

Insight into microtubule nucleation from tubulin-capping proteins

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Nucleation is one of the least understood steps of microtubule dynamics. It is a kinetically unfavorable process that is templated in the cell by the γ -tubulin ring complex or by preexisting microtubules; it also occurs in vitro from pure tubulin. Here we study the nucleation inhibition potency of natural or artificial proteins in connection with their binding mode to the longitudinal surface of α - or β -tubulin. The structure of tubulin-bound CopN, a *Chlamydia* protein that delays nucleation, suggests that this protein may interfere with two protofilaments at the (+) end of a nucleus. Designed ankyrin repeat proteins that share a binding mode similar to that of CopN also impede nucleation, whereas those that target only one protofilament do not. In addition, an a Rep protein predicted to target two protofilaments at the (-) end does not delay nucleation, pointing to different behaviors at both ends of the nucleus. Our results link the interference with protofilaments at the (+) end and the inhibition of nucleation.

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n eukaryotic cells, microtubules form different types of arrays to fulfill different functions. For instance, a microtubule aster organizes the cytoplasm in interphase, whereas the mitotic spindle of dividing cells ensures faithful chromosome segregation. Generating and maintaining these arrays require that both the formation and the length of microtubules be controlled in space and time (1, 2). Microtubule assembly proceeds in two main steps. First a nucleus forms, and then it elongates at its free ends. The microtubule elongation phase and the subsequent behavior of microtubules have been characterized mainly by the description of a dynamic instability mechanism, with alternating periods of slow growth and faster shortening (3). In comparison, microtubule nucleation has remained far less well described (4, 5). Although the issue is debated (6), nucleation is generally considered a kinetically unfavorable process. To overcome this kinetic barrier, in the cell, nucleation is templated by the γ -tubulin ring complex (γ -TuRC) (7) in combination with, for instance, XMAP215 family proteins (8, 9), but also by preexisting microtubules (1, 10). Nucleation is further assisted by microtubuleassociated proteins (2, 11, 12).

Whereas several models for in vitro spontaneous nucleation (from a pure tubulin solution) have been proposed (summarized in ref. 6), recent characterizations of the interaction between tubulin molecules in the microtubule have allowed narrowing down the possible nucleation process. Indeed, longitudinal contacts (between tubulins within a protofilament) have been shown to be stronger that lateral contacts (between adjacent protofilaments) in the core of the microtubule (13–15) and at its growing end (16). Building on these results, it is also likely that lateral contacts in the nucleus are weaker that longitudinal ones (5), in agreement with electron microscopy experiments from tubulin

solutions in the early steps of assembly (17, 18). However, the exact pathway of microtubule nucleation is not understood.

Here we link the binding mode of proteins targeting tubulin surfaces involved in longitudinal contacts to their ability to inhibit microtubule nucleation, focusing on the CopN protein from Chlamydia pneumoniae. CopN has been shown to interfere with microtubule growth (19, 20). While a mechanism of tubulin sequestration has been proposed for the inhibition of microtubule elongation (20, 21), CopN also delays nucleation, suggesting direct interference with formation of the nucleus (21). We have determined the structure of CopN bound to tubulin. Modeling indicates that CopN would cap a protofilament at the (+) end of a microtubule in a way that would also prevent the elongation of a neighboring protofilament. Among various possible mechanisms, this model and the comparison with β-tubulin-specific designed ankyrin repeat proteins (DARPins) (22, 23) raise the hypothesis that microtubule nucleation is inhibited by the simultaneous destabilization of two adjacent protofilaments.

Significance

Microtubules are involved in many key functions of eukaryotic cells, including cell division, intracellular transport, and cell shape. They are hollow tubes made of parallel filaments, themselves formed by the self-assembly of $\alpha\beta$ -tubulin molecules. Whereas microtubules lengthen and shorten from their ends dynamically, their birth, called nucleation, remains poorly understood. To gain information on this process, we have determined the structure of tubulin bound to CopN, a bacterial protein that delays nucleation. Together with the behavior of artificial tubulin-binding proteins, our results lead to the hypothesis that targeting two filaments at the fast-growing end of the microtubule inhibits nucleation. They also suggest different dynamics at both ends of the nucleus.

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Results

CopN Inhibits Microtubule Nucleation. In the presence of CopN, the assembly of tubulin in microtubules as monitored by turbidity is delayed compared with the control (21). This observation led us to propose that CopN interferes with microtubule nucleation. To confirm this hypothesis, we directly counted the number of microtubules obtained from tubulin:CopN and tubulin-alone samples, setting the concentration of "free" tubulin (not bound to CopN) constant. For convenience, this experiment was first performed with GMPCPP-tubulin, which leads to stable microtubules (24). As observed with GTP-tubulin (21), CopN also increased the lag phase of the GMPCPP-tubulin assembly (Fig. 1A) and consistently decreased the nucleation rate (Fig. 1 B and C). A similar trend was observed with microtubules assembled from GTP-tubulin (SI Appendix, Fig. S1). Because a similar



Fig. 1. CopN inhibits microtubule nucleation. (A) Microtubule assembly of GMPCPP-tubulin monitored by turbidity. The turbidity traces in PMg buffer of tubulin and tubulin:CopN samples at the indicated concentrations are shown. Here as in the other turbidity experiments, except where indicated otherwise, the temperature was switched from 5 °C to 37 °C after 1 min of recording time, and the arrowheads indicate the reverse temperature switch. (Inset) Zoom-in of the first 10 min of the assembly kinetics. (B) Microtubule nucleation monitored by TIRF microscopy. A few green fluorescent GMPCPP microtubule seeds were immobilized on the kinesin-1 heavy chain-coated TIRF coverslip to precisely determine the coverslip surface plane. Red fluorescent microtubules appeared in the tubulin control, whereas none formed in the tubulin:CopN sample after 30 min. (Scale bar: 10 µm.) (C) Immunofluorescence images of microtubules nucleated from GMPCPP-tubulin alone or in the presence of CopN and fixed at different time points, as indicated (Left), and quantification of the number of microtubules (Right). Data are the mean ± SD of the counts in four random microscope fields per condition. (Scale bar: 10 µm.) (D and E) Microtubule length variation during assembly. Error bars represent SD from at least ~1,000 microtubules (SI Appendix, Methods).

turbidity plateau level was obtained with tubulin and tubulin:CopN (Fig. 1*A*), and because fewer microtubules were formed in this latter case (Fig. 1*C*), we expected that they grew longer. To verify this hypothesis, we recorded the distributions of microtubule lengths formed in these conditions. We found that microtubules grew longer along with the increase in turbidity in the presence of CopN, whereas microtubule length did not vary significantly in the tubulin control (Fig. 1 *D* and *E*). This observation is in agreement with the high nucleation efficiency of GMPCPP-tubulin (24) at the expense of elongation (25). In the presence of CopN, fewer nuclei being formed, microtubule elongation is favored.

CopN Binds to the Longitudinal Surface of the Tubulin β Subunit. Having established that CopN interferes with microtubule nucleation, to gain insight into this mechanism, we aimed to determine the structure of CopN bound to tubulin. Because our attempts to crystallize this binary complex were unsuccessful, we considered using a tubulin-stabilizing protein as a crystallization chaperone. Because the two most commonly used tubulin crystallization helpers-stathmin-like domain (SLD) proteins and β-tubulin targeting DARPins—compete with CopN for tubulin binding (20, 21), we instead used α Rep proteins (26, 27) specific for α -tubulin (28). Among these proteins, the iiiA5 α Rep interacted with tubulin with a dissociation constant $(K_{\rm D})$ in the nanomolar range and made a ternary CopN-tubulin-iiiA5 complex (SI Appendix, Fig. S2). Using the $\Delta 84$ CopN construct (20) (Fig. 2A), which binds to tubulin with an affinity similar to that of full-length CopN (Fig. 2B) and delays microtubule assembly (Fig. 2C), we obtained crystals that diffract X-rays to 3.2-Å resolution (SI Appendix, Table S1). The structure was solved by molecular replacement, with two virtually identical CopN-tubulin-iiiA5 complexes in the asymmetric unit (rmsd after superposition, 0.36 Å over 1,273 C α atoms of the complex) (29).

As expected from the α Rep selection strategy (28), iiiA5 binds to α -tubulin (Fig. 2D). Also consistent with previous biochemical characterizations (21), the CopN protein, which comprises three repetitions of a five α -helix motif, interacts with tubulin through a positively charged surface involving the second and third helical motifs and targets the surface of β -tubulin that is involved in longitudinal contacts along a protofilament and is mostly acidic (Fig. 2D and *SI Appendix*, Fig. S3*A*). The tubulin-interacting area of CopN also agrees with previous mutational studies (21) (*SI Appendix*, Fig. S3). Interestingly, whereas CopN inhibits the tubulin nucleotide exchange (21), it does not obstruct the β -tubulin nucleotide-binding site, with the closest CopN atom approximately 9 Å away from GDP (*SI Appendix*, Fig. S4). This observation suggests that nucleotide exchange requires tubulin conformational changes that are restricted by CopN.

Finally, the structure of CopN-tubulin-iiiA5 points to some CopN flexibility. In addition to third helical motif movement with respect to the second motif on tubulin binding (*SI Appendix*, Fig. S5), the first helical repeat, which does not interact with tubulin (Fig. 2D) and is not much involved in crystal contacts, gains mobility in the ternary complex, as reflected by discontinuities in the electron density maps. As expected, the residues that are N-terminal to this helical motif are disordered, as was already the case in the structures of CopN not bound to tubulin. The same goes for the approximately 15 residues at the CopN C-terminal end.

Modeling of CopN at the "blunt" (+) end of a microtubule indicates that the CopN footprint on tubulin is restricted to the β subunit surface involved in longitudinal interactions (within a protofilament) (Fig. 2*E*). This binding mode suggests that CopN may interfere specifically with the growth at the microtubule (+) end, reminiscent of the mechanism of DARPins, which also target this β -tubulin surface (23). We investigated this possibility next.



Fig. 2. Structure of CopN-tubulin-iiiA5. (A) CopN constructs used in this study. (B) Isothermal titration calorimetry (ITC) analysis of the $\Delta 84$:tubulin interaction and comparison with CopN:tubulin. (C) Turbidity traces in M2G1 buffer supplemented with 0.5 mM GTP of 9 µM tubulin and of 30:21 µM tubulin:∆84 and tubulin:CopN samples. (Inset) Electron micrograph of a negatively stained tubulin:CopN sample at T = 25 min, showing the formation of microtubules. (Scale bar: 200 nm.) Additional electron micrographs are provided in SI Appendix, Fig. S15. (D) Overview of the complex crystallized. The three helical motifs of CopN are rendered each in a different shade of blue (cyan, light blue, and deeper blue, going from the N-terminal to the C-terminal motifs). (E) Model of CopN bound to a blunt (+) end of a microtubule. This model was obtained by superposing β -tubulin from CopNtubulin-iiiA5 to β -tubulin in the atomic model of a microtubule (PDB ID code 3JAS) (14). Three protofilaments are shown, with CopN modeled at the (+) end of the middle one (colored). Views are perpendicular to the microtubule axis (Left) and along the microtubule axis (Right).

CopN Inhibits Microtubule Elongation with a Specific Effect at the (+) End. The elongation of individual microtubules was imaged using a total internal reflection fluorescence microscopy (TIRFM) assay. Stabilized GMPCPP-microtubule seeds were immobilized through their interaction with motility-blocked kinesin-1 heavy chain molecules coated on the TIRFM coverslip. As expected, elongation from both ends of the seeds was observed when GTPtubulin (12 μ M) was introduced in the TIRFM chamber (Fig. 3*A*, *Upper*). With a mixture of 24:36 μ M CopN:tubulin, essentially no microtubule growth occurred (Fig. 3*A*, *Lower*). More interestingly, when a 3:23 μ M CopN:tubulin mixture was studied, microtubules elongated only at one end (Fig. 3*B*).

To identify which of the two ends lengthened, we first injected ATP. In these conditions, the microtubule seeds glided thanks to the (+) end-directed motility of kinesin-1 and allowed us to unambiguously identify their leading (-) and trailing (+) ends (Fig. 3B, Upper). We then injected AMP-PNP to block kinesin motility, followed by the CopN:tubulin sample. In this way, we identified the (-) end as the elongating end (Fig. 3B, Lower).

Finally, when a small amount of CopN was added to (dynamic) microtubules that emanated from the seeds (2:22 μ M CopN:tubulin), we observed that microtubules still displayed a dynamic behavior similar to that of control microtubules during the first 15 min of

recording, with no significant differences in growth and shrinking speeds or in catastrophe and rescue frequencies (most of which occurred at the seed) (Fig. 3C and SI Appendix, Fig. S6). However, when microtubules were observed after \sim 1 h of dynamic instability (Fig. 3D), they ended as polarized polymers, reproducing the (–) end elongation patterns observed in Fig. 3B. These results indicate that at this low CopN concentration, the effect becomes visible only after a prolonged period, and that repeated exposures of the (+) ends of GMPCPP seeds (owing to dynamic instability) result in a late blockade of (+) end-microtubule elongation, similar to what occurs when microtubule seeds are elongated in the presence of CopN.

Taken together, the foregoing experiments point to a specific inhibitory mechanism of CopN on elongation of dynamic microtubules at their (+) end. Therefore, the mechanism of CopN is more complex than previously proposed (20, 21) (*SI Appendix*, Fig. S7).

Although the modeling shown in Fig. 2*E* indicates that CopN interferes with longitudinal contacts within a protofilament, it does not point to interference with lateral contacts between protofilaments. Because this last feature is shared by the tubulin-targeting DARPin D1 and its high-affinity variants (23, 30), we investigated the interference of these DARPins with microtubule nucleation, using the optimized TM-3 DARPin as a reference. In conditions under which CopN (21) and $\Delta 84$ significantly delay



Fig. 3. CopN inhibits elongation at the (+) end of dynamic microtubules. (A) TIRFM images of microtubule elongation from GMPCPP seeds with tubulin alone (Top) or a mixture of CopN and tubulin (Bottom). At high concentration, CopN inhibits elongation at both microtubule ends in these TIRFM conditions. (Scale bar: 5 µm.) (B) At low CopN concentration, elongation is inhibited only at the (+) end. Microtubule seed polarity was determined through a sequential gliding and elongation assay (SI Appendix, Methods). (Top) Two images from the gliding sequence time lapse were superimposed to determine the gliding direction, the (-) end in front, (Bottom) The same field was recorded after elongation with a CopN+tubulin mixture (red). (C and D) Microtubules were elongated in the absence of CopN for 10 min. after which CopN was added into the chamber when appropriate. Microtubule dynamics were recorded for 15 min, showing dynamic instability in both control and CopN conditions, as shown on kymographs (C). (Scale bars: 5 µm for horizontal, 1 min for vertical.) Microtubules were also imaged at 45 min after the end of dynamics recording (D).

nucleation (Figs. 1 and 2*C*), turbidity experiments did not indicate that TM-3 has such an effect (Fig. 4*A* and *SI Appendix*, Fig. S8). These puzzling results give no indication of the mechanism of microtubule nucleation inhibition by CopN. We considered several hypotheses (schematized in *SI Appendix*, Fig. S9) to account for this activity.

Hypotheses for CopN Interference with Microtubule Nucleation. Several mechanisms may account for the inhibition of microtubule nucleation. A first possibility could be that CopN stabilizes tubulin in a conformation not compatible with either the formation or the elongation of the nucleus. If this conformation is different from the one(s) adopted during nucleation, the binding of CopN or of a CopN-tubulin complex to a nascent nucleus would destabilize it. Alternatively, CopN might have a higher affinity for tubulin molecules embedded in the nucleus than for tubulin in solution, thereby preventing elongation. Both cases imply that the structure of tubulin bound to CopN has unique features.

To evaluate the first possibility, we compared the structure of CopN-bound tubulin with that of tubulin bound to the TM-3 DARPin (*SI Appendix*, Table S1) (31), the binding site of which overlaps with that of CopN on the β subunit longitudinal surface (Fig. 4*B*). The conformations of tubulin are very close in both complexes, with an rmsd of 0.71 Å (842 tubulin Cas aligned). This low rmsd value reflects both a very similar relative orientation of the α and β subunits and a conserved conformation of tubulin bound to CopN is also very close to that of tubulin in complex with an SLD (*SI Appendix*, Fig. S10); therefore, we conclude from this analysis that the stabilization of a peculiar tubulin conformation by CopN is not a likely mechanism to explain the interference of this protein with microtubule nucleation.

A second possibility is the involvement of a mobile region of CopN. By definition, such a mechanism cannot be rationalized based on the crystal structure of the complex. In isolated CopN, the main flexible regions are the N-terminal (approximately 95 residues) and C-terminal (approximately 15 residues) ends (21). The disordered N-terminal region is narrowed down to approximately 10 residues in $\Delta 84$ (32), used to crystallize the CopN-tubulin-iiiA5 complex. To assess the contribution of the floppy ends of CopN to the inhibition of microtubule nucleation, we prepared $\Delta 92$, a construct starting at residue 93, and Δ Cter, the C-terminal counterpart construct ending at residue 385. These proteins lacked either the N-terminal or the C-terminal mobile region of CopN (Fig. 24). Both $\Delta 92$ and Δ Cter behaved like



Fig. 4. The TM-3 DARPin does not delay microtubule assembly, whereas both CopN and TM-3 recognize the same tubulin conformation. (A) Turbidity traces in M2G1 buffer supplemented with 0.5 mM GTP of tubulin:TM-3 samples and comparison with tubulin and tubulin:Δ84, as indicated; see also *SI Appendix*, Fig. S8. (B) The structure of tubulin from CopN-tubulin-iiiA5 (colored as in Fig. 2D; iiiA5 is not shown) superimposed on that of tubulin in complex with TM-3 (gray).

Fig. 5. The Δ 92 and Δ Cter CopN constructs delay microtubule nucleation. (*A*) Dissociation constants determined by ITC between tubulin and Δ 92 or Δ Cter. (*B*) Turbidity traces of 15 μ M tubulin (black curve) or of a mixture of 30 μ M tubulin with 15 μ M Δ 92, Δ Cter, or CopN in M2G1 buffer supplemented with 0.5 mM GTP. KCl (75 mM) was also added to the assembly buffer to prevent aggregation in the Δ 92 and Δ Cter samples.

CopN in terms of affinity for tubulin (Fig. 5A) and delay of microtubule assembly, as evaluated in the turbidity assay (Fig. 5B). These results rule out a main contribution of the disordered N- and Cterminal regions of CopN to microtubule nucleation inhibition.

A third hypothesis relies on the hydrolysis of GTP in tubulin molecules forming the nucleus, with an enhanced GTP hydrolysis rate leading to (GDP-tubulin) oligomers unproductive for nucleation (12, 33). Does CopN, which inhibits nucleotide exchange (21), favor GTP hydrolysis and then trap tubulin in a GDP-bound state? To address this question, we analyzed the variation of the nucleotide content of GTP-tubulin incubated alone or in the presence of excess CopN. Under conditions of microtubule assembly (Fig. 6A), GTP hydrolysis occurred in the sample without CopN (Fig. 6B), as expected (13, 34). In the presence of excess CopN, microtubule assembly was inhibited (Fig. 6A), and no GTP hydrolysis occurred after 90 min of incubation (Fig. 6B). The most likely explanation for this finding is that CopN, by preventing microtubule assembly, indirectly inhibits the associated GTP hydrolysis. We also studied the nucleotide content variation in nonassembly conditions (i.e., similar tubulin concentration, but incubation at room temperature in a glycerol-free buffer). In this case, GTPase activity developed on a time scale of hours, with no significant difference between the tubulin control and the CopN-tubulin complex (Fig. 6C).

Taken together, these results indicate that the mechanism of microtubule nucleation inhibition by CopN does not involve enhanced GTPase activity of tubulin. Consistently, CopN also inhibited tubulin assembly in the presence of the stable GTP analog GMPCPP (Fig. 1 and *SI Appendix*, Fig. S7).

To account for the CopN interference with microtubule nucleation, we considered a fourth hypothesis, as discussed next.

Interfering with the Elongation of Two Protofilaments at the Nucleus (+) **End Inhibits Microtubule Nucleation.** As shown in Fig. 2*E*, a CopN molecule positioned at the blunt (+) end of a microtubule is not expected to destabilize neighboring protofilaments. However, if these protofilaments are growing, clashes with the incoming tubulin heterodimers may occur, the closest interatomic distance being approximately 3 Å with tubulins positioned as in the microtubule core (Fig. 7*A*). In contrast, the same modeling but with TM-3 does not indicate such a steric hindrance (Fig. 7*B*). In other words, one CopN molecule may target the growth of two protofilaments, compared with one protofilament in the case of TM-3. This difference may provide the basis for the different effects of these proteins on microtubule nucleation.

To explore this hypothesis further, we expanded the study to other proteins that target one or the other end of microtubules. We first considered proteins that bind to α -tubulin. Because tubulin aggregation was observed in presence of the iiiA5 α Rep



Fig. 6. The CopN activity is not related to GTP hydrolysis by tubulin. (A) Turbidity traces of 20 μ M GTP-tubulin (without excess nucleotide) either alone or in the presence of 30 μ M CopN, incubated at 37 °C in M2G1 buffer. (B) Nucleotide content analysis by ion exchange chromatography of the samples of A. In the case of the GTP-tubulin assembly control, all tubulin was in the GDP form as soon as turbidity reached its maximum value (after approximately 3 min of incubation, marked by a star in A) and, as expected, at the end of the experiment (at T = 90 min) (n = 3). In the presence of CopN, only 0.5 \pm 0.5% (n = 2) of tubulin hydrolyzed its GTP at T = 90 min. a.u., arbitrary units. (C) Variation of the nucleotide content of 18 μ M GTP-tubulin incubated either alone or in presence of 30 μ M CopN, at room temperature and in a glycerol-free buffer. Error bars represent SD from two independent experiments. Student t test analysis did not show a significant difference between the two samples (P > 0.30).

(SI Appendix, Fig. S11), we instead studied two other α Reps that target the longitudinal surface of α -tubulin (28). One of these, iiH5, binds in a way that would prevent the elongation of two protofilaments when modeled at the microtubule (-) end: the one to which it would be bound and, through its protruding Ccap and last internal repeat, an adjacent one. In contrast, modeling predicts that only one protofilament would be impacted in the case of the iE5 α Rep (Fig. 7C). Therefore, iiH5 and iE5 are microtubule (-) end counterparts of the (+) end-targeting proteins CopN and TM-3, respectively. However, and in agreement with previous characterizations (28), neither of the two α Reps delayed microtubule nucleation in the turbidity assay (Fig. 7 D and E). A tandem repeat version of the iiH5 α Rep displayed the same behavior (SI Appendix, Fig. S12). The different effects of CopN and iiH5, both of which are predicted to interfere with the elongation of two adjacent protofilaments, are unlikely to stem from an affinity issue, given that the dissociation constant between tubulin and these proteins is in the same range of values (Fig. 2B) (21, 28). A possible explanation is that the protruding elements of iiH5 are mobile, although a Reps have been characterized as stable proteins (26). Another possibility is that the two ends of a nucleus exhibit different behaviors, in which case the binding of (potentially) disturbing proteins would have different consequences. Further experiments with additional endtargeting proteins are needed to clarify this point.

We next studied the effect of proteins that bind to the longitudinal surface of β -tubulin, in addition to CopN and TM-3. For this, we again used tubulin-binding DARPins for which the structure in complex with tubulin is available, namely D1 (23), D2 (35), and F3II (36) (SI Appendix, Fig. S13 and Table S1). D1 shares with TM-3 a very similar binding mode to tubulin. One major difference is that the C-terminal capping motif (C-cap) is disordered in TM-3. In D1, the C-cap is ordered and would come closer to an adjacent tubulin, but without leading to obvious clashes (Fig. 7B). When D1 was tested in a microtubule assembly assay under conditions in which part of the tubulin is in complex with the DARPin, the turbidity curve indicated that D1 delays microtubule assembly, but the effect is substantially less pronounced than that of CopN (Fig. 7F). This assay also indicated that tandem repeat DARPins built from TM-3 or D1 do not delay microtubule nucleation (SI Appendix, Fig. S12).

The D2 and F3II DARPins share a similar orientation on tubulin but are shifted by approximately 1.5 ankyrin repeats relative to each other (*SI Appendix*, Fig. S13). Consequently, whereas there would be severe steric hindrance between D2 modeled on a microtubule protofilament and an incoming tubulin bound to an adjacent one (Fig. 7*B*), predicting a strong impact on microtubule nucleation, the modeling of F3II on a microtubule (+) end did not indicate any disturbance with an adjacent growing protofilament (Fig. 7*B*), suggesting a different effect on nucleation. Consistently, D2 behaved similarly to CopN in the turbidity assay (Fig. 7*F*), whereas the turbidity curve in presence of F3II was similar to that of the tubulin control (Fig. 7*F* and *SI Appendix*, Fig. S14), indicating that this DARPin does not delay microtubule nucleation.

In summary, our results establish a link between the interference with protofilaments at the (+) end and the inhibition of microtubule nucleation.

Discussion

Nucleation is arguably one of the least understood steps of microtubule dynamics (4). With the use of proteins that target different tubulin epitopes exposed at both ends of a microtubule, our work provides insight into several characteristics of microtubule nucleation in the absence of nucleating factors. First, the turbidity assay did not point to any specific effect of proteins that target one single protofilament, as is the case of the α -tubulin binding α Rep iE5 and of the β -tubulin-specific DARPins TM-3 and F3II, leading to the hypothesis that such a binding mode at either end of a nucleus might not be sufficient to destabilize it.



Fig. 7. Model of the interference of CopN, DARPins, and αReps with elongating protofilaments, related to the inhibition of microtubule nucleation. (A) Model of CopN bound at the (+) end of a microtubule that elongates. Compared with Fig. 2E, the adjacent protofilament closest to bound CopN is elongated by one tubulin positioned as in a microtubule. Here and in B, views are from the outside of the microtubule, with the (+) end at the top of the figure. (B) Same as in A, but the modeling is with DARPins. In the case of D1/TM-3 (Left), the C-cap, which is ordered in D1 and disordered in TM-3, is shown in darker blue. (C) Models of α Reps bound to the microtubule (–) end and interference with elongating protofilaments. Views are from the interior of the microtubule. (D–F) Effect on microtubule assembly of α Reps and of DARPins. In D and F, the turbidity signal was recorded in M2G1 buffer with 0.5 mM GTP and compared with that of tubulin and tubulin:∆84 taken from Fig. 4A. In E, the buffer was supplemented with 75 mM KCl to prevent aggregation, and the tubulin and tubulin:CopN reference curves are from Fig. 5B. (D) iE5 does not delay microtubule nucleation. The assembly of 30 μ M tubulin with 20 μ M iE5 is similar to that of the 10 μ M tubulin control. (E) iiH5 does not delay microtubule nucleation. The assembly of tubulin:iiH5 samples at the indicated concentration (blue and orange curves) is shown. (F) Effects of D1, D2, and F3II DARPins. In each case, 30 µM tubulin was mixed with a DARPin concentration (~18 μ M) chosen so as to reach a turbidity signal at the plateau similar to that of the 10 μ M tubulin control. In the case of F3II, 20 μM tubulin and 18 μM F3II were incubated for 12.5 min in M2G1 buffer, followed by the addition of GTP and of 10 µM extra tubulin, corresponding to T = 0 of the curve (SI Appendix, Fig. S14).

After these proteins dissociate from the nucleus, protofilaments are able to regrow to sustain elongation. It should be noted that these proteins may be removed as a complex with tubulin, instead of dissociating by themselves. This is most likely the case for TM-3, because this DARPin dissociates very slowly from tubulin ($k_{off} < 0.3 \text{ min}^{-1}$) (30), compared with the off-rate constant of tubulin molecules from growing microtubule (+) end, which is three orders of magnitude faster (~15 s⁻¹) (37).

How does CopN inhibit microtubule nucleation? Based on the structure of the CopN-tubulin complex, we elaborated several hypotheses. Our experiments allowed us to exclude most of them. We end up with a scheme in which proteins that hinder the growth of two adjacent protofilaments at the microtubule (+) end delay nucleation. Experiments at the single protofilament resolution are needed to support this mechanism. We also have shown that among the proteins that interfere with two protofilaments, only those targeting the nucleus (+) end delay nucleation. These proteins include CopN and the D2 DARPin. Their (-) end counterpart iiH5 aRep has no influence on nucleation, as can be inferred from our turbidity assay. Although this last feature remains to be confirmed with additional proteins, these observations might point to a dissymmetry of the nucleus in terms of nucleation. The two ends of an elongating microtubule display different dynamic behaviors, with the (+) end growing faster and experiencing more frequent catastrophe events (38). Our results suggest that the polarity of microtubules also has implications starting in the early stages of nucleation.

The CopN protein is a *Chlamydia* effector secreted in the host cytoplasm during infection (39), but its function remains unclear (40). Our work raises the hypothesis that it acts as an inhibitor of microtubule nucleation. Relatedly, it might favor an array with

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fewer but longer microtubules. Because CopN binds to a tubulin surface at the opposite end to that targeted by the γ -TuRC (7), it might target γ -TuRC–templated nascent microtubules. However, the abundance of CopN is expected to be low (41), and the inhibitory effect on microtubule nucleation is not very efficient (21) (Figs. 1 and 2*C*). One possible way for CopN to have a significant effect on nucleation could be by concentrating in subcellular compartments, as has been shown in vitro for microtubule-associated centrosomal proteins (42). Further studies are needed to understand the effect of CopN on microtubules and its relevance in the *C. pneumoniae* infectious cycle.

Methods

Experimental procedures for the expression and purification of CopN, DARPin, and αRep proteins and for tubulin purification are described in *SI Appendix, Methods.* Microtubule assembly was monitored in bulk by turbidity and at the single microtubule level by TIRFM, as described in *SI Appendix, Methods.* Crystal structures of CopN-tubulin-iiiA5, tubulin–TM-3, and tubulin-F3II-R1 were determined as described in *SI Appendix, Methods.*

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