1 levels *in vivo* (Clark-Maguire and Mains, 1994a; Pintard et al., 2003). Furthermore, these variants might not be null for MT severing because the MT-severing assay used to characterize them was not highly sensitive (MT disassembly in *Xenopus* cells).

Several lines of indirect evidence suggest that MT severing might be required for spindle assembly. Electron tomography and 3D modelling of female meiotic spindles in the wild type and in *mei-1* null mutants lacking Katanin showed that Katanin converts long MT polymers into shorter MT fragments around chromatin. It was proposed that these shorter MT fragments could then act as ‘seeds’ for the nucleation of new MTs, thereby contributing to meiotic spindle assembly (Roll-Mecak and Vale, 2006; Srayko et al., 2006). Consistent with a role of MT severing in meiotic spindle assembly, genetic analysis revealed that γ -tubulin-dependent nucleation and MT severing provide redundant mechanisms to increase MT number during oocyte meiosis (McNally et al., 2006).

Despite considerable work, the exact contribution of Katanin MT severing to meiotic spindle assembly is still unclear, especially

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because there is currently no available expression system allowing a functional analysis of Katanin activity *in vivo*. In this study, we develop a unique strategy allowing the *in vivo* analysis of female meiotic spindle assembly in a living oocyte in the presence of MEI-1 variants null for MT severing. We show unequivocally that the MT-severing activity of Katanin is strictly required for meiotic spindle assembly. In addition, we show that MEI-2 provides MT-binding activity to the Katanin complex. Overall, our results reveal an essential role of Katanin MT severing in meiotic spindle assembly in living *C. elegans* oocytes.

RESULTS

Purified Katanin severs and bundles MTs *in vitro*

To biochemically characterize the *C. elegans* MEI-1/MEI-2 Katanin complex, its interaction with its substrate (MTs) and to determine the relative contributions of MEI-1 and MEI-2 subunits to Katanin activities, we co-expressed MEI-1 and MEI-2 from a bicistronic

vector in *E. coli* and purified the MEI-1/MEI-2 complex using double tag affinity chromatography (see the Materials and Methods). Through this approach, we obtained a highly purified MEI-1/MEI-2 complex (Fig. 1A, lane 2). To ascertain the relative contribution of MEI-1 and MEI-2 to the biochemical activities of the complex, we also purified MEI-1 and MEI-2 subunits alone (Fig. 1A, lanes 6 and 7, respectively).

Using this highly purified recombinant Katanin, we first asked whether this complex was active for MT-severing and MT-bundling activities. Using live, spinning-disk confocal microscopy, we monitored the severing of pre-assembled MTs labelled with Rhodamine-tubulin. MT severing was readily detectable specifically when Katanin was incubated with MTs in the presence of ATP (Fig. 1B), demonstrating that the purified Katanin complex is active *in vitro*. As a complementary and more quantitative approach, we monitored MT severing by following the fluorescence of DAPI-stained microtubules over time (Fig. 1C).

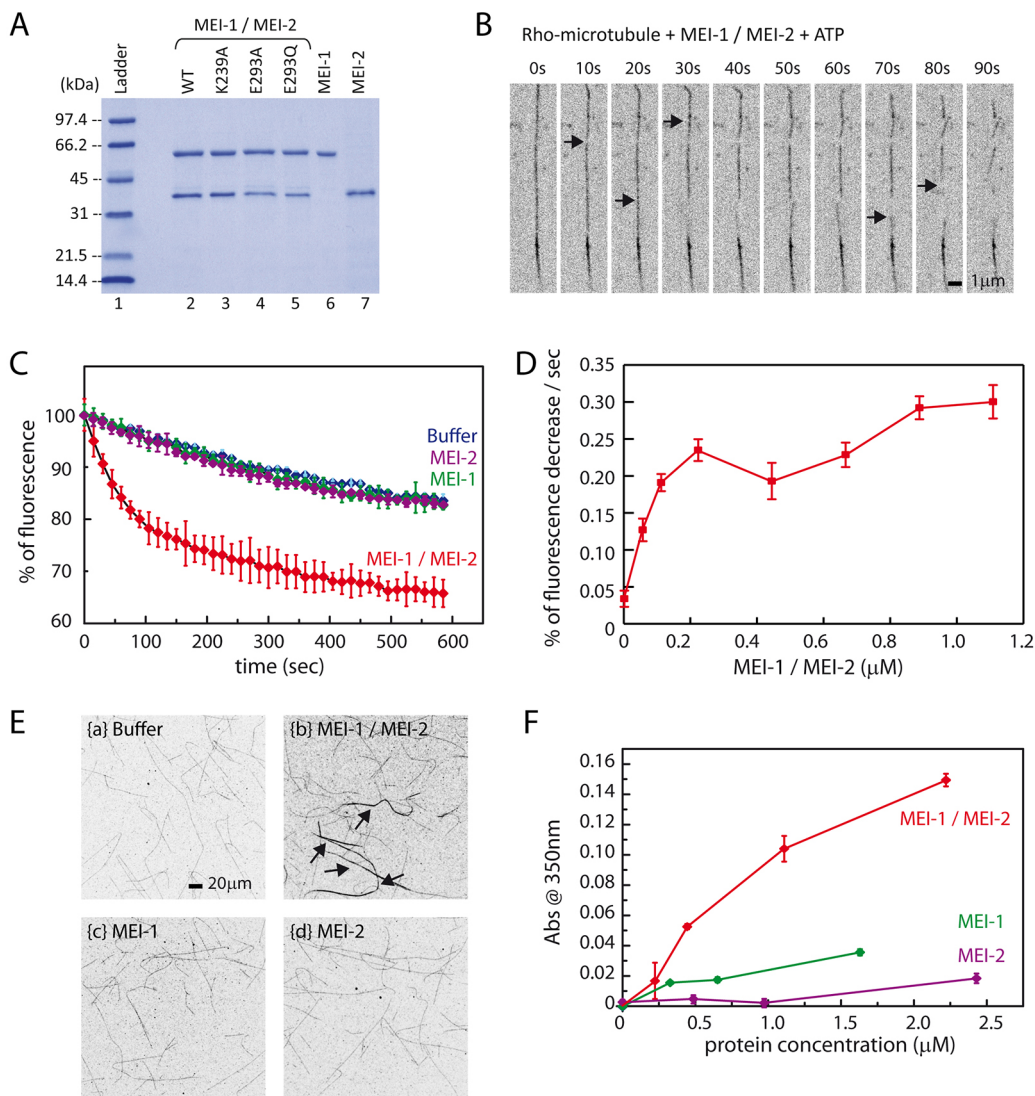


Fig. 1. Biochemical characterization of purified *C. elegans* Katanin. (A) Purified recombinant proteins analysed by Coomassie Blue-stained SDS-PAGE. (B) Microtubule-severing assay showing Rhodamine-labelled microtubules in the presence of MEI-1/MEI-2 and ATP. Arrows indicate initial microtubule-severing events identified, which in some cases, is followed by microtubule depolymerization. (C) Microtubule-severing activity of MEI-1/MEI-2, or MEI-1 or MEI-2 alone (mean±s.d.; $n \geq 5$). (D) Microtubule-severing activity of different concentrations of MEI-1/MEI-2 (mean±s.d.; $n=3$). (E). Microtubule-bundling activity of MEI-1/MEI-2, MEI-1 or MEI-2 alone. The experiment was reproduced at least five times giving similar results. Arrows indicate microtubules organized as bundle. (F) Quantitative microtubule-bundling activity of MEI-1/MEI-2, MEI-1 or MEI-2 alone (mean±s.d.; $n=5$).

DAPI fluorescence is greater for polymerized MTs than for free tubulin (Hartman et al., 1998; Heusele et al., 1987). After addition of Katanin and ATP, we observed a strong decrease in the fluorescence signal, indicative of MT severing (Fig. 1C,D). In contrast to the MEI-1/MEI-2 complex, neither MEI-1 nor MEI-2 added individually was able to sever MTs in these assays (Fig. 1C), indicating that both MEI-1 and MEI-2 subunits are required for MT-severing activity *in vitro*, which is consistent with previous observations (McNally et al., 2006).

We next investigated whether these purified proteins were able to bundle MTs. We incubated the purified proteins with fluorescently labeled MTs and used spinning-disk confocal microscopy to monitor the appearance of MT bundles. MEI-1/MEI-2 readily bundled MTs (Fig. 1Eb, arrowheads) whereas MEI-1 or MEI-2 individually had no effect on MT-bundling (Fig. 1Ec and d). We quantified the MT-bundling activity by turbidimetry (McNally and Vale, 1993). We observed a 10-fold increase in absorbance when MTs were incubated in the presence of MEI-1/MEI-2 but not in the presence of MEI-1 or MEI-2 alone (Fig. 1F), indicating that both MEI-1 and MEI-2 subunits are required for MT bundling.

Taken together, these results indicate that a highly purified Katanin complex bundles and severs MTs and that both MEI-1 and MEI-2 subunits are strictly required for these activities. These observations are fully consistent with the genetic data indicating that both *mei-1* and *mei-2* genes are essential for female meiosis in *C. elegans* oocytes (Mains et al., 1990; Srayko et al., 2000).

The MEI-2 subunit stabilizes the MEI-1 hexamer for optimal ATP-dependent MT severing

AAA+ ATPases oligomerize to form a nucleotide-binding pocket at the interface between two adjacent AAA+ subunits and to use ATP hydrolysis for substrate remodelling. We thus characterized the functional properties of this atypical hetero-oligomer (MEI-1/MEI-2) and, particularly, the respective contribution of each subunit to oligomer formation and ATP hydrolysis activities. We started by investigating the oligomer formation property of the complex, as it directly reflects subunit organization and formation of the nucleotide-binding pocket (Ogura and Wilkinson, 2001; Vale, 2000).

Using gel filtration, we analysed the oligomeric state of the purified proteins as a function of protein concentration (Fig. 2A) and/or in the presence of nucleotides (ATP or ADP) (Fig. 2C). We established that MEI-1/MEI-2 is in equilibrium between an apparent dimer (of MEI-1/MEI-2 heterodimer, 15 μ M) and an apparent octamer (of MEI-1/MEI-2 heterodimer, 75 μ M) (Fig. 2A). The amount of each protein, which was estimated by analysing the eluted fractions on SDS-PAGE and Coomassie Blue staining, is consistent with a stoichiometry of 1:1 of MEI-1:MEI-2 within the complex (Fig. 2B). In addition, for a fixed concentration of protein (15 μ M), the presence of nucleotides (ATP or ADP) similarly shifted the equilibrium towards the apparent octamer state (Fig. 2C, top panel). The elution of AAA+ ATPases as apparent octamers has been reported for the hexamer of various AAA+ proteins (e.g. PspF, ClpX) and is due to the intrinsic organization of the oligomer as an opened ring (Glynn et al., 2009; Joly et al., 2006; Rappas et al., 2005). Taken together, these results strongly suggest that the higher oligomeric form of the MEI-1/MEI-2 complex is a hexamer of MEI-1/MEI-2 dimers. Interestingly, dimer-hexamer equilibrium was not observed for MEI-1 alone, although the presence of ATP slightly shifted the elution volume of MEI-1 to a higher molecular weight complex (Fig. 2C, middle panel). In contrast to MEI-1, MEI-2 always behaved as an apparent 1/2-mer. These results suggest that

MEI-1 is able to form oligomers, which are stabilized by MEI-2. To further test this hypothesis, we measured the ATPase activity of the MEI-1/MEI-2 complex and of MEI-1 and MEI-2 alone (Fig. 2D). The ATPase activity of AAA+ proteins is a direct indication of the correct oligomer (hexamer) formation (Lupas and Martin, 2002; Ogura and Wilkinson, 2001; Vale, 2000). MEI-1 and MEI-1/MEI-2 presented similar ATPase activity (Fig. 2D). However, the rate of ATP hydrolysis was constant over a range of protein concentrations for MEI-1/MEI-2, whereas it drastically changed with protein concentration for MEI-1 alone. In particular, the ATPase activity of MEI-1 was negligible at lower protein concentrations (Fig. 2E). This result indicates that MEI-1 is not able to hydrolyse ATP at lower concentrations, most likely because the protein is not properly organized into an active hexamer. We conclude that MEI-1 is able to hydrolyse ATP, but the presence of MEI-2 stabilizes the oligomer for efficient ATPase activity.

The MEI-2 subunit is required for Katanin-microtubule interaction

MEI-1 alone can hydrolyse ATP but is not able to sever MTs, suggesting that MEI-2 might have a role in MT interaction. Therefore, we compared the ability of each subunit, or of the hetero-oligomer to interact with MTs. The purified proteins immobilized on nickel beads (6 \times His-MEI-1, 6 \times His-MEI-2 or 6 \times His-MEI-1/MEI-2) were incubated with pre-assembled MTs in the presence of ATP, or of the slowly hydrolysable ATP analog (ATP γ S). After extensive washes, the proteins were eluted from the beads and analysed by SDS-PAGE followed by Coomassie Blue staining (Fig. 3A). The amount of 6 \times His-MEI-2 retained on nickel beads was lower than the amount of MEI-2 retained in complex with 6 \times His-MEI-1 (Fig. 3A, lanes 3 and 4). This is expected given that MEI-2 is a 1/2-mer whereas MEI-1/MEI-2 is a hexamer. Nevertheless, both MEI-2 and MEI-1/MEI-2 captured MTs whereas MEI-1 alone did not (Fig. 3A, compare lanes 7,8 and 3,4 with 5,6). MT recovery using MEI-1/MEI-2 was decreased in the presence of ATP compared with ATP γ S (Fig. 3A, compare lanes 3 and 4), presumably because Katanin severed and released MTs in this condition, resulting in a lower MT-bound signal. Accordingly, MEI-2, which is unable to sever MTs alone, captured similar amounts of MTs in the presence of ATP or ATP γ S (Fig. 3A, compare lanes 7 and 8).

The finding that MEI-2 confers MT binding prompted us to evaluate the impact of MT binding on the ATPase activity of Katanin. We reasoned that MEI-2-mediated MT binding might stimulate the ATPase activity of the complex. Indeed, the presence of the substrate has been reported to stimulate the ATPase activity of several AAA+ ATPases (Johjima et al., 2015; McNally and Vale, 1993). We observed that the presence of MTs clearly stimulated the MEI-1/MEI-2 ATPase activity, whereas no stimulatory effect was observed with MEI-1 alone (Fig. 3B). These results suggest that the MEI-2-driven MT interaction might induce conformational changes within the Katanin complex, leading to an increase of its ATPase activity. We conclude that MEI-2 establishes a productive interaction between Katanin and MTs, leading to MT severing and release in the presence of ATP.

MT severing is essential *in vivo* for female meiotic spindle assembly in *C. elegans*

The exact contribution of MT severing to Katanin function *in vivo* is unknown. In particular, whether MT severing is required for bipolar female meiotic spindle assembly in *C. elegans* is a long-standing question that is still unanswered. Having characterized the precise

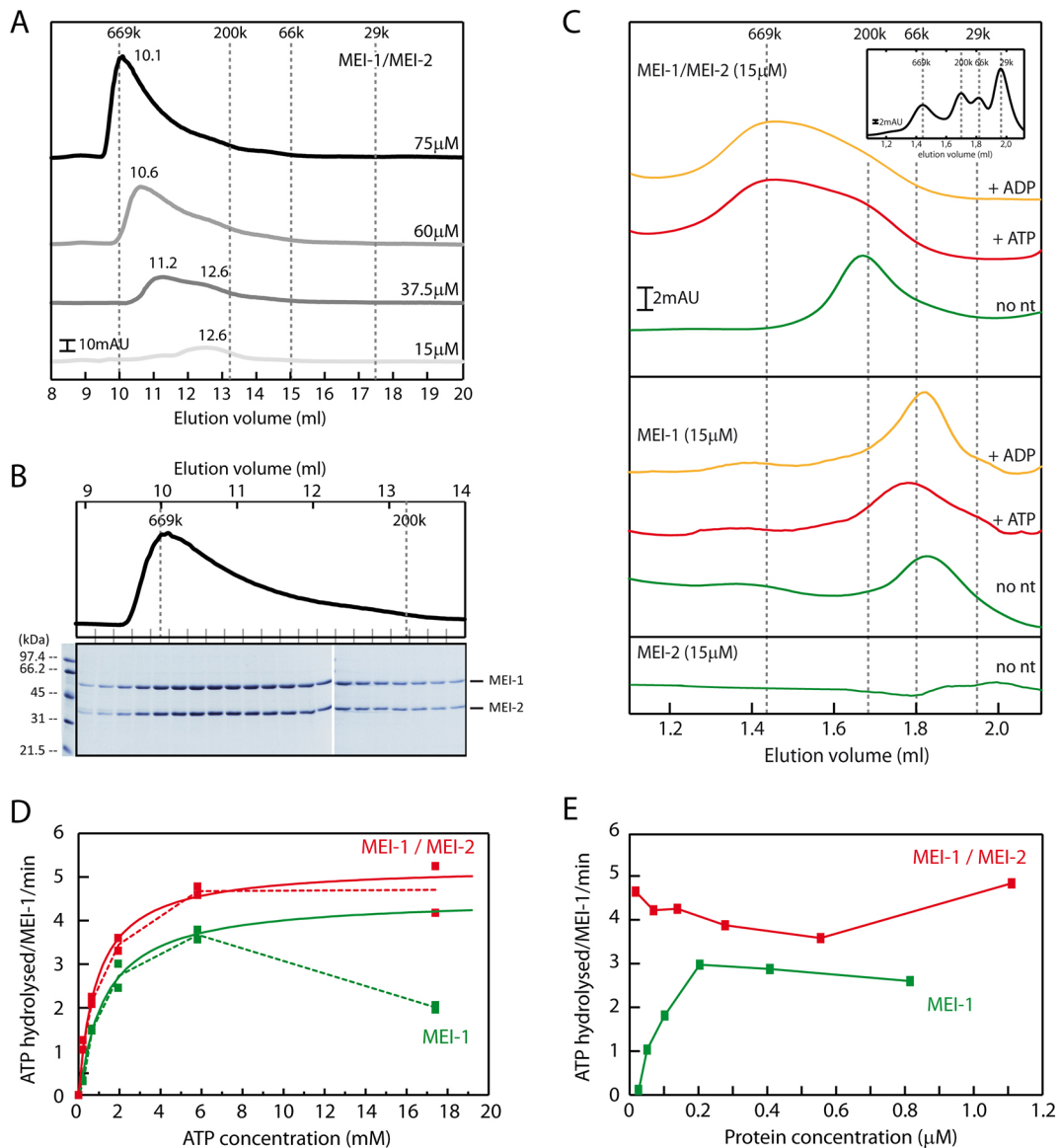


Fig. 2. Characterization of Katanin ATPase and oligomerization activities. (A) Chromatography profiles of different concentrations of MEI-1/MEI-2. Scale bar gives the scale of the ordinate axis; absorption unit (AU) corresponds to an A_{280} of 1. Experiment was reproduced at least three times giving similar results. (B) Elution fractions corresponding to the chromatography of 75 μM MEI-1/MEI-2 analysed by Coomassie Blue-stained 12% SDS-PAGE. (C) Chromatography profiles of MEI-1/MEI-2, MEI-1 alone or MEI-2 alone in the presence or absence of ATP or ADP. The column was calibrated using globular proteins (see inserted profile). The scale bar gives the scale of the ordinate axis; absorption unit (AU) corresponds to an A_{280} of 1. The experiment was reproduced at least three times giving similar results. (D) ATPase activity of 1 μM of purified MEI-1/MEI-2 or MEI-1 alone in the presence of different concentrations of ATP at 28°C. Dashed lines correspond to the mean of raw data and solid lines correspond to the Michaelis–Menten fit generated with Origin software. (E) ATPase activity of purified MEI-1/MEI-2 or MEI-1 alone (different concentrations as indicated) in the presence of 4 mM ATP at 28°C.

contribution of MEI-1 and MEI-2 subunits to MT-bundling and -severing activities of Katanin in *C. elegans*, we were in a unique situation to investigate the relative contribution of these two activities to meiotic spindle assembly in *C. elegans*. To this end, we designed Katanin variants where both activities were separated. Given that MT severing requires MT binding, we could only design MT-severing defective variants that retained their MT-bundling activity.

The AAA+ domain of MEI-1 is highly conserved between species (Fig. 4A,B). This is particularly striking for the Walker A (consensus sequence GxxGxGKT) and Walker B (consensus sequence hhhhDE, where h represents hydrophobic amino acid) that are responsible for ATP binding and hydrolysis, respectively.

We thus substituted highly conserved residues of MEI-1 Walker motifs: a Walker A variant (K239A) expected to alter ATP binding and two Walker B variants (E293Q: changing the side chain charge and E293A changing the side chain charge and length) that are predicted to be defective in ATP hydrolysis (Fig. 4B). As expected, the ATPase activity of purified Katanin harbouring these MEI-1 substitutions was severely reduced, although a residual activity could still be detected (Table 1). However, this remaining ATPase activity was clearly not sufficient to support MT severing *in vitro*. In contrast to wild-type Katanin, which induced a rapid decrease of fluorescence of DAPI-stained MTs, these variants had no effect on DAPI-stained MTs in these conditions (Fig. 4C). The lack of MT severing did not simply result from a lack of MT interaction because

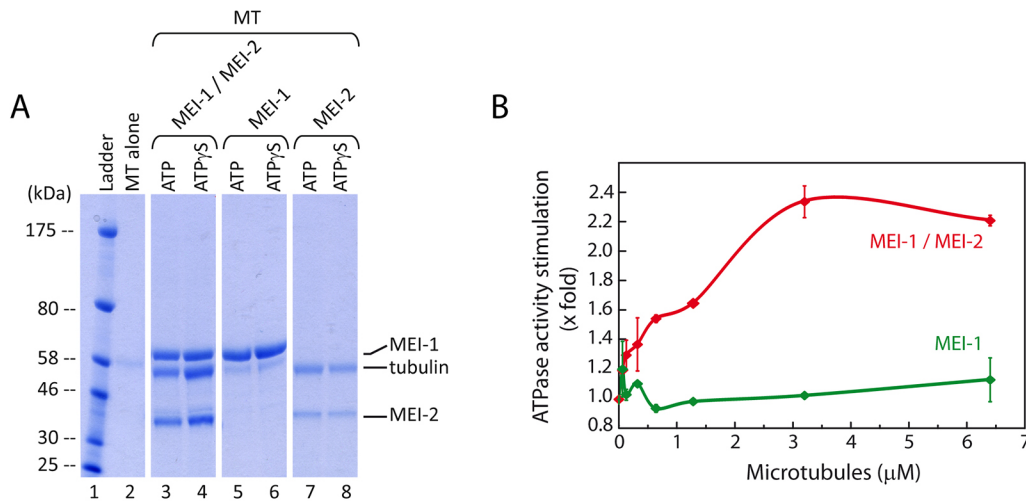


Fig. 3. MEI-2 confers microtubule binding to Katanin, which stimulates its ATPase activity. (A) Interaction between microtubules and MEI-1/MEI-2, MEI-1 or MEI-2 alone in the presence of 2 mM ATP or 2 mM ATP γ S. MEI-2 and MEI-1/MEI-2 were loaded on nickel beads in saturating conditions. The results presented here are from the same gel but the lanes have been rearranged for clarity. (B) ATPase activity of MEI-1/MEI-2 or MEI-1 alone in the presence of different concentrations of microtubules. Results are expressed in arbitrary units, with the condition with no microtubules set to 1 (mean \pm s.d.; $n=3$).

all the Katanin variants were able to bind and to bundle MTs *in vitro* (Fig. 4D,E). We thus conclude that these Katanin variants are specifically defective in MT severing.

We next introduced these ‘separation of function’ MEI-1 variants in worms to analyse the exact contribution of MT-severing activity to female meiotic spindle assembly. We inserted a *mei-1* transgene resistant to RNAi (*mei-1^R*) on chromosome II by MosSCI (Frøkjær-Jensen et al., 2008). We originally tagged MEI-1 with GFP at its N-terminus; however, whereas the GFP::MEI-1^R fusion protein was expressed at the expected size and detected on the meiotic spindle (data not shown), it failed to rescue the embryonic lethality resulting from endogenous *mei-1* depletion by RNAi, indicating that GFP::MEI-1^R is not functional. We thus generated a line expressing MEI-1 N-terminally tagged with the small FLAG epitope, to differentiate MEI-1^R produced from the transgene from endogenous, untagged MEI-1. To monitor transgene expression in the germline, we inserted the *FLAG::mei-1^R* transgene within an operon containing the Histone H2B fused to mCherry downstream of *FLAG::mei-1^R*, such that animals expressing mCherry::H2B in the germline also contain the FLAG::MEI-1^R transcript (Fig. 5A and Materials and Methods). Importantly, FLAG::MEI-1^R rescued the meiotic defects and associated lethality induced by RNAi-mediated depletion of endogenous *mei-1*, demonstrating the functionality of this fusion protein (Fig. 5B-D).

We next used a similar strategy to generate lines expressing the *mei-1^R* transgenes with the substitution on the Walker A and B motifs. The MEI-1 variants were detected at the expected size by western blot analysis with the exception of the Walker A variant (K239A), for which no protein could be detected (Fig. S3). This Katanin variant is unable to oligomerize in the presence of ATP (Fig. S2) and thus might be unstable *in vivo*. We then focused our phenotypic analysis on worms expressing the Walker B motif substitutions and used the animals containing the Walker A motif substitution as controls. We depleted endogenous *mei-1* by RNAi to assess the functionality of FLAG::MEI-1 E293Q and E293A variants. Whereas embryos expressing FLAG::MEI-1^R wild type (WT) were fully viable upon inactivation of endogenous *mei-1*, embryos expressing FLAG::MEI-1^R E293Q or E293A Walker B variants were embryonic lethal (Fig. 5B), most likely as a result of

defective meiosis. Accordingly, these embryos failed to assemble a bipolar meiotic spindle (Fig. 5C, compare a,c with e,f) and consequently produced abnormally large polar bodies (Fig. 5D), a phenotype reminiscent of *mei-1* null embryos (Fig. 5Cb and Da). The FLAG::MEI-1^R E293Q or E293A variants accumulated on the chromatin and around a mass of disorganized MTs, demonstrating that the defects in meiotic spindle assembly are not simply resulting from a lack of protein expression (Fig. 5C). FLAG::MEI-1^R WT localized to the meiotic spindle and to the meiotic chromosomes, similar to the endogenous MEI-1 (Fig. 5C) and supported bipolar meiotic spindle assembly.

Time-lapse spinning disk confocal microscopy of the first meiotic division in embryos expressing α -tubulin (TBA-2) fused to GFP and histone fused to mCherry confirmed these observations (Fig. 5E). Indeed, the assembly of a bipolar meiotic spindle was defective in embryos expressing the FLAG::MEI-1^R E293Q or E293A variants, in contrast to embryos expressing FLAG::MEI-1^R WT. No bipolar spindle assembly was detected in these embryos that resembled embryos lacking MEI-1 function (Fig. 5E). We conclude that the MEI-1 variants that are specifically defective in MT-severing fail to support meiotic spindle assembly.

The exact role of MT-severing for meiotic spindle assembly is currently unclear, but it has been proposed that MT-severing by Katanin might generate shorter MTs that would act as seeds for the polymerization of longer MTs (Roll-Mecak and Vale, 2006). We thus quantified MT density in embryos expressing the MT-severing deficient MEI-1 variants, from metaphase of

Table 1. ATPase activity: V_{max} and K_m constants of MEI-1 and MEI-1/MEI-2

	V_{max} (min ⁻¹)	K_m (mM)
MEI-1 WT/MEI-2	5.33 \pm 0.26	0.92 \pm 0.14
MEI-1 WT	4.66 \pm 0.42	1.45 \pm 0.36
MEI-1 K239A/MEI-2	2.55 \pm 0.41	2.94 \pm 1.39
MEI-1 E293A/MEI-2	1.54 \pm 0.36	2.19 \pm 0.96
MEI-1 E293Q/MEI-2	1.09 \pm 0.14	2.28 \pm 0.93

Values are mean \pm s.d. ATPase activity assay was performed at 28°C at least in triplicate.

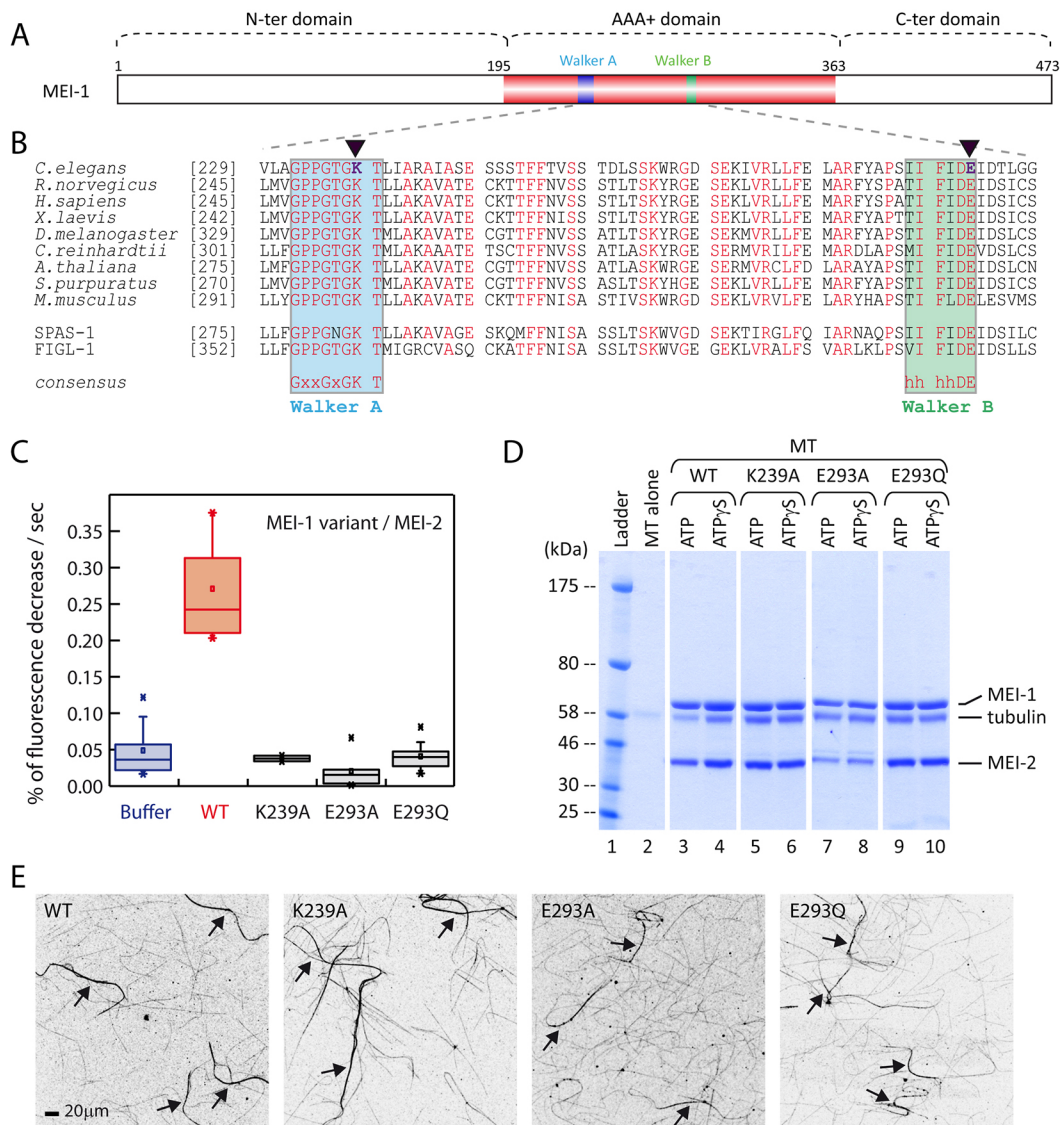


Fig. 4. Functional characterization of MEI-1 variants dissociating MT bundling and MT severing activities. (A) Schematic representation of *C. elegans* MEI-1 with Walker A and B motif in blue and green, respectively. (B) Multiple protein sequence alignments of the Walker A and B region between the Katanin p60 subunit from different species: *Caenorhabditis elegans* (P34808.1), *Rattus norvegicus* (AAH97929.1), *Homo sapiens* (NP_008975.1), *Xenopus laevis* (NP_001084226.1), *Drosophila melanogaster* (NP_524997.2), *Chlamydomonas reinhardtii* (EDP06327.1), *Arabidopsis thaliana* (BAB87822.1), *Strongylocentrotus purpuratus* (NP_999733.1) and *Mus musculus* (NP_081997.1); and *C. elegans* Spastin SPAS-1 (NP_001256115.1) and Fidgetin FIGL-1 (NP_504197.1). The residues in red are strictly conserved between all the Katanin p60 subunits aligned, with the Walker A and B residues substituted in this study indicated with black triangles. The consensus motifs are provided below the alignments. (C) Microtubule-severing activity of MEI-1/MEI-2 WT or variants. Box indicates s.d.; line, median value; whiskers, outliers; squares, mean; crosses, maximal and minimal values; $n=5$. (D) Interaction between microtubules and MEI-1/MEI-2 WT or variants in the presence of 2 mM ATP or 2 mM ATP γ S. (E) Microtubule-bundling activity of MEI-1/MEI-2 WT or variants. Experiment was reproduced at least five times giving similar results. Arrows indicate microtubules organized into bundles.

meiosis I (time, -10 min) to prophase of meiosis II (time, 0; Fig. 5F). Notably, in *mei-1(RNAi)* embryos or in *mei-1(RNAi)* embryos expressing the FLAG::MEI-1^R Walker B variants, spindle MT density was reduced at the time of meiotic spindle assembly as compared with the control (Fig. 5F; time -10 min), which is fully consistent with the hypothesis that MT severing contributes to meiotic spindle assembly by generating more MT polymers from an inefficient chromatin-based MT nucleation process (Srayko et al., 2006). These results also indicate that the MT-bundling activity of Katanin is not sufficient to organize and to assemble MTs into a bipolar meiotic spindle. We conclude that MT-severing activity of the Katanin is essential for female meiotic spindle assembly in *C. elegans*.

DISCUSSION

The *C. elegans* AAA+ ATPase Katanin, which is composed of MEI-1 and MEI-2 subunits, is essential for meiotic spindle assembly. However, the exact contribution of MEI-2 to Katanin activity, and the precise role of MT-severing for female meiotic spindle assembly, was unclear. In this study, we characterized the biochemical activities of *C. elegans* Katanin using a highly purified enzyme obtained from *E. coli*. We showed that the enzyme bundles and severs MTs. We established that the role of MEI-2 is to confer MT binding to *C. elegans* Katanin. Based on these results, we engineered Katanin variants that retain MT-bundling activity but that are defective in MT severing. Introducing these MEI-1 variants in worms allowed us to demonstrate that the

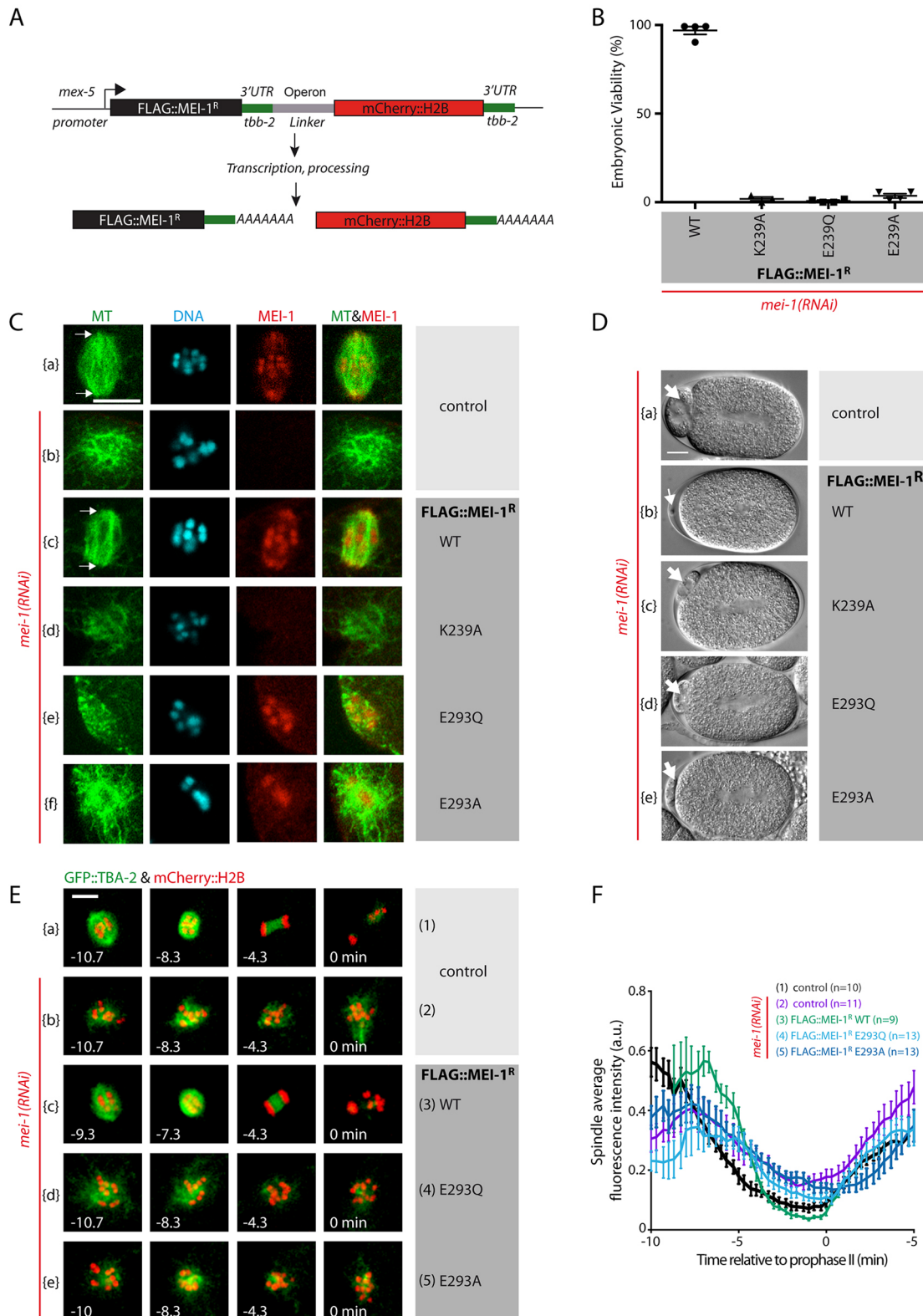


Fig. 5. *In vivo* characterization of Katanin variants in *C. elegans* meiotic embryos. (A) Schematics showing the strategy used for expressing an RNAi-resistant MEI-1 transgene (MEI-1^R) in the *C. elegans* germline detectable by fluorescent microscopy. The operon linker is the intercistronic region from the *gpd-2/gpd-3* operon. (B) Viability of embryos expressing FLAG::*mei-1*^R transgenes WT and mutants upon RNAi-mediated inactivation of endogenous *mei-1*. Error bars represent s.e.m.; *n*=4 experiments with 50 embryos each. (C) Confocal micrographs of early meiotic embryos stained for tubulin (green), MEI-1 (red) and DNA (DAPI, blue). Arrows indicate the meiotic spindle poles. (D) Differential interference contrast (DIC) images of one-cell embryos of the indicated genotype upon inactivation of endogenous *mei-1*. Arrows indicate polar bodies. (E) Live images of meiotic spindles of the indicated genotype carrying GFP:: α -Tubulin (green) and Histone2B::mCherry (red) transgenes. Individualization of the chromosomes at meiosis II (Prophase II) corresponds to time 0. (F) MT fluorescence intensity in meiotic spindles between metaphase I and prophase II of meiosis. Mean \pm s.e.m. Scale bars: 5 μ m (C,D,E).

MT-severing activity of Katanin is essential for female meiotic spindle assembly in *C. elegans*.

Genetic analyses have revealed an essential role of *mei-2* in female meiotic spindle assembly in *C. elegans* (Clandinin and Mains, 1993; Clark-Maguire and Mains, 1994b; Mains et al., 1990; Srayko et al., 2000); however, the exact contribution of MEI-2 to Katanin function was unknown. Here, we show that MEI-2, but not MEI-1, directly interacts with MTs *in vitro*, indicating that MEI-2 is responsible for the interaction of Katanin with MTs. However, whereas MEI-2 binds MTs, it does not bundle them, presumably because it contains only one binding interface with MTs. We hypothesize that the hexameric state provided by MEI-1 to the MEI-1/MEI-2 complex is imposing a geometrical arrangement of MEI-2 within the hexamer, exposing six potential MT-interacting motifs and providing a favourable environment for MT bundling. Most likely, one hexamer of MEI-1 can be ‘decorated’ with six MEI-2 subunits and interacts with at least two different MTs. This can be repeated all along the MTs, involving more and more MTs and so resulting in the formation of bundles of different size (as seen in Fig. 1E and Fig. 4E). Following this idea, if MEI-1 contained an intrinsic MT-binding domain, the MEI-1 hexamer would bundle MTs through its MT-binding domains, which is not what we observed. Nevertheless, the MEI-1 hexamer must also interact with MTs during the severing process. This interaction, which probably occurs between one MT and the central pore of the hexamer (Johjima et al., 2015), is most likely very transient and occurs only during MT severing in the presence of ATP, when the organization of the hexamer is optimal.

Kat60, p60 and KATNA1, the MEI-1 orthologues in *Drosophila*, sea urchins and humans, respectively, contain primary binding sites for MTs (Grode and Rogers, 2015; Hartman et al., 1998; McNally et al., 2000). These proteins interact with MTs through a three-helix bundle called the microtubule interacting and transport domain (MIT) located in the N-terminal part of the proteins (Iwaya et al., 2010). This domain is, however, not found in MEI-1 and MEI-1 alone does not bind MTs. In apparent contradiction to this observation, the N-terminal domain of MEI-1 (Nter-MEI-1) in *Xenopus* has been shown to interact with MTs when transfected in heterologous cells in culture (McNally and McNally, 2011). However, GST-MEI-2 was co-expressed with Nter-MEI-1 in these experiments (McNally and McNally, 2011). Given that the N-terminal part of MEI-1 is sufficient to bind MEI-2 (McNally and McNally, 2011) (and our unpublished results) and that MEI-2 binds MTs (this study), the binding of Nter-MEI-1 to MTs *in vivo* is likely to be mediated through MEI-2.

In all systems studied so far, the catalytic (AAA+) subunit is systematically associated with a regulatory subunit, suggesting an important and conserved function of the regulatory subunit, possibly in providing MT binding. Two evolutionarily conserved regulatory subunits have been identified: p80, which encodes a protein containing WD40 repeats in its N-terminal part, a proline-rich and a C-terminal domain containing the Con80 domain and a much smaller p80-like protein that contains only the C-terminal part, including the Con80 domain. Previous studies have established a role of the Con80 domain in MT-binding and Katanin activation (McNally et al., 2000). For instance, in sea urchins, whereas p60 alone exhibits MT-severing activity, MT severing is greatly stimulated by the Con80 domain (McNally et al., 2000). Likewise, KATNBL1 in humans, which contains only a Con80 domain, stimulates the MT-severing activity of the Katanin complex (Cheung et al., 2016). By contrast, the WD40 domain is dispensable for Katanin activity and rather plays a role in regulating the

subcellular localization of p60 (Hartman and Vale, 1999). MEI-2 lacks the WD40 domain but shares sequence similarities with the C-terminal Con80 domain, which suggests a conserved role of the accessory subunit in MT binding. It will be essential to further characterize the MT-interacting domains of the accessory subunits, particularly the MT-binding domain of MEI-2. Several *mei-2* mutations reducing Katanin function have been identified in *C. elegans* (Srayko et al., 2000) and it is plausible that some of these mutations alter MT binding.

Overall, our observations suggest a crucial and conserved role of the accessory subunit in Katanin function, not only in regulating its subcellular localization, but also in participating to the catalytic activity by providing substrate-binding site and by stabilizing the AAA+ ATPase subunit. Consistent with a prominent role of the accessory subunit in Katanin function, loss-of-function mutation of KATNBL1 in humans is associated with a syndrome of microcephaly, lissencephaly and short stature (Yigit et al., 2016). Loss-of-function mutation of KATNBL1 in mouse results in male sterility (O’Donnell et al., 2012). It will be useful to further dissect the role of the accessory subunits, and to address the relative contribution of p80 and p80-like subunits that are conserved from worms to humans. Such analysis should also facilitate the design of Katanin drug targets that exhibit a very specific range of action.

Beyond revealing the essential role of MEI-2 in providing MT binding to the Katanin complex, we demonstrate that the MT-severing activity of Katanin is essential for female meiotic spindle assembly in *C. elegans*. Whether MT severing is required, or not, for female meiotic spindle assembly is a long-standing question. Our experimental setting, allowing expression in worms of MEI-1 variants specifically defective in MT severing, allowed us to unequivocally address the contribution of the Katanin MT-severing activity for meiotic spindle assembly. We show that a complete absence of MT-severing activity prevents female meiotic spindle assembly, resulting in embryonic lethality. These observations clearly indicate that the MT-bundling activity of Katanin is not sufficient to assemble a meiotic spindle in the absence of the MT-severing activity.

A low level of Katanin-dependent MT-severing activity is, however, probably sufficient to support meiotic spindle assembly. Consistently, reducing MEI-1 levels by inducing its premature degradation during meiosis, in *egg-3(RNAi)* embryos (Johnson et al., 2009) or reducing its activity [as is probably the case for the MEI-1 P99L/P235S variant (McNally and McNally, 2011)], does not prevent meiotic spindle assembly but rather leads to the assembly of a long meiotic spindle. Conversely, high MT-severing activity during meiosis, as observed in *mei-1(ct46)* embryos expressing the non-degradable MEI-1 P99L form, leads to the formation of a short meiotic spindle. These results strongly suggest that a low level of MT severing is sufficient for meiotic spindle assembly, but that the extent of MT-severing influences spindle size [similar to what has been observed in *Xenopus*, where a correlation between meiotic spindle size and the level of Katanin activity is evident (Loughlin et al., 2011)].

Beyond its role in female meiotic spindle assembly in *C. elegans*, Katanin is emerging as an essential regulator of MT dynamics during cell division and differentiation in several systems. Accordingly, mutations of the catalytic AAA+ or the regulatory subunit are associated with several human diseases, including cancer. Understanding Katanin function and regulation in normal and pathological conditions will be important for the development of new therapeutic approaches.

MATERIALS AND METHODS

Plasmids

The list of plasmids used in this study is provided in Table S1. Plasmids designed to generate *C. elegans* lines expressing FLAG-MEI-1 fusion proteins were generated by direct assembly of PCR fragments, as described previously (Gibson et al., 2009). Some intermediate constructs were obtained by Gateway cloning according to the manufacturer's instructions (Invitrogen). All constructs were verified by DNA sequencing (MWG, Eurofins). The *mei-1* gDNA with a fragment of 1078 bp re-encoded to be RNAi resistant (52-1130 bp) was synthesized by GeneArt (Invitrogen). Amino acid substitutions were inserted by site-directed mutagenesis (QuikChange, Agilent Technologies).

Plasmid pNJ444 is derived from pRSF-Duet-1 and encodes 6×His-MEI-1 and STREP-MEI-2. pNJ450, pNJ452 and pNJ453 plasmids containing variants of MEI-1 were generated by mutating pNJ444 (Table S1). Plasmid pNJ440 is derived from pET28b+ and encodes 6×His-STREP-MEI-2. Plasmid pNJ420 is derived from the pET28b+ and encodes 6×His-MEI-1. All constructs were verified by DNA sequencing (MWG, Eurofins).

Nematode strains, culture conditions and RNAi

C. elegans strains were cultured and maintained using standard procedures (Brenner, 1974). Transgenic worms expressing FLAG::*mei-1^R* transgenes were generated by MosSCI using the strain EG6699 [ttTi5605 II; unc-119 (ed3) III; ox[Ex1578] (Frøkjær-Jensen et al., 2008). Correct insertion of the transgene was verified by PCR.

Protein purification

MEI-1 WT/MEI-2, MEI-1 K239A/MEI-2, MEI-1 E293A/MEI-2, MEI-1 E293Q/MEI-2 and MEI-2 were purified from BL21 (DE3) bacteria transformed with pNJ444, pNJ450, pNJ452, pNJ453 and pNJ440, respectively. We used small tags (6×His and Strep tags), which do not usually influence protein solubility, as compared with a larger GST or MBP tag. Briefly, 1 litre of LB medium was inoculated with an overnight culture (1% v/v) and grown at 37°C until OD_{600nm} of 0.4-0.6. The protein production was induced with 0.5 mM final concentration of isopropyl thio-β-D-galactoside (IPTG) for 3-4 h. After centrifugation, cells were resuspended in lysis buffer containing (50 mM Tris-HCl, pH 8.0, 500 mM NaCl and 5% glycerol) and broken by sonication. The supernatant was loaded onto a 5 ml StrepTrap HP (GE Healthcare) and after washing, the protein was eluted using 5 mM desthiobiotin in the lysis buffer. The protein eluate was directly loaded on a 1 ml HiTrap Chelating HP column (GE Healthcare) precharged with nickel and the bound proteins were eluted using imidazole in the lysis buffer after extensive washes. The buffer was exchanged for a storage buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl and 5% glycerol) using G25 columns and the proteins were snap frozen in liquid nitrogen and stored at -80°C.

MEI-1 was purified from BL21 (DE3) bacteria transformed with pNJ420 following the same protocol as above but the soluble fraction obtained after centrifugation was directly loaded on a 5 ml HiTrap Chelating HP column (GE Healthcare) precharged with nickel, followed by a G25 column using storage buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl and 5% glycerol) and snap frozen in liquid nitrogen and stored at -80°C.

Tubulin was purified from pig brain using four rounds of polymerization/depolymerization cycles, and resuspended in BRB80 buffer (80 mM PIPES-KOH, pH 6.8, 1 mM MgCl₂, 1 mM EGTA).

Rhodamine-labelled tubulin from porcine brain was purchased from Cytoskeleton. Protein concentration was estimated using the Lowry method (Lowry et al., 1951).

Taxol-stabilized microtubules

Tubulin polymerization was performed using tubulin purified from pig brain incubated in 80 mM PIPES-KOH pH 6.8, 100 mM KCl, 1 mM MgCl₂, 1 mM EGTA, 25% glycerol, 1 mM GTP for 1 h at 37°C and 20 μM paclitaxel (Sigma) was added for 30 min at 37°C to promote the assembly and the stabilization of long microtubules. Microtubules were pelleted at 90,000 g and resuspended in 80 mM PIPES-KOH, pH 6.8, 1 mM MgCl₂, 1 mM EGTA before use.

Microtubule binding

Magnetic nickel beads were incubated with a saturating amount of purified His-MEI-1/STREP-MEI-2 or His-MEI-1 or His-STREP-MEI-2 and then washed in the microtubule interaction buffer (50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 5% glycerol, 10 mM MgCl₂). The beads were then incubated with 2 μM Taxol-stabilized microtubules for 30 min at room temperature. After extensive washing of the beads with 100 volumes of microtubule interaction buffer, bound proteins were eluted using SDS Laemmli buffer. Proteins were analysed using 4-12% gradient SDS PAGE stained with Coomassie Blue.

Microtubule severing

Microscopy-based microtubule-severing assay

Flow chambers were assembled from coverslips using double-sided 3 M adhesive tape. The N-terminal part of an ATPase-dead Kinesin variant purified from *E. coli* was first flowed into the chamber and used as anchor points for microtubule binding. Taxol-stabilized microtubules (0.2 μM) assembled in the presence of Rhodamine-labelled tubulin were then flowed in the cell and after 2 min of incubation, the flow cell was extensively washed with 80 mM PIPES-KOH, pH 6.8, 1 mM MgCl₂, 1 mM EGTA to remove unbound microtubules. Then the buffer was changed for reaction buffer containing 50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 5% glycerol, 10 mM MgCl₂ in the presence of Katanin (from 0 to 2 μM) with or without nucleotide (1 mM ATP, ATPγS or ADP). The microtubule-severing reaction was followed using spinning disk confocal microscopy and images were processed and analysed using Fiji software (<https://fiji.sc/>).

DAPI-based microtubule-severing assay

For quantitative microtubule-severing assay, we used DAPI-stained microtubules obtained by polymerizing microtubules in the presence of 10 μM DAPI with Taxol. DAPI microtubules (0.2 μM) were mixed with either 0.2 μM MEI-1/MEI-2, MEI-1 or MEI-2 in 50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 5% glycerol, 10 mM MgCl₂ and 1 mM ATP. Fluorescence was monitored using SpectraMax2 and analysed using Origin Software. All experiments were repeated at least five times.

Microtubule bundling

Taxol-stabilized microtubules (0.2 μM) assembled in the presence of Rhodamine-labelled tubulin were incubated with 0.2 μM MEI-1/MEI-2, MEI-1 or MEI-2 for 5 min at 28°C in 50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 5% glycerol, 10 mM MgCl₂. The mix was directly spotted on a glass slide and observed by spinning disk confocal microscopy. Images were analysed using Fiji. For the absorbance-based assay, Taxol-stabilized microtubules (0.2 μM) were incubated as above and the absorbance at 350 nm was monitored using SpectraMax2. All experiments were repeated at least five times.

ATPase activity

Steady-state ATPase assays were used to monitor Katanin (WT or variants) ATPase activity. The assays were performed at 28°C in the presence of a NADH-coupled regeneration system (Nørby, 1988) in a 100 μl final volume, in buffer containing 25 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 1 mM DTT, 1 mM NADH, 10 mM phosphoenol pyruvate, 10 U/ml pyruvate kinase, 20 U/ml lactate dehydrogenase, ATP (from 0 mM to 20 mM) and Katanin or MEI-1 (from 0 to 2 μM). In the case of the MT-stimulated ATPase assay, a fixed 0.5 μM concentration of Katanin or MEI-1 was used and mixed with different concentrations of MTs (as indicated). The experiment was performed at least five times from two independent protein purifications.

Gel filtration

Different concentrations of Katanin (WT or variants) were incubated for 5 min at 4°C in buffer containing 50 mM Tris-HCl, pH 8.0, 500 mM NaCl and 10 mM MgCl₂, ±0.5 mM ATP or ADP. Samples (40 μl) were then injected onto a Superose 6 column (3.2×300 mm, 3.2 ml) (GE Healthcare) equilibrated with the sample buffer with or without nucleotides. Chromatography was performed on an AKTA system (GE Healthcare) at 4°C at a flow rate of 0.05 ml/min. Some gel filtration experiments were

performed on a Superdex 200 Column (10×300 mm, 24 ml) using the same protocol as previously described except that the flow rate was set at 0.5 ml/min. Columns were calibrated with a mix of globular proteins (Sigma): thyroglobulin (669 kDa), β -amylase (200 kDa), bovine serum albumin (66 kDa) and carbonic anhydrase (29 kDa). All experiments were repeated at least three times and the elution profiles obtained were similar.

Western blotting and antibodies

Western blot analysis was performed using standard procedures (Sambrook et al., 1989). Antibodies include home made rabbit polyclonal anti-MEI-1 (1:1000; Pintard et al., 2003), monoclonal mouse anti-actin (1:1000; MP Biomedicals, 69100), monoclonal mouse anti-tubulin (1:1000; Sigma, DM1A, T9026). HRP-conjugated anti-mouse and anti-rabbit secondary antibodies (Sigma) were used at 1:3000, and the signal was detected by chemiluminescence (Millipore).

In vivo imaging of meiotic divisions

Imaging of meiotic divisions was performed as described previously (Dumont et al., 2010) using a spinning disk confocal microscope. Adult worms were dissected in 4 μ l L-15 blastomere culture medium on a 24×60 mm coverslip mounted on a metal holder. The drop of medium was surrounded by a ring of petroleum jelly that serves as a spacer, thereby preventing compression of the embryos. A coverslip (18×18 mm) was placed on top to seal the chamber and prevent evaporation during filming. Live imaging was performed at 23°C using a spinning disk confocal head (CSU-X1; Yokogawa Corporation of America) mounted on a Ti-E inverted microscope (Nikon) equipped with 491 nm and 561 nm lasers (Roper Scientific) and a charge-coupled device camera (Coolsnap HQ2; Photometrics). Acquisition parameters were controlled by MetaMorph software (Molecular Devices). In all cases, a 60×, 1.4 NA PlanApochromat lens with 2×2 binning was used and four z-sections were collected at 2 μ m intervals every 20 s. In Fig. 5E, each image shown results from the maximal projection of three adjacent focal planes (performed with ImageJ or Fiji). Spindle average fluorescence intensities were quantified on sum-projections of four z-sections. A 28 pixel diameter ROI centred on chromosomes, which encompassed the entire meiotic spindle in all experimental conditions was drawn at each time point. The average fluorescence intensity in the GFP channel within this ROI was extracted and plotted against time. Error bars represent the s.e.m.

Immunofluorescence and microscopy

For immunofluorescence analysis, 10-15 worms were dissected in 7 μ l blastomere culture solution on subbing solution-coated slides using a 21-gauge needle to release embryos. An 18×18 mm coverslip was placed onto the drop and the slide was frozen on a block of metal precooled on packed dry ice. After 20 min, the coverslip was flicked off and the slide was plunged into -20°C methanol for 20 min. Slides were rehydrated in PBS for 5 min and incubated overnight at 4°C with 30 μ l primary antibodies in 3% BSA in PBS in a wet chamber. Affinity-purified rabbit MEI-1 antibody was used at a dilution of 1:500, mouse monoclonal α -tubulin antibody (DM1A; Sigma) was used at 1:400, and the secondary antibodies were coupled to Alexa Fluor 488 or Alexa Fluor 543 (Molecular Probes) used at 1:1000. Embryos were mounted in Vectashield mounting medium with DAPI. Fixed embryos were imaged using a LSM 710 confocal microscope (Zeiss) with 63× objectives. Captured images were processed using ImageJ and Adobe Photoshop.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

N.J. and L.P. conceived the study; N.J., J.D. and L.P. designed the experiments; N.J., L.M., E.G. and L.P. performed the experiments. All the authors took part in the interpretation of results and preparation of the manuscript.

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Supplementary information

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