Microtubule nucleation by γ-tubulin complexes

Justin M. Kollman, Andreas Merdes‡, Lionel Mourey§ and David A. Agard**

Abstract | Microtubule nucleation is regulated by the γ-tubulin ring complex (γTuRC) and related γ-tubulin complexes, providing spatial and temporal control over the initiation of microtubule growth. Recent structural work has shed light on the mechanism of γTuRC-based microtubule nucleation, confirming the long-standing hypothesis that the γTuRC functions as a microtubule template. The first crystallographic analysis of a non-γ-tubulin γTuRC component (γ-tubulin complex protein 4 (GCP4)) has resulted in a new appreciation of the relationships among all γTuRC proteins, leading to a refined model of their organization and function. The structures have also suggested an unexpected mechanism for regulating γTuRC activity via conformational modulation of the complex component GCP3. New experiments on γTuRC localization extend these insights, suggesting a direct link between its attachment at specific cellular sites and its activation.

Microtubule catastrophe
The rapid depolymerization of microtubules that occurs when GTP has been hydrolysed in all tubulin subunits up to the growing tip.

**Howard Hughes Medical Institute and Department of Biochemistry & Biophysics, University of California, San Francisco, San Francisco, California 94143, USA.*
‡Centre de Recherche en Pharmacologie-Santé, Unité Mixte de Recherche 2587 Centre National de la Recherche Scientifique–Pierre Fabre, 31400 Toulouse, France.
§Institut de Pharmacologie et de Biologie Structurale (IPBS), Centre National de la Recherche Scientifique (CNRS), and Université de Toulouse, Université Paul Sabatier, IPBS, Toulouse F-31077, France.
Correspondence to D.A.A.
e-mail: agard@msg.ucsf.edu
doi:10.1038/nrm3209
Published online
12 October 2011

The microtubule cytoskeleton is critically important for the spatial and temporal organization of eukaryotic cells, playing a central part in functions as diverse as intracellular transport, organelle positioning, motility, signalling and cell division. The ability to play this range of parts requires microtubules to be arranged in complex arrays that are capable of rapid reorganization. Microtubules themselves are highly dynamic polymers that switch between cycles of growth and depolymerization, and cells have evolved various ways to manipulate the basic polymer dynamics to achieve precise control of the organization and reorganization of the microtubule cytoskeleton. Although many different mechanisms are used to regulate microtubule dynamics, at a fundamental level, the cell achieves control by manipulating the rates of microtubule assembly and microtubule catastrophe, as well as the timing and location of the nucleation events that give rise to new microtubules.

Microtubules are hollow tubes of about 250 Å in diameter that are assembled from α-tubulin–β-tubulin (αβ-tubulin) heterodimers in a GTP-dependent manner (FIG. 1). The tubulin subunits make two types of filament contacts: longitudinal contacts run the length of the microtubule forming protofilaments, and lateral contacts between protofilaments (generally α-tubulin to α-tubulin and β-tubulin to β-tubulin) form the circumference of the microtubule[1,2] (FIG. 1a). Microtubule geometry is not fixed, however; the more-flexible lateral contacts can accommodate between 11 and 16 protofilaments[3], yielding microtubules of different diameter when assembled *in vitro* from purified tubulin[4]. *In vivo*, though, almost all microtubules have 13 protofilaments[5–7], suggesting that one level of cellular control involves defining unique microtubule geometry. The 13-fold symmetry is probably preferred because it is the only geometry in which protofilaments run straight along the microtubule length, as opposed to twisting around the microtubule, which allows processively tracking motor proteins to always remain on the same face of the structure. An unusual feature of 13-protofilament microtubules is that, as a consequence of their helical symmetry, a 'seam' is formed from lateral α-tubulin–β-tubulin interactions[8,9], which are generally presumed to be weaker than α-tubulin–α-tubulin or β-tubulin–β-tubulin lateral contacts. The mechanism by which cells ensure 13-protofilament geometry has long been a mystery.

Another key difference between microtubule assembly *in vivo* and *in vitro* is with regard to how new microtubules are initiated. *In vitro*, microtubule growth must proceed through small early assembly intermediates, for which disassembly is energetically favoured over assembly, resulting in slow initial growth[10]. After a sufficiently large oligomer has been achieved, microtubule growth becomes energetically favourable and the addition of tubulin heterodimers proceeds rapidly (FIG. 1b). Significantly, rather than relying on the spontaneous initiation of new microtubules, cells have evolved specialized nucleation sites *in vivo* that bypass the early, slower growth phase. These nucleation sites are largely found at microtubule-organizing centres (MTOCs).

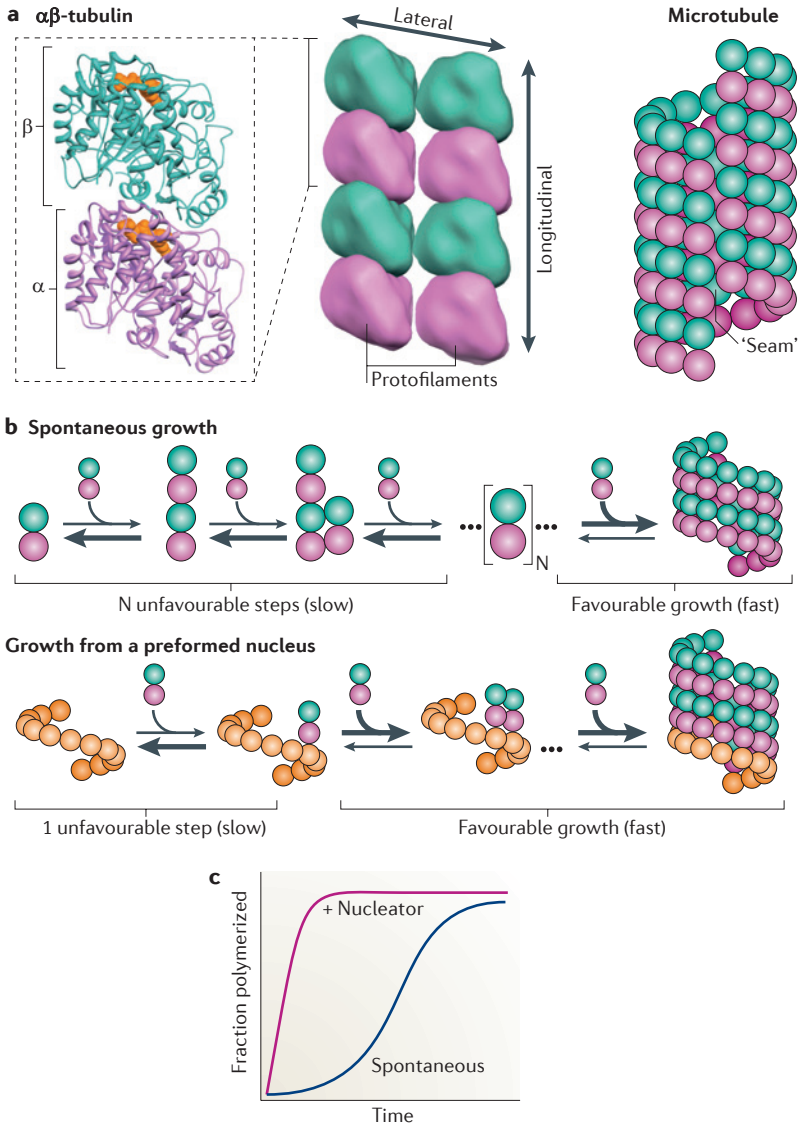


Figure 1 | **Microtubule assembly. a** | The α-tubulin–β-tubulin (αβ-tubulin) heterodimer is the fundamental repeating subunit of microtubules. When bound to GTP (shown in orange in the left panel), heterodimers come together through two types of contacts (indicated by double-headed arrows): GTP-mediated longitudinal contacts between α-tubulin and β-tubulin that form protofilaments, and lateral α-tubulin–α-tubulin and β-tubulin–β-tubulin contacts that form between protofilaments. The addition of tubulin subunits to this lattice yields the hollow microtubule. In 13-protofilament microtubules, a 'seam' is formed as a result of lateral α-tubulin–β-tubulin interactions. **b** | Spontaneous microtubule growth *in vitro* occurs in two stages: a relatively slow phase through unstable early assembly intermediates, and a rapid elongation phase. In early steps, the assembly energetics favour disassembly over assembly but, after a sufficiently large oligomer is formed by a variable number of steps (denoted here by N), assembly is energetically favoured and elongation proceeds rapidly. Whether disassembly or assembly is favoured by the assembly energetics is indicated by a bold arrow. *In vivo*, preformed nuclei allow microtubule growth to bypass the slow phase, providing spatial and temporal control over new microtubule growth. **c** | In bulk assembly assays, the presence of a nucleator causes rapid microtubule polymerization, bypassing the lag phase observed during spontaneous growth. Figure in part **b** is modified, with permission, from REF. 10 © (2008) The National Academy of Sciences, USA.

More than a century ago, the centrosome was identified as the primary MTOC in animal cells[11]. The centrosome, organized around a pair of centrioles, serves as the central anchor point for microtubules within the cell, defining a polar microtubule array[12]. In fungi, the functional analogue of the centrosome is the spindle pole body, which is a large multilayered structure embedded in the nuclear envelope that nucleates microtubules on both cytoplasmic and nuclear faces[13]. Plants, on the other hand, have no centrosome equivalent, but they nevertheless have highly organized acentrosomal microtubule arrays[14].

Despite the variation in MTOC morphology, all MTOCs rely on γ-tubulin, a homologue of α-tubulin and β-tubulin, for nucleating microtubules. γ-tubulin was first discovered in *Aspergillus nidulans* genetic screens as a suppressor of a β-tubulin mutation[15], and it was subsequently found localized at all MTOCs[16–21]. Purification of γ-tubulin from animal and yeast cells showed it to be part of larger complexes, which can directly nucleate microtubule growth *in vitro*[22–26]. γ-tubulin is essential for normal microtubule organization in every organism in which it has been studied, and it is nearly ubiquitous throughout the eukaryotes. Moreover, it is also involved in nucleation from non-MTOC sites within cells, such as nucleation that occurs through the chromosome-mediated nucleation pathway[27], and in plants[28], which lack centrosome-like structures, suggesting that it is critical for the initiation of all new microtubules *in vivo*.

In this Review, we focus on recent advances in our understanding of the mechanism of γ-tubulin-based microtubule nucleation. We begin with a brief review of the components of γ-tubulin complexes and previous models for their assembly and nucleation mechanism. We then describe recent structures of key γ-tubulin small complex (γTuSC) and γ-tubulin ring complex (γTuRC) components that led us to a new model for the organization of γ-tubulin complexes. We also explore the growing body of work on γ-tubulin complex localization, which increasingly seems to be linked with the regulation of nucleating activity.

The γTuSC and γTuRC nucleating complexes

Early biochemical characterization of γ-tubulin showed that it was part of larger complexes that did not include α-tubulin or β-tubulin. When γ-tubulin was purified from *Drosophila melanogaster* embryos or *Xenopus laevis* eggs, it was found to be part of a ~2.2 MDa complex with at least six other proteins: γ-tubulin complex protein 2 (GCP2), GCP3, GCP4, GCP5, GCP6 and NEDD1. The complex had a striking ring shape in electron micrographs, leading to the name γTuRC[24]. The γTuRC dissociates under high salt conditions to yield a stable 300 kDa subcomplex of γ-tubulin associated with GCP2 and GCP3, which is dubbed the γTuSC[29] (BOX 1). Importantly, purified γTuSC has a much lower microtubule-nucleating activity than intact γTuRC[29], suggesting that the assembly state of γ-tubulin is important in determining its activity.

Saccharomyces cerevisiae and closely related yeasts are unusual, as they appear to have lost all of the γTuRC-specific components, retaining only the γTuSC[25,26,30]. This supports the view that the γTuSC is the core of the nucleating machinery, sufficient in itself for proper microtubule organization. The apparent simplicity of the

budding yeast γ-tubulin complex has made it an attractive model for elucidating the mechanisms of microtubule nucleation. And yet, a noticeable contradiction has remained unresolved: budding yeast have only the weakly-nucleating γTuSC, yet are perfectly capable of nucleating microtubules.

The GCP family of γ-tubulin complex components. In addition to γ-tubulin, microtubule-nucleating complexes include five homologous GCPs[31–33] (BOX 1). The γTuSC consists of two copies of γ-tubulin and one each of GCP2 and GCP3. The γTuRC is composed of multiple copies of the γTuSC plus GCP4, GCP5 and GCP6. GCP2 and GCP3 are found in almost all eukaryotes and are essential for proper microtubule organization, suggesting that they form the core of the nucleating machinery. Most eukaryotes also possess GCP4 and GCP5, whereas GCP6 appears to be a recent addition in the animal and fungal lineages.

Although they constitute a unique family of homologous proteins, the overall sequence identity between GCPs is quite low (less than 15% identity overall in most comparisons between GCP family members) (J.M.K. and D.A.A., unpublished observations). Homology has only been confidently predicted in two short segments, the GRIP1 and GRIP2 motifs[31], which are unique to the GCPs. Almost nothing is known about the specific functions of these motifs, although it was speculated that they might participate in conserved protein–protein interactions[32]. The overall size of GCPs varies by more than twofold (ranging from ~70–210 kDa), as a result of numerous insertions and/or deletions, suggesting a different function for each family member. Outside of the GRIP1 and GRIP2 motifs that define the GCP family, no GCPs have other identifiable motifs conserved with other protein families.

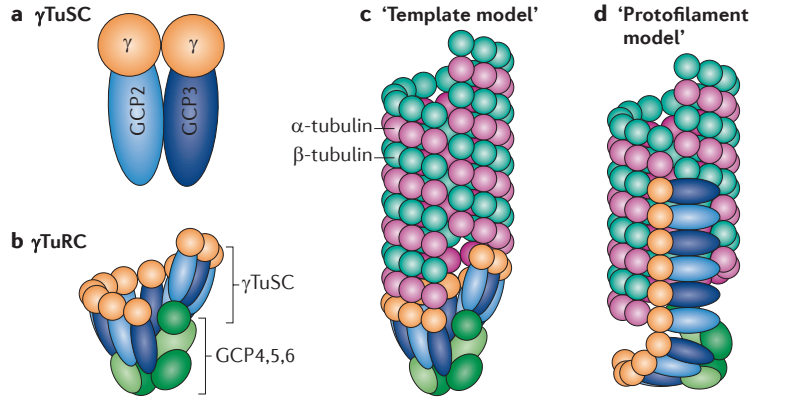
It is important to note that the various γ-tubulin complex components were initially described by different researchers in different organisms, leading at times to a confusing litany of names for homologous proteins. Here, we have adopted the generic GCP designation[33] for GCP2 to GCP6, and we prefer to limit its use to this family to indicate their common evolutionary origin. BOX 1 includes a list of the different names that have been used for each component.

Non-GCP family components of the γTuRC. Recently, two small proteins with no homology to the GCP family — mitotic-spindle organizing protein associated with a ring of γ-tubulin 1 (MOZART1) and MOZART2 — were described as integral γTuRC components in human cell lines[34,35]. It appears that, owing to their small size, these proteins were overlooked in earlier γTuRC pull-down experiments. When either protein is immunoprecipitated from cells, it is found in complex with all of the other γTuRC components. MOZART1, which is found in most eukaryotes, seems to have a role in γTuRC recruitment to MTOCs. MOZART2A and MOZART2B, which are found only in the deuterostome lineage, are specifically involved in γTuRC recruitment to interphase centrosomes but do not seem to play a part

Box 1 | γ-tubulin complexes and prior models for their assembly and action

The γ-tubulin small complex (γTuSC) is the conserved, essential core of the microtubule nucleating machinery, and it is found in nearly all eukaryotes. The γTuSC has two copies of γ-tubulin and one each of γ-tubulin complex protein 2 (GCP2) and GCP3 (see the figure, part **a**). In many eukaryotes, multiple γTuSCs assemble with GCP4, GCP5 and GCP6 into the γ-tubulin ring complex (γTuRC) (see the figure, part **b**). Previous models of γTuRC assembly suggested that GCP4, GCP5 and GCP6 together function as a cap-like scaffold for arranging multiple γTuSCs into a distinctive ring shape. This view depicts a model with six γTuSCs (12 γ-tubulins), which would leave a gap in the template, owing to the fact that microtubules are made up of 13 protofilaments. The most widely accepted model for the mechanism of γTuRC-based nucleation, the 'template model', suggests that the γTuRC acts as a template, presenting a ring of γ-tubulins that make longitudinal contacts with α-tubulin–β-tubulin (αβ-tubulin) (see the figure, part **c**). By contrast, the 'protofilament model' suggests that the γTuRC unfurls to present a γ-tubulin protofilament, which would nucleate through lateral contacts with αβ-tubulin (see the figure, part **d**).

A complete list of proteins that are thought to be part of the γTuSC and the γTuRC, including the more-recently identified proteins mitotic-spindle organizing protein associated with a ring of γ-tubulin 1 (MOZART1) and MOZART2, is given, along with alternative names for each protein (see the figure, part **e**). The five GCPs share regions of homology, although with very low levels of sequence identity (as low as 15% identity between GCP family members) (J.M.K. and D.A.A., unpublished observations). Two homologous regions of GCPs, GRIP1 and GRIP2, initially defined their homology[81] (see the figure, part **f**). Regions of more-distant homology were later shown to be more widely dispersed in the GCP sequences[32,50] (green shading in part **f** of the figure).

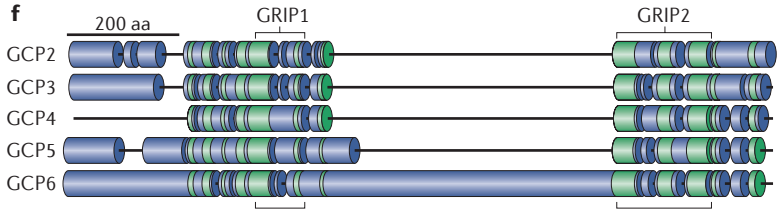


e

Protein	Distribution	Alternative names
γ-tubulin	All eukaryotes	TuB4, GTB1, TuG1
GCP2	All eukaryotes	DGRIP84, Xgrip110, Spc97, h103p, Alp4, GCPB
GCP3	All eukaryotes	DGRIP91, Xgrip109, Spc98, h104p, Alp6, GCPC
GCP4	Most eukaryotes	DGRIP75, Xgrip75, Gfh1, h76p, GCPD
GCP5	Most eukaryotes	DGRIP128, Xgrip133, Mod21, GCPE
GCP6	Animals and fungi	DGRIP163, Xgrip195, Alp16, GCPF
MOZART1	All eukaryotes	None
MOZART2A	Deuterostomes	GCP8A
MOZART2B	Deuterostomes	GCP8B

γTuSC, γTuRC

f



aa, amino acids; Alp, altered polarity protein; Gfh1, GCP4 homologue 1; GTB1, γ-tubulin 1; Spc, spindle pole body component; TuB4, tubulin β4; TuG1, tubulin γ1.

in γTuRC assembly. NEDD1 also frequently co-purifies with the γTuRC, but it does not appear to be an integral component of the complex. Rather, NEDD1 is a localization factor, important for both centrosomal and non-MTOC localization of the γTuRC, for example, within the mitotic spindle[36–38].

All of the core γTuRC components have been identified through co-purification, but it should be noted that a large number of proteins co-precipitate with the γTuRC at lower stoichiometries. Many of these may be factors that help the γTuRC attach to the MTOC or have transient roles in γTuRC regulation. However, given the recent experience with MOZART1 and MOZART2, it would not be surprising to find that our list of γTuRC components is incomplete, with additional integral γTuRC components yet to be discovered.

Stoichiometry of γTuRC components. The precise stoichiometry of γTuRC components remains unclear. A study in human cells showed that the complex contains multiple copies of the γTuSC components and GCP4, but only a single copy of GCP5 (no determination could be made about the copy number of GCP6)[32]. A more-recent study quantified the ratio of components in the human γTuRC, from gels of purified complex, and estimated the stoichiometry of the complex to be 14 copies of γ-tubulin, 12 copies of GCP2 or GCP3, 2–3 copies of GCP4 and a single copy of GCP5 (REF. 39). However, this quantification should be viewed as preliminary, as GCP6 was present at less than one copy per γTuRC, raising the possibility of heterogeneity in the sample. Interestingly, the stoichiometry implied by this study has more γ-tubulins than GCP2 and GCP3, suggesting that a small portion of γ-tubulin is incorporated into the γTuRC independently of the γTuSC.

γTuRC assembly and action: old models

It has been assumed that γ-tubulin nucleates by forming oligomers that mimic an early assembly intermediate of αβ-tubulin, with either lateral or longitudinal microtubule-like lattice contacts forming between γ-tubulins. Nucleation should then proceed through direct interactions of γ-tubulin with αβ-tubulin through lattice-like contacts. Generating models for the arrangement of γ-tubulin within the γTuRC and for the mechanism of γ-tubulin-based microtubule nucleation are therefore two aspects of the same problem. Lines of evidence from structural and biochemical studies have provided some insight into both problems.

Imaging of the γTuRC by electron microscopy — both two-dimensional images[24,29] and a low-resolution three-dimensional structure[40] — revealed a unique 'lock washer' shape, with repeating subunits around the circumference and a diameter and a helical pitch that are similar to those of a microtubule. γTuSCs were proposed to form the repeating wall of the ring. An apparent cap-like feature at the base of γTuRC, seen in the low-resolution structure, was thought to be formed from GCP4, GCP5 and GCP6. Given its position, the asymmetric cap was predicted to act as a scaffold for arranging γTuSCs into a defined ring shape (BOX 1).

In vitro, the γTuRC was shown to interact specifically with microtubule minus ends, at which it functions as a cap to prevent microtubule growth in the minus direction[41]. This was consistent with electron micrograph images showing closed structures at the ends of microtubules, whether nucleated by γTuRCs *in vitro*[40–42] or attached to MTOCs *in vivo*[43]. Synthesis of these data led to the 'template model', which suggests that the γ-tubulins in the γTuRC function as a microtubule template, making lateral contacts with each other around the ring and longitudinal contacts with α-tubulin (BOX 1, see the figure parts b and c).

Although the model is compelling in its simplicity, the experimental data were insufficient to define the specific number of γTuSCs in the ring, leading to questions as to how the pairs of γ-tubulins within the γTuSCs could nucleate microtubules with an odd number of protofilaments. Two possibilities were generally offered: six γTuSCs (12 γ-tubulins) might form an incomplete ring, leaving a gap at the location of the thirteenth protofilament, or seven γTuSCs (14 γ-tubulins) could form a ring with one extra γ-tubulin that does not interact with the microtubule.

An alternative hypothesis — the 'protofilament model' — was proposed early on, in which γ-tubulins would make longitudinal contacts with each other around the ring[44,45] (BOX 1, see the figure part d). This seemed reasonable, a priori, as longitudinal contacts are much stronger than lateral contacts, and rings of longitudinally-interacting tubulin, and its bacterial homologue FtsZ, have been observed. Moreover, electron micrographs of γTuRCs indicated that the structure might be quite flexible, suggesting that it could potentially unfurl to present a single protofilament of γ-tubulins that would nucleate through lateral contacts with α-tubulin and β-tubulin. However, the weight of evidence now strongly supports the template model.

Although a template mechanism of nucleation has been the dominant model for γTuRC function for over a decade, it has remained unproven, and several important questions have persisted. What is the mode of interaction (lateral or longitudinal) between γ-tubulin and αβ-tubulin? Why is γ-tubulin nucleating capacity weaker in the γTuSC than in the γTuRC, and how does *S. cerevisiae*, which only has the γTuSC, efficiently nucleate microtubules? How are 13-protofilament microtubules nucleated when γ-tubulins enter the complex in pairs through the γTuSC? And, finally, what are the structural and functional roles of the non γ-tubulin components of the γTuRC? Several recent advances have provided insight into these questions, generating a more complete framework for understanding γ-tubulin-based microtubule nucleation.

Structural insight into γTuRC function

A thorough, mechanistic understanding of microtubule nucleation by γ-tubulin will require a high-resolution structural model of the γTuRC. This is a daunting task. The large size and compositional complexity of the γTuRC have made it a challenging target for recombinant expression and, to date, only small quantities of

Microtubule-organizing centres
(MTOCs). Primary sites of microtubule nucleation in the cell, including centrosomes in animal cells and the spindle pole body in yeast.

Acentrosomal microtubule arrays
Ordered arrays of microtubules formed in the absence of a microtubule-organizing centre.

Chromosome-mediated nucleation
The pathway by which new microtubules are nucleated around chromosomes in response to a RAN gradient.

Deuterostome lineage
One of the two superphyla of more complex animals. It includes the echinoderms, chordates, hemichordates and xenoturbellida.

heterogeneous material have been purified from native sources (for example, *D. melanogaster* embryos[29], *X. laevis* eggs[24] and human cell lines[32]). An alternative strategy that has recently borne fruit has been to determine high-resolution structures of individual γTuRC components by crystallography and electron microscopy, and then to integrate these into a model of the γTuRC.

The γ-tubulin crystal structure. The crystal structure of monomeric human γ-tubulin was determined bound to GTP and to GDP[10,46]. γ-tubulin is very similar to α-tubulin and β-tubulin in its overall fold, which is consistent with the expectation that it is capable of making lattice-like contacts with the microtubule. Small differences on the microtubule lattice surfaces may give rise to differences in γ-tubulin interaction affinities at those sites, influencing the strength of γ-tubulin–γ-tubulin assembly interactions or γ-tubulin–microtubule interactions. Importantly, in the two γ-tubulin crystal forms, the individual γ-tubulins make lateral contacts with each other through the same contact region that αβ-tubulin use in microtubule lateral interactions, suggesting that this is their preferred mode of interaction. The crystal packing provided support for the template model of microtubule nucleation, which predicts lateral interactions between γ-tubulins and longitudinal interactions between γ-tubulin and αβ-tubulin.

The structure of the γTuSC. The structure of free *S. cerevisiae* γTuSC was initially determined at 25 Å by negative-stain single-particle electron microscopy (EM)[47] (its V-shaped structure was later confirmed at higher resolution by cryo-EM (see below)), and the subunit arrangement and the orientations of GCP2 and GCP3 in the structure were determined by direct labelling experiments[48]. The arms of the V-shaped structure are composed of GCP2 and GCP3, which have similar overall shapes and dimerize through their amino-terminal domains at the base of the V-shape. The tips of the V-shape contain γ-tubulin, which interacts with the carboxy-terminal domains of GCP2 and GCP3 (FIG. 2a). Surprisingly, the two γ-tubulins in the structure are held apart from each other, not making the anticipated lateral contacts that are required to match the microtubule lattice. This mismatch provides a partial explanation for the weaker nucleating activity of free γTuSC — each γ-tubulin remains totally independent, rather than forming a microtubule-like assembly intermediate that could facilitate microtubule assembly. Thus, the structure of the γTuSC suggests that it is in an 'off' state, which raises the possibility of regulation at the level of γTuSC conformation.

The γTuSC assembles with microtubule-like symmetry. Purified *S. cerevisiae* γTuSCs have a weak tendency to spontaneously assemble *in vitro* into ring-shaped structures that closely resemble the γTuRC[49]. The ring assemblies are only formed under a narrow range of buffer conditions, and their heterogeneity and instability made them an extremely challenging subject for structure determination. However, it was discovered that co-purification of the γTuSC with the N-terminal domain of the *S. cerevisiae* attachment factor spindle pole body component 110 (Spc110; which links the γTuSC to the core of the spindle pole body) dramatically stabilizes γTuSC assembly. So much so that, when associated with Spc110, γTuSC rings continue to grow, yielding extended helical filaments of laterally associated γTuSCs that are very well suited to cryo-EM reconstruction. The 8 Å structure of this γTuSC filament provided a breakthrough in our understanding of γTuSC assembly, with important implications for the mechanism of microtubule nucleation[49].

The most striking feature of the γTuSC oligomer structure is that there are six and a half γTuSCs per helical turn, owing to a half-subunit overlap between the first and seventh subunits (FIG. 2b). This gives 13 γ-tubulins per turn, matching the *in vivo* microtubule protofilament number, with a helical pitch that is very similar to that of a microtubule. There is remarkable similarity between a single ring of the γTuSC and the low-resolution structure of the γTuRC, strongly suggesting that γTuSC assemblies like these constitute the core of the γTuRC (FIG. 2c). This finding also resolved the paradox of how budding yeast efficiently nucleate microtubules with only the γTuSC — they can form γTuRC-like structures from γTuSCs alone.

The increased resolution of the γTuSC subunit structure allowed the precise orientation of each γ-tubulin to be determined. The minus ends of both γ-tubulins are buried in the interaction surface with GCP2 or GCP3, and their lateral surfaces are facing adjacent γ-tubulins. Moreover, each γ-tubulin plus end is fully exposed, strongly suggesting that this surface interacts via longitudinal contacts with the minus ends of αβ-tubulin. The combination of the γ-tubulin geometry and its orientation provides the strongest evidence to date that γ-tubulin complexes function as microtubule templates. Indeed, the γTuSC rings are likely to provide the constraint that ensures the creation of 13-protofilament microtubules *in vivo*. It is important to note that the 13-fold architecture of the oligomer is defined almost entirely by the conformations of, and interactions between, GCP2 and GCP3, with only minor contacts between γ-tubulins within the ring. The problem of how microtubule geometry with an odd number of protofilaments can be created from a template complex with an even number of subunits is also now resolved — the half-γTuSC overlap ensures that, at most, 13 γ-tubulins are exposed for interaction with αβ-tubulin.

Although the symmetry of γ-tubulin in γTuSC rings is similar to that of αβ-tubulin in microtubules, it is not a perfect match. There are no major conformational changes to the individual γTuSCs upon oligomerization; the two γ-tubulins within each γTuSC are still held apart. However, contacts between γ-tubulins of adjacent γTuSCs in the ring are nearly identical, in both their spacing and relative orientation, to microtubule lateral interactions, giving rise to an alternating pattern around the ring of contacting γ-tubulin pairs separated by gaps (FIG. 2d). It is important to note that the relative orientation of the γ-tubulins in the ring is determined

Single-particle electron microscopy
A method for combining two-dimensional images of molecules into a three-dimensional structure.

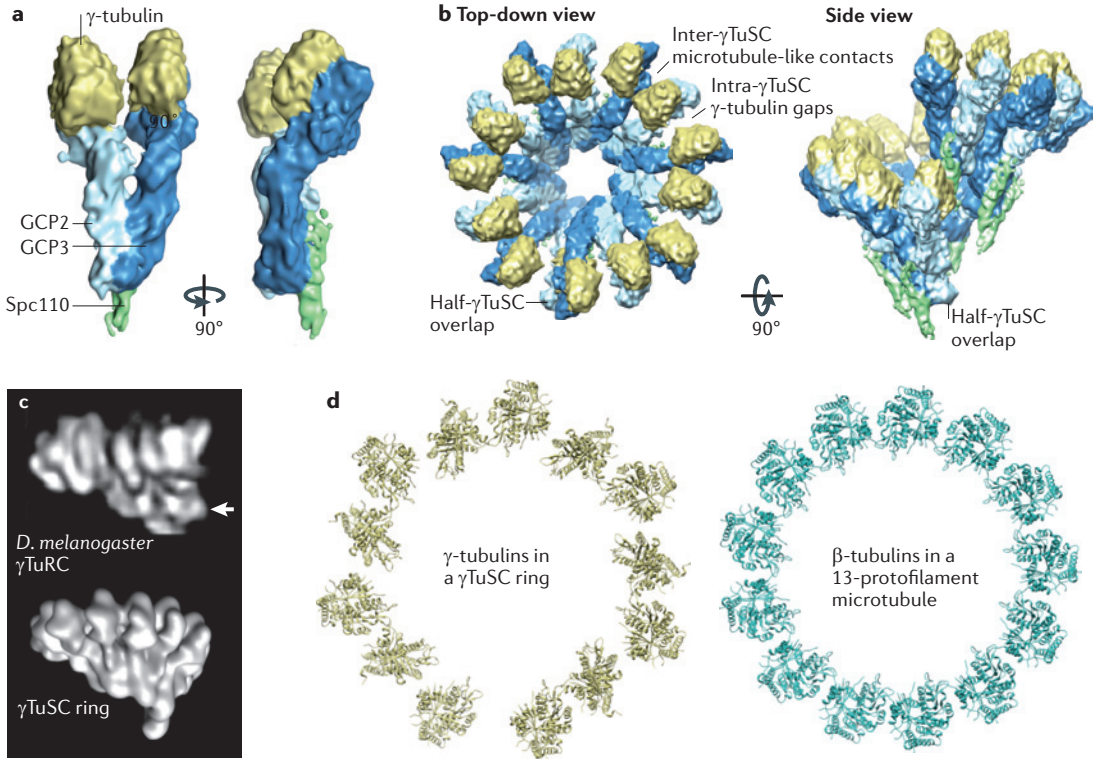


Figure 2 | **The structure of γTuSC. a** | The 8 Å cryo-electron microscopy (EM) structure of *Saccharomyces cerevisiae* γ-tubulin small complex (γTuSC) bound to the attachment factor spindle pole body component 110 (Spc110) is shown. This γTuSC is a single subunit of a large γTuSC oligomer (see panel **b**). In this view, the amino termini of γ-tubulin complex protein 2 (GCP2) and GCP3 are at the bottom, with their carboxy-terminal domains near the top interacting with γ-tubulin. In the structure, the two γ-tubulins are held apart from each other in a configuration that is incompatible with the microtubule lattice, which partially explains the relatively low nucleating capacity of free γTuSC relative to that of the γ-tubulin ring complex (γTuRC). **b** | Top-down and side views of the γTuSC ring are shown. The ring has six and a half γTuSCs per turn, which arise owing to a half-γTuSC overlap between the first and seventh subunits in the ring (see side view). This yields 13 γ-tubulins per turn, matching the *in vivo* microtubule protofilament number. The conformation of the γTuSC is unchanged in the ring structure, such that the intra-γTuSC gap between γ-tubulins remains. However, microtubule-like lateral interactions are observed between γ-tubulins at the inter-γTuSC interface. **c** | The low-resolution negative-stain EM reconstruction of a single *Drosophila melanogaster* γTuRC (top) closely resembles the γTuSC ring shown in panel **b**, rendered here at lower resolution for comparison (bottom). The region of the γTuRC originally interpreted as a GCP4–GCP5–GCP6 cap is indicated with an arrow; this region appears to correspond to the N-terminal regions of GCP2 and GCP3 instead. **d** | Comparison of γ-tubulin positions in γTuSC rings and α-tubulin–β-tubulin in the microtubule shows a mismatch in geometry, with alternating contacts and gaps in the γ-tubulin arrangement. Figures in parts **a**, **b** and **d** are modified, with permission, from REF. 49 © (2010) Macmillan Publishers Ltd. All rights reserved. Top image in part **c** is reproduced, with permission, from REF. 40 © (2010) Macmillan Publishers Ltd. All rights reserved.

primarily by interactions between GCP2 and GCP3, which have far greater surface areas in contact than the γ-tubulins.

The nucleating activity of the Spc110-stabilized oligomers was only slightly greater than the heterogeneous γTuSC rings assembled in the absence of Spc110 (REF. 49), and both had much lower nucleation levels than have been reported for the γTuRC[29]. However, under conditions in which the γTuSC remains monomeric, its nucleating activity was completely eliminated, suggesting that assembly of γTuSC rings is required even for low levels of nucleation activity[49]. The imprecise match between the γ-tubulin geometry and the microtubule geometry explains the modest levels of microtubule nucleation observed from the γTuSC oligomers, which probably arise from just the pairs of properly spaced γ-tubulins between the γTuSCs.

The GCP4 crystal structure: a model for the GCP family. A major advance toward the full understanding of γ-tubulin complexes was achieved recently by the determination of the crystal structure of human GCP4 (REF. 50). GCP4 has a unique fold, forming an elongated structure from five α-helical bundles, with a pronounced kink between the third and fourth bundle and a small domain flanking the fourth and fifth bundles (FIG. 3a). The crystal structure itself is incomplete, as it is missing several large loops because of their inherent flexibility. Nonetheless, GCP4 fits remarkably well into the γTuSC cryo-EM structure in the positions of GCP2 and GCP3, with only small adjustments necessary in the bend angle between the third and fourth helical bundles. The extraordinarily good match between GCP4 and GCP2 and GCP3 demonstrates an unexpectedly strong conservation of the overall fold of the GCP family proteins.

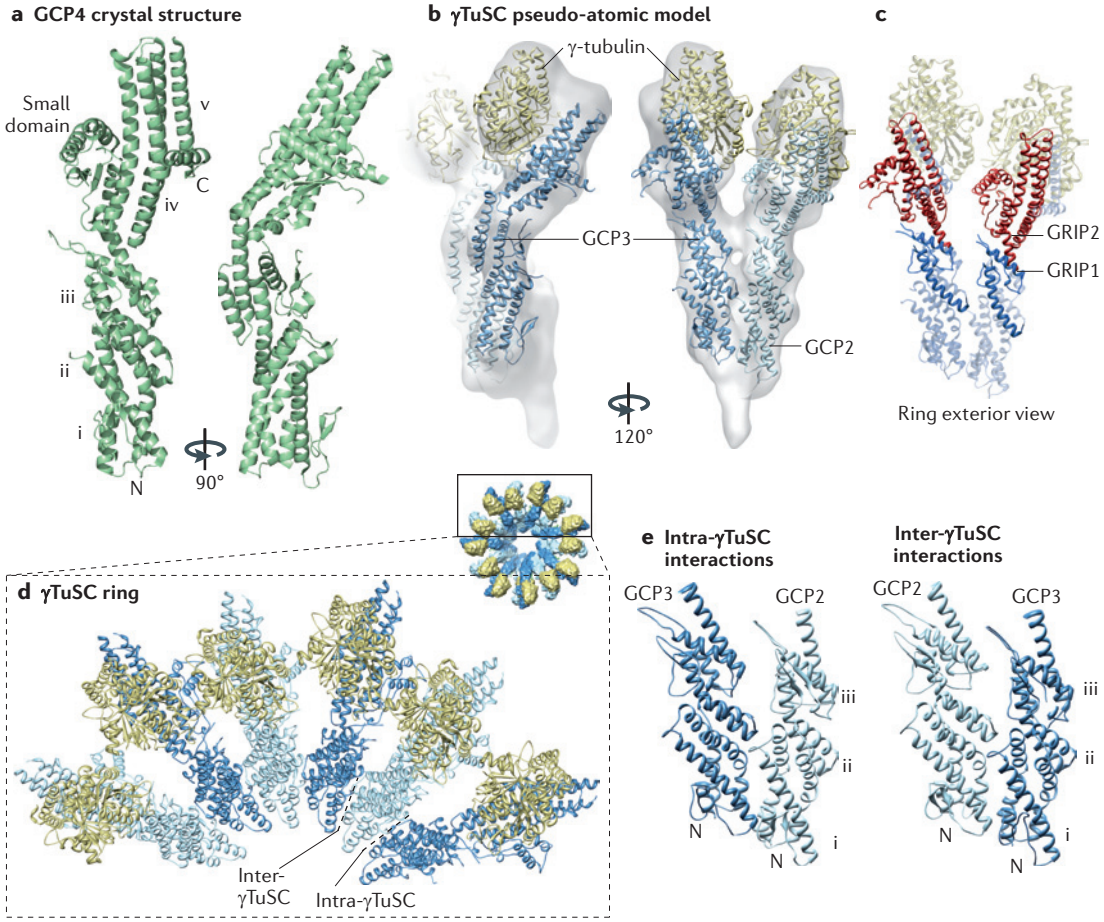


Figure 3 | **The GCP4 crystal structure defines the core structure of all of the GCPs. a** | The γ-tubulin complex protein 4 (GCP4) crystal structure is shown in two orthogonal views. In the view on the left, the five α-helical bundles (i–v), small domain, amino terminus and carboxyl terminus are labelled. The C-terminal domain, consisting of bundle iv, bundle v and the small domain, was shown to directly bind γ-tubulin. **b** | Two views of the pseudo-atomic model of the γ-tubulin small complex (γTuSC) are shown. The model was generated by fitting the γ-tubulin crystal structure (gold) and the GCP4 crystal structure (blue), as a stand-in for GCP2 and GCP3, into the cryo-electron microscopy (EM) reconstruction of the γTuSC (semi-transparent surface). The model reveals interaction surfaces between complex components. **c** | The model also shows the positions of the conserved GRIP1 and GRIP2 domains in GCP2 and GCP3 in the context of the full γTuSC. GRIP2 is clearly involved in γ-tubulin binding. The role of GRIP1 is more ambiguous; it forms part of the lateral contact surfaces between γTuSCs, as well as part of the faces of GCP2 and GCP3 that are exposed on the outside of the γTuSC ring. **d** | When the pseudo-atomic model from panel **b** is fit into the cryo-EM structure of the γTuSC ring (inset), it also reveals the surfaces of GCP2 and GCP3 that are important for oligomerization. γTuSCs interact with each other primarily through the sides of bundles i and ii. **e** | The N-terminal domains of GCP2 and GCP3 are shown making intra-γTuSC and inter-γTuSC contacts, with helical bundles i–iii labelled. Equivalent surfaces of the N-terminal domains of GCP2 and GCP3 are involved in both intra-γTuSC and inter-γTuSC interactions, indicating that a single assembly rule determines the organization of the ring structure. However, the affinities have been modulated such that the stronger intra-γTuSC interactions yield a stable complex, whereas the weaker inter-γTuSC interactions allow the assembly of γTuSCs into rings to be reversible. Figures in parts **b**, **d** and **e** are modified, with permission, from REF. 50

Previously, sequence homology had only been identified in the short GRIP1 and GRIP2 motifs of GCP family proteins[31–33] (BOX 1), but the structural similarity of GCP2 and GCP3 to GCP4 prompted a re-examination of sequence similarity. Using the GCP4 crystal structure and predicted secondary structures of the remaining GCPs as guides, a more accurate alignment of the entire family was possible, showing small islands of sequence conservation scattered throughout the proteins. The regions of strongest conservation were predominantly buried in the protein, defining a structural core, with highly variable loop regions allowing for numerous insertions and/or deletions. GCP4 is the shortest of the GCPs, being almost entirely composed of homologous regions. The strong conservation of the overall fold between GCP4 and GCP2 and GCP3, along with the more-expansive sequence homology that is now evident, allows us to use GCP4 as a model for the core of all the other GCPs.

This work also demonstrated a direct high-affinity interaction between GCP4 and γ-tubulin, showing not only structural but also functional conservation

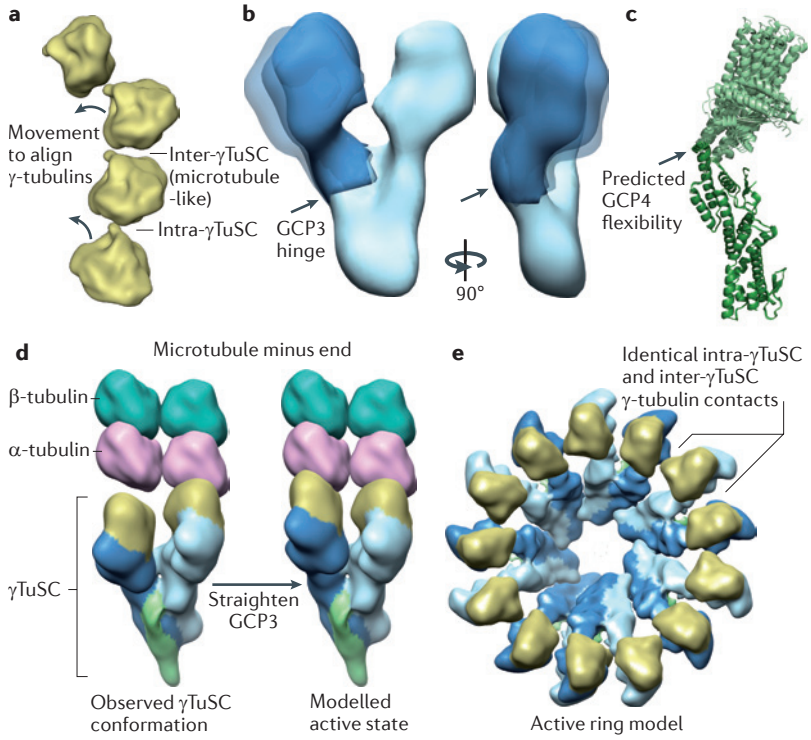


Figure 4 | **A model for the conformational activation of the γTuSC. a** | The γ-tubulins of two adjacent γ-tubulin small complexes (γTuSCs) from the γTuSC ring are shown in a top-down view. The inter-γTuSC contact is the same as a microtubule lateral contact, but the intra-γTuSC arrangement does not match the microtubule lattice. Arrows indicate the approximate motions that would align the intra-γTuSC contacts to match the microtubule lattice. **b** | The negative-stain electron microscopy reconstruction of free γTuSC revealed flexibility at a hinge point in γ-tubulin complex protein 3 (GCP3), resulting in varying distances between the two γ-tubulins. **c** | Normal mode analysis of the GCP4 crystal structure predicts flexibility at the indicated position, near the equivalent hinge point in GCP3. This suggests conservation of flexibility in the GCPs. **d** | A model for the conformational activation of the γTuSC through the straightening of GCP3. In the observed conformation, the two γ-tubulins are held apart so that they cannot both be making contacts with the microtubule. However, straightening at the GCP3 hinge point by 23˚ would close the intra-γTuSC γ-tubulin gaps, bringing all of the γ-tubulins in the ring to microtubule lattice-like spacing. **e** | In this modelled state, γ-tubulin in the ring would adopt perfect 13-protofilament microtubule geometry, serving as a potent microtubule nucleator. Figure in part **b** is modified, with permission, from REF. 47. Figure in part **c** is modified, with permission, from REF. 50 © (2010) Macmillan Publishers Ltd. All rights reserved. Figure in part **d** is modified, with permission, from REF. 49 © (2010) Macmillan Publishers Ltd. All rights reserved.

in the GCP family. The binding activity of GCP4 was localized within its C-terminal domain, which is precisely the region juxtaposed to γ-tubulin when GCP4 and γ-tubulin are fit into the γTuSC cryo-EM structure[49]. This is also consistent with the direct labelling experiments that showed the C-termini of GCP2 and GCP3 interacting with γ-tubulin[48]. Indeed, the surfaces involved in γ-tubulin binding are among the most conserved in the GCP family, and they include the GRIP2 motif. Earlier work with the *D. melanogaster* proteins had also suggested that γ-tubulin binds directly to GCP5 and GCP6 (REF. 36). The conservation of sequence and structure suggests that all of the GCPs directly bind γ-tubulin; as explored more fully below, this has important implications for understanding γTuRC organization.

Normal mode analysis
A computational method for predicting the flexibility of a protein structure based on its shape.

A pseudo-atomic model of the γTuSC. Using the GCP4 crystal structure as a template, homology models of GCP2 and GCP3 were generated and fit into the γTuSC cryo-EM structure, along with the crystal structure of γ-tubulin, to create a pseudo-atomic model of the γTuSC[50] (FIG. 3b). The γTuSC model predicts the surfaces involved in γ-tubulin–GCP2 and γ-tubulin–GCP3 interactions. The model also reveals the positions of the GCP GRIP1 and GRIP2 motifs and suggests functions for these motifs that were previously unknown (FIG. 3c). The GRIP2 motif is clearly part of the γ-tubulin-binding surface of GCPs, which is consistent with *in vitro* binding experiments using GCP4 and γ-tubulin. The role of GRIP1 is less clear; it forms part of the lateral interaction surfaces, suggesting that it has a role in γTuSC assembly. However, it also forms part of the surface of GCP2 and GCP3 that is exposed on the outer surface of the ring, suggesting that it may be a binding site for other proteins that interact with the γTuSC.

The pseudo-atomic model of the γTuSC also provides insight into the nature of assembly contacts in γTuSC oligomers (FIG. 3d). The intra-γTuSC and inter-γTuSC interactions between GCP2 and GCP3 are very similar: essentially, the interactions along the base of a γTuSC ring are the same all the way around and primarily involve contacts between helical bundles i and ii (FIG. 3e). There appears to be a single assembly rule guiding interactions between GCP2 and GCP3, whether within or between γTuSCs. Changes at these interaction surfaces seem to have tuned affinities to give very strong binding to hold together individual γTuSCs but weaker interactions that drive the reversible assembly of these γTuSCs into γTuSC rings.

Conformational regulation of the γTuSC

The mismatch between the γ-tubulins in γTuSC rings and microtubule geometry was interpreted as an 'off' state of the γTuSC, in which the nucleating complex is fully assembled but conformationally inactivated[49]. However, the γ-tubulins were arranged such that small movements could realign them into microtubule-like contacts (FIG. 4a). The key to conformational activation may lie in the inherent flexibility of GCP3, which was observed as a hinge-like motion in negative-stain EM reconstructions[47] (FIG. 4b). GCP4 was predicted by normal mode analysis to have a flex point at the position that is equivalent to the GCP3 hinge[50] (FIG. 4c). The GCP4 crystal structure provides a detailed view of the hinge point, allowing for a more precise model of the observed flexibility in GCP3, which appears to rely on rearrangement of hydrophobic interactions between the domains on either side of the hinge. Using the geometry of the 13-protofilament microtubule as a guide, we have developed a model for γTuSC activation, in which GCP3 straightens at its hinge point. This rearrangement in GCP3 is sufficient to bring the two γ-tubulins in γTuSC into the exact microtubule lattice spacing[49] (FIG. 4d). In the context of the γTuSC ring, straightening of GCP3 to close the gap between each pair of intra-γTuSC γ-tubulins would create a perfect template for microtubule assembly[49] (FIG. 4e).

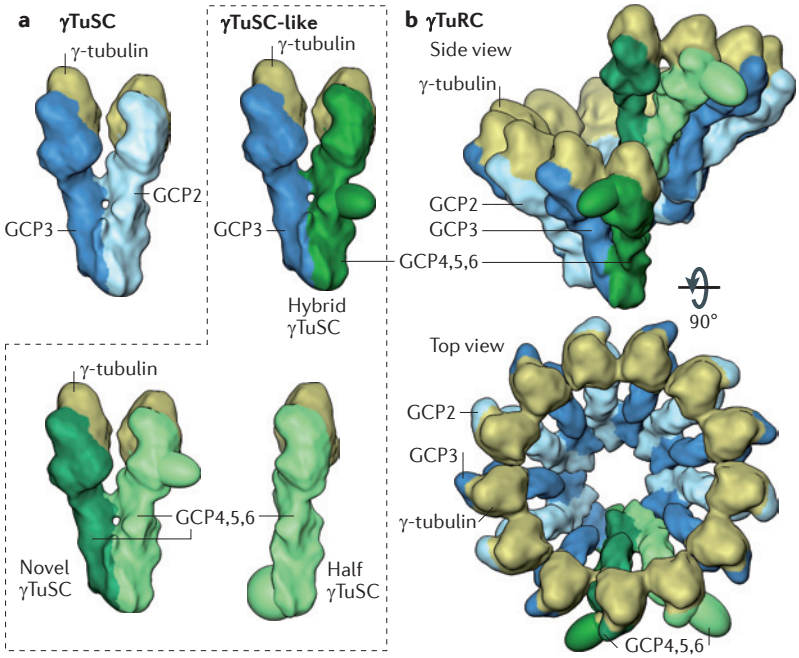


Figure 5 | **A revised model of γTuRC assembly. a** | The overall structure and ability to bind γ-tubulin are conserved between the γ-tubulin ring complex (γTuRC)-specific subunit γ-tubulin complex protein 4 (GCP4) and the γ-tubulin small complex (γTuSC) components GCP2 and GCP3, suggesting that all GCP family members act as γTuSC-like components. The γTuRC-specific GCPs (GCP4, GCP5 and GCP6; all represented by green shapes) may function in one of three γTuSC-like complexes: as hybrid γTuSCs with GCP2 or GCP3 (shown in blue); interacting with each other to form novel γTuSC-like complexes; or as γ-tubulin-binding half γTuSCs. **b** | Through conserved lateral interactions, GCP4, GCP5 and GCP6 could be directly incorporated into the ring structure, as opposed to forming a cap structure as in previous models (BOX 1). Although GCP4, GCP5 and GCP6 might incorporate at any position within the ring, it is most attractive to think of them interacting at the ends, where they might function to initiate or terminate ring formation and to stabilize the ring at the overlap. A cartoon of this model was also presented in REF. 49.

It remains to be tested whether such a conformational change in GCP3 is possible and, if so, what might mediate the rearrangement. One possible mechanism is post-translational modification of γTuSC components; indeed, all three of the γTuSC components are phosphorylated at different points during the cell cycle by different kinases, including cyclin-dependent kinase 1 (CDK1) and MPS1 (also known as TTK)[51–53]. Another possibility is that the conformation is changed through allosteric interactions with γTuSC-binding proteins. Although less likely, nucleotide binding and hydrolysis by γ-tubulin may also have a role in regulating the conformation of the complex.

Another possibility is that the predicted conformational change occurs only after microtubule growth has begun. That is, perhaps pairs of protofilaments begin to grow from the properly-spaced γ-tubulins between γTuSCs, and then lateral association of the nascent protofilaments drives the straightening of GCP3. Regulation might then be achieved by modification of the stiffness of the GCP3 hinge. However, growth in this way would seem to be much less favourable than growth from a properly-formed γ-tubulin nucleus with the correct geometry; and, in this case, the γTuRC would function more as a minus-end anchor than as a nucleator.

Conformational regulation of nucleating activity is not an entirely new concept. A very similar mechanism is at play in actin nucleation by the actin-related protein 2/3 (ARP2/3) complex. In this example, the nucleating complex is assembled with the actin homologues ARP2 and ARP3 held apart from each other[54]. The complex is then activated by a structural rearrangement that brings ARP2 and ARP3 together with filamentous actin (F-actin)-like contacts, creating a nucleus for actin filament growth[55,56]. It is striking that evolution seems to have converged on similar mechanisms for regulating nucleation activity in these two very different filament systems.

A new model of γTuRC assembly

The recent progress in understanding γ-tubulin complex structures has led us directly to a revised γTuRC model. As described above, previous models of γTuRC assembly posited a repeating ring of γTuSCs organized by a scaffolding cap composed of GCP4, GCP5 and GCP6 (BOX 1). The roles of GCP4, GCP5 and GCP6 in our model of γTuRC assembly must be revisited in light of several important findings. First, the γTuSC spontaneously assembles ring structures with microtubule-like symmetry without GCP4, GCP5 and GCP6 (FIG. 2), negating the necessity of a scaffolding role for these three proteins. Second, the overall structure and ability to bind γ-tubulin is conserved in GCP2, GCP3 and GCP4 (FIG. 3), suggesting that all of the GCPs directly bind γ-tubulin. Third, a single GCP assembly rule appears to define interactions between GCPs (FIG. 3e), suggesting that all of the GCPs assemble into the γTuRC through equivalent conserved surfaces.

Structural roles of GCP4, GCP5 and GCP6. In light of these findings, we propose a new model for the γTuRC structure, in which GCP4, GCP5 and GCP6 are incorporated directly into the ring structure, each binding directly to γ-tubulin (FIG. 5). This model nicely explains why the observed ratio of γ-tubulin to GCP2 and GCP3 is greater than one[39]. Based on the γTuSC ring structure, the region at the base of the earlier γTuRC structure, which was originally interpreted as a scaffolding cap, appears to consist of the N-terminal regions of the GCPs (FIG. 2c). Indeed, the similarity between the γTuRC structures and the γTuSC ring structure is quite striking, suggesting that the entire γTuRC consists of a ring of γTuSC-like structures.

In the model, GCP4, GCP5 and GCP6 interact with each other, and with GCP2 and GCP3, via the lateral GCP assembly rule. One can imagine GCP4, GCP5 and GCP6 acting as γTuSC-like complexes in one of three modes: as half γTuSCs with a single GCP binding one γ-tubulin; as hybrid γTuSCs, in which a γTuRC-specific GCP replaces GCP2 or GCP3 in the γTuSC; or as completely novel γTuSCs composed of two γTuRC-specific GCPs (FIG. 5a). Different GCPs may assemble through different modes. High-resolution homology modelling of the other GCPs based on the GCP4 crystal structure may prove useful in determining which GCPs directly interact with each other, as well as the potential

limitations on assembly interactions at some surfaces (that is, insertions at some positions near lateral interaction surfaces might be predicted to interfere with further assembly in that direction). γ-tubulin-bound GCP4, GCP5 and GCP6 could then substitute for γTuSC GCPs within the ring by the GCP assembly rule (FIG. 5b).

The positions of GCP4, GCP5 and GCP6 within the ring are unclear. Although they could potentially insert at any position in the ring, some indirect evidence suggests that the three interact directly with each other. Loss of any one of GCP4, GCP5 or GCP6 destabilizes γTuRCs[57–61], suggesting that these GCPs function as a unit to stabilize a well-defined ring. Studies in *A. nidulans*[59] and *Schizosaccharomyces pombe*[62] have also demonstrated a hierarchical localization dependence for GCP4, GCP5 and GCP6, suggesting that they directly interact with each other in γTuRC. In our view, the best place to position GCP4, GCP5 and GCP6 would be at the ends of the ring, where the half-γTuSC overlap occurs. In this location, they could efficiently initiate or terminate γTuSC assembly and could stabilize the ring by interacting with each other across the overlap. By interacting with each other at the ends of the ring, GCP4, GCP5 and GCP6 would also be able to define a single ring-shaped structure, as opposed to the elongated helical filaments that can be formed from the γTuSC alone.

The γTuSC oligomer structure did not reveal how many γTuSCs are required to form a functional microtubule nucleation site — it was consistent with both previous models, with either 12 γ-tubulins and a gap or 14 γ-tubulins and an overlap. A consequence of our model, with GCP4, GCP5 and GCP6 at opposite ends of the ring but interacting with each other, is the prediction that the γTuRC will have an overlap, allowing GCP4, GCP5 and GCP6 to be close enough to interact while also ensuring the formation of a well-defined ring.

In the model, GCP4, GCP5 and GCP6 define the position of the microtubule seam, where αβ-tubulin lateral interactions occur; at this position, a single lateral interaction would be formed between γ-tubulin and α-tubulin. Direct stabilization of the weaker α-tubulin–β-tubulin lateral contacts at the seam could potentially play a part in the nucleation mechanism of the γTuRC. It should also be noted that the new γTuRC model is only consistent with nucleation of a B-lattice configuration (α-tubulin–α-tubulin and β-tubulin–β-tubulin lateral interactions, with the exception of the α-tubulin–β-tubulin interactions at the seam, as depicted in FIG. 1a) and not with an A-lattice configuration (α-tubulin–β-tubulin lateral interactions at each site in the microtubule).

Although the overall structure and γ-tubulin-binding function of the GCP family proteins are conserved, there remains a great deal of variation within the family, largely in the form of multiple insertions and/or deletions within the sequences (BOX 1). These regions are likely to be responsible for unique functionality of the GCPs, and they could serve to alter assembly interactions to ensure incorporation at unique sites within the ring and to act as unique attachment sites to confer γTuRC-specific localization.

Roles of GCPs in γTuRC localization. A clear distinction exists between the γTuRC components that are required for its centrosomal and its spindle localization. Depletion of either GCP2 or GCP3 from *D. melanogaster* S2 cells eliminates the localization of γ-tubulin at centrosomes and spindles and results in gross abnormalities in microtubule organization. However, depletion of GCP4, GCP5 and GCP6 — either singly or all three simultaneously — eliminates the spindle, but not centrosomal, localization of γ-tubulin in S2 cells, as well as in the yeast *A. nidulans*[57,59,63]. Surprisingly, the cells depleted of GCP4, GCP5 and GCP6 are still able to nucleate microtubules from the centrosome and to assemble mitotic spindles. This is perhaps less puzzling in light of the ability of the γTuSC to assemble ring structures without GCP4, GCP5 and GCP6 (REF. 49). These rings, although less stable without GCP4, GCP5 and GCP6, would then be bound to the centrosome through a γTuSC-specific attachment, where they could nucleate microtubules.

γTuRC attachment and activation

In animal cells, the majority of the γTuRCs (80%) are soluble in the cytoplasm[64]. However, its nucleating activity seems to be limited to specific locations in the cell, such as the centrosome or spindle pole body, or within the mitotic spindle. Although a considerable number of proteins are known to bind to cytoplasmic γTuRCs in both interphase and mitosis, including NEDD1, MOZART1, MOZART2A, MOZART2B and NME[34–39], none of them appears to be sufficient to stimulate nucleation. This raises the possibility that binding of γ-tubulin complexes by attachment factors directly induces their nucleating activity. As discussed above, one level of activation probably involves a conformational change in GCP3 to reorganize the γ-tubulin geometry; direct binding of attachment factors may allosterically induce the predicted conformational change in GCP3.

Attachment factors can be roughly categorized in two groups, centrosomal (or spindle pole body) and non-centrosomal, and are discussed below.

Centrosomal attachment factors. The primary mode of centrosomal attachment seems to be through interaction with γTuSC components, as γTuSC centrosomal localization is unaffected by the absence of other γTuRC components. This is demonstrated in budding yeast that lack GCP4, GCP5 and GCP6, and also by the knockdown of these GCPs either singly or together in animal cells. This suggests a conserved mechanism for direct γTuSC attachment to MTOCs, analogous to the way in which the attachment factor Spc110 links the γTuSC to the spindle pole body in budding yeast (FIG. 6a). When fully-assembled γTuRCs are present, there may also be redundant mechanisms for centrosomal attachment that function through the γTuRC-specific proteins (FIG. 6b).

In the case of budding yeast, direct binding to the attachment factor Spc110 was not sufficient to fully activate the γTuSC *in vitro*, although this may have been due to the use of a truncated form of Spc110 (REF. 49). In animal cells, several centrosomal proteins have been described to bind or activate γ-tubulin complexes,

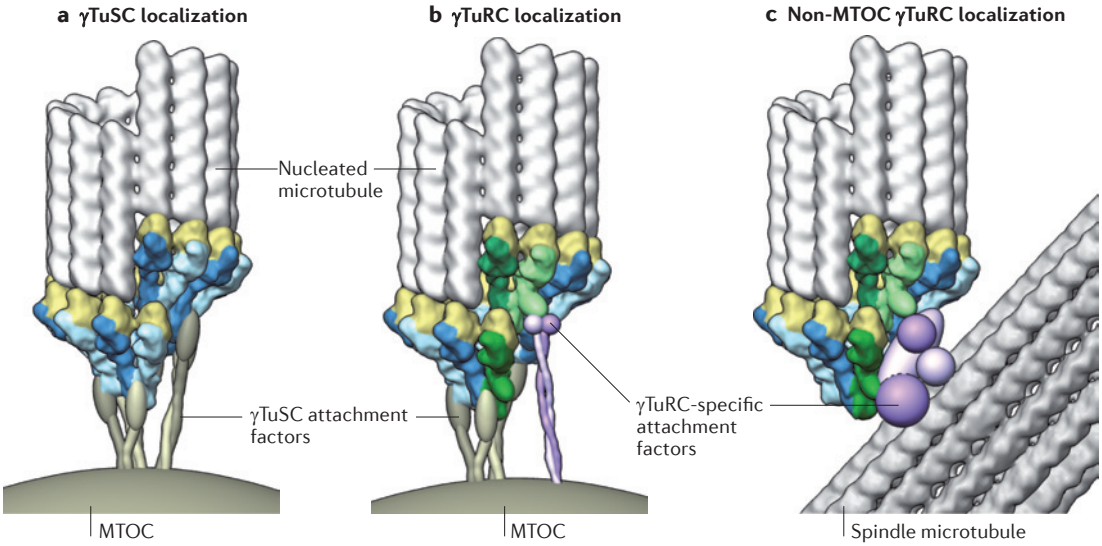


Figure 6 | **Modes of γTuSC- and γTuRC-specific attachment. a** | A conserved mechanism exists for direct γ-tubulin small complex (γTuSC) attachment to the microtubule-organizing centre (MTOC). In budding yeast, the γTuSC is attached to the nuclear face of the MTOC by spindle pole body component 110 (Spc110), which serves not only to localize the γTuSC but also to promote its assembly into rings. **b** | In organisms with complete γ-tubulin ring complexes (γTuRCs), an analogous means of γTuSC-mediated attachment must exist, as γTuSC localizes at the MTOC even when all of the γTuRC-specific components (γ-tubulin complex protein 4 (GCP4), GCP5 and GCP6; shown in green) are depleted. Redundant γTuRC-specific attachment factors may also exist at the MTOC (shown in purple). **c** | Localization of nucleating complexes at non-MTOC sites within the cell is largely dependent on the presence of all three γTuRC-specific proteins. For example, γTuRC localization to existing microtubules within the mitotic spindle by augmin (shown in purple) requires GCP4, GCP5 and GCP6. Figure is modified, with permission, from REF. 49

including pericentrin, centrosome- and Golgi-localized PKN-associated protein (CG-NAP; also known as AKAP450), ninein, and centrosomal protein of 192 kDa (CEP192)[65–69]. These are all large structural proteins that form coiled-coil interactions, and, based on reconstructions of the pericentriolar material in which γTuRCs are embedded, all are putative scaffolding components of this fibrous pericentriolar matrix[70]. For some of these proteins, an interaction with GCP2 and GCP3 has been proposed, but it is unclear whether these interactions are direct or indirect[65,66].

Non-centrosomal attachment factors. In contrast to γTuSC-mediated localization at MTOCs, attachment of γ-tubulin complexes at other sites appears to depend largely on the γTuRC-specific GCPs (FIG. 6c). The recently discovered eight-subunit augmin complex is a non-centrosomal γTuRC attachment factor that is important for γTuRC localization within the mitotic spindle[63,71–75]. Depletion of augmin components leads to loss of γTuRC localization within the spindle but does not affect its centrosomal localization[63,72,73,76]. Depletion of GCP4, GCP5, GCP6 or NEDD1 also results in loss of γ-tubulin localization within the spindle[37,57], suggesting that augmin may interact with the γTuRC through one or all of these components[77].

Based on these data, it has been proposed that augmin links γTuRCs to the surface of spindle microtubules, where they function as secondary nucleation sites for additional spindle microtubules[71]. A similar function has been suggested for mitochondrial translation optimization 1 (Mto1), a γTuRC attachment factor that binds along microtubules in fission yeast cytoplasmic arrays[62]. The regular arrangement of microtubule arrays that result from Mto1 or augmin sites in fission yeast, *D. melanogaster* and human cells, suggests that the γTuRC is bound to the microtubules in a defined geometry which dictates the orientation of freshly nucleated microtubules. This would be consistent with observations in the acentrosomal microtubule arrays of plants, where the γTuRC is recruited to the surface of existing microtubules and nucleates new microtubules with a well-defined branch angle[28,78].

A clear link between the localization of the γTuRC and the activation of nucleation was demonstrated in *S. pombe*: when the cytoplasmic attachment factor Mto1 is deleted, cytoplasmic microtubule nucleation is completely abolished[62]. Other studies suggest similar activation ability for a class of proteins that includes Mto1, centrosomin in *D. melanogaster*, and CDK5 regulatory subunit-associated protein 2 (CDK5RAP2) and myomegalin in vertebrates[79]. In contrast to Mto1, which is a specific cytoplasmic attachment factor, centrosomin, CDK5RAP2 and myomegalin are found both at the centrosome and in the cytoplasm, and they may therefore participate in both centrosomal and cytoplasmic recruitment of γTuRCs. All of these proteins are related by the presence of a motif of ~60 amino acids, which has been dubbed the γTuRC-mediated nucleation activator (γTuNA) motif[39]. Overexpression of protein fragments containing γTuNA strongly induces cytoplasmic microtubule nucleation in a γ-tubulin-dependent manner in

human and *D. melanogaster* cells[39,80]. Moreover, γTuNA itself directly binds the γTuRC and greatly enhances its ability to nucleate microtubules *in vitro*, providing a direct functional link between the localization and the activation of the γTuRC. It remains unclear how, and via which γTuRC components, γTuNA induces microtubule activation, as binding seems to occur only if the intact γTuRC is present[39].

Conclusions

The recent structural studies described above have enhanced our understanding of γ-tubulin based microtubule nucleation. γ-tubulin complexes have been shown to form microtubule templates that almost certainly nucleate microtubules through longitudinal contacts with α-tubulin and β-tubulin. This activity appears to be regulated, at least in part, through the conformation of GCP3. Which proteins modulate γTuRC activity, and by what mechanism, remains a pressing question in understanding γTuRC regulation. Increasingly, it seems that the attachment, both centrosomal and non-centrosomal, of the γTuRC is correlated with an increase in its nucleating activity; the observation that the small γTuNA motif enhances γTuRC nucleation activity provides another tool for understanding the mechanism of attachment-factor based enhancement and whether this correlates directly with the predicted change in GCP3.

Another major problem to solve in understanding γ-tubulin complex function is the role of nucleotide binding and hydrolysis in nucleation. γ-tubulin and β-tubulin have similar affinities and basal hydrolysis rates for GTP. However, it remains an open question whether the formation of longitudinal contacts with α-tubulin stimulates hydrolysis of the GTP bound by γ-tubulin (as it does for GTP-bound β-tubulin), and whether hydrolysis weakens the α-tubulin–γ-tubulin interaction (as it does with the α-tubulin–β-tubulin interaction). For example, complete hydrolysis of GTP on the γTuRC could facilitate the release of bound microtubules.

Our revised model for γTuRC assembly, with GCP4, GCP5, and GCP6 interacting with the γTuSC as part of the ring itself, provides a new framework for future studies aimed at elucidating the mechanistic basis of γTuRC function, regulation and localization. In particular, it will now be important to determine the individual functions of GCP4, GCP5 and GCP6, the specific interactions they make with each other and with the γTuSC, and their positions within the γTuRC. To this end, structural work and modelling of individual components, as well as a higher resolution structure of the γTuRC itself, will be necessary to provide an accurate pseudo-atomic model of the entire γTuRC. This model will doubtless prove to be invaluable in generating specific, testable hypotheses about γTuRC function and regulation.

1. Nogales, E., Whittaker, M., Milligan, R. A. & Downing, K. H. High-resolution model of the microtubule. *Cell* **96**, 79–88 (1999).
2. Nogales, E., Wolf, S. G. & Downing, K. H. Structure of the αβ tubulin dimer by electron crystallography. *Nature* **391**, 199–203 (1998).
3. Chretien, D. & Wade, R. H. New data on the microtubule surface lattice. *Biol. Cell* **71**, 161–174 (1991).
4. Sui, H. & Downing, K. H. Structural basis of interprotofilament interaction and lateral deformation of microtubules. *Structure* **18**, 1022–1031 (2010).
5. Ledbetter, M. C. & Porter, K. R. Morphology of microtubules of plant cell. *Science* **144**, 872–874 (1964).
6. Tilney, L. G. *et al.* Microtubules: evidence for 13 protofilaments. *J. Cell Biol.* **59**, 267–275 (1973).
7. Evans, L., Mitchison, T. & Kirschner, M. Influence of the centrosome on the structure of nucleated microtubules. *J. Cell Biol.* **100**, 1185–1191 (1985).
8. Mandelkow, E. M., Schultheiss, R., Rapp, R., Muller, M. & Mandelkow, E. On the surface lattice of microtubules: helix starts, protofilament number, seam, and handedness. *J. Cell Biol.* **102**, 1067–1073 (1986).
9. McEwen, B. & Edelstein, S. J. Evidence for a mixed lattice in microtubules reassembled *in vitro*. *J. Mol. Biol.* **139**, 123–145 (1980).
10. Rice, L. M., Montabana, E. A. & Agard, D. A. The lattice as allosteric effector: structural studies of αβ- and γ-tubulin clarify the role of GTP in microtubule assembly. *Proc. Natl Acad. Sci. USA* **105**, 5378–5383 (2008).
11. Wilson, E. B. *The Cell in Development and Heredity* (Macmillan, New York, 1928).
12. Azimzadeh, J. & Bornens, M. Structure and duplication of the centrosome. *J. Cell Sci.* **120**, 2139–2142 (2007).
13. Jaspersen, S. L. & Winey, M. The budding yeast spindle pole body: structure, duplication, and function. *Annu. Rev. Cell Dev. Biol.* **20**, 1–28 (2004).
14. Wasteneys, G. O. & Ambrose, J. C. Spatial organization of plant cortical microtubules: close encounters of the 2D kind. *Trends Cell Biol.* **19**, 62–71 (2009).
15. Oakley, C. E. & Oakley, B. R. Identification of γ-tubulin, a new member of the tubulin superfamily encoded by *mipA* gene of *Aspergillus nidulans*. *Nature* **338**, 662–664 (1989).
16. Oakley, B. R., Oakley, C. E., Yoon, Y. & Jung, M. K. γ-tubulin is a component of the spindle pole body that is essential for microtubule function in *Aspergillus nidulans*. *Cell* **61**, 1289–1301 (1990).
17. Zheng, Y., Jung, M. K. & Oakley, B. R. γ-tubulin is present in *Drosophila melanogaster* and *Homo sapiens* and is associated with the centrosome. *Cell* **65**, 817–823 (1991).
18. Stearns, T., Evans, L. & Kirschner, M. γ-tubulin is a highly conserved component of the centrosome. *Cell* **65**, 825–836 (1991).
19. Sobel, S. G. & Snyder, M. A highly divergent γ-tubulin gene is essential for cell growth and proper microtubule organization in *Saccharomyces cerevisiae*. *J. Cell Biol.* **131**, 1775–1788 (1995).
20. Horio, T. *et al.* The fission yeast γ-tubulin is essential for mitosis and is localized at microtubule organizing centers. *J. Cell Sci.* **99**, 693–700 (1991).
21. Spang, A., Geissler, S., Grein, K. & Schiebel, E. γ-tubulin-like Tub4p of *Saccharomyces cerevisiae* is associated with the spindle pole body substructures that organize microtubules and is required for mitotic spindle formation. *J. Cell Biol.* **134**, 429–441 (1996).
22. Raff, J. W., Kellogg, D. R. & Alberts, B. M. *Drosophila* γ-tubulin is part of a complex containing two previously identified centrosomal MAPs. *J. Cell Biol.* **121**, 823–835 (1993).
23. Stearns, T. & Kirschner, M. *In vitro* reconstitution of centrosome assembly and function: the central role of γ-tubulin. *Cell* **76**, 623–637 (1994).
24. Zheng, Y., Wong, M. L., Alberts, B. & Mitchison, T. Nucleation of microtubule assembly by a γ-tubulin-containing ring complex. *Nature* **378**, 578–583 (1995).
25. Knop, M., Pereira, G., Geissler, S., Grein, K. & Schiebel, E. The spindle pole body component Spc97p interacts with the γ-tubulin of *Saccharomyces cerevisiae* and functions in microtubule organization and spindle pole body duplication. *EMBO J.* **16**, 1550–1564 (1997).
26. Geissler, S. *et al.* The spindle pole body component Spc98p interacts with the γ-tubulin-like Tub4p of *Saccharomyces cerevisiae* at the sites of microtubule attachment. *EMBO J.* **15**, 3899–3911 (1996).
27. Mishra, R. K., Chakraborty, P., Arnaoutov, A., Fontoura, B. M. & Dasso, M. The Nup107–160 complex and γ-TuRC regulate microtubule polymerization at kinetochores. *Nature Cell Biol.* **12**, 164–169.
28. Murata, T. *et al.* Microtubule-dependent microtubule nucleation based on recruitment of γ-tubulin in higher plants. *Nature Cell Biol.* **7**, 961–968 (2005).
29. Oegema, K. *et al.* Characterization of two related *Drosophila* γ-tubulin complexes that differ in their ability to nucleate microtubules. *J. Cell Biol.* **144**, 721–733 (1999).
30. Vinh, D. B., Kern, J. W., Hancock, W. O., Howard, J. & Davis, T. N. Reconstitution and characterization of budding yeast γ-tubulin complex. *Mol. Biol. Cell* **13**, 1144–1157 (2002).
31. Gunawardane, R. N. *et al.* Characterization and reconstitution of *Drosophila* γ-tubulin ring complex subunits. *J. Cell Biol.* **151**, 1513–1524 (2000).
32. Murphy, S. M. *et al.* GCP5 and GCP6: two new members of the human γ-tubulin complex. *Mol. Biol. Cell* **12**, 3340–3352 (2001).
33. Murphy, S. M., Urbani, L. & Stearns, T. The mammalian γ-tubulin complex contains homologues of the yeast spindle pole body components Spc97p and Spc98p. *J. Cell Biol.* **141**, 663–674 (1998).
34. Teixido-Travesa, N. *et al.* The γTuRC revisited: a comparative analysis of interphase and mitotic human γTuRC redefines the set of core components and identifies the novel subunit GCP8. *Mol. Biol. Cell* **21**, 3963–3972 (2010).
Describes a new core γTuRC component, MOZART2 (referred to here as GCP8), and shows that it is necessary for interphase localization of the γTuRC to the centrosome but not for γTuRC assembly.
35. Hutchins, J. R. *et al.* Systematic analysis of human protein complexes identifies chromosome segregation proteins. *Science* **328**, 593–599 (2010).
Identifies two new γTuRC components, MOZART1 and MOZART2, and shows that MOZART1 depletion results in abnormal spindles and reduced γ-tubulin recruitment to mitotic spindle poles. This suggests a role for MOZART1 in γTuRC localization.
36. Gunawardane, R. N., Martin, O. C. & Zheng, Y. Characterization of a new γTuRC subunit with WD repeats. *Mol. Biol. Cell* **14**, 1017–1026 (2003).

37. Luders, J., Patel, U. K. & Stearns, T. GCP-WD is a γ-tubulin targeting factor required for centrosomal and chromatin-mediated microtubule nucleation. *Nature Cell Biol.* **8**, 137–147 (2006).
38. Haren, L. *et al.* NEDD1-dependent recruitment of the γ-tubulin ring complex to the centrosome is necessary for centriole duplication and spindle assembly. *J. Cell Biol.* **172**, 505–515 (2006).
39. Choi, Y. K., Liu, P., Sze, S. K., Dai, C. & Qi, R. Z. CDK5RAP2 stimulates microtubule nucleation by the γ-tubulin ring complex. *J. Cell Biol.* **191**, 1089–1095 (2010).
Shows that a class of γTuRC attachment factors, which includes CDK5RAP2, centrosomin, Mto1 and myomegalin, also stimulates γTuRC activity both *in vivo* and *in vitro*. Also demonstrates that a short sequence motif, γTuNA, directly binds the γTuSC and is sufficient to stimulate microtubule nucleation.
40. Moritz, M., Braunfeld, M. B., Guenebaut, V., Heuser, J. & Agard, D. A. Structure of the γ-tubulin ring complex: a template for microtubule nucleation. *Nature Cell Biol.* **2**, 365–370 (2000).
41. Wiese, C. & Zheng, Y. A new function for the γ-tubulin ring complex as a microtubule minus-end cap. *Nature Cell Biol.* **2**, 358–364 (2000).
42. Keating, T. J. & Borisy, G. G. Immunostructural evidence for the template mechanism of microtubule nucleation. *Nature Cell Biol.* **2**, 352–357 (2000).
43. Byers, B., Shriver, K. & Goetsch, L. The role of spindle pole bodies and modified microtubule ends in the initiation of microtubule assembly in *Saccharomyces cerevisiae*. *J. Cell Sci.* **30**, 331–352 (1978).
44. Erickson, H. P. & Stoffler, D. Protofilaments and rings, two conformations of the tubulin family conserved from bacterial FtsZ to α/β and γtubulin. *J. Cell Biol.* **135**, 5–8 (1996).
45. Erickson, H. P. γ-tubulin nucleation: template or protofilament? *Nature Cell Biol.* **2**, E93–E96 (2000).
46. Aldaz, H., Rice, L. M., Stearns, T. & Agard, D. A. Insights into microtubule nucleation from the crystal structure of human γ-tubulin. *Nature* **435**, 523–527 (2005).
47. Kollman, J. M. *et al.* The structure of the γ-tubulin small complex: implications of its architecture and flexibility for microtubule nucleation. *Mol. Biol. Cell* **19**, 207–215 (2008).
48. Choy, R. M., Kollman, J. M., Zelter, A., Davis, T. N. & Agard, D. A. Localization and orientation of the γ-tubulin small complex components using protein tags as labels for single particle EM. *J. Struct. Biol.* **168**, 571–574 (2009).
49. Kollman, J. M., Polka, J. K., Zelter, A., Davis, T. N. & Agard, D. A. Microtubule nucleating γ-TuSC assembles structures with 13-fold microtubule-like symmetry. *Nature* **466**, 879–882 (2010).
Demonstrates the ability of the γTuSC to spontaneously assemble into rings, and presents the structure of γTuSC rings, which have 13-fold symmetry but are in an 'off' state owing to the conformation of γTuSC components.
50. Guillet, V. *et al.* Crystal structure of γ-tubulin complex protein GCP4 provides insight into microtubule nucleation. *Nature Struct. Mol. Biol.* **18**, 915–919 (2011).
Presents the first atomic structure of a GCP, GCP4, which is very similar to GCP2 and GCP3 in the γTuSC, providing the basis for a pseudo-atomic model of the γTuSC and a new model for γTuRC organization.
51. Keck, J. M. *et al.* A cell cycle phosphoproteome of the yeast centrosome. *Science* **332**, 1557–1561 (2011).
52. Vogel, J. *et al.* Phosphorylation of γ-tubulin regulates microtubule organization in budding yeast. *Dev. Cell* **1**, 621–631 (2001).
53. Lin, T. C. *et al.* Phosphorylation of the yeast γ-tubulin Tub4 regulates microtubule function. *PLoS ONE* **6**, e19700 (2011).
54. Robinson, R. C. *et al.* Crystal structure of Arp2/3 complex. *Science* **294**, 1679–1684 (2001).
55. Volkmann, N. *et al.* Structure of Arp2/3 complex in its activated state and in actin filament branch junctions. *Science* **293**, 2456–2459 (2001).
56. Rodal, A. A. *et al.* Conformational changes in the Arp2/3 complex leading to actin nucleation. *Nature Struct. Mol. Biol.* **12**, 26–31 (2005).
57. Verollet, C. *et al. Drosophila melanogaster* γ-TuRC is dispensable for targeting γ-tubulin to the centrosome and microtubule nucleation. *J. Cell Biol.* **172**, 517–528 (2006).
58. Vogt, N., Koch, I., Schwarz, H., Schnorrer, F. & Nusslein-Volhard, C. The γTuRC components Grip75 and Grip128 have an essential microtubule-anchoring function in the *Drosophila* germline. *Development* **133**, 3963–3972 (2006).
59. Xiong, Y. & Oakley, B. R. *In vivo* analysis of the functions of γ-tubulin-complex proteins. *J. Cell Sci.* **122**, 4218–4227 (2009).
Demonstrates a hierarchical organization of γTuRC-specific GCPs in *A. nidulans*.
60. Zhang, L., Keating, T. J., Wilde, A., Borisy, G. G. & Zheng, Y. The role of Xgrip210 in γ-tubulin ring complex assembly and centrosome recruitment. *J. Cell Biol.* **151**, 1525–1536 (2000).
61. Izumi, N., Fumoto, K., Izumi, S. & Kikuchi, A. GSK-3β regulates proper mitotic spindle formation in cooperation with a component of the γ-tubulin ring complex, GCP5. *J. Biol. Chem.* **283**, 12981–12991 (2008).
62. Anders, A., Lourenco, P. C. & Sawin, K. E. Noncore components of the fission yeast γ-tubulin complex. *Mol. Biol. Cell* **17**, 5075–5093 (2006).
63. Goshima, G. *et al.* Genes required for mitotic spindle assembly in *Drosophila* S2 cells. *Science* **316**, 417–421 (2007).
64. Moudjou, M., Bordes, N., Paintrand, M. & Bornens, M. γ-tubulin in mammalian cells: the centrosomal and the cytosolic forms. *J. Cell Sci.* **109**, 875–887 (1996).
65. Takahashi, M., Yamagiwa, A., Nishimura, T., Mukai, H. & Ono, Y. Centrosomal proteins CG-NAP and kendrin provide microtubule nucleation sites by anchoring γ-tubulin ring complex. *Mol. Biol. Cell* **13**, 3235–3245 (2002).
66. Zimmerman, W. C., Sillibourne, J., Rosa, J. & Doxsey, S. J. Mitosis-specific anchoring of γ tubulin complexes by pericentrin controls spindle organization and mitotic entry. *Mol. Biol. Cell* **15**, 3642–3657 (2004).
67. Delgehyr, N., Sillibourne, J. & Bornens, M. Microtubule nucleation and anchoring at the centrosome are independent processes linked by ninein function. *J. Cell Sci.* **118**, 1565–1575 (2005).
68. Gomez-Ferreria, M. A. *et al.* Human Cep192 is required for mitotic centrosome and spindle assembly. *Curr. Biol.* **17**, 1960–1966 (2007).
69. Zhu, F. *et al.* The mammalian SPD-2 ortholog Cep192 regulates centrosome biogenesis. *Curr. Biol.* **18**, 136–141 (2008).
70. Moritz, M., Braunfeld, M. B., Sedat, J. W., Alberts, B. & Agard, D. A. Microtubule nucleation by γ-tubulin-containing rings in the centrosome. *Nature* **378**, 638–640 (1995).
71. Goshima, G., Mayer, M., Zhang, N., Stuurman, N. & Vale, R. D. Augmin: a protein complex required for centrosome-independent microtubule generation within the spindle. *J. Cell Biol.* **181**, 421–429 (2008).
Describes the augmin complex, showing that it is critical for γTuRC-specific localization within the mitotic spindle, which leads to amplification of microtubules within the spindle.
72. Lawo, S. *et al.* HAUS, the 8-subunit human augmin complex, regulates centrosome and spindle integrity. *Curr. Biol.* **19**, 816–826 (2009).
73. Uehara, R. *et al.* The augmin complex plays a critical role in spindle microtubule generation for mitotic progression and cytokinesis in human cells. *Proc. Natl Acad. Sci. USA* **106**, 6998–7003 (2009).
74. Meireles, A. M., Fisher, K. H., Colombie, N., Wakefield, J. G. & Ohkura, H. Wac: a new Augmin subunit required for chromosome alignment but not for acentrosomal microtubule assembly in female meiosis. *J. Cell Biol.* **184**, 777–784 (2009).
75. Wainman, A. *et al.* A new augmin subunit, Msd1, demonstrates the importance of mitotic spindle-templated microtubule nucleation in the absence of functioning centrosomes. *Genes Dev.* **23**, 1876–1881 (2009).
76. Bucciarelli, E. *et al. Drosophila* Dgt6 interacts with Ndc80, Msps/XMAP215, and γ-tubulin to promote kinetochore-driven MT formation. *Curr. Biol.* **19**, 1839–1845 (2009).
77. Zhu, H., Coppinger, J. A., Jang, C. Y., Yates, J. R., 3rd & Fang, G. FAM29A promotes microtubule amplification via recruitment of the NEDD1–γ-tubulin complex to the mitotic spindle. *J. Cell Biol.* **183**, 835–48 (2008).
78. Nakamura, M., Ehrhardt, D. W. & Hashimoto, T. Microtubule and katanin-dependent dynamics of microtubule nucleation complexes in the acentrosomal *Arabidopsis* cortical array. *Nature Cell Biol.* **12**, 1064–1070 (2010).
79. Sawin, K. E., Lourenco, P. C. & Snaith, H. A. Microtubule nucleation at non-spindle pole body microtubule-organizing centers requires fission yeast centrosomin-related protein Mod20p. *Curr. Biol.* **14**, 763–775 (2004).
80. Terada, Y., Uetake, Y. & Kuriyama, R. Interaction of Aurora-A and centrosomin at the microtubule-nucleating site in *Drosophila* and mammalian cells. *J. Cell Biol.* **162**, 757–763 (2003).
81. Wiese, C. & Zheng, Y. Microtubule nucleation: γ-tubulin and beyond. *J. Cell Sci.* **119**, 4143–4153 (2006).

Competing interests statement
The authors declare no competing financial interests.

FURTHER INFORMATION
Andreas Merdes's homepage: http://www-cbd.ups-tlse.fr/spip.php?rubrique37
Lionel Mourey's homepage: http://www.ipbs.fr/?-Biophysique-structurale-Lionel-
David A. Agard's homepage: http://www.msg.ucsf.edu/agard
ALL LINKS ARE ACTIVE IN THE ONLINE PDF