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The *C. elegans* homologue of the spastic paraplegia protein, spastin, disassembles microtubules

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Abstract

Mutations in human spastin (SPG4) cause an autosomal dominant form of hereditary spastic paraplegia. Sequence analysis revealed that spastin contains the AAA (ATPases associated with diverse cellular activities) domain in the C-terminal region. Recently, it was reported that spastin interacts dynamically with microtubules and displays microtubule-severing activity. A plausible *Caenorhabditis elegans* homologue of spastin (SPAS-1) has been identified by homology search and phylogenetic analyses. To understand the function of the spastin homologue, we characterized the *spas-1* deletion mutant and analyzed *spas-1* expression regulation in *C. elegans*. SPAS-1 was localized with cytoskeletons at the perinuclear region. We found that microtubules were intensely stained at the centrosomal region in the deletion mutant. Furthermore, overexpression of SPAS-1 caused disassembly of microtubule network in cultured cells, while ATPase-deficient SPAS-1 did not. These results indicate that *C. elegans* SPAS-1 plays an important role in microtubule dynamics. We also found that two kinds of products were generated from *spas-1* by alternative splicing in a developmental stage-dependent manner.

Keywords: AAA ATPase; *C. elegans*; Chaperone; Hereditary spastic paraplegia; Microtubule; Spastin

Hereditary spastic paraplegia (HSP) is characterized by progressive weakness, spasticity, and loss of the vibratory sense in the lower limbs, and caused by the specific degeneration of the corticospinal tracts, the longest axons in humans [1,2]. More than 20 genetic loci have been identified, and most cases of the autosomal dominant (AD) form of the disease are due to the mutations in the *SPG4* gene encoding spastin [1–4]. Sequence analysis revealed that spastin contains the AAA (ATPases associated with diverse cellular activities) domain in the C-terminal region [3]. In patients, a variety of mutations are scattered along the coding region of the spastin gene, but mostly located in the ATPase domain [3,5]. Transcript analysis of patients' specimen showed that the amount of mutant mRNA was drastically reduced. Therefore, it has been proposed that haploinsufficiency could be a mechanism to lead AD-HSP caused by the mutation in the spastin gene [3,5–7].

A role for spastin in microtubule (MT) dynamics has emerged recently [8]. When wild-type spastin was overexpressed, MT disassembly was observed in transfected cells. In contrast, overexpression of ATPase-defective spastin induced constitutive binding to MTs and the MT-bundle formation [9,10]. More recently, two studies have demonstrated that purified spastin from human and *Drosophila* showed a MT severing activity as similar to that katanin has it [8,11–13]. Recent studies have also demonstrated that *Drosophila* spastin regulates synaptic MT stability [10,14] and that zebrafish spastin is essential for axon outgrowth in the embryo [15]. These results altogether suggest that defects in MT severing activity of mutant spastin are a cause of axonal degeneration in the human disease.

To elucidate the cellular function of spastin and if possible, to establish a disease model, we have studied the *C.*

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elegans spastin homologue C24B5.2, named SPAS-1. In this study, we demonstrate that SPAS-1 plays an important role in MT dynamics.

Materials and methods

C. elegans strains. *Caenorhabditis elegans* Bristol strain N2 was used as the wild-type. The *spas-1*(*tm683*) deletion mutant was kindly provided from Dr. S. Mitani. To exclude unexpected additional mutations, deletion mutant was out-crossed five times. Worms were maintained at 20 °C using standard protocols as described previously [16].

Plasmid construction. The genomic DNA fragment of the entire *spas-1* gene including expression regulatory signals was amplified with primers 5′-GCAACTGCAGCAAGCACGGTGTGGGAATCAGAATGG-3′ and 5′-GCAACTGCAGCGAGCAGTTGCTGCTTTGGAAACTC-3′, and was cloned into the TOPO TA Cloning vector (Invitrogen), yielding pCKX6001. The upstream regulatory region of *spas-1* was amplified by PCR using pCKX6001 as a template and the following primers 5′-GCAACTGCAGCAAGCACGGTGTGGGAATCAGAATGG-3′ and 5′-CCTTTTGAAAAGGCGAACATTGGAACTGAAAATTTAATAC-3′. The full-length cDNA fragments for *spas-1* were amplified by PCR using yk118h2 and yk735e10, generous gifts from Dr. Y. Kohara, as a template and the following primers 5′-GTATTAAATTTTCAGTTCCAATGTTCGCCTTTTCAAAAGGTC-3′ and 5′-AGAACCCGGGTTAGCAACCGAAACTTCGAG-3′. The regulatory fragment and each cDNA fragment of *spas-1* were combined and cloned into the plasmid pPD49.26 (a kind gift from Dr. A. Fire), yielding pCKX6013 and pCKX6014. Plasmid DNAs were column purified (Qiagen) and filtered through SUPREC-02 (Takara) before microinjection.

Immunofluorescence microscopy. The slides containing embryos were placed on a dry ice block for 10 min, freeze-cracked and fixed by methanol/acetone. Embryos were rehydrated with a blocking solution (5% skim milk, 5% BSA in PBST) for 90 min at room temperature, and incubated with the primary antibody [FITC-conjugated anti-α-tubulin antibody (DM1A; Sigma) or affinity-purified rabbit anti-SPAS-1 antibody] overnight at 4 °C and then with the secondary antibody for 2 h at room temperature. 4,6-Diamidino-2-phenylindole (DAPI) was added to a final concentration of 2 μg/ml, and the sample was mounted for immunofluorescence microscopy.

Cell fractionation. Embryos were collected by bleaching mixed-stage worms in a 0.8 N NaOH, 8% hypochlorite solution. The homogenates of embryos were prepared and fractionated as described previously [17], and analyzed by Western blotting. Protein concentration was determined by BCA Protein Assay Kit (Pierce). Histone H4 and HSP90 were detected by anti-histone H4 antibody (sc-10810; Santa Cruz Biotechnology) and anti-HSP90 antibody (Sigma), and used as a marker for the nuclear fraction and the cytosolic fraction, respectively. Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer Life Science) was used for detection.

RT-PCR. mRNA was isolated using Fast Track Kit (Invitrogen) and its concentration was determined by absorbance of 260 nm. First strand cDNA was synthesized using SuperScript II (Invitrogen) and a region of *spas-1* spanning exons 2–5 was amplified using the following primers: FW 5′-GAAATCAGAGATAAACGAC-3′ and RV 5′-GTGCAGCTCGATTTACTGG-3′ (see Fig. 2B). Cycling conditions for PCR were as follows: 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, followed by 72 °C for 7 min.

Cell culture, transfection, and immunocytochemistry. Full-length cDNA fragments for *spas-1, figl-1, mei-1,* and *vps-4* were amplified from yk735e10, yk61d2, yk479e10, and yk1312f11, respectively, and cloned into pcDNA3 (Invitrogen). PCR primers were designed to express N-terminally FLAG-epitope tagged fusion proteins. Site-directed mutagenesis in *spas-1* was carried out by using Quick Change II XL Site-directed mutagenesis Kit (Stratagene). Introduction of desired mutation was confirmed by DNA sequencing. HEK293 cells were cultured in DMEM supplemented with 10% FBS and were transfected by using FuGENE6 (Roche) according to the manufacturer's instructions. Cells were fixed with 4% paraformaldehyde and treated with a Cy3-conjugated anti-FLAG antibody (Sigma) and an FITC-conjugated anti-α-tubulin antibody for immunofluorescence microscopy.

Results and discussion

Identification of the C. elegans spastin orthologue

Together with fidgetin, Vps4p/SKD1, katanin, and Msp1, spastin belongs to the meiotic subfamily of AAA proteins [18,19]. *Caenorhabditis elegans* C24B5.2 has been shown to locate closest to spastin on the phylogenetic tree [18,20]. Therefore we named C24B5.2 SPAS-1. When the amino acid sequences of spastin homologues from human, mouse, fly, and *C. elegans* were aligned, it was found that *C. elegans* SPAS-1 does not contain a MIT (microtubule-interacting and trafficking) domain [21], although their AAA domains are highly homologous (data not shown). Note that the MIT domain can be found in proteins involved in MT binding and intracellular transport, but its precise function is unclear.

Expression analysis of spas-1

To examine the localization of *spas-1* mRNA, we conducted *in situ* hybridization with RNA probe of *spas-1*. In young adult hermaphrodites, *spas-1* was expressed in the whole bodies, particularly intestine, gonad, and vulva (data not shown). We next examined cellular localization of SPAS-1 in early embryos. SPAS-1 was localized to the perinuclear and cytoplasmic regions in wild-type embryos by means of immunofluorescence technique (Fig. 1A). Furthermore, SPAS-1 was primarily detected in the fraction that contains nuclei and cytoskeletons, when cell lysates were fractionated into three fractions (Fig. 1B). It should be mentioned that a role for spastin in MT dynamics has recently emerged [8]. Therefore, it is possible that *C. elegans* spastin homologue SPAS-1 may be localized with cytoskeletons at the perinuclear region.

To know the expression pattern of SPAS-1 during different developmental stages (embryos, L3–L4 larvae, and mixed stage), we performed Western blot analysis using the affinity-purified rabbit polyclonal antibodies. Two bands were detected at 50 and 57 kDa in wild-type worms (Fig. 2A) but not in the deletion mutant (Fig. 2C), suggesting that both bands are products of *spas-1*. There are several possibilities, processing, modification, and alternative splicing, to produce two kinds of proteins. To examine these possibilities, we have determined sequences of several *spas-1* cDNA clones. We found that there are two types of *spas-1* transcript: one consists of nine exons and encodes 57 kDa (SPAS-1L), and the other consists of eight exons resulted from skipping the fourth exon and encodes 50 kDa (SPAS-1S) (Fig. 2B). To further confirm these results, we carried out RT-PCR with a pair of primers shown in Fig. 2B. As shown in Fig. 2D, two PCR products were obtained and their sizes completely matched expected ones. These are in good agreement with the result of Wes-

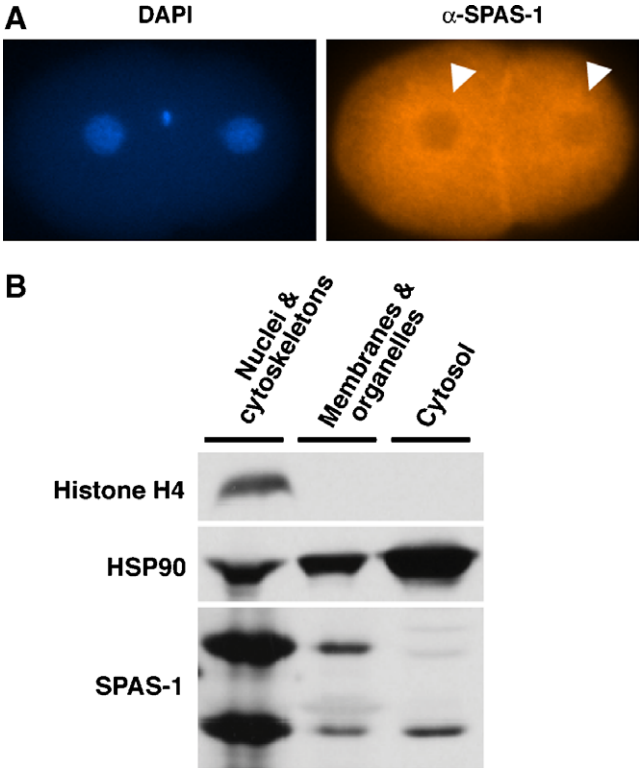

Fig. 1. Expression of SPAS-1. (A) Early embryos were stained with DAPI and anti-SPAS-1 antibody. SPAS-1 was localized to the perinuclear and cytoplasmic regions in embryos, denoted with arrowheads. (B) Embryos were homogenized and fractionated into three fractions as described previously [17]. SPAS-1 was primarily detected in the nuclear fraction, which contains nuclei and cytoskeletons. Histone H4 and HSP90 were used as a marker for the nuclear fraction and the cytosolic fraction, respectively.

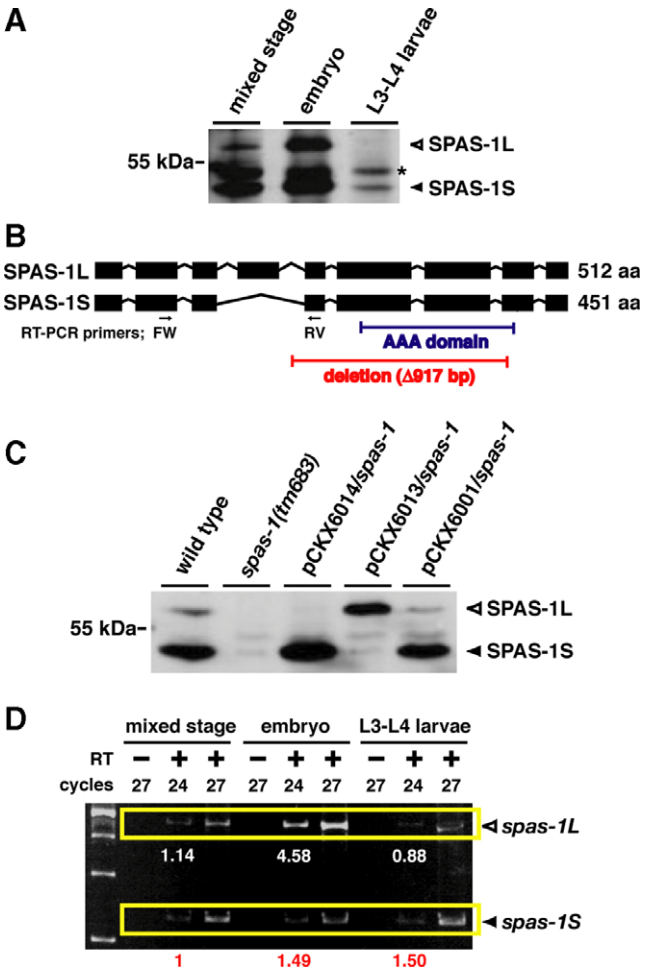

Fig. 2. Alternating splicing of *spas-1*. (A) Western blotting analysis of SPAS-1 at different growth stages. Two bands were detected at 57 (SPAS-1L) and 50 kDa (SPAS-1S), denoted with open and closed arrowheads. Asterisk: nonspecific band. (B) Schematic representation of *C. elegans* SPAS-1. SPAS-1 has two isoforms (L and S); L form contains exon 4 and S form does not. Regions of the AAA domain and the deletion are also shown. (C) SPAS-1L and SPAS-1S were expressed in the *spas-1*(*tm683*) deletion mutant microinjected with pCKX6013, pCKX6014 or pCKX6001. Open and closed arrowheads: SPAS-1L and SPAS-1S. (D) mRNA prepared from each developmental stage was used for RT-PCR. Reactions without reverse-transcription (RT−) were used as a negative control. PCR was cycled 24 or 27 times. Each band was quantitatively analyzed and the ratios were indicated.

tern blotting analysis as shown in Fig. 2A. These results suggest that alternative splicing occurred on *spas-1* transcripts. We then expressed each cDNA product in the deletion mutant. As shown in Fig. 2C, these two bands clearly corresponded to the product from each cDNA. Furthermore, when the genomic DNA fragment containing the *spas-1* gene was expressed, two bands were observed as same as that observed in the wild-type (Fig. 2C). These results together unambiguously demonstrate that SPAS-1 has two isoforms generated by alternative splicing, but not by post-translational processing or modification. It is interesting to mention that in human, three *spastin* mRNA splice variants, Δexon 4, 8, and 15, were identified, but their functional divergence was not revealed [6]. On the other hand, spastin subcellular localization is regulated through the usage of different translation start sites in human and mouse [8,22]. Thus, the functional analysis of several isoforms might be a key to understand disease mechanisms.

We further analyzed alternative splicing of *spas-1* during different developmental stages by means of RT-PCR and found that the L form appeared predominant at the embryonic stage, while the S form existed nearly constantly at all the developmental stages (Fig. 2D). These results suggest that alternative splicing occurred in a stage-specific manner. SPAS-1L may play an important role at early developmental stage, although deduced amino acid sequences of exon 4 did not show significant homology to known domains and motifs.

Characterization of the deletion mutant of spas-1

To understand the function of the *C. elegans* spastin homologue SPAS-1, we characterized the deletion mutant of *spas-1*(*tm683*), which has a 917 bp deletion in the *spas-1* gene (see Fig. 2B). The deletion mutant showed a slightly slow growth, a reduction of brood size to 80% (196 eggs for mutant comparing with 257 eggs for wild-type in average), abnormal oogenesis and a multivulva phenotype, while it

did not show any detectable defects on motility, defecation cycles, and life span (data not shown). These results suggest that *spas-1* may play a role in developmental processes, especially in oogenesis.

As shown in Fig. 1, SPAS-1 was suggested to localize with cytoskeletons at the perinuclear region. It has been recently reported that spastin interacts dynamically with MTs and displays a MT severing activity [8–12]. Therefore, we examined MT structure in early embryos of the *spas-1*(*tm683*) mutant. During the first cell division in embryos, MT network was nicely formed in the wild-type (Fig. 3 upper panel). However, in the deletion mutant, MTs were intensely stained at the centrosomal region, although daughter chromosomes were separated adequately (Fig. 3 lower panel). These results suggested that *C. elegans* SPAS-1 may be involved in MT dynamics as spastin from human, mouse, and fly is so.

SPAS-1 is involved in MT dynamics

To further demonstrate this possibility, we examined if MT network is disassembled when SPAS-1 is expressed in cultured cells. As clearly shown in Fig. 4A, when SPAS-1 is expressed in HEK293 cells, MT network disappeared. This is fully consistent with results of human spastin as reported previously [9]. As described above, SPAS-1 is classified into the meiotic subfamily containing MEI-1, FIGL-1, and VPS-4 [18–20]. We then examined whether these proteins of the meiotic subfamily are also involved in disassembly of MT network. As shown in Fig. 4A, besides SPAS-1, only MEI-1 partially disassembled the MT network. MEI-1, a *C. elegans* homologue of human katanin, has been shown to be required for MT disassembly in meiosis and to require MEI-2 for its full activity [23]. Our result is in good agreement with this notion. Fidgetin, whose *C. elegans* homologue is FIGL-1 [20], was reported to localize in nucleus and was suggested to play a regulatory role in signaling pathways of developmental processes [24]. FIGL-1 was also solely localized in nucleus and did not show any change in MT network (Fig. 4A). VPS-4, whose mammalian homologue is Vps4p/SKD1, which has been reported to be required for endosomal trafficking and morphology [25], was also suggested not to be involved in MT dynamics (Fig. 4A).

Many mutations in *spastin* have been identified from HSP patients so far. Among them, most of missense mutations are located in the AAA domain [3,5], implying the importance of the ATPase activity for spastin's function. The Lys residue in Walker A motif and the Glu residue in Walker B motif are involved in ATP binding and hydrolysis, respectively. The conserved aromatic residue in the pore region is proposed to be involved in substrate binding and its translocation [26]. We then examined effects of three such mutations (Walker A mutation K224R, Walker B mutation E278Q and pore mutation W251A) on the disassembly activity of MT network. As shown in Fig. 4B, in all cases MT disassembly activity was abolished, indicating that ATPase activity is definitely required for disassembly of MT network. The importance of the pore residue is also consistent with the recent report [27]. It is important to mention that SPAS-1^{E278Q} colocalized with MT, but was not dispersed in the cytoplasm. This result demonstrates that ATP-binding of SPAS-1 is sufficient for binding with MTs, but not for dissociation from them. Such MT bundling has also been observed with ATP hydrolysis-defective mutant spastins [8,12]. These results suggest that spastin plays an important role in regulation of MT dynamics by severing and bundling. It is thus possible that mutant spastins disturb the balance of MT dynamics and cause pathological effects in a dominant negative fashion. The notion that SPG4 is the autosomal dominant form supports this idea. We are now trying to establish transgenic worms expressing these mutant SPAS-1 proteins and to examine whether or not they serve as an HSP model. Further bio-

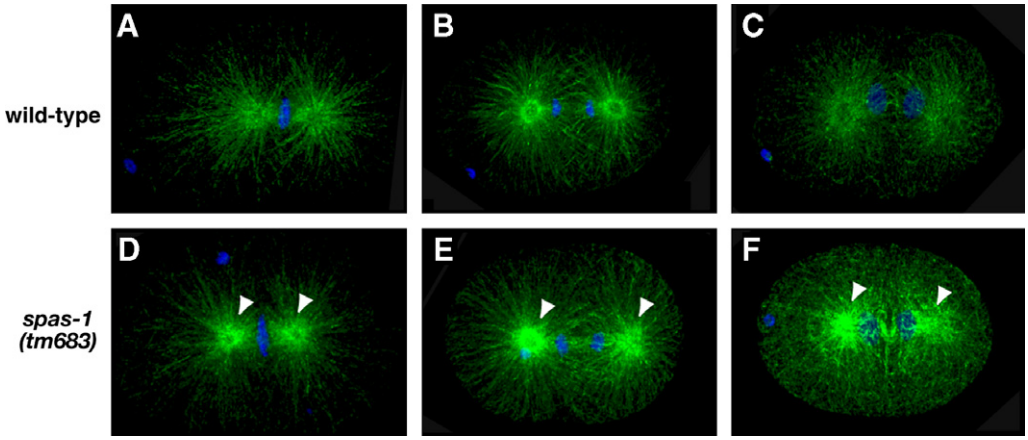

Fig. 3. Effects of the lack of SPAS-1 on MT dynamics. MTs (green) in early embryos of wild-type (A–C) and deletion mutant (D–F) were observed under the immunofluorescence microscopy. Chromosomes were stained with DAPI (blue). Embryos of metaphase (A,D), anaphase (B,E), and telophase (C,F) are shown. MTs were intensely stained at the centrosomal region (with arrowheads) in the *spas-1*(*tm683*) deletion mutant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

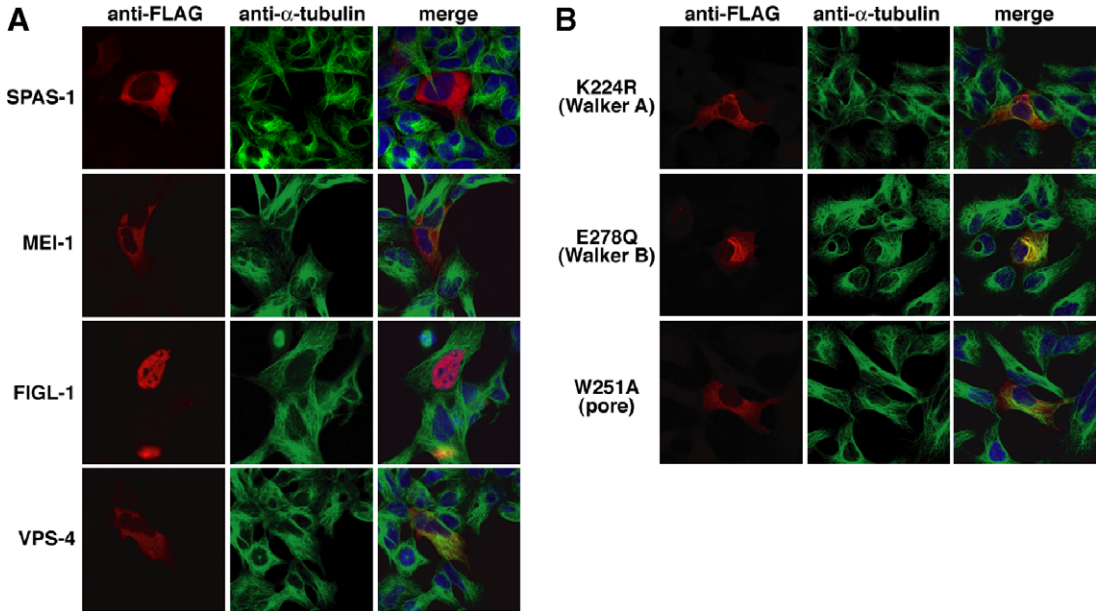

Fig. 4. MT disassembly activity of SPAS-1. (A) FLAG-epitope tagged *C. elegans* SPAS-1, MEI-1, FIGL-1, and VPS-4 were overexpressed in HEK293 cells. Transfected cells were immunostained with anti-FLAG (left) and anti-α-tubulin (middle) antibodies. Merged images were also shown (right). (B) FLAG-epitope tagged mutated SPAS-1 proteins (K224R, E278Q, and W251A) were overexpressed in HEK293 cells. None of mutants possessed MT disassembly activity.

chemical studies will be required to reveal precise mechanisms of MT severing and bundling.

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