The where, when and how of microtubule nucleation – one ring to rule them all

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Summary

The function of microtubules depends on their arrangement into highly ordered arrays. Spatio-temporal control over the formation of new microtubules and regulation of their properties are central to the organization of these arrays. The nucleation of new microtubules requires γ-tubulin, an essential protein that assembles into multi-subunit complexes and is found in all eukaryotic organisms. However, the way in which γ-tubulin complexes are regulated and how this affects nucleation and, potentially, microtubule behavior, is poorly understood. γ-tubulin has been found in complexes of various sizes but several lines of evidence suggest that only large, ring-shaped complexes function as efficient microtubule nucleators. Human γ-tubulin ring complexes (γTuRCs) are composed of γ-tubulin and the γ-tubulin complex components (GCPs) 2, 3, 4, 5 and 6, which are members of a conserved protein family. Recent work has identified additional unrelated γTuRC subunits, as well as a large number of more transient γTuRC interactors. In this Commentary, we discuss the regulation of γTuRC-dependent microtubule nucleation as a key mechanism of microtubule organization. Specifically, we focus on the regulatory roles of the γTuRC subunits and interactors and present an overview of other mechanisms that regulate γTuRC-dependent microtubule nucleation and organization.

Key words: Microtubule organization, Microtubule nucleation, γ-tubulin ring complex, MTOC, Centrosome, Spindle

Introduction

Microtubules are hollow cylindrical polymers that are assembled from heterodimers composed of α- and β-tubulin. The longitudinal orientation of the tubulin dimers provides microtubules with an intrinsic polarity, with α-tubulin facing the so-called minus end and β-tubulin the so-called plus end. In vivo the minus end is relatively stable, whereas the plus end is highly dynamic (Jiang and Akhmanova, 2011). Microtubules provide tracks for the transport of molecules or organelles, mediate the segregation of chromosomes during meiotic and mitotic divisions, and serve as building blocks of flagella and motile cilia (Ishikawa and Marshall, 2011; Kapitein and Hoogenraad, 2011; Walczak and Heald, 2008). All microtubule-dependent processes share the requirement for the microtubules to be organized in arrays with defined geometry. This is achieved using two complementary strategies. The first strategy involves regulation of existing microtubules by controlling their elongation, stabilization, transport, sliding and bundling, as well as their severing and disassembly (Jiang and Akhmanova, 2011; Roll-Mecak and McNally, 2010). The second strategy is the regulation of microtubule nucleation, which determines where, when and how polymerization of new microtubules is initiated.

Microtubule nucleation is typically spatially restricted to microtubule-organizing centers (MTOCs) (Lüders and Stearns, 2007). The main MTOC in animal cells is the centrosome, a small spherical structure that comprises a central pair of centrioles surrounded by the pericentriolar material (PCM) (Azimzadeh and Bornens, 2007; Bornens, 2012). Depending on the cell type, nucleation activity is additionally associated with other sites (Bartolini and Gundersen, 2006; Lüders and Stearns, 2007). Each type of MTOC has a size, shape and distribution that is suitable for the organization of a particular type of microtubule array.

Temporal control of microtubule nucleation is achieved by coupling the regulation of nucleation site assembly and/or activation to cell cycle progression or a specific time point during a cellular differentiation program. For example, additional centrosomal nucleation sites are assembled at the G2-M transition to generate larger more-active centrosomes that help in the organization of the spindle poles, whereas cell differentiation is frequently coordinated with gradual centrosome inactivation and transfer of nucleation sites to other cellular structures (Lüders and Stearns, 2007). Nucleation sites might also be able to modulate the properties of the nucleated microtubules, for example, by imposing constraints on the structure of the microtubule (Evans et al., 1985) or by loading regulatory proteins onto the microtubule lattice (Cuschieri et al., 2006; Zimmerman and Chang, 2005).

In this Commentary, we will highlight the control over microtubule nucleation as a fundamental regulatory strategy for the assembly of highly ordered microtubule arrays and discuss the γ-tubulin ring complex (γTuRC), a multi-subunit protein complex that nucleates microtubule polymerization, as the key to this regulation. We will provide an overview of known and potential mechanisms that modulate γTuRC function and discuss how this regulatory framework affects microtubule organization.

The main microtubule nucleator – the γTuRC

Microtubule polymerization occurs spontaneously in vitro, but under physiological conditions this process requires a nucleator that mimics or stabilizes a small microtubule seed formed from multiple α-tubulin–β-tubulin heterodimers. A well-known microtubule

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nucleator is γ-tubulin, which localizes to all known MTOCs and is required for their function. In *Drosophila*, *Xenopus* and humans γ-tubulin assembles into γTuRCs, which are the main cellular microtubule nucleators (Moritz et al., 1995; Moritz et al., 1998; Murphy et al., 2001; Murphy et al., 1998; Oegema et al., 1999; Zheng et al., 1995). In addition to nucleation, these complexes have also been implicated in microtubule stabilization by capping the minus ends (Anders and Sawin, 2011; Wiese and Zheng, 2000) and in the modulation of microtubule-plus-end dynamics (Bouissou et al., 2009).

Nucleation activity has also been described for transforming acidic coiled coil (TACC) family proteins, several microtubule plus-end-binding proteins (Rusan and Rogers, 2009) and, during mitosis, for RanGTP-activated factors, such as TPX2 (Clarke and Zhang, 2008; Gruss and Vernos, 2004). Future research will show whether all these proteins function as true nucleators or have a role later in the assembly process, for example by stabilizing short microtubule fragments or promoting the addition of microtubule subunits at the plus ends.

An important question is how the γTuRC nucleates microtubules. Recent data strongly support the so-called template nucleation model, which proposes that the helical arrangement of γ-tubulin molecules in the γTuRC matches the symmetry of a microtubule and thereby provides an assembly platform for α-tubulin–β-tubulin heterodimers through longitudinal contacts between γ- and α-tubulin. Nucleation models and other structural aspects of γTuRCs have been covered in an excellent recent review (Kollman et al., 2011). Here, we will only briefly discuss γTuRC structure and will focus on what is known about the involvement of γTuRC subunits in the regulation of microtubule nucleation.

Molecular composition of γTuRCs

Early work has suggested that, apart from γ-tubulin, all γTuRC core subunits, termed γ-tubulin complex proteins (GCPs, also known as TUBGCPs in humans), belong to a conserved protein family (Fig. 1A) (Gunawardane et al., 2000; Murphy et al., 2001). Highly conserved sequences in GCPs 2–6 were initially described as γ-tubulin ring protein (Grip) motifs (Gunawardane et al., 2000), but the sequence similarity extends beyond these motifs and, on the basis of insight obtained from the GCP4 crystal structure, we will refer to the conserved regions as N- and C-terminal 'Grip domains' (Fig. 1A) (Guillet et al., 2011). More-recent studies have identified

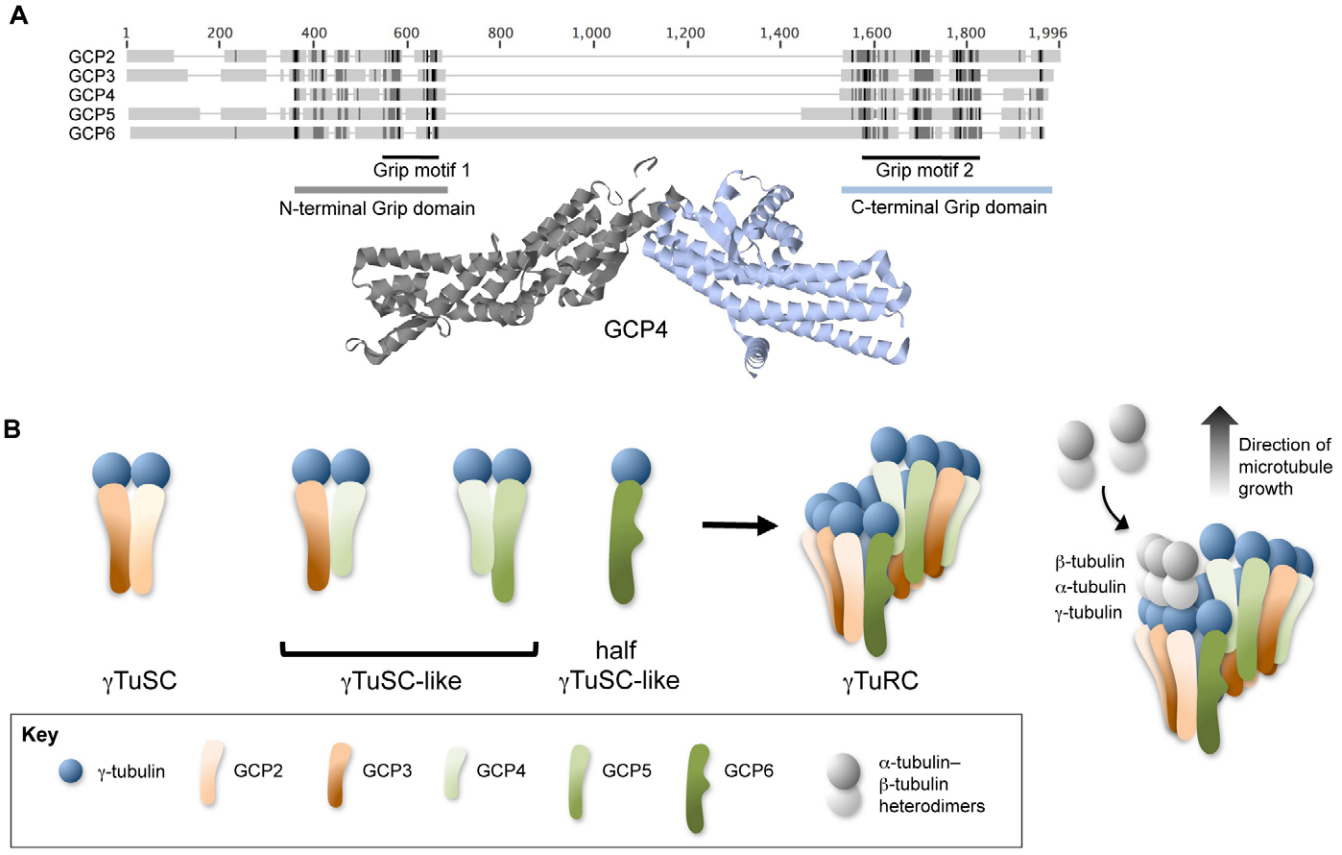

Fig. 1. Structural features of Grip-GCPs and their roles in γTuRC assembly. (**A**) An alignment of human GCP2, GCP3, GCP4, GCP5 and GCP6 using the MUSCLE algorithm within Geneious software. Conserved regions are indicated by gray shading, with the darker regions corresponding to a higher degree of conservation. On the basis of information obtained from the GCP4 crystal structure, which is shown as a ribbon representation below the alignment, one can define N- and C-terminal Grip domains that contain the previously identified Grip motifs. (**B**) Speculative model of γTuRC assembly. γTuSCs are composed of GCP2 and GCP3 (shades of orange) and two molecules of γ-tubulin (blue). γTuSC-like complexes are assembled by replacement of GCP2 and/or GCP3 with GCP4, GCP5 and/or GCP6 (shades of green). Half complexes are composed of a single molecule of GCP4, GCP5 or GCP6 interacting with γ-tubulin. All complexes participate in the formation of the γTuRC ring structure. Nucleation of microtubule polymerization involves longitudinal interactions of α-tubulin–β-tubulin heterodimers with γ-tubulin in the γTuRC (template nucleation model).

additional γTuRC subunits that are not related to these five GCP family members (Fig. 2; Table 1) (Choi et al., 2010; Gunawardane et al., 2003; Haren et al., 2006; Hutchins et al., 2010; Lüders et al., 2006; Teixido-Travesa et al., 2010). We define as 'γTuRC core components' all proteins that co-purify with γTuRCs at amounts that are similar to the GCP family members, co-fractionate with γTuRCs in sucrose gradients and colocalize with γ-tubulin in cells. We will refer to core subunits in general as 'GCPs' and to the Grip-domain-containing GCPs 2–6 as 'Grip-GCPs'. All other γTuRC-associated proteins will be considered interactors, which might bind to γTuRCs less tightly or bind only under certain cellular conditions.

Grip-GCPs

Depletion of γ-tubulin or any of the Grip-GCPs destabilizes γTuRC in sucrose gradients, suggesting that they all have important structural roles (Izumi et al., 2008; Vérollet et al., 2006; Vogt et al., 2006; Xiong and Oakley, 2009; Zhang et al., 2000). γTuRCs are formed by the helical arrangement of smaller Y-shaped subcomplexes, the so-called γ-tubulin small complexes (γTuSCs), which are composed of two molecules of γ-tubulin and one molecule each of GCP2 and GCP3 (Fig. 1B; Box 1). Recent work has suggested that the conserved regions in the five Grip-GCPs form a structural core that is common to all Grip-GCPs (Fig. 1A), and that GCP4, GCP5 and GCP6 might be part of the γTuRC ring structure by substituting for GCP2 or GCP3 at specific positions to function, for example, as ring assembly initiators or terminators (Fig. 1B) (Guillet et al., 2011; Kollman et al., 2011).

Other GCPs

Human γTuRCs contain several core subunits that are not related to Grip-GCPs (Fig. 2; Table 1) and might have regulatory instead of structural roles. Indeed, two of them, GCP-WD (also known as

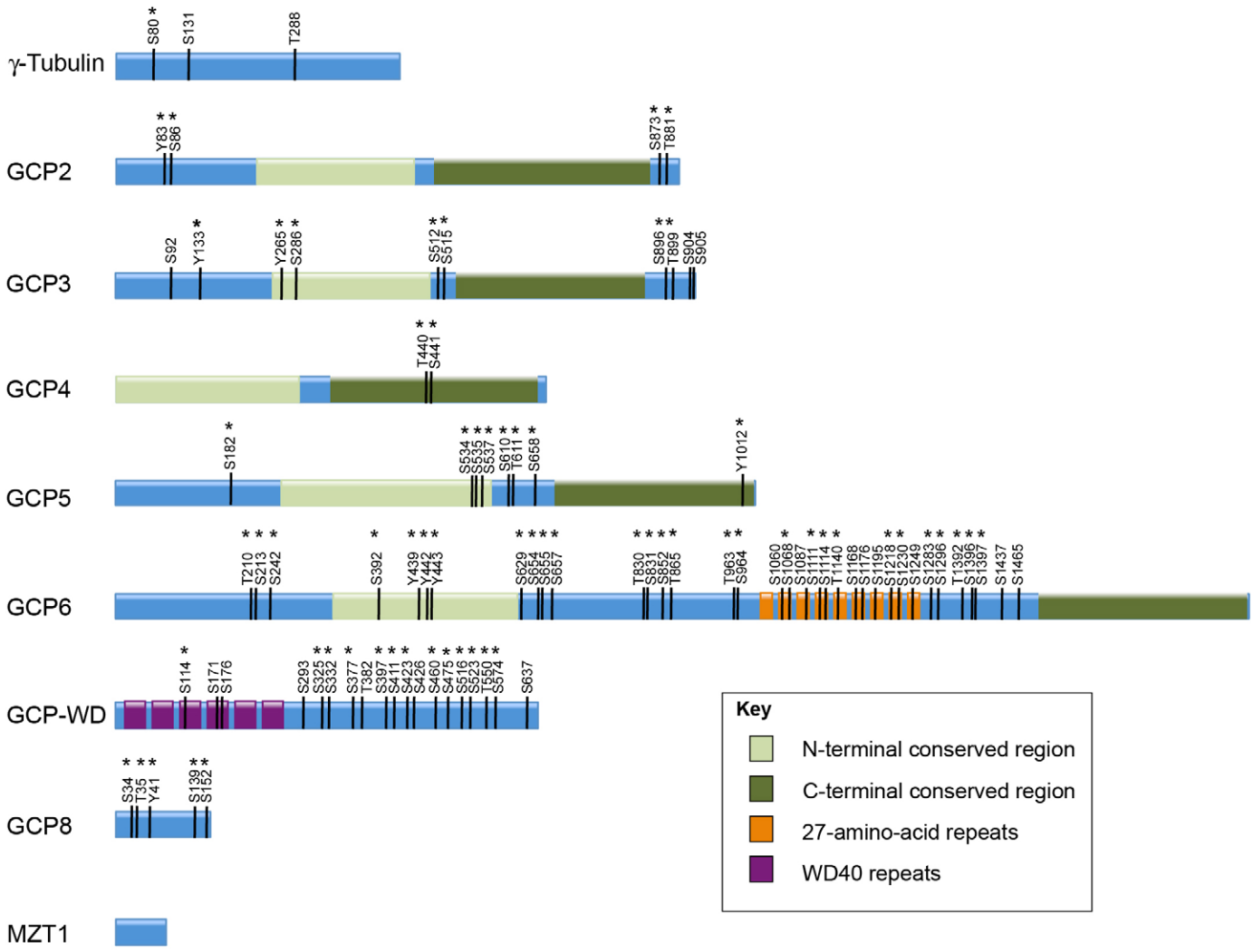

Fig. 2. γTuRC core components domains and phosphorylation sites. Human γ-tubulin and GCPs are shown in schematic diagrams that are scaled from their molecular mass, domains and sequence motifs are colored. The positions of phosphorylation sites that have been identified in studies referenced in the text and from proteomic analyses are indicated. Sites that have been identified in vivo are marked by an asterisk (Beausoleil et al., 2004; Daub et al., 2008; Dephoure et al., 2008; Hegemann et al., 2011; Hornbeck et al., 2004; Imami et al., 2008; Mayya et al., 2009; Olsen et al., 2010; Oppermann et al., 2009; Rigbolt et al., 2011; Rikova et al., 2007; Rush et al., 2005; Van Hoof et al., 2009; Wang et al., 2008).

Table 1. Core subunits of human γTuRCs and their properties

Official gene symbol	GCP nomenclature	Mr	*X. tropicalis*	*D. melanogaster*	*A. thaliana*	*A. nidulans*	*S. pombe*	*S. cerevisiae*	Required for γTuRC assembly and/or stability	Comments
TUBG1 and *TUBG2*	γ-tubulin 1 and γ-tubulin 2	51.1	+	+	+	+	+	+	Yes	Component of γTuSC
TUBGCP2	GCP2	102.5	+	+	+	+	+	+	Yes	Component of γTuSC
TUBGCP3	GCP3	103.6	+	+	+	+	+	+	Yes	Component of γTuSC
TUBGCP4	GCP4	76.1	+	+	+	+	+	–	Yes	Minor role in *A. nidulans* and *S. pombe*
TUBGCP5	GCP5	118.3	+	+	+	+	+	–	Yes	Minor role in *A. nidulans* and *S. pombe*
TUBGCP6	GCP6	200.5	+	+	+	+	+	–	Yes	Minor role in *A. nidulans* and *S. pombe*
NEDD1	GCP-WD	71.9	+	+	+	–	–	–	No	Centrosome and spindle targeting factor
MZT2A and *MZT2B*	GCP8A and GCP8B	16.2	+	–	–	–	–	–	No	Role in interphase-specific centrosome targeting
MZT1	GCP9	8.5	+	+	+	+	+	–	?	Required for bipolar spindle assembly

+, component is present; –, component is not present; ?, unknown; Mr, molecular mass in kDa.

NEDD1) and GCP8 (also known as MOZART2), have been shown to be non-essential for γTuRC assembly (Gunawardane et al., 2003; Haren et al., 2006; Lüders et al., 2006; Teixido-Travesa et al., 2010).

GCP-WD is a γTuRC-targeting factor that is indispensable for mitotic and meiotic spindle assembly and progression. It is found in animals and plants but not in fungi (Gunawardane et al., 2003; Haren et al., 2006; Lüders et al., 2006). The C-terminal half of GCP-WD mediates its oligomerization and binding to the γTuRC through the direct interaction with γ-tubulin. The N-terminal WD40 repeats, which are predicted to form the blades of a β-propeller structure, are required to target the γTuRC to centrosomes and other non-centrosomal MTOCs (Haren et al., 2006; Liu and Wiese, 2008; Lüders et al., 2006; Ma et al., 2010; Manning et al., 2010; Zeng et al., 2009).

GCP8 is a small protein that is conserved in deuterostomes but does not contain any known domains or sequence motifs (Choi et al., 2010; Hutchins et al., 2010; Teixido-Travesa et al., 2010). Homologs are also found in the unicellular green alga *Micromonas* and in *Hymenoptera* but, curiously, not in other plants or insects. GCP8 specifically contributes to γTuRC recruitment to and microtubule nucleation at interphase centrosomes, but has no obvious role during mitosis (Teixido-Travesa et al., 2010).

Another γTuRC core subunit is MOZART1 (Hutchins et al., 2010; Teixido-Travesa et al., 2010). In human cells, MOZART1 is required for recruitment of γTuRC to mitotic centrosomes and for bipolar spindle assembly (Hutchins et al., 2010). Similarly, plant MOZART1, which binds to GCP3 and localizes to active cortical nucleation sites in interphase, is required for proper spindle assembly and chromosome segregation during mitosis (Janski et al., 2012; Nakamura et al., 2012). However, none of these studies have analyzed whether MOZART1 has a role in γTuRC assembly and/or stability. Interestingly, MOZART1 is conserved in fission yeast but not in budding yeast, which might indicate a function involving γTuRC-like complexes.

Other γTuRC-associated proteins

Purified human γTuRCs contain two additional proteins, the nucleoside-diphosphate kinase family member NDK7 (also known as NME7), which functions in ciliary transport and motility (Lai et al., 2011; Vogel et al., 2010), and LGALS3BP (for lectin, galactoside-binding, soluble, 3 binding protein), which might have a role in cell–cell and cell–matrix interactions (Table 1) (Choi et al., 2010; Hutchins et al., 2010; Teixido-Travesa et al., 2010). However, it is currently unknown whether NDK7 and LGALS3BP qualify as γTuRC core subunits and what their role in the γTuRC is.

Structural versus regulatory γTuRC subunits

Grip-GCPs and γ-tubulin are considered essential for the γTuRC structure. Regulatory functions have been suggested for some of

Box 1. Organism-specific differences in composition and size of γ-tubulin complexes

γ-tubulin and members of the GCP family can assemble into complexes of various sizes. Early work in budding yeast has identified γ-tubulin complexes as heterotetramers that are composed of two molecules of γ-tubulin and one molecule each of the only two GCP family members present in budding yeast, GCP2 and GCP3. Such complexes are now commonly referred to as γ-tubulin small complexes (γTuSCs). In *Drosophila* and vertebrates, γ-tubulin also forms much larger assemblies, termed γ-tubulin ring complexes (γTuRCs). In addition to γ-tubulin, GCP2 and GCP3, γTuRCs contain three additional GCP family members (termed GCP4, GCP5 and GCP6 in humans). These proteins are also found in fungi other than budding yeast. However, in these organisms γ-tubulin complexes that are larger in size than the γTuSC appear to be less abundant or less stable than the γTuRCs in higher eukaryotes. Whereas γTuRC is considered to be a more active nucleator than γTuSC, the γTuRC-specific GCP4, GCP5 and GCP6 in fungi are not essential for viability, which suggests that in some organisms γTuSC subunits alone can support microtubule nucleation.

Below, we outline the types and sizes of γ-tubulin complexes in the soluble cellular fraction in different organisms.

Homo sapiens*, *Xenopus laevis: some smaller complexes, but mostly γTuRC (~32S) (Moritz et al., 1995; Moritz et al., 1998; Murphy et al., 2001; Murphy et al., 1998; Oegema et al., 1999; Zheng et al., 1995).

Drosophila melanogaster: γTuSC (~10–13S) and γTuRC (>31S) (Moritz et al., 1995; Moritz et al., 1998; Murphy et al., 2001; Murphy et al., 1998; Oegema et al., 1999; Zheng et al., 1995).

Aspergillus nidulans: mostly small complexes (~7–14S), some larger complexes (~21S) (Xiong and Oakley, 2009).

Schizosaccharomyces pombe: gel filtration analysis under low ionic strength buffer conditions showed large (>2000 kDa) complexes. However, sucrose gradient fractionation under more physiological buffer conditions revealed mostly small, γTuSC-sized complexes (~8–9S) (Anders et al., 2006; Fujita et al., 2002; Venkatram et al., 2004).

Saccharomyces cerevisiae: only γTuSC (~12S) (Vinh et al., 2002).

these proteins because their mutation or RNAi-mediated depletion can alter microtubule stability and dynamics (Bouissou et al., 2009; Fujita et al., 2002; Jung et al., 2001; Paluh et al., 2000; Tange et al., 2004; Zimmerman and Chang, 2005). However, altered microtubule dynamics might also be an indirect effect of changes in microtubule nucleation in a closed system (Gregoretti et al., 2006; Sawin et al., 2004). As we will discuss in the following section, insight into γTuRC regulation has primarily been obtained from the analysis of non-structural γTuRC subunits and interactors.

Regulation of the γTuRC through associated proteins

Several γTuRC-associated proteins have been implicated in γTuRC regulation, frequently by mediating subcellular targeting of the complex to specific MTOCs (Fig. 2; Table 2).

Targeting to centrosomes

It has been proposed that several centrosomal proteins, including pericentrin (Zimmerman et al., 2004), AKAP450 (also known as CG-NAP or AKAP9) (Takahashi et al., 2002) and CDK5RAP2 (also known as Cep215) (Fong et al., 2008) recruit γTuRC to centrosomes. However, as integral components of the PCM, these proteins are important for centrosome structure and therefore might also indirectly affect γTuRC recruitment (Graser et al., 2007; Haren et al., 2009; Lee and Rhee, 2011).

In human cells, the γTuRC subunit GCP-WD is the attachment factor that lies most proximal to the γTuRC. GCP-WD is indispensable for the centrosomal localization of γ-tubulin in interphase and mitosis, but, unlike other subunits of the complex, it localizes to centrosomes independently of the γTuRC (Haren et al., 2006; Lüders et al., 2006). The γTuRC subunit GCP8 contributes to γ-tubulin recruitment to interphase centrosomes, but the centrosomal localization of GCP8 itself also depends on GCP-WD (Teixido-Travesa et al., 2010).

In addition to GCP-WD, centrosomal targeting of γ-tubulin in humans requires an intact γTuRC (N. T.-T., J. R. and J. L., unpublished observations; Izumi et al., 2008). By contrast, depletion of GCP-WD, GCP4, GCP5 and GCP6 in *Drosophila* does not abolish centrosomal recruitment of γ-tubulin and microtubule nucleation (Vérollet et al., 2006). Similarly, GCP4, GCP5 and GCP6 in *Aspergillus nidulans* and *Schizosaccharomyces pombe* are not essential for viability and are dispensable for γ-tubulin recruitment and microtubule nucleation at spindle pole bodies. Moreover, *Saccharomyces cerevisiae* naturally lacks orthologs of GCP4, GCP5 and GCP6, which demonstrates that the γTuSC proteins alone have the ability to assemble nucleation sites in some species (Anders et al., 2006; Fujita et al., 2002; Venkatram et al., 2004; Xiong and Oakley, 2009). How does the γTuSC, which is a very poor nucleator in vitro (Oegema et al., 1999), support microtubule nucleation in cells? It is possible that in the aforementioned scenarios γTuSCs still form ring-like assemblies, but only following their interaction with centrosomes or spindle pole bodies. This view is supported by the observation that a fragment of budding yeast Spc110, which links γTuSCs to the spindle pole body, promotes assembly of ring-like γTuSC oligomers in vitro (Kollman et al., 2010). The presence of GCP4, GCP5 and GCP6 and the ability to assemble γTuRCs might, thus, be important for nucleation from certain types of MTOCs.

Targeting to non-centrosomal sites

Whereas centrosome targeting of the γTuRC is clearly crucial for centrosomal microtubule organization, ~80% of the total cellular γ-tubulin is present in the non-centrosomal cytosolic fraction, which suggests that γ-tubulin might also function at other cellular sites (Fig. 3A) (Moudjou et al., 1996).

During mitosis both chromatin-generated RanGTP and the chromosomal passenger complex (CPC) independently promote microtubule assembly around mitotic chromosomes (Clarke and Zhang, 2008; Maresca et al., 2009). Whereas the γTuRC is clearly required for nucleation at these non-centrosomal sites (Groen et al., 2009; Lüders et al., 2006), no direct regulatory link to RanGTP or the CPC has been established. Interestingly, it is the kinetochores rather than general chromatin that have the dominant role in microtubule formation and spindle assembly (O'Connell et al., 2009). Although γ-tubulin is known to localize to kinetochore-bound microtubules, a recent study has suggested that the NUP107–NUP160 (for nuclear pore complex protein 107 and 160, respectively) complex recruits γTuRC to kinetochores independently of microtubules (Mishra et al., 2010). However, those authors did not demonstrate the absence of microtubules in

Table 2. Selected γTuRC interactors and their proposed roles

Interactor	Role	References
Pericentrin	Binds directly to GCP2 and GCP3; provides a scaffold for tethering the γTuRC to mitotic centrosomes	(Lee and Rhee, 2011; Takahashi et al., 2002; Zimmerman et al., 2004)
CDK5RAP2	Provides a scaffold for tethering the γTuRC; activates γTuRC nucleation activity	(Barr et al., 2010; Choi et al., 2010; Fong et al., 2008)
AKAP9	Binds directly to GCP2 and GCP3; provides a scaffold for tethering the γTuRC to mitotic centrosomes and Golgi	(Takahashi et al., 2002; Zimmerman et al., 2004)
TRiC chaperonin	Promotes folding of γ-tubulin and GCP-WD	(Melki et al., 1993; Teixido-Travesa et al., 2010; Yam et al., 2008)
HCA66	Required for stability of the γTuSC subunits γ-tubulin, GCP2 and GCP3	(Fant et al., 2009b)
Keratin	Binds directly to GCP6 and assembles γ-tubulin-containing nucleation sites in the apical domain of epithelial cells; interaction with GCP6 is disrupted by CDK1-dependent GCP6 phosphorylation	(Oriolo et al., 2007)
Augmin complex	Enhanced interaction with γTuRC during mitosis; recruits γTuRC to spindle microtubules through GCP-WD to promote intra-spindle microtubule generation	(Goshima et al., 2008; Lawo et al., 2009; Uehara et al., 2009; Zhu et al., 2008)
Nup107–Nup160 complex	Tethers γTuRCs to unattached kinetochores to support nucleation of kinetochore microtubules	(Mishra et al., 2010)
Plk1	Binds and phosphorylates GCP-WD subunit in mitosis; might contribute to γTuRC recruitment to the centrosome	(Haren et al., 2009; Johmura et al., 2011; Zhang et al., 2009)
SADB	Associates with and phosphorylates γ-tubulin to regulate centriole duplication	(Alvarado-Kristensson et al., 2009)
Plk4	Binds and phosphorylates GCP6 to regulate centriole duplication	(Bahtz et al., 2012)
Syc and Src family tyrosine kinases	Associate with γTuRCs and phosphorylate γTuRC-associated proteins to promote microtubule nucleation	(Dráberová et al., 1999; Kukharskyy et al., 2004; Macurek et al., 2008; Sulimenko et al., 2006)
BRCA1	E3 ligase activity ubiquitylates γ-tubulin to inhibit centrosomal nucleation activity	(Sankaran et al., 2005; Starita et al., 2004)
NME7	Candidate γTuRC subunit; NDP kinase with function in motile cilia, the role in γTuRC is unknown	(Choi et al., 2010; Hutchins et al., 2010; Ikeda, 2010; Teixido-Travesa et al., 2010; Vogel et al., 2010)
LGALS3BP	Candidate γTuRC subunit; potential roles in cell–cell and cell–matrix interaction, and cell migration, the role in γTuRC is unknown	(Grassadonia et al., 2004; Hutchins et al., 2010; Teixido-Travesa et al., 2010)

their experiments. Nucleation of microtubules by kinetochore-bound γTuRC would result in microtubules with plus ends that lie distal to the kinetochore, and this reversed orientation would have to be corrected by a specific mechanism. So far, such a mechanism has been described only in budding yeast, but in this case the kinetochore-associated nucleator is Stu2, a protein related to ch-TOG (also known as CKAP5) and not γ-tubulin (Kitamura et al., 2010). Further investigation is thus needed to resolve these issues.

During mitosis the γTuRC is also targeted to spindle microtubules, and expression of a GCP-WD mutant that specifically disrupts targeting of γTuRC to spindles, but not to centrosomes, interferes with proper spindle assembly and reduces microtubule density in the spindle (Lüders et al., 2006). On the basis of this finding, the so-called amplification model has been proposed: during spindle assembly γTuRCs might associate laterally with previously formed microtubules to nucleate additional microtubules (Fig. 3A) (Lüders et al., 2006; Lüders and Stearns, 2007). The identification of augmin, a multi-subunit protein complex that recruits γTuRC to spindle microtubules through the adaptor GCP-WD, has provided important molecular insight into this pathway (Goshima and Kimura, 2010). However, microtubule nucleation by γ-tubulin complexes that are laterally bound to existing microtubules, as described in plants and in fission yeast (Janson et al., 2005; Murata et al., 2005), has not yet been described in vertebrates.

In *Drosophila* S2 cells the GCP-WD ortholog is required for the localization of γTuRCs along interphase microtubules to regulate microtubule plus-end dynamics. The molecular details of this regulation remain unclear, but the targeting also requires *Drosophila* GCP4, which suggests that this process involves γTuRC instead of γTuSC (Bouissou et al., 2009).

Another non-centrosomal MTOC is the Golgi complex. Microtubules that are nucleated at the Golgi complex help in the positioning of Golgi stacks and contribute to the overall organization of the Golgi complex (Kodani and Sütterlin, 2009; Miller et al., 2009). Interestingly, AKAP450 and CDK5RAP2, which have both been described as γTuRC-tethering factors at the centrosome, also localize to the Golgi, and AKAP450 has been shown to recruit the γTuRC to the cis-Golgi compartment (Rivero et al., 2009; Wang et al., 2010).

In some cases Grip-GCPs have also been implicated in γTuRC targeting. Orthologs of GCP4 and GCP5 target γTuRCs to non-centrosomal MTOCs during *Drosophila* oogenesis (Vogt et al., 2006). In mammalian epithelial cells GCP6 mediates γTuRC localization to the apical submembrane region through its interaction with keratin (Oriolo et al., 2007).

Modulators of γTuRC nucleation activity

For microtubule nucleation to occur predominantly at MTOCs and not at random sites in the cytoplasm, where a substantial number of γTuRCs are also present, cells require a regulatory mechanism in addition to specific targeting of γTuRCs. One possibility is that efficient nucleation requires activation of γTuRCs and that the activating molecules are only present at MTOCs (Fig. 3B). Such an activator might be the centrosomal scaffold protein CDK5RAP2, which contains a sequence motif that mediates binding to the γ-tubulin complex and is conserved in related γ-tubulin tethering proteins in *Drosophila* and fission yeast (namely Cnn, and Mto1 and Pcp1, respectively) (Fong et al., 2008; Sawin et al., 2004). Full-length CDK5RAP2, or a fragment comprising the conserved motif, stimulates microtubule nucleation by γTuRCs in the cytoplasm of

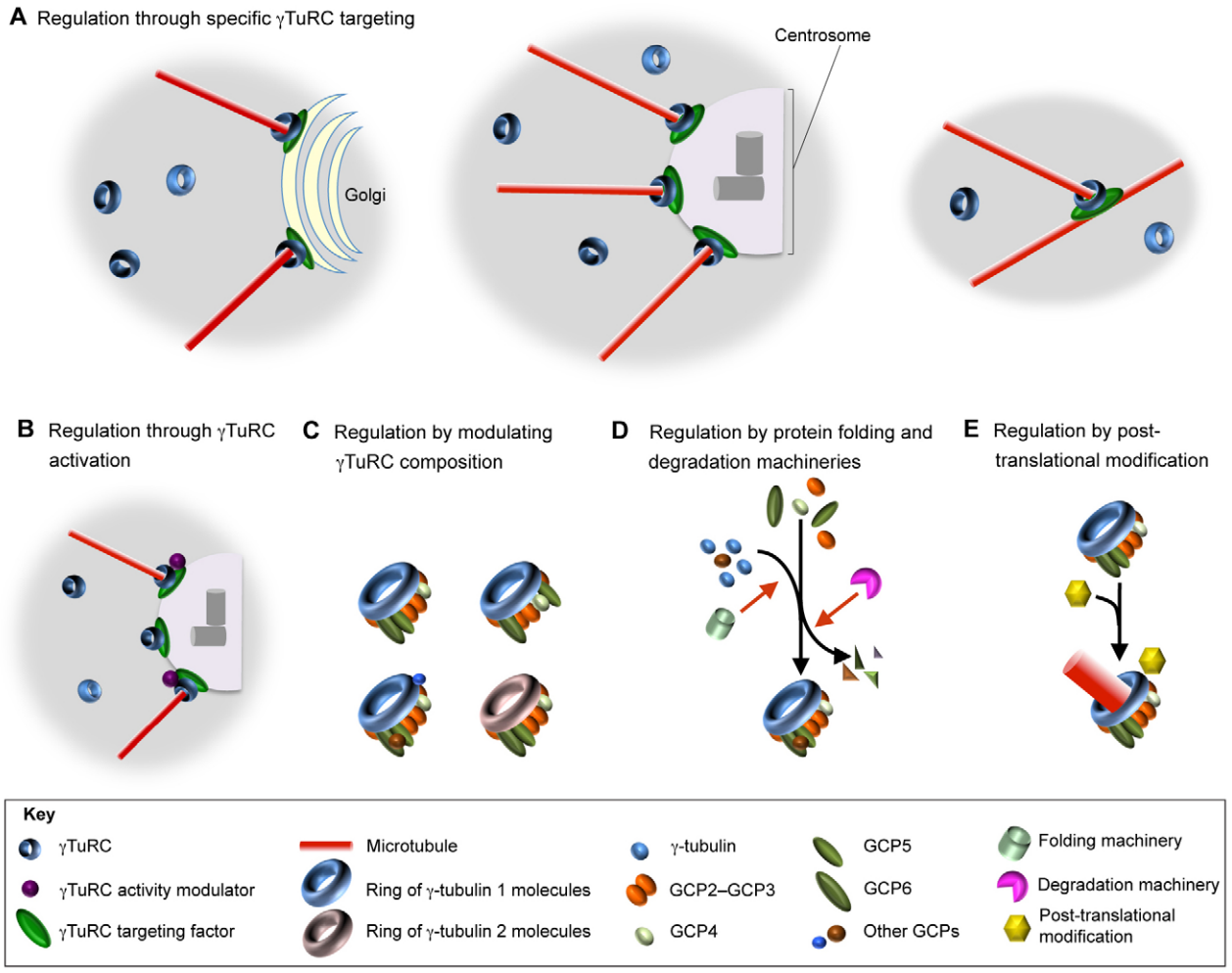

Fig. 3. Mechanisms of γTuRC regulation. (**A**) γTuRC-associated targeting factors (green) direct the complex to different subcellular locations as indicated. In addition, the targeting factor could also function as an activator of the γTuRC nucleation activity. (**B**) Activity modulators (purple) might stimulate the nucleation activity of γTuRCs following the interaction with MTOCs. (**C**) Grip-GCPs might occupy fixed positions in the γTuRC structure. Alternatively, Grip-GCPs might be interchangeable and thus generate γTuRCs with distinct properties. Incorporation of γ-tubulin and Grip-GCP isoforms and other GCPs creates additional variability. (**D**) By controlling biogenesis and stability of specific γTuRC subunits, protein folding and degradation machineries could modulate assembly and function of γTuRCs. (**E**) Post-translational modifications could regulate the nucleation activity of γTuRCs (shown as an example), the stability of subunits, their assembly into complexes and/or γTuRC localization.

cells and from isolated γTuRCs in vitro (Choi et al., 2010). This result is consistent with the idea that most of the cytoplasmic γTuRCs are not associated with an activator. Indeed, very little or no CDK5RAP2 co-purifies with cytoplasmic γTuRCs (Hutchins et al., 2010; Teixido-Travesa et al., 2010). Activation of the γTuRC might involve a conformational switch, possibly mediated by a flexible hinge region in GCP3, which would adjust the position of γ-tubulin molecules in the γTuRC to more accurately match the geometry of the microtubule lattice (Kollman et al., 2011).

Modulation of the core subunit composition

Human γTuRCs contain a single GCP5 molecule and multiple copies of other Grip-GCPs, but their exact stoichiometry is unknown (Murphy et al., 2001). The stoichiometry of Grip-GCPs is similar in γTuRCs from asynchronous and mitotic HeLa cells (Teixido-Travesa et al., 2010), but GCP6 is absent from a fraction of γTuRCs that are associated with a recombinant CDK5RAP2 fragment (Choi et al., 2010). Some γ-tubulin complexes also seem to lack GCP-WD (Choi et al., 2010; Nakamura et al., 2012). Taken together these findings suggest that distinct γTuRC subpopulations exist. The model that all Grip-GCPs occupy specific positions in the ring (Kollman et al., 2011) could be extended by assuming that Grip-GCPs, at least in some positions, are interchangeable. This would allow the assembly of γTuRCs with variable stoichiometries of Grip-GCPs (Fig. 3C). Incorporation of γ-tubulin and Grip-GCP isoforms, as well as other GCPs, would create additional variability to generate subpopulations of γTuRCs with roles that are specific to a certain cell cycle stage or cell type (Nakamura et al., 2012; Raynaud-Messina et al., 2001; Tavosanis et al., 1997; Vinopal et al., 2012; Wiese, 2008; Wilson et al., 1997; Yuba-Kubo et al., 2005).

Regulation through protein folding and degradation machineries

The biogenesis and function of α- and β-tubulins are regulated by folding and degradation machineries (Lundin et al., 2010).

Similarly, both γ-tubulin and GCP-WD have been identified as substrates of the chaperonin TRiC (TCP1-ring complex, also known as CCT), which co-purifies with γTuRCs isolated from HeLa cells (Melki et al., 1993; Teixido-Travesa et al., 2010; Yam et al., 2008). By controlling the availability of key subunits, folding and degradation machineries could modulate γTuRC assembly and/or function (Fig. 3D). In addition to folding, the chaperonin complex could also be involved in the incorporation of subunits into γTuSC or γTuRC. A similar role has been proposed for the protein HCA66 [also known as U3 small nucleolar RNA-associated protein 6 (UTP6)]. Depletion of HCA66 destabilizes γ-tubulin, GCP2 and GCP3, and interferes with assembly and function of γTuSC and γTuRC (Fant et al., 2009a).

The role of GTP

GTP binding by α- and β-tubulin, and GTP hydrolysis by β-tubulin promote polymerization and the so-called 'dynamic instability' of microtubules (Desai and Mitchison, 1997). Similarly, GTP binding and/or hydrolysis by γ-tubulin could modulate γTuRC nucleation activity or the properties of the nucleated microtubule. Indeed, analysis of the γ-tubulin nucleotide-binding domain in fungi has identified mutations that are lethal or affect microtubule dynamics (Hendrickson et al., 2001; Jung et al., 2001). However, neither monomeric γ-tubulin nor the γTuSC undergo a major conformational change in response to the γ-tubulin nucleotide state (Kollman et al., 2008; Rice et al., 2008). Thus, further work is required to solve this issue.

In summary, γTuRC-associated proteins are able to control targeting, assembly, composition and activity of γTuRCs. However, these functions also involve post-translational modifications, which we will discuss in the following section.

Regulation of the γTuRC by posttranslational modification

Most of the γTuRC subunits are phosphorylated (Fig. 2). In many cases phosphorylation occurs specifically in mitosis or depends on mitotic kinases such as cyclin-dependent kinase 1 (CDK1), Polo-like kinase 1 (PLK1) and Aurora A.

Phosphorylation and centrosome maturation

During centrosome maturation at the G2-M transition, centrosomes increase their size and nucleation activity to 'prepare' for their role as mitotic spindle organizers. PLK1, which is a major regulator of this process, and several other mitotic kinases, including Aurora A and NIMA-family kinases, promote centrosomal accumulation of γ-tubulin (Barr and Gergely, 2007; Barr et al., 2004; O'Regan et al., 2007). In flies, centrosome maturation (as well as γ-tubulin recruitment) depends on only two proteins: Cnn, a fly homolog of CDK5RAP2, and Plk1, which is required for Cnn phosphorylation in mitosis (Dobbelaere et al., 2008). By contrast, identification of a PLK1 substrate that directly controls γTuRC recruitment in vertebrates has proven to be difficult. Importantly, PLK1 promotes centrosomal recruitment not only of γTuRCs but also of several structural PCM proteins, including Cep192, pericentrin and CDK5RAP2 (Haren et al., 2009; Lee and Rhee, 2011; Santamaria et al., 2011), and all of these proteins are phosphorylated in vivo in a PLK1-dependent manner (Kettenbach et al., 2011; Santamaria et al., 2011). Therefore, in vertebrates, PLK1 probably controls γTuRC recruitment through more than one mechanism, which includes the regulation of centrosome size through phosphorylation of structural PCM proteins (Haren et al., 2009; Lee and Rhee, 2011).

Phosphorylation of γ-tubulin and Grip-GCPs

Phosphorylation of γ-tubulin was first studied in budding yeast. Phosphorylation of a conserved tyrosine near the γ-tubulin C-terminus during the G1 phase regulates microtubule organization by promoting astral microtubule assembly (Vogel et al., 2001). It has been shown that Ser360 in γ-tubulin, which is conserved in humans, is phosphorylated at spindle pole bodies by Cdk1 (Keck et al., 2011). Yeast expressing γ-tubulin with a Ser360Asp mutation that mimics phosphorylation of this site are viable at low temperatures but display spindle defects involving changes in anaphase spindle microtubule dynamics (Keck et al., 2011). At higher temperatures cells arrest in mitosis with short bipolar spindles containing disorganized microtubules. However, these defects seem to be caused, in part, by destabilization of γ-tubulin (Lin et al., 2011). Characterization of additional phosphorylation sites, which were identified in yeast γTuSC subunits that are bound to spindle pole bodies (Keck et al., 2011; Lin et al., 2011) and present in the cytoplasm (Lin et al., 2011), might provide further insight into the regulation of γ-tubulin complexes.

In human cells, the serine/threonine protein kinase SADB (also known as BRSK1) phosphorylates γ-tubulin at the conserved Ser131 residue to control centrosome duplication, possibly by regulating γTuRC-dependent nucleation of centriolar microtubules (Alvarado-Kristensson et al., 2009). In addition, centriole duplication requires phosphorylation of GCP6 by Plk4, a known regulator of centriole biogenesis (Bahtz et al., 2012). The mechanism, by which these phosphorylation events are linked to centriole biogenesis, has not been revealed. In epithelial cells, CDK1 phosphorylates GCP6 to disrupt the interaction of γTuRCs with keratin and to remove the complexes from the apical domain, which might be important for the remodeling of the microtubule array on mitotic entry (Oriolo et al., 2007). In vitro, human GCP5 is a substrate for glycogen synthase kinase 3 beta (GSK3β), which negatively regulates the amount of γ-tubulin at mitotic centrosomes (Izumi et al., 2008). It is unclear, however, whether this regulation occurs at the level of GCP5 or involves other PCM components. Human GCP2, GCP3 and GCP4 also contain multiple phosphorylation sites, but none of these have been functionally characterized (Hegemann et al., 2011; Kettenbach et al., 2011; Santamaria et al., 2011). The regulation of γ-tubulin complexes also involves members of the Syc and Src family kinases, but substrates have not been identified (Colello et al., 2010; Kukharskyy et al., 2004; Macurek et al., 2008; Sulimenko et al., 2006).

In summary, the γ-tubulin phosphorylation sites studied so far affect the stability of γ-tubulin, the properties of microtubules, and specific γTuRC-dependent processes, but do not seem to control microtubule nucleation activity per se. Phosphorylation of Grip-GCPs is still poorly characterized and in a few cases controls γTuRC localization. However, because Grip-GCPs coordinate the arrangement of γ-tubulin molecules in the γTuRC, phosphorylation of Grip-GCPs could also regulate a conformational change that might be required for γTuRC activation (Kollman et al., 2011).

Phosphorylation of GCP-WD

GCP-WD is phosphorylated on multiple sites in vivo and both CDK1 and PLK1 contribute to its phosphorylation in mitosis

(Haren et al., 2009; Johmura et al., 2011; Lüders et al., 2006; Santamaria et al., 2011). Mutation of the CDK1 consensus site at Ser411 (in isoform B; Ser418 in the longer isoform A) to alanine disrupts the interaction of GCP-WD with augmin and, in a manner that is similar to augmin depletion, abolishes localization of γTuRC to spindle microtubules and intra-spindle microtubule generation (Johmura et al., 2011; Lüders et al., 2006; Uehara et al., 2009). Ser460 and Thr550 on GCP-WD are phosphorylated by CDK1 to mediate interaction with the PLK1 polo box (Haren et al., 2009; Johmura et al., 2011; Zhang et al., 2009). Interestingly, PLK1 bound to Ser460 on GCP-WD seems to indirectly control spindle binding of GCP-WD through phosphorylation of a subunit of the augmin complex, HAUS8 (also known as Hice1) (Johmura et al., 2011). Mutating Thr550 or a group of four PLK1-dependent phosphorylation sites, identified in vitro, to alanine weakens binding of GCP-WD to γ-tubulin and moderately reduces localization of γTuRC to centrosomes (Zhang et al., 2009). However, this phenomenon was not observed in a previous analysis of the Thr550Ala mutation (Haren et al., 2009), which suggests that PLK1 binding to Thr550 is not essential for targeting of γTuRCs to mitotic centrosomes. Importantly, none of these phosphorylation sites is required for the PLK1-dependent accumulation of GCP-WD at mitotic centrosomes, which suggests that there are PLK1 substrates upstream of GCP-WD (Haren et al., 2009; Zhang et al., 2009). One of these substrates has recently been shown to be the NIMA-family kinase Nek9 (Sdelci et al., 2012), which is activated by Plk1 at mitotic centrosomes. Active Nek9 phosphorylates GCP-WD at Ser377, which is key to recruiting γTuRC to mitotic centrosomes and assembling a functional bipolar spindle.

Taken together, the available data suggest that GCP-WD phosphorylation mutants affect the localization of γTuRCs to specific nucleation sites, which is consistent with the function of this protein as a γTuRC-targeting factor.

Other post-translational modifications

Whereas α- and β-tubulin are heavily altered by a range of modifications, including acetylation, glycylation and glutamylation, such modifications have not been described for γ-tubulin. However, it is known that GCP2 is modified by acetylation at Lys827 but the function of this modification remains to be determined (Choudhary et al., 2009). The ubiquitin ligase activity of the breast cancer type 1 susceptibility protein (BRCA1) ubiquitylates γ-tubulin and reduces γ-tubulin localization and nucleation activity at centrosomes (Parvin, 2009). Ubiquitylation of other γTuRC subunits occurs but has not been functionally characterized (Kim et al., 2011; Wagner et al., 2011; Xu et al., 2010).

Conclusions

At more than 15 years after the discovery of the γTuRC, we are only just beginning to unravel how this remarkable molecular machine is regulated. Progress has been made in understanding the targeting of γ-tubulin complexes to various MTOCs in different cell types and organisms. However, we still know very little about the regulation of γTuRC nucleation activity. Apart from studying the activation of γTuRC by interacting proteins, a functional characterization of phosphorylation sites in γTuRC subunits will be required. Similarly, a systematic structural and functional analysis of γ-tubulin mutants is necessary to clarify the role of nucleotides in the regulation of the γTuRC.

Another important issue that still needs to be addressed is the characterization of potential γTuRC subpopulations that might differ in composition or post-translational modification. γTuRC subpopulations could be obtained from different cell types and/or cell cycle stages through pull down of various different subunits using antibodies or affinity tags. By using single-molecule imaging methods, it might be possible to compare the subunit composition of individual γTuRCs in vitro and in vivo.

Before we can fully comprehend how γTuRC-associated proteins, post-translational modifications, nucleotides, and other factors affect microtubule nucleation, we need a better mechanistic understanding of this process. Careful structural and functional analysis of γTuRCs in vitro, ideally reconstituted from purified recombinant proteins, will be crucial to achieving this goal. An important milestone would be the development of novel tools and assays that would allow γTuRC-mediated nucleation to be distinguished from stabilization and elongation of microtubules.

Together these studies will help us achieve a more complete picture of where, when and how the γTuRC microtubules in vivo to generate microtubule arrays of great structural and functional diversity.

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