Novel septin 9 repeat motifs altered in neuralgic amyotrophy bind and bundle microtubules

Xiaobo Bai, Jonathan R. Bowen, Tara K. Knox, Kaifeng Zhou, Manuela Pendziwiat, Gregor Kuhlenbäumer, Charles V. Sindelar, and Elias T. Spiliotis

1Department of Biology, Drexel University, Philadelphia, PA 19104
2Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06520
3Institute of Experimental Medicine, University of Kiel, 24105 Kiel, Germany

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Correspondence to Elias T. Spiliotis: ets33@drexel.edu

Abbreviations used in this paper: CTT, C-terminal tail; HNA, hereditary neuralgic amyotrophy; MAP, microtubule-associated protein; MT, microtubule; SEPT9, septin 9.

Introduction

Septins are hetero-oligomeric GTP-binding proteins that assemble into nonpolar filamentous structures, which are essential for microtubule (MT)-dependent cell processes such as mitosis and vesicle transport (Spiliotis, 2010; Saarikangas and Barral, 2011; Mostowy and Cossart, 2012). In mammalian cells, septin depletion affects MT organization, dynamics, and post-translational modifications (Spiliotis et al., 2008; Bowen et al., 2011), but how septins interact with MTs is unknown. Septin 9 (SEPT9) is a ubiquitously expressed septin that caps the ends of septin heteromers (Füchtbauer et al., 2011; Kim et al., 2011; Sellin et al., 2011). Alternative splicing and translation start sites give rise to SEPT9 isoforms, which differ in the length and sequence of their N terminus, which is critical for the association of septin heteromers with MTs (Sellin et al., 2011). Missense mutations in the N terminus of the SEPT9 are genetically linked to hereditary neuralgic amyotrophy (HNA), an autosomal-dominant neuropathy. The mechanism of SEPT9 interaction with MTs and the molecular basis of HNA are unknown. Here, we show that the N-terminal domain of SEPT9 contains the novel repeat motifs K/R-x-x-E/D and R/K-R-x-E, which bind and bundle MTs by interacting with the acidic C-terminal tails of β-tubulin. Alanine scanning mutagenesis revealed that the K/R-R/x-x-E/D motifs pair electrostatically with one another and the tails of β-tubulin, enabling septin–septin interactions that link MTs together. SEPT9 isoforms lacking repeat motifs or containing the HNA-linked mutation R88W, which maps to the R/K-R-x-E motif, diminished intracellular MT bundling and impaired asymmetric neurite growth in PC-12 cells. Thus, the SEPT9 repeat motifs bind and bundle MTs, and thereby promote asymmetric neurite growth. These results provide the first insight into the mechanism of septin interaction with MTs and the molecular and cellular basis of HNA.

Results and discussion

The basic N-terminal domain of SEPT9 binds and bundles MTs by interacting with the acidic C-terminal tails of β-tubulin

To determine how SEPT9 interacts with MTs, we first sought to identify the SEPT9 domain that binds MTs using in vitro cosedimentation assays. Recombinant His-tagged fragments were made from the longest SEPT9_i1 isoform (SEPT9-FL; Fig. 1 A), which consists of the GTP-binding domain (aa 283–586; SEPT9-G) and a structurally disordered N-terminal tail (aa 1–283; SEPT9-N; Fig. S1 A) with a basic region (aa 1–142; SEPT9-B). Abnormal expression of SEPT9 isoforms has also been reported in many cancers (Connolly et al., 2011a). Overexpression of SEPT9 isoforms induces oncogenic phenotypes including resistance to the anti-cancer drug paclitaxel, which stabilizes and bundles MTs (Orr et al., 2003; Gonzalez et al., 2007; Chacko et al., 2012). Unraveling the mechanism of SEPT9 interaction with MTs could point to the molecular roles of SEPT9 in these disorders and lead to therapeutic strategies.
proline-rich acidic domain (aa 143–283; SEPT9-A). SEPT9-FL co-sedimented with MTs (Fig. 1, B and J) and the apparent \(K_d\) value was 3.2 \(\mu\)M (Fig. S1 B). In contrast to SEPT9-G and SEPT9-A, which pelleted with MTs weakly (Fig. 1, D–F and J), SEPT9-N and SEPT9-B bound MTs similarly to SEPT9-FL (Fig. 1, C, E, and J).

Next, low speed MT pelleting and visual MT assays were used to examine whether SEPT9 can bundle MTs. At low speed (8,000 g), MT sedimentation was increased by SEPT9-FL, SEPT9-N, and SEPT9-B (Fig. 1, G–I and K), but SEPT9-G and SEPT9-A had no effect (Fig. 1, H, I, and K). Incubation of fluorescent MTs with SEPT9-FL showed a marked increase in the length, thickness, and brightness of MT bundles (Fig. 1, L, N, and O). This bundling effect was independently confirmed by negative stain EM. MTs were arranged in bundles and doublets of parallel tubules that made tight contacts (Fig. 1 M). Elongated MT bundles were also observed with fluorescent MTs that were stabilized with the nonhydrolyzable GTP analogue GMP-CPP (Fig. S1 C). Decoration of paclitaxel- and GMP-CPP-stabilized MT bundles with recombinant GFP-tagged SEPT9_i1 demonstrated that SEPT9 binds and bundles MTs similarly to SEPT9-FL (Fig. 1, L, N, and O). These data indicate that SEPT9 binds and bundles MTs via its basic N-terminal domain. Because SEPT9 forms hetero-oligomeric complexes with other septins (e.g., SEPT7, SEPT6), we purified SEPT9 in the presence of SEPT6 and tested how SEPT6 affects MT bundling compared with SEPT9 alone or SEPT6/7. SEPT6/7 induced low speed sedimentation of MTs at a concentration (0.2 \(\mu\)M) that neither SEPT9 nor SEPT2/6/7 had an effect (Fig. S1, J and K). Thus, SEPT9 appears to be a key subunit for the bundling of MTs by septin complexes.

Electrostatic interactions between positively charged domains and the negatively charged C-terminal tails (CTTs) of tubulin underlie the mechanism by which some microtubule-associated proteins (MAPs) and kinesin motors associate with MTs (Amos and Schlieper, 2005; Marx et al., 2006; Akhmanova and Steinmetz, 2008). Although we were not able to identify any known motifs, the N terminus of SEPT9_i1 contains multiple repeats of the tetrapeptide sequences K/R-x-x-E/D and R/K-R-x-E (Fig. 3, A and B), which are often flanked by proline or serine residues. By truncating the basic N-terminal domain of SEPT9_i1, we determined that aa 61–113 comprises the most minimal region of SEPT9_i1 that binds and bundles MTs (Fig. S2, A–C) and contains multiple repeats of the identified motifs.

We set out to elucidate the role of these novel motifs in MT binding and bundling by alanine scanning mutagenesis. The first half of the SEPT9_i1(61–113) peptide contains a region (R1) of three K/R-x-x-E/D motifs and the second half (R2) consists of a K-x-x-E motif flanked by two R-R-x-E motifs (Fig. 3 C); neither R1 nor R2 alone bound or bundled MTs. Alanine substitution of the basic residues in two or all three of the R1 motifs resulted in an \(~50\%\) decrease in MT binding (Fig. 3 D and Fig. S2 D). A similar decrease was observed by mutating the basic residues of the R2 motifs (Fig. 3 D and Fig. S2 E). We assessed the effects of the same mutations in MT bundling using a low speed sedimentation assay. Strikingly, alanine substitutions of the basic residues of the R-R-x-E motifs of R2 decreased MT bundling, but mutating the basic residues of the K/R-x-x-E/D motifs of R1 did not decrease MT bundling (Fig. 3 E; Fig. S2, F and G). These data indicate that although the basic residues of both K/R-x-x-E/D and R-R-x-E contribute to MT binding, the arginine residues of the R-R-x-E motifs are uniquely critical for MT bundling.

Next, we assessed the role of the acidic residues. Alanine substitutions of the acidic residues in one or two of the motifs of either R1 or R2 resulted in a moderate \((~30\%\) increase of MT-SEPT9_i1(61–113) binding (Fig. 3 F and Fig. S2 H). MT bundling, however, decreased by \(~50\%\) when the acidic residues in two of the K/R-x-x-E/D motifs of the R1 region were mutated (Fig. 3 G and Fig. S2 I). In contrast, alanine substitutions of glutamate in the R-R-x-E motifs of the R2 region resulted in only a marginal decrease in MT bundling (Fig. 3 G and Fig. S2 I). These results show that although the acidic residues dampen somewhat the electrostatic affinity of all motifs for the CTTs of \(\beta\)-tubulin, the acidic residues of the K/R-x-x-E/D motifs (R1)
Figure 1. **SEPT9 binds and bundles MTs via a basic N-terminal domain.** (A) Sequence and domains of SEPT9_i1. (B–F) Coomassie-stained SDS-PAGE gels of the supernatant (S) and pellet (P) fractions after high speed (39,000 g) sedimentation of pre-polymerized paclitaxel-stabilized MTs with domains of SEPT9_i1. (G–I) Low speed (8,000 g) sedimentation of MTs in the presence of SEPT9_i1 domains. (J and K) Graphs show percentages of total protein pelleted with MTs at 39,000 g (J) and percentage of total MTs pelleted at 8,000 g (K) in three independent experiments. (L) Images show X-rhodamine–labeled MTs after mixing with recombinant SEPT9 fragments. Bars, 10 µm. (M) Negative stain EM images of MTs before and after mixing with SEPT9_i1. Arrows and arrowheads point to MT bundles and doublets, respectively. (N and O) Plots show the intensity of X-rhodamine fluorescence per micron of MT (O; n = 100) and the length of MTs (P; n = 100) per condition.
Figure 2. **SEPT9 interacts with the acidic C-terminal tails of β-tubulin.** (A) Gel shows supernatant (S) and pellet (P) fractions after sedimentation (39,000 g) of SEPT9-FL with untreated and subtilisin-treated MTs (S-MTs). Graph shows percentage of total SEPT9-FL in the S and P fractions. (B) Images show untreated and subtilisin-treated X-rhodamine–labeled MTs after mixing with SEPT9-FL. Bars, 10 µm. (C and D) Plots show the fluorescence intensity per micron of MT (C; n = 50) and the length of MTs (D; n = 50). (E and F) Plots show the fraction of SEPT9-FL pelleted with MTs in the presence of increasing concentrations of α-tubulin, βII-tubulin, and scrambled CTT peptide relative to no peptide in three independent experiments. Gels show the pellet fractions after MT pelleting with SEPT9-FL in the presence of increasing concentrations of peptides. (G) Gels show the pellet fractions after sedimentation of MTs with SEPT9-FL in the presence of β, βII-, and βIII-tubulin CTT peptides. Graph shows the fraction of SEPT9-FL pelleted in the presence of peptides relative to no peptide in three independent experiments. (H) Increasing amounts of bovine brain tubulin was separated by 7.5% SDS-PAGE and transferred to nitrocellulose membranes, which were stained with Ponceau S red. Membranes were blotted with DM1A and TUB2.1 antibodies against α- and β-tubulin, respectively, and overlaid with His-tagged SEPT9-FL and SEPT2, which were detected with anti-His antibodies.
We hypothesized that SEPT9 isoforms that lack K/R-R/x-x-E/D motifs from their N terminus could affect MT bundling and asymmetric neurite growth. Among the known SEPT9 protein isoforms, SEPT9_i4 lacks the N-terminal 164 aa and therefore most of the MT-binding and -bundling repeat motifs of SEPT9_i1. To test if the absence of these motifs affects intracellular MT bundling, we expressed GFP-tagged chimeras of these isoforms in MDCK cells, in which septins were previously shown to colocalize with MTs (Spiliotis et al., 2008; Bowen et al., 2011). We found that SEPT9_i1 colocalized strongly with perinuclear MT bundles, whereas SEPT9_i4 was distributed at peripheral lamellae showing little to no colocalization with MTs (Fig. 4, A and C). Upon treatment with paclitaxel, an MT-stabilizing drug that induces MT bundling, SEPT9_i1 colocalized with MT bundles extensively, whereas SEPT9_i4 showed little colocalization with MTs (Fig. 4, B and C). Interestingly, paclitaxel-treated cells that expressed SEPT9_i4 cells appeared to have less number of long and straight MT bundles (Fig. 4 B). This effect was quantified by measuring the relative percentage of MTs with fluorescence intensities 5× or 10× greater than the mean intensity of the

are uniquely critical for MT bundling. Because the basic residues of the R-R-x-E motif are also uniquely involved in MT bundling, we posit that the extra arginine residues of the R-R-x-E motifs allow for additional interactions with the acidic residues of the K/R-x-x-E/D motifs, enabling homophilic trans interactions between SEPT9_i1(61–113) peptides that cross-link MTs into bundles (Fig. 3 H). Consistent with this model, a GST-tagged version of the N terminus of SEPT9 bound His-tagged SEPT9-N and SEPT9-FL (Fig. S2, J and K). Thus, homophilic trans interactions between the N termini of SEPT9 are likely to facilitate septin and MT cross-linking.

SEPT9 repeat motifs are required for intracellular MT bundling and the generation of neurite asymmetry

Given the mechanistic role of the K/R-R-x-x-E/D motifs in MT binding and bundling in vitro, we examined the functional significance of these motifs for intracellular MT bundling and neuronal morphogenesis, which requires MT bundling by MAPs (e.g., tau, MAP1B) that support the asymmetric growth of neuronal protrusions termed neurites (Caceres and Kosik, 1990; Teng et al., 2001; Feltrin et al., 2012). We hypothesized that SEPT9 isoforms that lack K/R-R-x-x-E/D motifs from their N terminus could affect MT bundling and asymmetric neurite growth. Among the known SEPT9 protein isoforms, SEPT9_i4 lacks the N-terminal 164 aa and therefore most of the MT-binding and -bundling repeat motifs of SEPT9_i1. To test if the absence of these motifs affects intracellular MT bundling, we expressed GFP-tagged chimeras of these isoforms in MDCK cells, in which septins were previously shown to colocalize with MTs (Spiliotis et al., 2008; Bowen et al., 2011). We found that SEPT9_i1 colocalized strongly with perinuclear MT bundles, whereas SEPT9_i4 was distributed at peripheral lamellae showing little to no colocalization with MTs (Fig. 4, A and C). Upon treatment with paclitaxel, an MT-stabilizing drug that induces MT bundling, SEPT9_i1 colocalized with MT bundles extensively, whereas SEPT9_i4 showed little colocalization with MTs (Fig. 4, B and C). Interestingly, paclitaxel-treated cells that expressed SEPT9_i4 cells appeared to have less number of long and straight MT bundles (Fig. 4 B). This effect was quantified by measuring the relative percentage of MTs with fluorescence intensities 5× or 10× greater than the mean intensity of the

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MT bundling by novel SEPT9 repeat motifs K/R-x-x-E/D and R/K-R-x-E. (A and B) WebLogo alignments of eleven hexapeptide sequences containing the K/R-x-x-E/D motif (A) and six sequences containing the R/K-R-x-E motif (B) within the N terminus (aa 1–286) of SEPT9_i1. The height of each residue indicates the frequency of its presence at the indicated positions. (C) Sequence of aa 61–113 of SEPT9_i1. The blue and red stars highlight the basic and acidic residues mutated to alanine. (D and E) Graphs show percentage of total SEPT9_i1(61–113) [wild-type and basic residue mutants] pelleted with MTs at 39,000 g (D), and percentage of total tubulin pelleted at 8,000 g (E) in three independent experiments. Representative gels are shown in Fig. S2. (F and G) Graphs show percentage of total 61–113 peptide (wild-type and acidic residue mutants) pelleted with MTs at 39,000 g (F), and percentage of total tubulin pelleted at 8,000 g (G) in three independent experiments. Representative gels are shown in Fig. S2. (H) Schematic shows a model of electrostatic interactions between the acidic (red) CTTs of tubulin and the basic (blue) residues of the SEPT9 repeat motifs. MT cross-linking is achieved by interactions between the acidic and basic residues of the K/R-x-x-E/D and R/K-R-x-E motifs, respectively.
Figure 4. **SEPT9 repeat motifs are required for MT bundling and asymmetric neurite growth.** (A and B) Maximal projections of 3D confocal microscopy images of MDCK cells expressing GFP-tagged SEPT9_i1 and SEPT9_i4 before (A) and after (B) treatment with 5 µM paclitaxel for 1.5 h. (C) Manders coefficients for the colocalization of GFP-tagged SEPT9_i1 and SEPT9_i4 with MTs in MDCK cells (n = 15). High and low colocalization are indicated by coefficients >0.5 and <0.5, respectively. (D and E) Plots show the fluorescence intensity of putative MT bundles with 5× and 10× the mean intensity of single MTs as percentage of total MT intensity in MDCK cells (n = 15) before (D) or after (E) treatment with paclitaxel. (F) Phase-contrast images show PC12 cells transfected with GFP and GFP-tagged SEPT9_i1 and SEPT9_i4 after a 2- and 3-d NGF treatment. Insets show GFP fluorescence in inverted monochrome. Bars, 10 µm. (G) Graph shows percentage of PC12 cells (n = 90) with one or more neurites. Pooled data from three independent experiments are shown.
dimmest single peripheral MTs, using a segmentation analysis of 3D confocal image stacks as previously published (Sammak and Borisy, 1988; Bowen et al., 2011). MDCK cells that expressed SEPT9_i4-GFP had consistently lower percentage of 5× or 10× MTs compared with control and SEPT9_i1-GFP in the presence or absence of paclitaxel (Fig. 4, D and E). A lower percentage of MT bundles was also observed in fibroblasts from Sept9 knockout mice at steady-state and after treatment with paclitaxel (Fig. S3). Thus, SEPT9 is critical for the intracellular organization of MTs into bundles.

During neuronal morphogenesis, MT bundling supports the asymmetric growth of a neurite, which extends faster and longer than other neurites to become the presumptive axon. To test whether SEPT9_i1 and the K/R-R/x-x-E/D motifs affect neurite growth, we assayed for NGF-induced neurite formation in PC12 cells. After 48 and 72 h of NGF treatment, the percentage of GFP-SEPT9_i1-expressing cells with single neurites was respectively fivefold and twofold higher than control cells (Fig. 4, F and G). In contrast to this amplification of asymmetric neurite growth by SEPT9_i1, expression of SEPT9_i4 had the opposite effect, increasing the percentage of cells with more than three neurites by twofold after 48 h of NGF treatment (Fig. 4, F and G). Notably, after 72 h of NGF exposure, the percentage of SEPT9_i4-expressing cells with single neurites was twofold lower than control cells (Fig. 4 G) and the average neurite length was shorter than the length of single neurites in SEPT9_i1-expressing cells (2.3 vs. 3.3 times the cell width). These results indicate that the MT-bundling properties of SEPT9 promote asymmetric neurite growth, which depends on the presence of K/R-R/x-x-E/D motifs within the N-terminal sequence of SEPT9.

HNA-linked mutation R88W impairs MT bundling and asymmetric neurite growth
Alterations in the N-terminal sequence of SEPT9 have been genetically linked to HNA, a neuralgic disorder whose molecular etiology remains unknown (Kuhlenbäumer et al., 2005; Hannibal et al., 2009; van Alfen, 2011). Several HNA patients carry the mutation R88W in isoform 3 of SEPT9 (Hannibal et al., 2011; de Almeida Marques et al., 2012). SEPT9 molecules lack C-terminal tails, but our data indicate that their N-terminal extensions could mediate trans interactions by electrostatic pairing between the acidic and basic residues of the K/R-x-x/E/D and R/K-R-x-E motifs, respectively. SEPT9-mediated MT cross-linking could be further reinforced by the trans interactions between the C-terminal coiled-coil domains of septins mediate the pairing of septin filaments (John et al., 2007; Bertin et al., 2008; DeMay et al., 2011; de Almeida Marques et al., 2012). Although SEPT2 does not bind MTs (Fig. S1), previous work indicates that SEPT7 and possibly SEPT6 interact with MTs (Hu et al., 2012; Moon et al., 2013). These subunits, however, lack the repeat motifs of SEPT9.

Septins colocalize with MTs in various cell types, but the physiological significance of this interaction is poorly understood. Here, we have found that SEPT9 and its N-terminal repeat motifs affect intracellular MT bundling and asymmetric neurite growth. Importantly, expression of SEPT9 isoforms with deletions (SEPT9_i4) and mutations (HNA-linked R88W) in the repeated K/R-R/x-x/E/D sequence motifs impair MT bundling and asymmetric neurite growth. Interestingly, cancer cells that overexpress SEPT9_i4 have increased resistance to the drug paclitaxel (Chacko et al., 2012). It is unknown if paclitaxel-mediated MT bundling contributes to the cytotoxicity of the drug, but our data indicate that SEPT9 and its repeat motifs are partly required for paclitaxel-induced MT bundling. This role of SEPT9, however, could vary between different cell types. Given the preferential interaction of SEPT9 with β1-tubulin, variations in the expression of β-tubulin isotypes could affect septin association with MTs. In summary, our results suggest that alterations in SEPT9 isoform expression trigger changes in the intracellular organization and function of MTs, and thereby could contribute to the pathology of HNA and cancer.

Materials and methods

Cells, peptides, and plasmids
MDCKIII/G cells were maintained in low glucose DME media (Sigma-Aldrich) supplemented with 10% FBS [Cell Generation] and 1 g/liter NaHCO₃. MDCK cells were transfected with the plasmids pEGFP-SEPT9_i1.
Industries) was used to take a skin sample from the extensor surface of the upper arm. After removal of the subcutaneous fatty tissue, skin explants were cultured in high glucose DME media with 20% FBS for 5–7 d. Skin fibroblasts that grew out of the explants were isolated and passaged. Experiments with R88W and control 1 fibroblasts were performed at passages 7–11 and 9–13, respectively. The peptides NH2-CEVGVDSVEGEGEEE-GEEY-COOH (α-tubulin CTT), NH2-CQETAEEYQDEEQGEADAEDFG-COOH (control, scrambled βII-tubulin CTT), and NH2-CQYQDATAEEEEDFEGEAEEEA-COOH (βI tubulin CTT) were purchased at >95% purity from GenScript. The peptides NH2-CQYQDATADEQGEFEEEEGEDEA-COOH (βII-tubulin CTT) and NH2-CQYQDATAEEEEEYEDDEESEAQPK-COOH (βIII-tubulin CTT) were purchased at >95% purity from Life－Tein, LLC.

His-SEPT9_i1-expressing plasmid was constructed by PCR amplifying human SEPT9_i1 (NP_001106963) using the primers 5′-CGTAAGCTTG-CATGAAGAAGTCTTACTC-3′ and 5′-GTACTCGAGCTACATCTCTGGG-GGC-3′ and cloning the amplified fragment into the HindIII and XhoI sites of pEGFP-SEPT9_v4, which encode respectively for GFP-tagged SEPT9_i1 and SEPT9_i4 and were constructed by PCR amplification of SEPT9_v1 (AF189713) and SEPT9_v4 (AJ312322) from normal breast tissue cDNA and insertion into pEGFP-C2 (Connolly et al., 2011b). PC-12 cells were maintained in high glucose DME supplemented with 6% donor-defined equine serum (Hyclone), 6% defined bovine calf serum (Hyclone), and 1 g/liter NaHCO3. PC-12 differentiation and neurite growth were induced after 24 h of transfection by incubation in low serum (1% horse serum, 1% bovine calf serum) DME containing 100 ng/ml 2.5S NGF (Harlan Biosciences). Embryonic fibroblasts derived from Sept9cond/cond and Sept9del/del mice were provided by E.-M. Füchtbauer (University of Aarhus, Aarhus, Denmark) and maintained in DME with 10% FBS as described previously (Füchtbauer et al., 2011). Dermal cells were derived from skin biopsies taken from a healthy individual (control 1) and an HNA patient with the SEPT9_i3_R88W mutation (c.262C>T). Both subjects provided written informed consents and the biopsy protocol was approved by the University of Kiel ethics committee. Under local anesthesia, a standard skin-biopsy punch (diameter 3 mm; Kai Industries) was used to take a skin sample from the extensor surface of the upper arm. After removal of the subcutaneous fatty tissue, skin explants were cultured in high glucose DME media with 20% FBS for 5–7 d. Skin fibroblasts that grew out of the explants were isolated and passaged. Experiments with R88W and control 1 fibroblasts were performed at passages 7–11 and 9–13, respectively. The peptides NH2-CEVGVDSVEGEGEEE-GEEY-COOH (α-tubulin CTT), NH2-CQETAEEYQDEEQGEADAEDFG-COOH (control, scrambled βII-tubulin CTT), and NH2-CQYQDATAEEEEDFEGEAEEEA-COOH (βI tubulin CTT) were purchased at >95% purity from GenScript. The peptides NH2-CQYQDATADEQGEFEEEEGEDEA-COOH (βII-tubulin CTT) and NH2-CQYQDATAEEEEEYEDDEESEAQPK-COOH (βIII-tubulin CTT) were purchased at >95% purity from Life－Tein, LLC.

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His-SEPT9_i1-expressing plasmid was constructed by PCR amplifying human SEPT9_i1 (NP_001106963) using the primers 5′-CGTAAGCTTG-CATGAAGAAGTCTTACTC-3′ and 5′-GTACTCGAGCTACATCTCTGGG-GGC-3′ and cloning the amplified fragment into the HindIII and XhoI sites of...
pET28a(+), His-GFP-SEPT9_i1-expressing plasmid was constructed by PCR amplifying GFP-SEPT9-i1 from pEGFP-C2-SEPT9_v1 (see above) using the primers 5'-TCGAGCTCATTGATGACAGGGC-3' and 5'-CTAGTCAGCTTCATTGGGGCCG-3' and inserting the fragment into the HindIII and Xhol sites of pET28a(+). His-SEPT9_i1-expressing plasmid was made by PCR amplifying human SEPT9_v3 (NP_006614) using the primers 5'-TCCGGATCCATGTCGGTCAGTGTC-3' and 5'-ACTAAGCTTTAAGGAGCGAGCTCGAG-3' and cloning the amplified fragment into the BamHI and HindIII sites of pET-28a(+). Plasmids expressing His-SEPT9-N (aa 1–142 of SEPT9_i1), His-SEPT9_B (aa 1–283 of SEPT9_i1) and His-SEPT9-G were constructed by PCR amplifying SEPT9-N and SEPT9-G and inserting into HindIII and Xhol sites of pGEX-EXT.

For the bacterial expression of septin heterotrimers, the bicistronic plasmid pSEPT6-HSEPT7 was constructed for the simultaneous expression of nontagged [NT] SEPT6 and His-tagged SEPT7. First, His-SEPT7-expressing plasmid was made by PCR amplifying the rat SEPT7 transcript variant 2 (NM_133179) using the primer 5'-TCCGGATCCATGTCGGTCAGTGTC-3' and 5'-TCGAAAGCTTCCATGTCGCCCGG-3' and cloning the amplified fragment into the BamHI and HindIII sites of pET28a(+). Plasmids expressing His-SEPT7–expressing plasmid. The plasmids encoding for NT-SEPT2 and NT-SEPT9_i1 were made from pET plasmids expressing His-SEPT9_i1 (see above) and His-SEPT2, which was constructed by PCR amplifying mouse SEPT2 (NP_006614) using the primer 5'-TCGAGCTCATTGATGACAGGGC-3' and 5'-CTAGTCAGCTTCATTGGGGCCG-3' and cloning the amplified fragment into the BamHI and Xhol sites of pET28a(+). His-SEPT9_i1-expressing plasmid. The plasmids encoding for NT-SEPT2 and NT-SEPT9_i1-expressing plasmid.

Expression and purification of recombinant proteins

Plasmids encoding for recombinant proteins were transformed into Escherichia coli BL21 (DE3) (Invitrogen). After bacterial cultures reached an OD600 of 0.8, protein expression was induced with 0.5 mM IPTG for 16 h at 18°C. Bacteria were centrifuged at 5,000 rpm for 5 min at 4°C. Pellets were resuspended in buffer containing 1% Triton X-100, 50 mM Tris, pH 8.0, 150 mM NaCl, 10% glycerol, and 10 mM imidazole, and lysed using a French pressure cell (1,280 psi). Cell lysates were clarified by centrifugation at 14,000 g for 30 min at 4°C. Supernatants were loaded on columns containing 500 µl Ni-NTA beads (Qiagen). Columns were washed with 50 mM Tris, pH 8, 300 mM NaCl, 10% glycerol, and 10 mM imidazole, and eluted with 50 mM Tris, pH 8, 300 mM NaCl, 10% glycerol, and 10 mM imidazole. Gels were stained with Coomassie Brilliant Blue. Gels were scanned and protein band densities were quantified with the Odyssey infrared scanning system (LI-COR Biosciences).

For visualization of MT bundling by negative stain electron microscopy, X-hodamine-labeled bovine brain tubulin (10 µM, Cytoskeleton, Inc.) was polymerized in G-PERM buffer containing 80 µM paclitaxel. MTs were incubated with recombinant proteins (20 nM) in G-PEM buffer containing 20 µM glutathione agaro 4B beads for 15 min. After washing three times with GST binding buffer, beads were incubated with 20 µM His-SEPT2, His-SEPT9_N, or His-SEPT9_G for 1.5 h. Beads were washed with GST binding buffer five times before resuspended with loading buffer and boiled. Samples were loaded into 10% SDS-PAGE gels and stained with Coomassie Brilliant Blue.

Western blots and overlay assays

Bovine brain tubulin (>99% pure; Cytoskeleton, Inc.) was separated by 7.5% SDS-PAGE and transferred to PROTRAN B85 nitrocellulose membrane (Whatman), which were stained with Pronase S (Sigma-Aldrich) and scanned with an ImageLaser 2.0 (Carbox). After incubation with MBP, proteins were washed with dH2O, membranes were blotted with mouse antibody DM1A against α-tubulin (1:100,000, Sigma-Aldrich) and TUB2.1 antibody against β-tubulin (1:100,000, Sigma-Aldrich), and secondary Alexa Fluor 680 goat anti-mouse IgG (1:15,000, Invitrogen). For blot overlay assays, membranes were incubated for 1 h at 4°C in blocking buffer (10 mM Tris HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% Tween 20, 5% nonfat dry milk, and 0.05% BSA). Subsequently, membranes were overlayed with His-SEPT9_i1.
Cells (MDCK and skin fibroblasts) treated with paclitaxel (Sigma-Aldrich) or carboxyfluorescein (Dojindo, Kumamoto, Japan) were fixed with 4% paraformaldehyde and 0.5% glutaraldehyde (Electron Microscopy Sciences) and 0.05% glutaraldehyde (Electron Microscopy Sciences), and 0.5% Triton X-100. Microtubules were stained with mouse antibody DM1A against α-tubulin (Sigma-Aldrich) and donkey DyLight 594-conjugated F(ab)2; to mouse IgG (Jackson ImmunoResearch Laboratories, Inc.). Samples were mounted with FluorSave (EMD Millipore) and imaged on a laser-scanning confocal microscope (FluoView 1000; Olympus) using a Plan Apochromat 60x/1.4 NA objective. Serial optical sections were acquired from the bottom to the top of each cell at 0.2-µm steps. Each z-stack was imported into Slidebook 5.0 software for colocalization and MT fluorescence analyses. Background fluorescence was removed and fluorescence intensity segmentation was used to mask MTs and septin filaments, and colocalization values were automatically calculated using the Manders algorithm of the Slidebook 5.0 software. To determine the fraction of MT bundles relative to total MTs, we first measured the mean fluorescence intensity per pixel for the dimmest single MTs found in the periphery of each cell. Subsequently, MTs and MT regions with pixel values over 5 or 10 times the single MT pixel value were masked. The sum fluorescent intensity of these putative MT bundles was calculated as a fraction of the total MT fluorescence intensity in a 3D stack of images using Slidebook 5.0 software.

PC-12 cells were fixed with PHEM buffer (60 mM Pipes-KOH, pH 6.9, 25 mM Hepes, 10 mM EDTA, and 2 mM MgCl2) containing 3% paraformaldehyde (Electron Microscopy Sciences) and 5% sucrose. Cells were permeabilized with 0.5% Triton X-100 and stained for α-tubulin and imaged on a fluorescent microscope (Axio Observer; Carl Zeiss) equipped with a Plan-Apo 63x/1.4 NA objective, a deep-cooled CCD camera (ORCA-AG; Hamamatsu Photonics), and Slidebook 5.0 software. Microtubule-positive protrusions with lengths longer than the diameter of the cell soma were scored as neurites.

Statistical analysis and prediction of intrinsic disorder

Datasets were plotted in box-and-whisker diagrams. The bold horizontal line marks the median value and the bottom and top of each box corresponds respectively to the 25th (Q1) and 75th (Q3) percentiles of the range of values shown. Whisker ends correspond to the minimum and maximum values of each dataset. Values 1.5 times more than the Q3 value or 1.5 times less than the Q1 value were considered statistical outliers and were plotted outside the whisker portions of the diagram. Kollman-Crippen-Smirnov tests were performed to assess the normal distribution of each dataset and unpaired Student’s t-tests were used to derive P-values for normally distributed datasets with equal standard deviations (SDs). The Welch test was used to compare datasets with unequal SDs and the Mann-Whitney test was used to compare datasets, which were not normally distributed.

The prediction of intrinsic disorder of SEPT9 was performed by combining results from GlobPlot (http://globplot.embl.de/), DisEMBL (http://dis.embl.de/), DIPRED2 (http://bioinf.cs.ucl.ac.uk/psipred/?disopred=1), IUPRED (http://iupred.enzim.hu/), DISPRO (http://www.ics.ucl.ac.uk/~balldig/dispro.html), OnD-CRF (http://babel.ucmp.umu.se/ond-crff/), and DRIFFR (http://www.sbc.su.se/~maccoll/dffr/). The predicted intrinsic disorder of every amino acid was set to 1 or 0 for values that were above or below the intrinsic disorder cutoff points of each algo (Seeger et al., 2012).

Online supplemental material

Fig. S1 shows the predicted intrinsic disorder of SEPT9 and its conservation statistic, and the effects of GMP-CP and ionic strength on the interaction of SEPT9 with MTs. Fig. S2 shows the MT-bundling and tubulin-binding properties of SEPT9 and all of its mutants. Fig. S3 shows that SEPT9 is partly required for taxol-mediated MT bundling. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201308068/DC1.

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