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Regulation of microtubule plus end dynamics by septin 9

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Abstract

Septins are GTP-binding proteins that associate with the microtubule (MT) and actin cytoskeleton. Septins affect MT organization and posttranslational modifications, but their role in MT dynamics is less understood. Here, we reconstituted MT dynamics in the presence of the MT-binding septin (SEPT9) using an in vitro cell-free assay, which images the polymerization of tubulin from guanosine-5'-[(α,β)-methylene]triphosphate (GMPCPP)-stabilized MT seeds. We found that submicromolar concentrations of SEPT9 suppress MT catastrophe and enhance the growth of MT plus ends to great lengths, while low micromolar concentrations of SEPT9 stabilize MTs by inhibiting dynamic instability. We show that SEPT9 associates preferentially with the lattice of GMPCPP-stabilized MT seeds and surprisingly recruits soluble tubulin to the MT lattice. Notably, the effects of SEPT9 on MT dynamics are dependent on its G-G dimerization interface, which is formed by the pockets of the GTP-binding domains. A mutation (H530D) that disrupts G-G dimerization abrogates the effects of SEPT9 on MT dynamics and diminishes its ability to recruit tubulin to the MT lattice. Taken together, these results suggest that SEPT9 promotes the formation and maintenance of long stable MTs through a mechanism that may involve recruitment of unpolymerized tubulin to the MT lattice.

Keywords

microtubule catastrophe and rescue; microtubule plus ends; microtubule stability; microtubules; SEPT9; septins

1 | INTRODUCTION

Septins are filamentous GTP-binding proteins, which associate with the microtubule (MT) and actin cytoskeleton (Spiliotis, 2018). In mammalian cells, septins colocalize with subsets of MTs and are required for MT-dependent functions in mitosis (Estey, Di Ciano-Oliveira, Froese, Bejide, & Trimble, 2010; Menon et al., 2014; Spiliotis, Kinoshita, & Nelson, 2005), vesicle trafficking (Spiliotis, Hunt, Hu, Kinoshita, & Nelson, 2008) and the formation of protrusive structures such as cilia (Ghossoub et al., 2013) and axon branches (Hu et al., 2012). In addition, septins have been implicated in the formation of MT-based nanotube-like protrusions, which are caused by bacterial toxins, and micro-tentacle protrusions that tumor cells develop in the absence of matrix attachments (Nolke et al., 2016; Ostevold et al., 2017).

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SUPPORTING INFORMATION

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Septin 9 (SEPT9) was the first septin observed to colocalize and associate with MTs directly (Nagata et al., 2003; Surka, Tsang, & Trimble, 2002). Notably, SEPT9 depletion resulted in thinning of the MT network, suggesting a role in MT dynamics and organization (Nagata et al., 2003). Subsequent work revealed that SEPT9 binds and bundles MTs via its unique N-terminal extension, which contains a repeat motif sequence that interacts with the C-terminal tails of β -tubulin (Bai et al., 2013). Despite this knowledge, it is unknown whether SEPT9 impacts directly the dynamic instability of MTs.

A number of studies have implicated septins in the regulation of MT dynamics. Septin 7 (SEPT7) knock-down was first shown to increase MT acetylation, a posttranslational modification associated with MT stability and longevity (Janke & Montagnac, 2017; Kremer, Haystead, & Macara, 2005). This effect was attributed to an increased interaction of MTs with the MT-associated protein 4 (MAP4), which is inhibited by septins (Kremer et al., 2005). Because MAP4 stabilizes MTs, it was suggested that septins enhance MT dynamics (Kremer et al., 2005). Subsequent work showed that SEPT7 interacts directly with the histone deacetylase 6 (HDAC6), mediating the deacetylation of tubulin (Ageta-Ishihara et al., 2013). In SEPT7-depleted neurons, increased levels of MT acetylation correlated with reduced rates of MT plus end growth (Ageta-Ishihara et al., 2013). In epithelial cells, SEPT2 depletion was shown to enhance MT catastrophe, indicating that MTs are less stable upon septin depletion (Bowen, Hwang, Bai, Roy, & Spiliotis, 2011). Compounded by new evidence of a direct interaction between septins and the MT plus end tracking protein EB1 (Nolke et al., 2016), these findings have added little clarity on how MT dynamics are affected by septins.

In vitro reconstitution of MT dynamics with total internal reflection fluorescence (TIRF) microscopy has provided valuable insights into the regulation of MTs by MAPs (Gell et al., 2010). In these assays, the effects of purified recombinant proteins can be assayed directly without the caveats of protein depletion or overexpression. Given that septins compete with MAPs (e.g., MAP4) and affect MT posttranslational modifications, we sought to test if septins affect MT dynamics in cell-free TIRF assays. Here, we reconstituted MT dynamics in the presence of purified recombinant SEPT9, which associates with MTs directly. We report that SEPT9 enhances the growth and length of MT plus ends by suppressing MT catastrophe, and stabilize MTs in a concentration-dependent manner. Notably, SEPT9 associates preferentially with MT segments that contain tubulin bound to GMPCPP, a slowly hydrolyzable analog of GTP, and recruits soluble tubulin onto the lattice of MTs. We show that the dimeric G-G interface of SEPT9, which is formed by the GTP-binding pockets, is critical for its ability to recruit tubulin and affect MT dynamics. Overall, our results demonstrate that MT growth and stability are directly enhanced by SEPT9, and suggest a mechanism that may involve recruitment of unpolymerized tubulin to the MT lattice.

2 | RESULTS AND DISCUSSION

2.1 | SEPT9 enhances MT plus end growth

Using TIRF microscopy, we sought to test if SEPT9 (SEPT9_i1) affects the dynamics of the plus- and minus-ends of MTs that extend from stable seeds, which were made with a mix of HiLyte488- and biotin-labeled tubulin in the presence of guanosine-5'-[(α,β)-

methyleno]triphosphate (GMPCPP), a slowly hydrolyzable analog of GTP, and immobilized to glass chambers (Figure 1a,b). MT growth and shrinkage was imaged after perfusion of a mixture of Rhodamine-labeled and unlabeled tubulin (10 μ M) with purified recombinant SEPT9 (Figure 1c). At concentrations of hundreds of nanomolar, which is the physiological range of septin concentration in cells (Hein et al., 2015; Itzhak et al., 2017), SEPT9 had a subtle and variable effect on the rates of MT plus end growth and depolymerization (Figure 1d,e). In contrast, SEPT9 (400–800 nM) had a pronounced effect on catastrophe frequencies, which were reduced by ~40%, and MT plus end lengths increased by more than two-fold (Figure 1f,g). Kymographs demonstrated that higher concentrations of SEPT9 result in persistent growth of MT plus ends to great lengths (Figure 1h–k). Thus, SEPT9 appears to promote MT plus end growth by reducing catastrophe frequency. Note that as previously reported (Bowne-Anderson, Zanic, Kauer, & Howard, 2013), rescue events (switch from MT shrinkage to growth) are seldom in these in vitro assays and not amenable to meaningful analysis.

By definition, MT minus-ends grow slower than plus ends. Indeed, the average rate of MT minus-end growth was ~75% slower and minus-ends grew to an average length, which was less than half of the length of plus ends (Figure 1l,o). Increasing concentrations of SEPT9 (10–800 nM) had no effect on minus-end growth rates (Figure 1l). Similarly, depolymerization rates and catastrophe frequencies remained unchanged (Figure 1m,n). A subtle increase in MT minus-end length was observed in the presence of 800 nM SEPT9, but lengths were not altered at any other submicromolar concentrations of SEPT9 (Figure 1o). Hence, submicromolar concentrations of SEPT9 do not appear to impact significantly the dynamics of MT minus-ends.

2.2 | Regulation of MT dynamics requires dimerization of SEPT9 through its G-G interface

Septins oligomerize into higher order structures by dimerizing in tandem through two interfaces that involve interactions between the GTP-binding pockets (G-G interface) and their N- and C-terminal domains (N-C interface) (Sirajuddin et al., 2007; Valadares, Muniz Pereira, Ulian Araujo, & Garratt, 2017). SEPT9 possesses a long N-terminal tail, which is unique among all septins and mediates the interaction with MTs (Bai et al., 2013). Although no structural information exists for the N-terminal tail of SEPT9, crystal structures of the GTP-binding domain of SEPT9 reveal a G-G dimerization interface, which involves residues that are highly conserved across the GTP-binding domains of septins (Figure 2a,b).

To test if G-G dimerization is critical for the regulation of MT dynamics by SEPT9, we mutated a conserved histidine residue into aspartate (H530D), a mutation that was previously shown to abrogate the G-G dimerization of SEPT2 (Sirajuddin et al., 2007). Gel filtration analysis of the wild type SEPT9 showed a peak, which corresponds to a molecular weight of 258 kD (Figure 2c,d). This indicates that SEPT9 exists as a tetramer or alternatively as a dimer as the long (283 aa) and disordered N-terminal domain of SEPT9 could increase its hydrodynamic radius and apparent molecular weight (Bai et al., 2013; Erickson, 2009; Uversky, 2002). The H530D mutation shifted the peak of SEPT9 to fractions of lower molecular weight (Figure 2c,d), which indicated that the mutation disrupted the G-G dimerization of SEPT9 (Figure 2c,d).

Using TIRF microscopy, we tested whether SEPT9_i1(H530D) affects MT plus end dynamics at a concentration (800 nM), which had a maximum impact for wild type SEPT9. We found that SEPT9_i1 (H530D) did not have a significant impact on the rates of MT plus-end growth and depolymerization (Figure 2e,f). Notably, in contrast to the wild type SEPT9_i1 which markedly affects catastrophe frequencies and MT lengths, SEPT9_i1(H530D) did not affect these parameters (Figure 2g,h). These results suggest that dimerization of SEPT9 through its G-G interface is critical for modulating the dynamics of MT plus ends.

2.3 | SEPT9 associates preferentially with GMPCPP-stabilized MTs

To gain an insight into how SEPT9 affects MT dynamics, we sought to visualize the interaction of recombinant SEPT9 with GMPCPP-stabilized MT seeds and their dynamic ends. We imaged MT dynamics with TIRF microscopy after adding a mix of HiLight488-labeled and unlabeled tubulin (10 μ M) with recombinant mCherry-SEPT9 (400–800 nM) to HiLyte-647-labeled MT seeds. Time-lapse imaging of mCherry-SEPT9 revealed three different modes of interaction with MTs: (a) stable association with segments of MT seeds and the lattice of dynamic plus-end segments (Figure 3a,b), (b) transient association with the tips of MT plus end segments (Figure 3c), and (c) diffusive movements along the lattice of MT plus end segments (Figure 3d). On the GMPCPP-stabilized seeds, mCherry-SEPT9 binding was rather stable, persisting for several minutes (Figure 3a,c). On occasion, SEPT9 associated stably with the lattice of growing plus-end segments (Figure 3b) and transiently with plus-end tips (Figure 3c). In addition, SEPT9 exhibited diffusive movements along the lattice of MT plus end segments (Figure 3d).

To determine whether SEPT9 has a preferential mode of interaction with MTs, we quantified the localization of mCherry-SEPT9_i1 on MT seeds and plus end segments. On the majority of septin-decorated MTs (>60%), mCherry-SEPT9_i1 associated with MT seeds exclusively (Figure 3e). In a significant fraction of MTs, mCherry-SEPT9_i1 was found on both the seeds and the lattice of dynamic plus end segments (5–15%) or on the latter only (~20%), while it was largely absent from minus-end segments (Figure 3e). Quantification of the mean intensity of mCherry-SEPT9 along the entire length of MT seeds and the lattice of plus end segments showed that at submicromolar concentrations, SEPT9 is more concentrated on MT seeds (Figure 3f,g). Interestingly, at ten-fold higher concentrations (4–8 μ M), mCherry-SEPT9_i1 did not exhibit any preference between the lattices of the GMPCPP seeds and plus-end segments, which were completely decorated with SEPT9 (Figure 3f,g). Time-lapse imaging of mCherry-SEPT9 at the concentration of 1.5 μ M showed a preferential and gradual accumulation of SEPT9 to the MT seed (Figure S1a–c). At higher concentrations (2–3 μ M), SEPT9 was similarly observed to gradually accumulate to the MT seed and MT end segments after their growth (Figure S1d–i). Taken together, these results suggest that SEPT9_i1 has a stronger affinity for MT segments that contain GTP-bound tubulin, which is predominately found at the tips of MT plus-ends and areas of the MT lattice that undergo repair after damage or contain remnants of GTP-tubulin (Dimitrov et al., 2008; Schaedel et al., 2015).

2.4 | SEPT9 recruits tubulin to the MT lattice

In time-lapse movies of MT dynamics and mCherry-SEPT9_i1, we observed that soluble HiLyte488-tubulin, which is normally added to the ends of GMPCPP HiLyte647-labeled seeds, was present on MT seed segments that contained mCherry-SEPT9_i1 (arrows in Figure 3a,b). Given that MT seeds are stable polymers that do not exchange any tubulin, we examined whether tubulin recruitment to stable GMPCPP seeds was dependent on SEPT9. We quantified the mean fluorescence intensity of soluble HiLyte488-tubulin on the lattice of MT seeds in the absence and presence of mCherry-SEPT9_i1. We found that the levels of HiLyte488-tubulin increased on MT seeds with increasing concentrations of mCherry-SEPT9_i1 (Figures 3f,h and S1). Notably, HiLyte488-tubulin decorated the entire length of MT seeds in the presence of $>1 \mu\text{M}$ concentrations of mCherry-SEPT9_i1 (Figure 3f,h) and was quantitatively more concentrated compared to submicromolar concentrations of mCherry-SEPT9_i1 (Figure 3h). Recruitment of soluble tubulin to stable MT seeds was also reproduced with increasing concentrations of recombinant nonfluorescent SEPT9_i1 (Figure 4a). Hence, SEPT9 appears to recruit soluble tubulin onto the MT lattice, which may enhance tubulin incorporation into the dynamic plus ends facilitating persistent growth.

Given that the H530D mutation of SEPT9, which disrupts G-G dimerization, abrogates the effects of SEPT9 on MT growth and length (Figure 2), we asked if the mutation also affects the recruitment of soluble tubulin to MT seeds. In contrast to the wild type SEPT9_i1, tubulin recruitment to MT seeds was markedly reduced in the presence of SEPT9_i1(H530D) (Figure 4a). This reduction was evident at both submicromolar (400–800 nM) and micromolar (4–8 μM) concentrations of SEPT9 (Figure 4a).

Because there is a significant recruitment of soluble tubulin to MT seeds in the presence of micromolar concentrations of SEPT9_i1 (H530D), we tested whether the mutation in the G-G dimerization domain impacted SEPT9_i1 functionally at these higher concentrations (4–8 μM). We found that micromolar concentrations of wild type SEPT9 inhibited the dynamic instability of MTs. MTs were observed to grow and stabilize without any further growth/shrinkage or stabilize to a certain length after a brief depolymerization event (Figure 4c, arrows). These stable MTs comprised 60–70% of total MTs that grew from stable GMPCPP seeds (Figure 4b). The remaining 30–40% MTs were more dynamic and underwent multiple rounds of growth and shrinkage. In the presence of the SEPT9_i1(H530D) mutant, the fraction of dynamic MTs increased dramatically and MTs exhibited phases of growth and shrinkage, which were more typical of the dynamic instability of MTs in the absence of SEPT9 or at lower concentrations of SEPT9 (Figure 4b,d). Therefore, it appears that micromolar concentrations of SEPT9_i1 stabilize MTs in a manner that in part depends on G-G dimerization and the recruitment of soluble tubulin to the MT lattice.

Previous studies have inferred roles for septins in MT dynamics by interpreting results from septin depletion in different cell types and using acetylation as a marker of MT stability (Ageta-Ishihara et al., 2013; Bowen et al., 2011; Kremer et al., 2005; Menon et al., 2014). Here, we used an in vitro cell-free assay to test if MT dynamics is directly impacted by SEPT9, a septin that binds MTs directly (Bai et al., 2013). We found that SEPT9 enhances MT lengths by suppressing the catastrophe of MT plus ends. This effect is reminiscent of previous observations in Madin-Darby canine kidney (MDCK) epithelial cells, which

showed that MT catastrophe is the main parameter that is significantly affected by knock-down of SEPT2 (Bowen et al., 2011). While SEPT2 does not associate with MTs directly (Bai et al., 2013), it colocalizes with SEPT9 on the MTs of MDCK cells and SEPT2 depletion affects SEPT9 expression and localization (Dolat et al., 2014). Our results indicate that MT plus ends are most markedly affected by SEPT9, but we cannot rule out a possible effect on MT minus-ends as higher concentrations ($> 1 \mu\text{M}$) of SEPT9 were occasionally observed to stabilize minus-ends (Figure S1).

Surprisingly, SEPT9 appears to recruit tubulin to the MT lattice, which may provide a mechanism for inhibiting MT catastrophe. While presently this is a speculation, SEPT9 may function similar to the cytoplasmic linker associated protein (CLASP), a MT rescue factor that recruits tubulin dimers (Al-Bassam et al., 2010; Lawrence, Arpag, Norris, & Zanic, 2018). Similar to SEPT9, CLASP associates preferentially with GMPCPP-stabilized MTs and forms clusters, which are largely stationary. On the MT lattice, CLASP is thought to present GTP-bound tubulin to depolymerizing protofilaments, which become stable and/or regain growth. Hence, SEPT9 may similarly suppress MT catastrophe by creating islands of GTP-bound tubulin, which are thought to arrest MT depolymerization and resume MT growth (Aumeier et al., 2016; de Forges et al., 2016; Dimitrov et al., 2008; Schaedel et al., 2015; Tropini, Roth, Zanic, Gardner, & Howard, 2012).

In previous studies, we found that SEPT9 binds and bundles taxol-stabilized MTs through electrostatic interactions between the repeat motifs of its N-terminal basic domain and the acidic C-terminal tails of polymerized tubulin (Bai et al., 2013). Here, SEPT9 associates preferentially with the GMPCPP-bound MT lattice, which conformationally resembles the state of a taxol-stabilized GDP-bound lattice (Alushin et al., 2014). While the G domain of SEPT9 does not appear to associate with polymerized tubulin (Bai et al., 2013), the G-G dimerization interface is critical for the recruitment of unpolymerized tubulin to the MT lattice. Hence, SEPT9 may utilize its N-terminal and G domains differentially for binding to the MT lattice and unpolymerized tubulin dimers, respectively. Alternatively, oligomerization through the G-G interface might be critical for simultaneous binding of the N-terminus of SEPT9 to tubulin dimers and the MT lattice. Given that higher concentrations ($8 \mu\text{M}$) of SEPT9(H530D) can result in tubulin recruitment to MT seeds and increased MT stability, the N-terminal and GTP-binding domains of SEPT9 appear to function cooperatively in the binding of tubulin dimers. Moreover, at higher concentrations, where SEPT9 covers the MT lattice uniformly, individual protofilaments might be locked together and become structurally stabilized by the N-terminal domain of SEPT9 independently of the G-G interface and the recruitment of tubulin dimers. These possibilities presume that SEPT9 binds along the longitudinal axis of a MT protofilament, but it is also plausible that SEPT9 binds tubulin subunits of adjacent protofilaments, crosslinking them together along the wide axis of the MT lattice. Hence, dimerization via the G-G interface may serve the purpose of crosslinking adjacent protofilaments.

Septins (SEPT2, SEPT6 and SEPT7) were recently reported to interact directly with the MT plus end tracking protein EB1 (Nolke et al., 2016). While septins have never been reported to track on MT plus ends, this finding suggests that MT plus ends can be captured by membrane- or cytoskeleton-bound septins. Given that SEPT9 forms heteromeric complexes

with SEPT2/6/7 (Kim, Froese, Estey, & Trimble, 2011; Sellin, Sandblad, Stenmark, & Gullberg, 2011), it is possible that SEPT9 presents tubulin dimers to MT plus ends that come in contact with SEPT2/6/7. Therefore, SEPT9 may promote MT plus end growth at the base of membrane protrusions or subsets of actin filaments or MTs, which are enriched with septins (Spiliotis, 2018). In addition to promoting the incorporation of tubulin dimers to plus ends, SEPT9 may also contribute to the self-renewal of long-lived MTs by mediating the addition of tubulin dimers onto sites of MT lattice damage or stress (de Forges et al., 2016; Schaedel et al., 2015). Unfortunately, purification of SEPT9 in complex with SEPT2/6/7 is technically challenging and does not yield sufficient quantities for in vitro experimentation, so more work is required to determine how the heteromeric SEPT2/6/7/9 impacts MT dynamics. Future studies will tackle this problem and further explore the mechanism by which SEPT9 promotes MT growth and stability, and its implications for the spatial control of MT dynamics.

3 | MATERIALS AND METHODS

3.1 | Plasmids

His-SEPT9_i1 was constructed as described before (Bai et al., 2013). His-SEPT9_i1 H530D mutant was made according to KAPA Biosystems Site-directed mutagenesis protocol. Briefly, His-SEPT9_i1 was amplified with KAPA HiFi HotStart DNA polymerase (KK2502; KAPA BIOSYSTEMS) using the primers 5'-CCATCGAAGTTGAAAACACCACAGACTGTGAGTTTG-3' and 5'-CAAATCAGAGTCTGTGGTGTTCAACTTCGATGG-3'. The amplified PCR product was treated with DpnI (R0176L; New England Biolabs) for 1 h at 37 °C. DpnI-treated product was heat inactivated at 80 °C for 20 min and subsequently was transformed in *E. coli* DH5a cells. His-mCherry-SEPT9_i1 was made by two-step cloning into the pET28a(+) vector as recently published (Karasmanis et al., 2018).

3.2 | Protein expression and purification

Recombinant His-tagged SEPT9_i1, SEPT9_i1(H530D) and Hismcherry-SEPT9_i1 were transformed into *E. coli* BL21 (DE3) (Invitrogen). Bacterial cultures were grown to OD₆₀₀ of 0.5 and induced with 0.5 mM IPTG for 5 h at 22 °C or 0.2 mM IPTG for 16 h at 18 °C for fluorescence tagged SEPT9_i1. Cultures were centrifuged at 4,000 rpm for 20 min at 4 °C. Pellets were resuspended in lysis buffer containing 50 mM Tris pH 8.0, 150 mM NaCl, 10% glycerol, 1 mM PMSF, 1 mg/mL lysozyme and 10 mM imidazole and lysed by sonication (10 sets of 15 pulses on ice with 30 s interval between each set). Cell lysates were centrifuged at 13,000 rpm for 30 min at 4 °C and passed through a 0.45 µm pore filter. Supernatants were loaded onto gravity flow columns with Ni-NTA agarose beads (745,400.25; Macherey-Nagel), which were equilibrated with 10 mL lysis buffer. Columns were washed with 30 mL washing buffer (50 mM Tris pH 8.0, 300 mM NaCl, 10% glycerol, 10 mM imidazole). Proteins were eluted in elution buffer containing 50 mM Tris pH 8.0, 150 mM NaCl, 10% glycerol and 250 mM imidazole, and dialyzed overnight in BRB80 (80 mM Pipes pH 6.9, 2 mM MgCl₂, 1 mM EGTA). His-tagged mCherry-SEPT9_i1 was further purified using an AKTA FPLC system (GE Healthcare) with a Superdex 200 10/300 GL (Amersham Biosciences) gel filtration column.

3.3 | Size exclusion chromatography

Gel filtration was performed in an AKTA FPLC system (GE Healthcare) with a Superdex 200 10/300 GL (Amersham Biosciences) gel filtration column. Recombinant proteins were centrifuged at 13,000 rpm for 10 min at 4 °C. Protein samples (26 μ M) were loaded onto a 500 μ L loop and injected to the gel filtration column pre-equilibrated with 50 mL buffer containing 50 mM Tris pH 8.0, 150 mM NaCl and 10% glycerol between each run. Proteins were eluted at a flow rate of 0.3 mL/min and collected in 0.5 mL fractions. Collected fractions were analyzed by SDS-PAGE and Coomassie staining. The column was calibrated with gel filtration calibration MWGF100 standards (Sigma), which contain Dextran blue for void volume determination, thyroglobulin, apoferritin, β -amylase, alcohol dehydrogenase, albumin and carbonic anhydrase. For protein size determination, a standard curve was generated.

3.4 | TIRF assays of MT dynamics

Slides and glass coverslips were acid washed as described previously (Tanenbaum, Vale, & McKenney, 2013). Imaging chambers were prepared using two strips of parafilm, instead of a double-sided tape, and heat was applied to melt the parafilm strips onto the glass slide and coverslip of the chamber (Reid et al., 2016). The flow chamber was incubated with the following solutions: 1% Pluronic F-127 for 5 min, 5 mg/mL Biotin-BSA (A8549–10MG; Sigma-Aldrich) for 5 min, 0.5 mg/mL Neutravidin (A2666; Invitrogen) for 5 min and ~60–80 nM GMPCPP-stabilized MT seeds for 15 min. MT seeds were made from a tubulin mix, which contained 11.5% biotin-tubulin (T333P-A, Cytoskeleton Inc.), 77% unlabeled tubulin (T240-A, Cytoskeleton Inc.) and 11.5% HiLyte488-tubulin (TL488M-A, Cytoskeleton Inc.) or 10% HiLyte647-tubulin (TL670M-A, Cytoskeleton Inc.). The tubulin mix (20 μ M) was combined with 1 mM GMPCPP (NU-405 L; Jena Bioscience) in BRB80 (80 mM Pipes pH 6.9, 2 mM $MgCl_2$, 1 mM EGTA) and incubated at 37 °C for 30 min. After incubation, seeds were diluted in BRB80 and centrifuged at 100,000 g (Optima TL100; Beckman Coulter) for 15 min. Pelleted MT seeds were resuspended in 60 μ L of BRB80. Prior to the assay, chambers were incubated with blocking buffer (BRB80, 1% Pluronic F-127, 1 mg/mL BSA) for 5 min. MT polymerization was initiated by introducing a mix of 10 μ M tubulin, which contained 6.6% X-Rhodamine-tubulin (TL620M-A; Cytoskeleton) or HiLyte488-tubulin (TL488M-A; Cytoskeleton), 1 mM GTP, 0.1 mg/mL BSA, 0.1% Pluronic F-127, 0.1% κ -casein and 0.2% methyl cellulose. This mix was supplemented with an oxygen scavenging system (0.5 mg/mL glucose oxidase, 0.1 mg/mL catalase, 4.5 mg/mL D-glucose, 70 mM β -mercaptoethanol) in BRB80 and recombinant SEPT9 proteins. Imaging chambers were sealed with vacuum grease prior to imaging at 37 °C.

TIRF microscopy was performed with the DeltaVision OMX V4 inverted microscope (GE Healthcare) equipped with 60x/1.49 NA TIRF objective lens (Olympus), motorized stage, sCMOS pco.edge cameras (PCO), stage top incubator with temperature controller and the softWoRx software. Dynamic MTs and MT seeds were imaged every 5 s (Figures 1–3 and S1) or 2 s (Figure 4) for 15 min using 488 nm, 568 nm and 642 nm lasers with 100 ms exposure time.

3.5 | Image and statistical analyses

Analysis of MT dynamics and fluorescence intensity measurements were performed in ImageJ/Fiji. Time-lapse movies were bleach corrected using the bleach correction plugin. To quantify MT dynamics, kymographs were generated using the KymographBuilder plugin. To estimate growth and depolymerization rates, manually segmented lines were drawn on the trace of growing or shrinking MT plus or minus-ends, respectively. The Velocity measurement tool macro was used to calculate speed measurements. Catastrophe frequencies were measured by dividing the total number of catastrophe events by the total time spent growing.

To measure the fluorescence intensity of mCherry-SEPT9_i1 (Figure 3), line masks of equal width (three pixels) were made along the GMPCPP-stabilized seeds and the entire length of the lattice of plus end segments. Using the Fiji software, the average pixel intensity was derived after subtraction of the average pixel intensity of background fluorescence, which was quantified by taking the mean value from three regions that did not contain any MTs. The same method was used to measure the average pixel intensity of soluble fluorescent tubulin on GMPCPP-stabilized seeds (Figures 3h and 4). Fluorescence intensities in the line scans of Figure S1 were derived using the plot profile function of the Fiji software.

Statistical analysis was performed using GraphPad Prism software. Each data set was tested for normal distribution using the Kolmogorov–Smirnov test. Mean, SEM, and p values were derived using Student's t test for normally distributed data and the Mann–Whitney U test for non-normally distributed data.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

SEPT9 promotes the growth of MT plus ends. (a) Schematic shows the TIRF microscopy assay of MT dynamics. MT seeds were made with a mix of unlabeled tubulin and HiLyte488- and biotinylated-tubulin in the presence of GMPCPP and immobilized to glass chambers. To visualize the growth and shrinkage of MT minus and plus ends, a mix of soluble rhodamine-labeled and unlabeled tubulin (10 μ M) was added. (b) TIRF microscopy image of MT seed with polymerized minus and plus ends. (c) Coomassie-stained SDS-PAGE gel of the purified recombinant SEPT9_i1. (d-g) Dot plots show the mean ($\pm$ SEM) rates of MT plus end polymerization (d) and depolymerization (e), as well as the frequency of MT plus end catastrophe (f) and length of growth (g) in the absence of SEPT9_i1 ($n = 69$) or presence of 10 nM ($n = 85$), 100 nM ($n = 72$), 200 nM ($n = 73$), 400 nM ($n = 61$) and 800 nM ($n = 89$) of SEPT9_i1. (h-k) Kymographs show representative growth and shrinkage events of MT minus and plus ends (red) that extend from MT seeds (green) in the presence of 0 nM (h), 200 nM (i), 400 nM (j) and 800 nM (k) of SEPT9_i1. (l-o) Dot plots show the mean ($\pm$ SEM) rates of MT minus-end polymerization (l) and depolymerization (m), as well as the frequency of MT minus-end catastrophe (n) and length of growth (o) in the absence of SEPT9_i1 ($n = 48$) or presence of 10 nM ($n = 48$), 100 nM ($n = 36$), 200 nM ($n = 25$), 400

nM ($n = 32$) and 800 nM ($n = 51$) of SEPT9_i1. Ns: nonsignificant ($p > .05$). $*p < .05$, $**p < .01$, $***p < .001$, $****p < .0001$ [Color figure can be viewed at wileyonlinelibrary.com]

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FIGURE 2.

Regulation of MT plus end dynamics by SEPT9 requires a dynamic G-G interface of dimerization. (a) Amino acid sequences of SEPT2 (aa 251–274) and SEPT9 (aa 511–534) show the positions of the highly conserved histidine (H) and tryptophan (W) residues, whose interaction is critical for the maintenance of the G-G interface of dimerization. (b) The ribbon cartoons show the position of these residues in the X-ray crystal structure of SEPT2 (PDB: 3FTQ) and SEPT9 (PDB: 4YQF). (c–d) Coomassie-stained SDS-PAGE gels (c) of the fractions obtained from size-exclusion chromatography (Superdex 200) of SEPT9_i1 (top) and SEPT9_i1(H530D) (bottom). Chromatogram (d) shows that SEPT9_i1(H530D) elutes in volumes of lower size compared to the wild type SEPT9_i1. (e–h) Dot plots show the mean ($\pm$ SEM) rates of MT plus end polymerization (e) and depolymerization (f), as well as the frequency of MT plus end catastrophe (g) and length of growth (h) in the absence of SEPT9_i1 ($n = 48$) or in the presence of 800 nM of wild type SEPT9_i1 ($n = 37$) or SEPT9_i1(H530D) ($n = 37$). Ns: nonsignificant ($p > .05$). * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$ [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 3.

SEPT9 associates preferentially with GMPCPP-bound MTs. (a–b) Kymographs show the localization of mCherry-SEPT9_i1 (800 nM) to the lattice of stable GMPCPP MT seeds (a; magenta or dashed outline) and the lattice of dynamic MT plus end segments (b; green). Arrows point to sites of co-accumulation of mCherry-SEPT9_i1 and tubulin. (c–d) Kymographs show transient association of mCherry-SEPT9_i1 (400 nM) with the tip of a MT plus end (c) and diffusive movement of mCherry-SEPT9_i1 along the lattice of growing plus ends (d). (e) Bar graph shows the percentage of septin-decorated MTs, which contained mCherry-SEPT9_i1 only on the stable seed portion (magenta) or the plus end segment (green; lattice and tips), and on both seeds and plus end segments (blue). The percentage of MTs with mCherry-SEPT9_i1 localizing only to the minus-ends was very small (red). Quantification was performed for 400 nM ($n = 129$) and 800 nM ($n = 107$) of mCherry-SEPT9_i1. (f) Images show representative examples of HiLyte647-labeled MT seeds (magenta) after polymerization reactions with HiLyte488-tubulin (green) and mCherry-SEPT9_i1 at the indicated concentrations. MT seeds are outlined with dotted lines. Note that mCherry-SEPT9_i1 ($1 \mu\text{M}$) binds preferentially to the MT seed. Scale bars, $5 \mu\text{m}$. (g) Dot

plots show the mean ($\pm$ SEM) fluorescence intensity of mCherry-SEPT9_i1 on the seed and plus end (lattice and tips) portion of MTs. Quantification was performed from static images of MTs after 15 min of polymerization in the presence of 400 nM ($n = 42$), 800 nM ($n = 51$), 4 μ M ($n = 43$) and 8 μ M ($n = 45$) of mCherry-SEPT9_i1. (h) Dot plots show the mean fluorescence intensity ($\pm$ SEM) of soluble HiLyte488-tubulin on HiLyte647-labeled MT seeds in the presence of 0 nM ($n = 52$), 400 nM ($n = 42$), 800 nM ($n = 51$), 4 μ M ($n = 43$) and 8 μ M ($n = 45$) of mCherry-SEPT9_i1. Ns: nonsignificant ($p > .05$). * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$ [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 4.

SEPT9 recruits tubulin to the MT lattice and stabilizes MTs. (a) Dot plots show the mean ($\pm$ SEM) fluorescence intensity of soluble Rhodamine-tubulin on HiLyte488-labeled MT seeds in the presence of 400 nM ($n = 72$), 800 nM ($n = 64$), 4 μ M ($n = 72$) and 8 μ M ($n = 73$) of wild type SEPT9_i1 or 400 nM ($n = 73$), 800 nM ($n = 72$), 4 μ M ($n = 72$) and 8 μ M ($n = 72$) of SEPT9_i1(H530D). (b) Bar graphs show the percentage of stable and dynamic MTs in the presence of wild type SEPT9_i1 ($n = 25$ –28) or SEPT9_i1(H530D) ($n = 24$ –26). (c–d) Kymographs of tubulin polymerization from GMPCPP seeds in the presence of 4 or 8 μ M of wild type SEPT9_i1 (c) and SEPT9_i1(H530D) (d). Arrows point to the stabilized length of the MT plus ends and small arrowheads point to events of growth and shrinkage. Tubulin channel is shown in red and inverted monochrome. ns: nonsignificant ($p > .05$). * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$ [Color figure can be viewed at wileyonlinelibrary.com]