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Microtubule nucleation at the centrosome and beyond

Sabine Petry and Ronald D. Vale

The overall organization of the microtubule cytoskeleton depends critically on where and when new microtubules are nucleated. Here we review recent work on factors that are involved in localizing and potentially activating γ-tubulin, the key microtubule nucleating protein.

Microtubules, 25 nm polar filaments assembled from α/β-tubulin heterodimers, permeate the cytoplasm of virtually all eukaryotic cells. They serve as the chief structural component of the mitotic spindle in dividing cells and provide roadways for motor-driven transport to position the majority of membrane-bounded organelles in animal cells[1]. Microtubules also work in conjunction with the actin cytoskeleton and signalling systems to establish and maintain cell polarity[2].

Microtubule assembly occurs *in vitro* when the concentrations of pure α/β-tubulin heterodimers exceed a 'critical concentration'. However, they rarely spontaneously assemble in cells and instead require a nucleating factor to initiate polymerization. Microtubules are nucleated from the centrosome, which was originally believed to be the sole cellular structure from which microtubules are nucleated and was thus termed the microtubule organizing centre (MTOC). Microtubules were proposed to then dissociate or sever from the centrosome for transport to other locations in the cell by molecular motors. Although severing from the centrosome and motor-driven transport are likely to occur in many cells[3], it is becoming clear that microtubule nucleation also takes place in other cellular locations. Indeed, many cells (egg meiotic and plant cells, for instance) lack classical centrosomes or can function without them. The list of non-centrosomal MTOCs is now considerable, and still expanding: during cell division, microtubules originate from spindle microtubules[4], kinetochores[5] and in the vicinity of chromatin[6]; and during interphase, microtubules are made at the nuclear envelope[7], the Golgi apparatus[8,9], pre-existing microtubules[10,11] and the plasma membrane[12]. Collectively, these non-centrosomal sites seem to play an important role in governing cellular microtubule architecture.

The best-characterized microtubule nucleating factors are protein complexes of γ-tubulin and several associated proteins. The yeast γ-tubulin small complex (γ-TuSC) consists of 2 γ-tubulin molecules and one each of γ-tubulin complex proteins (GCP) 2 and 3 (refs. 13–15), and is capable of moderately facilitating microtubule nucleation[16]. In higher eukaryotes, the larger γ-tubulin ring complex (γ-TuRC), named for its characteristic shape observed in negative-stain electron microscopy (EM) images[17], is a marginally more efficient microtubule nucleator[14]. Both the γ-TuSC and γ-TuRC complexes are thought to form a ring-like template that binds the α-tubulin subunit, which is exposed at the microtubule minus end. Thus, microtubules grow with a defined polarity from γ-tubulin complexes, with their minus ends anchored on the ring of γ-tubulin subunits and the plus end of the microtubule extending into the cytoplasm.

In this Perspective, we focus on the latest research and emerging questions concerning the regulation of γ-tubulin activity. Recent work has uncovered some of the factors that dock γ-tubulin to different structures in the cell. Furthermore, purified γ-tubulin complexes seem not to be constitutively active, suggesting that the efficiency of γ-tubulin-mediated microtubule nucleation is tuned by regulatory factors, a few of which have been identified.

Structural insight into γ-TuSC and γ-TuRC

Under certain conditions, the yeast γ-TuSC tetramer assembles into transient spiral-like structures, in which the first turn shares similar features with the γ-TuRC ring[18]. The helical symmetry made it possible to apply averaging techniques, resulting in a medium-resolution cryo-EM reconstruction at 8 Å; this revealed new details on how γ-tubulin, GCP2 and GCP3 are arranged within γ-TuSC[16]. In contrast, much less is known about the architecture of γ-TuRC. In 2000, three landmark papers reported that it forms a cone-like cap at the microtubule minus end following microtubule nucleation[19–21]. Based on prior results and gold labelling of individual subunits, the γ-TuRC-specific subunits (GCP4, 5 and 6), which are essential for γ-TuRC formation, were first hypothesized to bind to GCP2 and 3 to form the distal tip of the γ-TuRC cap. However, the crystal structure of GCP4 (ref. 22) indicates another possibility. All GCPs are characterized by an N-terminal Grip1 and a C-terminal Grip2 motif, and GCP4 can bind directly to γ-tubulin *in vitro* via its Grip2 motif[22]. This suggests that GCP4, and perhaps also GCP5 and 6, which are closely related to GCP4 in their sequence and domain organization, might be directly incorporated into the γ-TuRC ring and contact γ-tubulin, instead of only forming the distal end of the complex (Fig. 1). Consistent with this idea, modelling of the GCP4 crystal structure into the EM reconstruction of the yeast γ-TuSC provided a good fit and revealed a potential hinge point in the middle of the structure. Straightening this hinge might decrease the spacing between γ-tubulin molecules within the γ-TuSC ring and bring the complex into a hypothesized nucleation-competent conformation[22], which would be assumed when bound to a microtubule[23]. When γ-TuSC is artificially trapped in this closed state, its nucleation capacity is increased, further supporting the idea of this allosteric switch[23]. An even higher increase in microtubule nucleation

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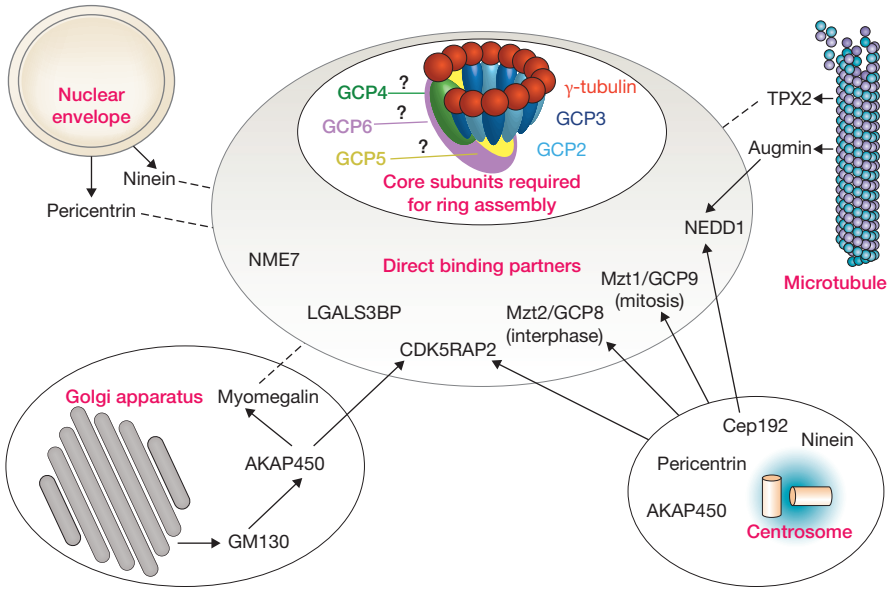

Figure 1 A schematic diagram of γ-TuRC composition and its connections to various microtubule nucleation pathways in an animal cell. Inner central circle: The core subunits γ-tubulin and GCP2–6 are required for γ-TuRC ring formation. Recent structural evidence suggests that GCP4 to 6 may be directly incorporated into the γ-TuRC ring instead of forming only its very tip. However, the exact location of GCP4–6 is not known (question marks). Outer central circle: Other proteins have been co-purified with γ-TuRC and thus directly bind γ-TuRC *in vitro*. These proteins may not be constitutive subunits, because it is not yet clear whether all γ-TuRC populations contain them. These include the kinase NME7, CDK5RAP2, Mzt1, Mzt2 and NEDD1, which are described in the main text, as well as the protein LGALS3BP, the precise role of which remains to be defined. All other indicated proteins have been shown to target γ-TuRC to different MTOCs (nuclear envelope, Golgi apparatus, centrosome and MTs). Dashed lines indicate an interaction with γ-TuRC where the interacting subunit is not yet known, whereas solid arrows indicate recruitment through an identified protein or subunit. Interactions or recruitment can occur for the whole MTOC (line/arrow from MTOC border) or with individual proteins (line/arrow pointing to the protein name).

activity could be achieved with tubulin of the same species as γ-TuRC (*Saccharomyces cerevisiae*) instead of porcine brain tubulin, which is used traditionally[23]. However, higher-resolution structures of the complete γ-TuRC (similar to those achieved for the γ-TuSC spiral), both before and after microtubule nucleation, will be needed to understand how GCP4, 5 and 6 are arranged in the complex, and how γ-TuRC facilitates microtubule nucleation (Table 1).

Localization and activation factors for yeast γ-tubulin complexes

γ-Tubulin complexes must be correctly localized and activated in cells. This process is perhaps best understood in yeast, which has a relatively simple microtubule cytoskeleton made up of approximately 40 filaments. Spc110 appears to be a critical protein both for localizing and activating the complex, as shown in *S. cerevisiae*. The N-terminus of Spc110, which induces a ring-like assembly of γ-TuSC as discussed above[18], contains two conserved motifs important for microtubule nucleation activation: the conserved CM1 motif[24] and a newly discovered Spc110/Pcp1 motif (SPM), which is also present in the fission yeast spindle body protein Pcp1 and human pericentrin[25]. Both the CM1 and SPM motifs, and phosphorylation by the cell-cycle-regulating kinases Mps1 and Cdk in the central region of Spc110, are important for promoting Spc110 oligomerization, but only phosphorylation and the SPM motif seem to control microtubule nucleation[25].

Other proteins that interact with γ-tubulin complexes contain only the CM1 motif; for example, Spc72, Mto1, centrosomin, CDK5RAP2 and myomegalin[25]. Among the best characterized of these is the *Schizosaccharomyces pombe* Mto1 protein, which forms a complex with Mto2. The C-terminus of Mto1 localises the γ-tubulin complex to several cytoplasmic MTOCs and the spindle pole body, and its Nterminal CM1 motif, in conjunction with Mto2, also promotes the microtubule nucleation activity of γ-tubulin complexes[26]. The roles in recruitment and activation of γ-tubulin could be separated in minimal Mto1/2 versions, and are therefore independent of one another[26]. Active nucleation complexes are likely to contain 13 copies each of Mto1 and Mto2, along with a γtubulin complex[26]. Lynch *et al.* also showed that multimerization of Mto1/2 may induce multimerization of γ-TuSC[26]. However, how and to what extent the Mto1/2 complex activates yeast γ-tubulin remains to be determined.

In summary, the microtubule nucleation and localization of the yeast γ-tubulin complex is regulated by interacting factors and activating phosphorylations. It is currently not clear whether individual proteins encode all these functionalities (for example, Spc110), or whether multiple activating factors are required simultaneously. It will be important to assess the extent to which these factors activate γTuSC on their own versus in various combinations, and, most importantly, how activation is achieved. This will require quantitative *in vitro* studies to complement the elegant *in vivo* studies that have been performed in yeast. Finally, an EM structure of a fully activated yeast γ-TuSC assembly, in conjunction with the current medium-resolution structure of the yeast γ-TuSC spiral, will provide useful insights into the mechanism of activation.

Localization and activation factors of higher eukaryote γ-tubulin complexes

Higher eukaryotic cells can have up to 1,000 times more microtubules than yeast. Not surprisingly, the regulation of microtubule nucleation seems more complex. Besides the GRIP-motif-containing GCPs that build γ-TuRC, additional proteins co-purify with the complex, many of which may play a role in its localization and activation (Fig. 1 and Table 1). Perhaps the best studied is the WD-domain protein of 71 kDa (Dgp71WD), which co-purifies with *Drosophila melanogaster* γTuRC. Depletion of this protein, or its *Xenopus laevis* orthologue NEDD1, impairs γ-tubulin recruitment to spindle microtubules[27,28]. Depletion or inhibition of the human orthologue GCP-WD abolishes γ-TuRC localization and microtubule nucleation at the centrosome and chromatin[28,29]. Differential phosphorylation is likely to control the sites to which NEDD1 recruits γ-TuRC[30,31], yet it remains unclear whether GCP-WD also stimulates the microtubule nucleation activity of γ-TuRC. A second localization factor pair is MOZART2A/B (mitotic-spindle organizing protein associated with a ring of γtubulin, 16.2 kDa), which has recently been renamed GCP8A/B. These highly related proteins (98% sequence identity) were co-purified with γ-TuRC from human cells in two independent studies[32,33]. GCP8B co-localizes with γ-TuRC and is necessary for its

Table 1 Overview of γ-TuRC complexes and the assays that have been performed

γ-Tubulin complex			Subunit	Co-purification with γ-TuRC	Binding site on γ-TuRC	Effect on *in vitro* nucleation activity
γ-TuSC	Core γ-TuRC with subunits required for ring assembly	Extended γ-TuRC with direct binding partners	γ-Tubulin	✓	First ring layer and GCPs	γ-TuSC sufficient for nucleation
			GCP2	✓	γ-Tubulin, GCP3	
			GCP3	✓	γ-Tubulin, GCP2	
			GCP4	✓	γ-Tubulin, other?	γ-TuRC nucleation greater than γ-TuSC
			GCP5	✓	ND	
			GCP6	✓	ND	
			NEDD1	✓	ND	ND
			NME7	✓	ND	2.5-fold enhancement for γ-TuRC
			CDK5RAP2	✓	ND	7.5-fold enhancement for γ-TuRC
			Mzt2/GCP8	✓	ND	ND
			Mzt1/GCP9	✓	GCP3, other?	ND
			LGALS3BP	✓	ND	ND

For γ-TuSC, co-purification has been achieved, its *in vitro* nucleation activity has been tested, the binding sites for subunits γ-tubulin, GCP2 and GCP3 have been identified, and a medium-resolution structure has been solved by electron microscopy (EM). In contrast, the exact binding sites for GCP4, 5 and 6 are not clear, and the existing EM structure is at low resolution. Only a few co-purified binding partners have been tested for the activity effect on γ-TuRC, and it remains unclear how they interact with the complex. So far, NME7[40] and CDK5RAP2[39] have been shown to enhance the MT nucleation activity of γ-TuRC, but the mechanisms remain unclear. It is also unknown whether the activation mechanism is conserved for all MT nucleation pathways (see Fig. 1). ND, not determined.

recruitment to interphase centrosomes downstream of GCP-WD[33]. MOZART1 (renamed GCP9), a third localization factor that shares weak sequence similarity to MOZART2, also seems to be involved in γ-tubulin recruitment to centrosomes in mitotic cells, and its depletion leads to mitotic spindle defects[32].

Other proteins implicated in the recruitment of γ-TuRC to centrosomes include pericentrin[34] and AKAP450 (also known as CGNAP or AKAP9)[35]. These two proteins share a so-called PACT domain at their C-terminus for centrosome targeting, and they interact with γ-TuRC through their N-terminus[36]. Furthermore, Cep192 recruits GCP-WD/NEDD1[37], and ninein-like proteins play a role in γ-TuRC recruitment to centrosomes during interphase[38].

A γ-TuRC-associated protein with nucleation-activating function is the nucleoside-diphosphate kinase NME7[32,33,39]. This protein localizes to most sites of interphase microtubule nucleation, and to both centrosomes and the mitotic spindle during mitosis; yet its depletion does not interfere with the centrosomal localization of γ-TuRC (or CDK5RAP2; see below)[40]. NME7 increases the nucleation capacity of γ-TuRC *in vitro* by ~2.5-fold, which is dependent on its kinase activity with as-yet unknown targets. Thus, NME7 is a mild modulator of γ-TuRC activity.

The best characterized activator factor for γ-TuRC is CDK5RAP2, which binds to it and is involved in its centrosomal attachment[39]. *In vitro*, CDK5RAP2 activates γ-TuRC-mediated microtubule nucleation by ~7-fold[39]. The activation function of the large CDK5RAP2 protein is contained within in the ~5.5 kDa γ-tubulin complex binding domain (termed γ-TuNA; γ-TuRC-mediated nucleation activator), which is also conserved in the γ-tubulin complex tethering proteins centrosomin (*Drosophila*) and Mto1p and Pcp1p (*S. pombe*)[39]. Currently, there is little understanding of the mechanism of γ-TuRC activation and localization by this very small domain.

Non-centrosomal microtubule nucleation centres

The majority of work on microtubule nucleation has involved centrosomes or spindle pole bodies. However, it is clear that microtubules arise from membranous organelles, chromosomes and other sites in the cell, in a γ-tubulin-dependent manner. Here, we briefly describe some of the best-understood non-centrosomal microtubule nucleation processes in animal cells.

Nuclear membrane. Several cell types exhibit microtubule nucleation at the nuclear membrane, and this is best characterized in skeletal muscle cells[7]. γ-Tubulin and the centrosomal proteins pericentrin and ninein are redistributed to the nuclear envelope following skeletal muscle differentiation[41], and nuclei of non-muscle cells acquire the ability to bind centrosomal proteins when fused to differentiating myoblasts[42]. In addition, GCP9 is important for γ-tubulin localization to the nuclear envelope[43], and local microtubule formation can be blocked by an inhibitory γ-tubulin antibody[41]. The nuclei of plant cells also bind γ-tubulin and GCP2/3 (ref. 44) and are able to nucleate microtubules from their surfaces[45]. It has been suggested that microtubules generated at the nuclear membrane play a role in establishing nuclear shape and positioning nuclear pore complexes[43]. However, in both plant and animal cells, the overall mechanisms of γ-tubulin localization and activation at the nuclear membrane remain poorly understood.

Golgi apparatus. The Golgi apparatus of animal cells typically faces the leading edge or the axis of secretion of the cell. Microtubules can be generated from the Golgi in a γ-tubulin-dependent manner; some interphase cells, such as RPE-1 cells, generate about half of their microtubule cytoskeleton from Golgi MTOCs[8,9]. Golgi-nucleated microtubules have been proposed to play an important role in the fusion of membranes into a continuous Golgi ribbon[46]. On a molecular level, several proteins that anchor γ-TuRC to pericentriolar material also co-localize with the cis-Golgi, such as myomegalin[47]. The cis-Golgi-specific protein GM130 recruits AKAP450[48], which in turn recruits CDK5RAP2[49]. Therefore, GM130 may be a key regulatory factor of the Golgi MTOC; however, details of how microtubule nucleation from the Golgi is regulated and activated remain unclear.

Chromatin and kinetochores. Ran-GTP greatly activates microtubule generation near chromatin[6,50] by releasing a few dozen spindle assembly factors (SAFs) after nuclear envelope breakdown, which were proposed to contribute to microtubule formation and stabilization[50,51]. However, their exact function remains unclear. In particular, whether they stabilize or generate microtubules, and whether they belong to overlapping

or separate microtubule nucleation pathways, is not known. The chromosomal passenger complex (CPC) can compensate for the Ran-GTP gradient under certain conditions by generating microtubules from centromeric chromatin[52]. Both the Ran-GTP and CPC pathways, along with the Nup107-160 complexes[53], contribute to microtubule formation at kinetochores[54], which may assist in kinetochore capture[54].

Microtubule-dependent microtubule nucleation. In addition to nucleation from large organelles such as centrosomes, nuclei and the Golgi, microtubules also can be nucleated from a rather simple source: a pre-existing microtubule. This was initially observed in interphase plant and yeast cells. In plant cells, microtubules were seen to branch off existing microtubules at the cell cortex with a characteristic angle of ~40° and 0°, in a γ-tubulin-dependent manner[10,55,] whereas in *S pombe,* microtubule nucleation produces daughter microtubules that are anti-parallel to the mother microtubule (at a 180° angle)[11].

Shortly after these observations, new evidence indicated that microtubules are generated within the body of spindles[56,57] and not only at centrosomes and chromosomes, as was previously thought. As γ-tubulin was localized to the body of the spindle, it was a candidate for nucleating these new microtubules, and several genes were found to specifically target γ-tubulin to spindle microtubules but not to centrosomes[58]. These proteins were shown to form an 8-subunit complex (called augmin) in *Drosophila*[59] and human cells[60,61]. Recently, progress has been made in deciphering the interactions of subunits within a recombinant augmin complex[62]. Depletion of augmin gives rise to spindle defects in animal cells and a reduction in microtubule density within the spindle[60,61]. Although absent from both *S. pombe* and *S. cerevisiae*, augmin also is found in plants and plays an important role in the formation of the plant spindle and the microtubule-rich phragmoplast[63–65], and in triggering microtubule-dependent microtubule nucleation at the plant cortex[66].

Although it is clear that new microtubules arise within the spindle, the very high microtubule density precluded direct observations of whether new microtubules were being templated from pre-existing ones. Cell-free extracts of *Xenopus* eggs in conjunction with total internal fluorescence (TIRF) microscopy circumvented this roadblock. By simultaneously imaging microtubules with fluorescent tubulin and newly growing microtubule ends, it was possible to demonstrate directly that newly forming microtubules grew from the wall of pre-existing ones[4]. The branch angle between the daughter and mother microtubule was usually shallow, and in many cases the newly nucleated microtubules grew parallel to the template microtubule. Therefore, this nucleating mechanism preserves the polarity of the template filament. Microtubule-dependent microtubule nucleation provides an effective means of amplifying their numbers quickly with the same polarity, which appears to be important for the organization of a large and polar microtubule-rich structure such as a spindle. The cell-free *Xenopus* extract assay also allowed the identification of γ-tubulin, augmin and TPX2 (targeting factor of Xlkp2) as key molecular factors needed for microtubule branching. How augmin, TPX2 and γ-TuRC work together to induce branching microtubule nucleation, and the structure of the protein complex at the branch point, remain important and unanswered questions.

Conclusion and outlook

Interesting parallels can be drawn between actin and microtubule nucleation. Although the actin nucleator Arp2/3 was discovered after γ-tubulin[67], the complete Arp2/3 complex has been crystallized, its structure on a mother filament has been deduced by high-resolution cryo-EM, and the mechanism of activating Arp2/3 by WAVE and WASp complexes has been dissected in great detail[68]. In addition, several other actin nucleators (such as formins and SPIRE) and their mechanisms have been uncovered[68]. This work on actin nucleation foreshadows what remains to be discovered regarding microtubule nucleation.

Although γ-tubulin is generally regarded as the universal microtubule nucleator, it remains possible that additional microtubule nucleating proteins exist. For example, depletion of γ-tubulin by RNA interference (RNAi) in *Caenorhabditis elegans*[69] and *Drosophila* interphase cells[70] still allowed for the formation of an microtubule cytoskeleton, albeit in an abnormal form and with reduced microtubule filament numbers. However, it is difficult to ascertain whether the microtubules present in γ-tubulin RNAi were created from residual γ-tubulin or resulted from the activity of another nucleating process. A candidate for an alternative nucleating factor is the microtubule polymerase XMAP215, which can partially rescue the depletion of γ-tubulin in acentrosomal spindles[71] and has been shown to promote microtubule nucleating activity *in vitro*[72]. Similarly, the protein TPX2 can nucleate microtubules from purified tubulin *in vitro*[73]. However, the promotion of microtubule nucleating activity of these factors is not fully γ-tubulin-independent[7271], and recent evidence supports a model in which these microtubule-associated proteins control the kinetics of microtubule nucleation from γ-TuRC or pre-existing microtubule plus ends[74], leaving open the question of whether they act as true nucleators. Finally, microtubules themselves could serve as seeds for nucleation, which can be generated through the severing activity of katanin or spastin[75].

Many of the upcoming challenges for understanding the mechanism of microtubule nucleation will require biochemistry and structural studies. The current structural work on γ-TuSC has been very illuminating, but a high-resolution view of the 'active nucleating state' of both γ-TuSC and γ-TuRC is still needed. In addition, genetics and biochemistry have identified many interacting proteins of these complexes, highlighted in this review, which have been implicated in localizing and/or activating γ-tubulin. However, little is known about the upstream mechanisms ensuring that these proteins activate microtubule nucleation at the correct location and time. There are clear indications that signalling pathways involving phosphorylation cascades or Ran-GTP impinge on γ-tubulin complexes, but the details are crudely understood[51]. Whether proteins other than γ-tubulin can nucleate microtubules in cells also remains an open question. The field will need to address these questions to further understand how microtubule nucleation is initiated and regulated to ensure that it occurs at the correct time and place to create the microtubule networks fundamental to the activities of eukaryotic cells.

ADDITIONAL INFORMATION
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COMPETING FINANCIAL INTERESTS
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