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Microtubule nucleating γ TuSC assembles structures with 13-fold microtubule-like symmetry

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Abstract

Microtubules are nucleated *in vivo* by γ -tubulin complexes. The 300 kDa γ -tubulin small complex (γ TuSC), consisting of two molecules of γ -tubulin and one copy each of the accessory proteins Spc97p and Spc98p, is the conserved, essential core of the microtubule nucleating machinery^{1,2}. In metazoa multiple γ TuSCs assemble with other proteins into γ -tubulin ring complexes (γ TuRCs). The structure of γ TuRC suggested that it functions as a microtubule template^{2–5}. Because each γ TuSC contains two molecules of γ -tubulin, it was assumed that the γ TuRC-specific proteins are required to organize γ TuSCs to match thirteen-fold microtubule symmetry. Here, we show that γ TuSC forms rings even in the absence of other γ TuRC components. The yeast adaptor protein Spc110p stabilizes the rings into extended filaments and is required for oligomer formation under physiological buffer conditions. The 8Å cryo-EM reconstruction of the filament reveals thirteen γ -tubulins per turn, matching microtubule symmetry, with plus ends exposed for interaction with microtubules, implying that one turn of the filament constitutes a microtubule template. The domain structures of Spc97p and Spc98p suggest functions for conserved sequence motifs, with implications for the γ TuRC-specific proteins. The γ TuSC filaments nucleate microtubules at a low level, and the structure provides a strong hypothesis for how nucleation is regulated, converting this less active form to a potent nucleator.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Author Contributions J.M.K. purified and prepared samples for EM, collected cryo-EM data, determined the structure, and performed microtubule nucleation experiments. J.K.P. explored γ -TuSC assembly conditions and prepared and imaged capped microtubules. A.Z. designed and cloned expression constructs, and generated and tested baculovirus strains. D.A.A and J.M.K. designed experiments and analyzed data. J.M.K., D.A.A. and T.N.D. wrote the paper. All the authors discussed the results and commented on the manuscript.

Author Information The cryo-EM reconstruction has been deposited with the Electron Microscopy Database with the accession code 1731. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests.

Microtubules assembled *in vitro* have a broad distribution of protofilament numbers centred around fourteen⁶. However, microtubules nucleated in cells have mostly thirteen protofilaments⁷, suggesting that γ -tubulin complexes constrain microtubule geometry. Thirteen-fold symmetry is likely preferred as it allows the protofilaments to run straight along the microtubule (as opposed to being twisted in other protofilament symmetries), allowing motor proteins tracking processively to remain on one face of the microtubule. It has generally been assumed that γ TuRC-specific proteins form a cap-like structure that establishes thirteen-fold symmetry by providing a scaffold for γ TuSC assembly. How γ TuSC is organized in organisms like *Saccharomyces cerevisiae*, which lack all of the γ TuRC-specific proteins, has remained an open question.

The sequence and structural similarity between γ - and α/β -tubulin suggested that nucleation results from microtubule-like contacts between γ - and α/β -tubulin⁸. The microtubule lattice consists of lateral and longitudinal contacts^{9,10}; the stronger longitudinal contacts define microtubule polarity, with “plus” and “minus” ends (Supplementary Fig. 1). The template model for microtubule nucleation predicts that γ -tubulins interact laterally to form a ring which makes longitudinal contacts with α/β -tubulin^{2–5} minus ends; alternative models that predict lateral interactions between γ - and α/β -tubulin¹¹ have not been definitively ruled out.

Our previous 25 Å structure of *Saccharomyces* γ TuSC was determined in buffer conditions that yielded predominantly monomeric complexes^{12,13}. Here, we show that buffer conditions that promote microtubule growth (BRB80: low salt, pH 6.9) also promote spontaneous assembly of γ TuSCs into rings similar to *Drosophila* γ TuRCs^{2,4} (Fig. 1a,b). Ring formation was sensitive to both salt and pH (Supplementary Fig. 2a–c). The γ TuSC rings bound microtubules, and many of these microtubule ends are capped (Fig. 1c), similar to microtubules nucleated *in vivo*^{14,15} or from γ TuRCs *in vitro*^{3,4,16}. Spontaneous assembly suggests that ring formation is an intrinsic property of γ TuSC, and not dependent on γ TuRC-specific proteins.

The N-terminal 220 residues of Spc110p (Spc110p^{1–220}), which attaches γ TuSC to the nuclear face of the yeast spindle pole body, dramatically increased the stability and length of γ TuSC assemblies (Fig. 1d). Copurification with Spc110p^{1–220} yielded a continuum of γ TuSC oligomers ranging from dimers to long, well-ordered helical filaments, even under conditions where γ TuSC alone fails to assemble (Supplementary Fig. 2d–f). We determined the three-dimensional structure of γ TuSC filaments from cryo-electron micrographs (Fig. 2a), using iterative helical real space reconstruction, a single particle approach to helical structure determination¹⁷. The resolution of the reconstruction, which included about 25,000 γ TuSC subunits, was estimated at 8 Å by the Fourier shell correlation 0.5 cutoff (Fig. 2b).

The filament is a single spiral of laterally associated γ TuSCs, without contact between layers (Fig. 2c,d; Supplementary Movie 1). The helical symmetry (54.3° rotation and 22.2 Å rise per subunit) gives rise to just over six and a half γ TuSCs – or thirteen γ -tubulins – per turn, with a half γ TuSC overlap. Each turn of helix forms a lock-washer shape similar to γ TuRC4 (Fig. 2e). The thirteen-fold γ -tubulin symmetry of the filament is dictated largely by the

extensive lateral interactions between Spc97p and Spc98p of adjacent γ TuSCs (Figure 2e, Supplementary Fig. 6a), locking in the lateral tubulin contacts which on their own are flexible enough to accommodate a range of different symmetries. We propose that a γ TuSC assembly very similar to a single ring from the filament provides the constraint that limits microtubules to 13 protofilaments in all eukaryotes *in vivo*⁷.

γ TuSC in the filament is remarkably similar to free γ TuSC^{13,18} (Fig. 2f, Supplementary Fig. 3), indicating that oligomerization does not induce large scale conformational changes. The 8 Å structure provides new insight into the domain architecture of Spc97p and Spc98p. Spc97p and Spc98p dimerize at their N-terminal ends nearest the helical axis, and have extended central domains connecting to C-terminal γ -tubulin binding domains. The central domain of Spc98p is kinked, at a position previously shown to be the site of limited hinge-like flexibility¹³. The masses of the domains determined from the cryo-EM map provide a rough estimate of their boundaries in each sequence, indicating the positions of the grip1 and grip2 motifs, conserved in all γ -tubulin complex proteins¹⁹ (Supplementary Fig. 4a,b). The grip2 motif covers nearly half of the C-terminal domains, strongly suggesting that it is important for γ -tubulin binding. The grip1 motif is in the central domain, near inter- γ TuSC contacts and the kink in Spc98p. We tentatively assign Spc110p¹⁻²²⁰ to a ridge of density running along the exterior face of γ TuSC in the filament, making contacts primarily with Spc98p (Supplementary Fig. 3). The resolution of the reconstruction appears to be nonuniform, as tubes of alpha helical density are clear in the N-terminal domains of Spc97p and Spc98p at the core of the structure, while secondary structure features are not well defined in the peripheral density where γ -tubulin is located (Supplementary Fig. 5). The lower effective resolution in the γ -tubulin regions may be due to limited flexibility in the weak connections between the central and C-terminal domains of Spc97p and Spc98p.

The human γ -tubulin crystal structure²⁰ was fit into the density in the regions previously assigned to γ -tubulin¹³ (Fig. 3a,b). The minus end longitudinal surface of γ -tubulin is completely buried in the interface with Spc97p/Spc98p. The lateral contacts between γ -tubulins of neighbouring γ TuSCs are nearly identical to microtubule lateral contacts. The two γ -tubulins within each γ TuSC are skewed slightly apart, in a configuration incompatible with the microtubule lattice (Fig 3b,c), as observed in the free γ TuSC structure¹³. This symmetry gives rise to an alternating pattern of γ -tubulin pairs with microtubule-like lateral spacing separated by gaps, generating a staggered mismatch with the microtubule lattice (Fig. 3c–e). The only microtubule lattice surface of γ -tubulin fully exposed in the filament is the plus end face, favouring a model in which γ -tubulin makes longitudinal contacts with α/β -tubulin. This, combined with the thirteen-fold γ -tubulin symmetry, provides the strongest evidence to date to support a γ -tubulin template mechanism for microtubule nucleation.

We tested the capacity of γ TuSC oligomers to nucleate microtubules (Fig. 4). At pH 6.9 both γ TuSC alone and γ TuSC filaments provided modest levels of nucleation, slightly higher for γ TuSC filaments than γ TuSC alone. At pH 7.5 γ TuSC alone does not nucleate microtubules, whereas the filaments retain a low level of nucleation. As γ TuSC rings do not form at pH 7.5, but γ TuSC filaments remain intact (Supplementary Fig. 2b,c), these results suggest that γ TuSC nucleation activity is assembly-dependent. The levels of nucleation

observed are consistent with previous measurements for γ TuSC_{2,21}, but less robust than seen with γ TuRC_{2,5}, suggesting that assembly alone is insufficient to fully activate γ TuSC nucleating activity. The arrangement of γ -tubulin in γ TuSC oligomers provides a structural explanation for their relatively modest nucleating activity. Nucleation likely arises from the inter- γ TuSC γ -tubulin pairs, which have the correct microtubule lattice spacing. Simulations indicate that a γ TuSC assembly in which all of the γ -tubulins make lateral microtubule-like contacts would provide greatly enhanced nucleation (L. Rice, personal communication).

The structure provides a clear hypothesis for how nucleation could be fully activated. We previously predicted that bending at the flexible kink in Spc98p is required to bring the intra- γ TuSC γ -tubulins to the microtubule spacing^{13,18}. We speculated that γ TuSC assembly might drive this change, but that clearly is not the case – a similar conformational change is still required in γ TuSC rings. In the lower resolution γ TuSC structure we predicted that the movement would be a closure of the gap between γ -tubulins; here we see that the movement must be more perpendicular to the edge of the ring, bringing γ -tubulin in toward the helical axis. A rotation of 23° about the kink in Spc98p would reposition γ -tubulin by 26 Å, bringing it to the microtubule lattice spacing (Supplementary Fig. 7, Movies 2 and 3). The staggered γ -tubulin arrangement likely serves a regulatory function, maintaining γ TuSC oligomers in a low activity state until a signal (protein binding, post-translational modification, etc.) directs the rearrangements in Spc98p necessary to form a template with exact microtubule lattice geometry. Although less likely, rearrangement of the γ -tubulins could be induced by binding of α/β -tubulin. In such a model γ TuSC would function primarily as a cap for stabilizing and localizing microtubule minus ends, rather than as a strong nucleator.

The dramatic enhancement of γ TuSC oligomer stability by Spc110p, combined with its role in γ TuSC localization, likely serves to ensure that microtubule template assembly in yeast occurs only at the spindle pole body. We propose a general model for microtubule nucleation in which Spc110p or its functional equivalent directly attaches γ TuSC to microtubule organizing centres, promoting template assembly. A subsequent activation step then fully activates nucleation by rearranging the γ -tubulin network (Fig. 5a). In a template with seven γ TuSCs, the location of the half γ TuSC overlap defines the position of the 13 protofilament microtubule seam; a single lateral contact between γ -tubulin and α -tubulin would be made at the overlap, as well. It is unclear how many γ TuSCs are required to nucleate a microtubule – an incomplete ring may be sufficient to initiate growth.

The γ TuSC filament structure provides unique insight into the roles of γ TuRC-specific proteins. Our results clearly show that γ TuSC assembly alone establishes thirteen-fold γ -tubulin symmetry, indicating that the γ TuRC-specific proteins are not required as a scaffold. This is consistent with the observation that all of the γ TuRC-specific proteins can be depleted without affecting centrosomal microtubule nucleation of thirteen protofilament microtubules^{22,23}. While not required as a scaffold, the γ TuRC-specific proteins may serve to stabilize the ring structure or fully activate nucleation activity, and they are essential for γ TuRC localization at non-centrosomal sites, as in augmin-dependent binding within the mitotic spindle²⁴ (Fig. 5b).

We suggested above that the grip1 and grip2 motifs, conserved in all the γ TuRC-specific proteins, are involved in ring assembly contacts and γ -tubulin binding, respectively. This raises the intriguing possibility that the γ TuRC-specific proteins may each bind γ -tubulin and substitute for Spc97p or Spc98p in the ring itself. To do this, they might form hybrid γ TuSCs with one of the γ TuRC-specific proteins plus Spc97p or Spc98p, alternative γ TuSCs with two different γ TuRC-specific proteins, or unique half γ TuSCs (Supplementary Fig. 8). Such alternative γ TuSCs might serve to initiate or terminate γ TuSC oligomerization, or to stabilize the ring at the overlapping ends, while providing unique attachment sites in the structure of the ring itself.

Methods Summary

γ TuSC was co-expressed with GST-Spc110p¹⁻²²⁰ as described^{13,21}, except that complexes were eluted by cleavage of the GST tag with TEV protease as the final purification step. γ TuSC rings were formed by 30 minute incubation on ice after dilution to 0.2 μ M in BRB80 (80 mM PIPES pH 6.9, 1mM EGTA, 1mM MgCl₂). Nucleation assays were performed essentially as described²¹. The cryo-EM reconstruction was performed essentially as described by Egelman¹⁷ and Sasche, et al.²⁵.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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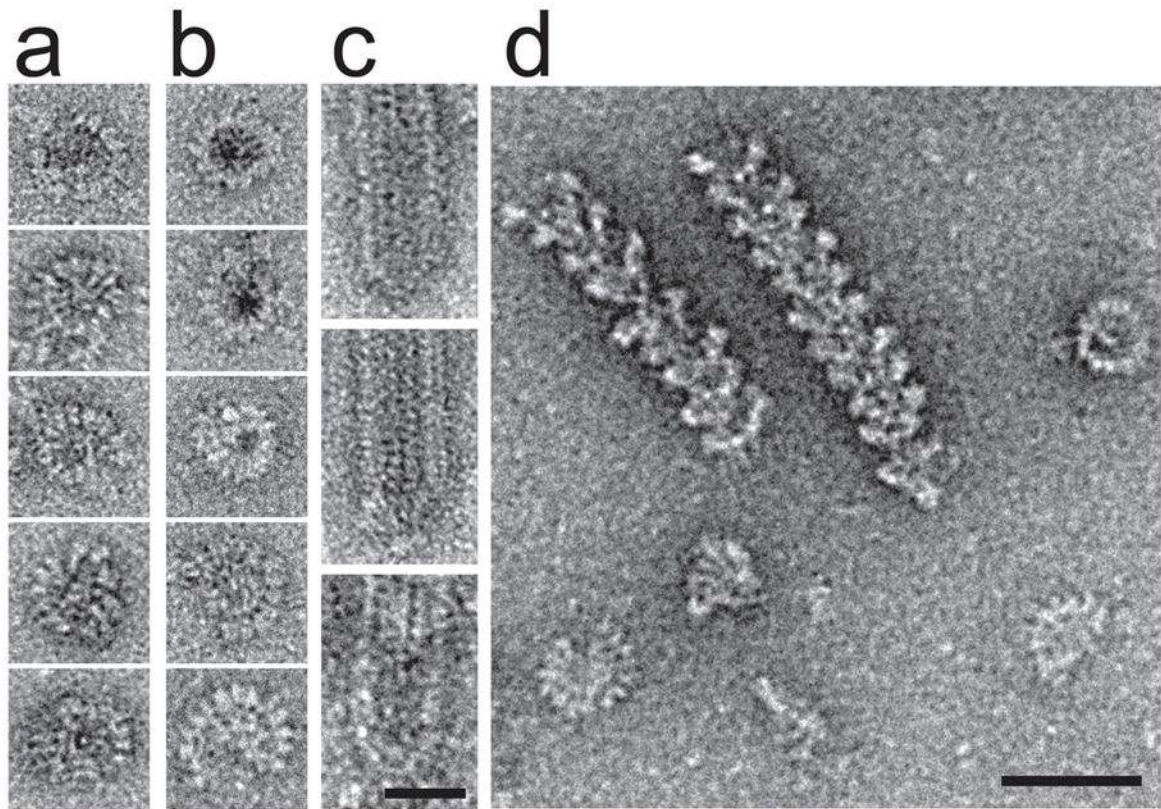


Figure 1. γ TuSC oligomers form spontaneously and are stabilized by Spc110p

a) Ring-like structures were observed in negative stain electron micrographs of *Saccharomyces* γ TuSC at pH 6.9. b) γ TuRC purified from *Drosophila* embryos is similar in shape and size to the γ TuSC rings. c) Capped ends were observed on microtubules grown in the presence of pre-formed γ TuSC rings. Scalebar for a, b, and c, 25 nm. d) Negative stain images of γ TuSC filaments formed upon copurification with Spc110p¹⁻²²⁰. Scalebar, 50 nm.

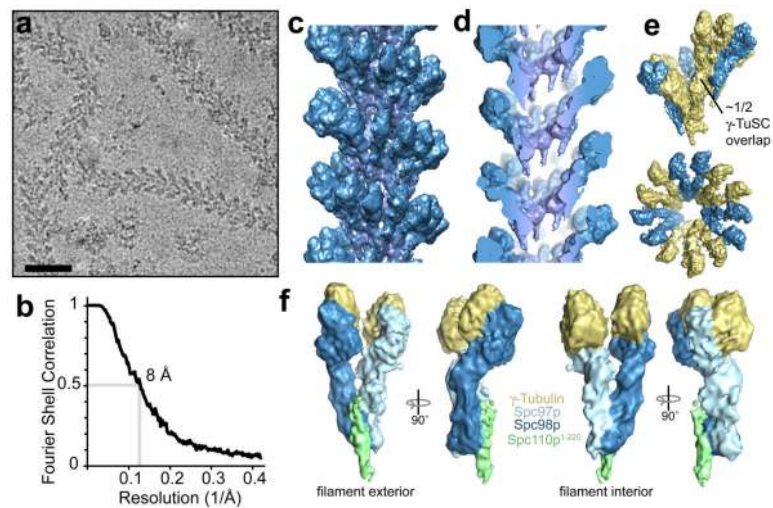


Figure 2. γ TuSC filament structure

a) Cryo-electron micrograph of γ TuSC filaments. Scalebar, 50 nm. b) The resolution of the structure is estimated at 8 Å by the Fourier shell correlation. c) A segment of the reconstructed filament filtered to 8 Å. d) A cutaway view of the filament, illustrating the lack of connection between helical layers. e) One turn of the helix, coloured by γ TuSC. The filament has six and a half γ TuSCs per turn, with a half γ TuSC overlap. f) A single γ TuSC/Spc110p¹⁻²²⁰ subunit from the filament, with the approximate boundaries between the individual proteins indicated by colour.

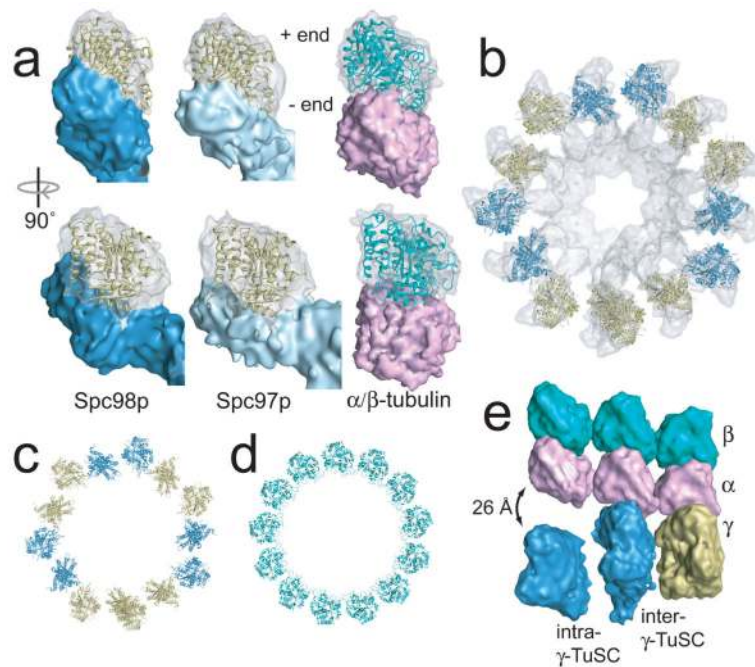


Figure 3. γ -tubulin in the γ TuSC filament has a geometry similar to 13-protofilament microtubules

a) The EM density of γ -tubulin (transparent) and the Spc97p/Spc98p binding domains (opaque) are shown with the γ -tubulin crystal structure fit in the density. The density is tilted relative to the helical axis so that in each case the plus end is vertical. An α/β -tubulin heterodimer is shown with simulated EM density for comparison. b) Thirteen γ -tubulins fit into one turn of the filament. Neighbouring γ -tubulins in the same γ TuSC are the same colour. c) The γ -tubulin symmetry is similar to the symmetry of a 13-protofilament microtubule (d), but separated intra- γ TuSC γ -tubulins alternate with contacting inter- γ TuSC γ -tubulins. e) To illustrate the mismatch between geometries laterally-interacting α/β -tubulin heterodimers were aligned to the filament so that the central α/β -tubulin makes longitudinal contacts to γ -tubulin.

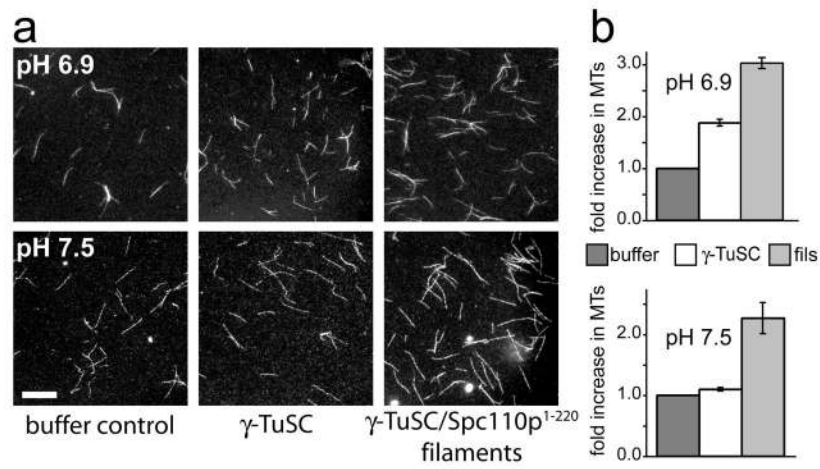


Figure 4. γ TuSC oligomers nucleate microtubules at low levels

a) Fluorescence micrographs of rhodamine labelled microtubules assembled at pH 6.9 or 7.5 in the presence of buffer, γ TuSC, or γ TuSC/Spc110p¹⁻²²⁰ filaments (final γ TuSC concentration 150 nM). Scalebar, 5 μ M. b) The mean number of microtubules per field is shown for nucleation at pH 6.9 (n=5) and pH 7.5 (n=3). Error bars represent the standard error of the mean.

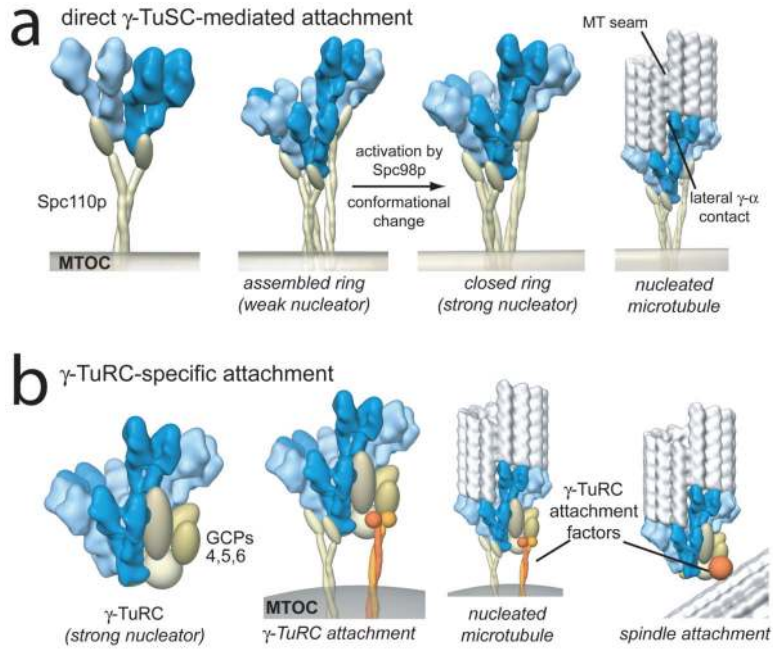


Figure 5. Models of nucleation complex attachment and activation

a) In the absence of γ TuRC-specific components, as in *Saccharomyces*, Spc110p, or its equivalent, directly attaches γ TuSC to microtubule organizing centres, promoting ring assembly. We hypothesize a conformational change in Spc98p promotes nucleation by rearranging γ -tubulin into an exact microtubule template. b) In organisms with complete γ TuRCs, active complexes attach to organizing centres directly via γ TuSCs, or potentially through unique sites in the γ TuRC-specific components. Localization of γ TuRCs at non-MTOC locations, for example within the mitotic spindle, is mediated through the γ TuRC-specific proteins. In both scenarios, γ TuSC interactions define the geometry of the nucleating template.