PROBLEMS & PARADIGMS
Prospects & Overviews

The gamma-tubulin ring complex: Deciphering the molecular organization and assembly mechanism of a major vertebrate microtubule nucleator

Anna Böhler | Bram J.A. Vermeulen | Martin Würtz | Erik Zupa | Stefan Pfeffer | Elmar Schiebel

Zentrum für Molekulare Biologie der Universität Heidelberg, Heidelberg, Germany

Correspondence
Stefan Pfeffer,
Email: s.pfeffer@zmbh.uni-heidelberg.de, or Elmar Schiebel,
Email: e.schiebel@zmbh.uni-heidelberg.de

Anna Böhler, Bram J.A. Vermeulen, Martin Würtz and Erik Zupa contributed equally to this work.

Abstract
Microtubules are protein cylinders with functions in cell motility, signal sensing, cell organization, intracellular transport, and chromosome segregation. One of the key properties of microtubules is their dynamic architecture, allowing them to grow and shrink in length by adding or removing copies of their basic subunit, the heterodimer αβ-tubulin. In higher eukaryotes, de novo assembly of microtubules from αβ-tubulin is initiated by a 2 MDa multi-subunit complex, the gamma-tubulin ring complex (γ-TuRC). For many years, the structure of the γ-TuRC and the function of its subunits remained enigmatic, although structural data from the much simpler yeast counterpart, the γ-tubulin small complex (γ-TuSC), were available. Two recent breakthroughs in the field, high-resolution structural analysis and recombinant reconstitution of the complex, have revolutionized our knowledge about the architecture and function of the γ-TuRC and will form the basis for addressing outstanding questions about biogenesis and regulation of this essential microtubule organizer.

KEYWORDS
gamma-tubulin ring complex, gamma-tubulin small complex, gamma-tubulin, microtubule nucleation, microtubules, recombinant gamma-tubulin ring complex

INTRODUCTION
The heterodimer αβ-tubulin assembles de novo into microtubules, which are protein cylinders with a diameter of 25 nm consisting of strings of αβ-tubulin, so-called protofilaments. All αβ-tubulin protofilaments have the same αβ-tubulin repeat organization, rendering microtubules intrinsically polar structures with a fast-growing microtubule plus end that exposes β-tubulin and a slower growing microtubule minus end that is terminated by α-tubulin.[1] Microtubules are assembled spontaneously in vitro above a critical αβ-tubulin concentration, which ranges between 1–20 μM depending on the αβ-tubulin source.[2]
FIGURE 1  Overall structure and domain organization of human GCPs. (A) GCP4 (brown) is composed of an N-terminal GRIP1 domain and a C-terminal GRIP2 domain, which interacts with γ-tubulin (yellow). (B) Domain organization of GCP2-6. All sequences are aligned according to the GRIP1 domains. Residue numbering is given.

The formation of the first αβ-tubulin oligomers is the rate-limiting step in non-templated microtubule nucleation, followed by closure of the growing αβ-tubulin sheet into a cylinder. Microtubules assembled spontaneously in vitro can have different protofilament numbers, ranging from 9 to 16, resulting in varying microtubule diameters. In contrast, complexes that contain the protein γ-tubulin, a member of the tubulin superfamily, specifically promote cellular assembly of microtubules with exactly 13 protofilaments below the critical αβ-tubulin concentration, even though there are exceptions with differing protofilament numbers in several organisms. These γ-tubulin complexes (γ-TuCs) expose 13 γ-tubulin molecules that are spatially organized in a spiral partially recapitulating microtubule geometry, suggesting that they function as a structural template. During the nucleation reaction, αβ-tubulin heterodimers are likely recruited to γ-TuCs via α-tubulin/γ-tubulin interaction and thereby positioned in a defined 3D arrangement that supports formation of lateral interactions between neighboring αβ-tubulin heterodimers. In addition, γ-TuCs determine the cellular sites of microtubule nucleation and stabilize the microtubule minus end.

In Saccharomyces cerevisiae, two paralogous γ-TuC component proteins (GCP), termed ‘GCP2’ and ‘GCP3’, bind one copy of γ-tubulin each via their C-terminal γ-tubulin ring protein 2 (GRIP2) domain (Figure 1A) and assemble into the heterotetrmeric γ-tubulin small complex (γ-TuSC) by laterally interacting via their N-terminal GRIP1 domains. Seven γ-TuSCs assemble into a left-handed γ-TuSC spiral upon binding to the Centrosomin Motif 1 (CM1) of the spindle pole body (SPB) component Spc110 at the SPB, the yeast equivalent of the centrosome. In the closed conformation of the γ-TuSC spiral (see below), γ-tubulin molecules are arranged with the same helical parameters as αβ-tubulins in microtubules, forming an ideal structural template for the assembly of microtubules. Since in this arrangement, the γ-tubulin subunits bound to GCP2 at position 1 and GCP3 at position 14 superimpose along the helical axis, only 13 γ-tubulin molecules are available for αβ-tubulin recruitment, consistent with the nucleation of microtubules with 13 αβ-tubulin protofilaments.

Many eukaryotes, for example Schizosaccharomyces pombe, Aspergillus nidulans and Drosophila melanogaster, not only contain the γ-TuSC/CM1 system, but also the compositionally more complex and much larger γ-tubulin ring complex (γ-TuRC) (Table 1). In addition to the γ-TuSC subunits γ-tubulin, GCP2 and GCP3, the γ-TuRC contains three additional GCP paralogues, GCP4, GCP5, and GCP6. While all GCP proteins contain the functionally essential GRIP1 and GRIP2 domains, they differ in the length of N-terminal extensions and spacer regions between the GRIP1 and GRIP2 domains (Figure 1B). Deletion and depletion analysis revealed that the γ-TuSC system is the principal microtubule nucleator in S. pombe, A. nidulans and D. melanogaster, while the γ-TuRC-specific components are dispensable for viability on the cell level. In contrast, all GCPs are important for cell viability in human haploid cell lines, as demonstrated by gene knockout experiments, indicating that the full γ-TuRC system is likely essential for microtubule nucleation in vertebrates. Conversely, to our knowledge, there is no evidence for a function of the oligomerised γ-TuSC in microtubule nucleation in human cells. An additional component of the microtubule nucleation machinery in many eukaryotes is the mitotic spindle organizing protein 1 (MZT1), which integrates into both the γ-TuSC and γ-TuRC. While MZT1 is essential in most organisms, it exhibits tissue specific expression and incorporation into the γ-TuRC in D. melanogaster.

In interphase cells, the pre-assembled γ-TuRC is located in the cytoplasm and is recruited to the pericentriolar material (PCM) of centrosomes via the adaptor protein NEDD1 (neural precursor cell expressed, developmentally down-regulated 1) that interacts with CEP192 (centrosomal protein of 192 kDa) and by the CM1-containing CDK5RAP2 (CDK5 regulatory subunit-associated protein 2), also known as CEP215, NEDD1 and CDK5RAP2 probably have distinct functions in microtubule anchoring and...
nucleation, respectively. At the beginning of mitosis, the PCM is expanding\cite{28} and thereby recruits additional γ-TuRCs, which contributes to the increased microtubule nucleation activity that is needed for mitotic spindle assembly.\cite{29} In addition, the augmin complex targets the γ-TuRC to the surface of preformed microtubules to promote microtubule branching.\cite{30} The growth of branched microtubules is enhanced by the activity of TPX2 (targeting protein for Xklp2), which has been proposed to lead to the formation of a phase-separated liquid ‘compartment’ along microtubules that is enriched in αβ-tubulin.\cite{31,32}

### TOWARDS STRUCTURAL UNDERSTANDING OF THE VERTEBRATE γ-TuRC

Proteomic analysis of the purified native human γ-TuRC identified γ-tubulin, GCP2 to GCP6, MZT1 and MZT2, the nucleoside diphosphate kinase 7 (NME7) and the protein NEDD1 as constituents of the complex.\cite{27,33} siRNA-mediated knockdown experiments of γ-TuRC subunits suggested that GCP2-6 are essential for the integrity of the γ-TuRC, while this is not the case for NME7 and NEDD1.\cite{34,35} siRNA depletion in combination with sucrose gradient analysis of the γ-TuRC resulted in conflicting data on whether or not MZT1 fulfils a structural role in the complex.\cite{34,36} NME7 was proposed to promote γ-TuRC-mediated nucleation activity.\cite{35} Notably, proteomic and biochemical analyses indicated an unequal stoichiometry of the GCP proteins in the complex, raising the question of whether there is one uniform γ-TuRC architecture comprising the GCP variants in different but defined copy numbers, or whether there are various co-existing γ-TuRC populations at different abundance that result in apparent unequal stoichiometry of GCPs when analyzed in bulk.\cite{27,33,37}

Based on the lock washer-like appearance of the γ-TuRC when imaged by cryo-electron tomography, it was proposed that a ring of γ-TuSC units formed by GCP2 and GCP3 coordinates the γ-tubulin molecules and thus underlies microtubule nucleation activity.\cite{38} In contrast, the low copy number components GCP4, 5 and 6 were proposed to merely stabilize the γ-TuSC oligomer by forming a cap on the complex. This model had to be revised when GCP4 was shown to have a functional GRIP2 domain that interacts with γ-tubulin and to be structurally highly similar to yeast GCP2 and GCP3,\cite{39} which cumulatively suggested that GCP4 to GCP6 actively contribute to organizing the γ-tubulin ring structure and thus to microtubule formation. However, because of missing high-resolution structural data of the complete γ-TuRC, its architecture remained a mystery.

This gap was filled when three complementary high-resolution cryo-EM structures of the vertebrate γ-TuRC were published beginning of 2020.\cite{40–42} The GCP subunit order in the γ-TuRC was consistently determined to be GCP2-GCP4-GCP5-GCP6-GCP3-GCP2 (Figure 2A), resulting in an open left-handed spiral with 14 γ-tubulin/GCP spokes. In all three γ-TuRC reconstructions, a structural scaffold termed the "luminal bridge" was observed to line the interior of the complex. This scaffold is composed of the N-termini of GCP6 and GCP3 (either from position 6 or 8), two molecules of MZT1 and, surprisingly, one molecule of actin (Figure 2B).\cite{43,44} Actin is structurally embedded into the γ-TuRC by multiple interactions, comprising a first interface towards a structural module formed by the very N-terminus of GCP6 and one copy of MZT1, and a second interface between the actin D-loop and the γ-tubulin coordinated by GCP3 at position 2 (Figure 2B).\cite{40,43} Stable integration of actin into the γ-TuRC is reminiscent of actin and actin-related proteins (Arps) being structural components in other large complexes, such as the motor protein dynein, chromatin remodeling factors (INO80, SWR1, SWI/SNF) and the histone acetyltransferase NuA4. In the aforementioned complexes, actin and Arps fulfill diverse functions, such as complex recruitment to nucleosomes to facilitate chromatin remodeling and promoting ATPase activity of the catalytic subunit.\cite{45} The function of actin in the γ-TuRC is probably not in actin polymerization\cite{40} but rather in assembly or structural stabilization of the γ-TuRC. Experiments affecting the specific incorporation

### TABLE 1 Composition of γ-TuSC and γ-TuRC in selected organisms

| Homo sapiens | Xenopus laevis | Drosophila melanogaster | Schizosaccharomyces pombe | Aspergillus nidulans | Candida albicans | Saccharomyces cerevisiae |
|-------------|----------------|------------------------|--------------------------|---------------------|-----------------|------------------------|
| GCP2*       | Xgrip110       | Dgrip84                | Alp4                     | GCPB                | Spc97           | Spc97                  |
| GCP3*       | Xgrip109       | Dgrip91                | Alp6                     | GCPC                | Spc98           | Spc98                  |
| GCP4*       | Xgrip76        | Dgrip75                | Gfh1                     | GCPD                | –               | –                      |
| GCP5*       | Xgrip133       | Dgrip128               | Mod21                    | GCPE                | –               | –                      |
| GCP6*       | Xgrip210       | Dgrip163               | Alp16                    | GCPF                | –               | –                      |
| γ-tubulin*  | Xtubg1         | γTub23C/γTub37C       | Tug1/Tubg1               | mipA                | Tub4            | Tub4                   |
| MZT1*       | MZT1           | Mzt1                   | Mzt1                     | MztA                | Mzt1            | –                      |
| MZT2        | MZT2           | –                      | –                        | –                   | –               | –                      |
| Actin       | Actin          | n.d.                   | n.d.                     | n.d.                | –               | –                      |
| NME7        | NME7           | n.d.                   | –                        | –                   | –               | –                      |
| NEDD1       | NEDD1          | Dgp71WD                | –                        | –                   | –               | –                      |

n.d. = not determined, – = not abundant or not identified, * = required for γ-TuRC integrity.\cite{34,36}
FIGURE 2 Molecular architecture of the vertebrate γ-TuRC. (A) Cryo-EM reconstruction of the human γ-TuRC (EMD-21073) with superposed atomic models for all resolved components (PDB-6V6S). Coloring as indicated. The γ-TuRC spokes have been numbered from 1 to 14. (B) Structure of the luminal bridge (EMD-21984, PDB-6X0U), composed of the GCP6 and GCP3 N-termini, actin and two copies of MZT1. Actin interacts with the γ-tubulin copy coordinated at spoke 2 (D-loop interface) and the GCP6 N-terminus (actin-GCP6 interface). Coloring as indicated. (C) Differential accessibility of the γ-tubulin surface. Extended C-terminal helices sterically restrict accessibility of γ-tubulin copies associated with GCP3 (PDB-6V6S). Only the C-terminal helices of GCP2 and GCP3 are colored. Otherwise coloring as in A. (D) Structural basis for CM1 motif recruitment to the γ-TuRC (EMD-21985, PDB-6X0V). The CM1 motif binds to spokes 12 and 13 on the exterior of the γ-TuRC supported by a structural module of MZT2 and a GCP2 N-terminus. Coloring as indicated.

MULTIFACTORIAL REGULATION OF MICROTUBULE NUCLEATION ACTIVITY

Microtubule nucleation activity is spatially and temporally highly controlled. In particular, the microtubule nucleation activity of centrosomes is strongly enhanced at the beginning of mitosis. Whether this is achieved exclusively by elevated γ-TuRC recruitment to the pericentriolar material (PCM) of centrosomes or is additionally supported by an increase in specific microtubule nucleation activity of individual γ-TuRCs is currently unclear. In any case, it has been established that...
phosphorylation of NEDD1 by polo kinase PLK1 is critical for targeting of γ-tubulin to the centrosome and to the spindle at the onset of mitosis. Whether the NME7 subunit of the γ-TuRC is involved in regulating the microtubule nucleation activity of the γ-TuRC remains under debate. Although detected in the samples by mass spectrometry with ~0.9 subunit per γ-TuRC, NME7 could not be localized in γ-TuRC structures and consequently, there is no structural data potentially explaining why NME7 depletion affects the microtubule nucleation activity of the γ-TuRC.

The microtubule polymerase XMAP215 (Xenopus microtubule assembly protein with 215 kDa), named ch-TOG (colonic hepatic tumor-overexpressed gene) in humans, was proposed to bind to the γ-TuRC via its C-terminal region. γ-TuRC-associated XMAP215/ch-TOG probably recruits αβ-tubulin subunits to the γ-tubulin spiral of the γ-TuRC and perhaps even to the first growing αβ-tubulin oligomers, thus enhancing the microtubule nucleation activity of the γ-TuRC. Being coordinated by structurally different GCP variants that probably regulate the microtubule nucleation activity of the γ-TuRC,

| Study                  | Source                           | Overexpressed proteins | Tagged γ-TuRC proteins | Affinity purification basis | Complex elution | Additional purification steps |
|------------------------|----------------------------------|------------------------|------------------------|-----------------------------|-----------------|-----------------------------|
| Liu et al., 2020[40]   | Native, X. laevis egg extract    | –                      | –                      | γ-tubulin antibody-coupled | –               | –                           |
| Wieczorek et al., 2020[42] | Native, HeLa S3 cells          | GFP-γ-TuNA (CM1 Domain – aa 51–100 – of CDK5RAP2) | –                      | GFP nanobody column         | Proteolytic digestion (PreScission protease) | Sucrose gradient centrifugation |
| Consolati et al., 2020[41] | Native, HeLa-Kyoto cells        | GCP2-BAP (biotin acceptor peptide) | GCP2-TEV-mTagBFP-BAP    | Strepavidin munein matrix column | D-biotin      | Size exclusion chromatography |
| Zimmermann et al., 2020[40] | Recombinant, insect cells (Sf9) | γ-tubulin, MZT1, MZT2, GCP2, GCP3, GCP4, GCP5, GCP6, RUVBL1, RUVBL2 | GCP6-3C–Twin-Strep GCP3-His6 | Strep-Tactin XT resin       | Proteolytic digestion (3C protease) | –                           |
| Wieczorek et al., 2021[59] | Recombinant, insect cells (Sf9) | γ-tubulin, MZT1, MZT2, GCP2, GCP3, GCP4, GCP5, GCP6, β-actin, NEDD1 | ZZ-TEV-MZT2-EGFP γ-tubulin-TEV-His6 | NHStrap column coupled to rabbit IgG | Proteolytic digestion (TEV protease) | Size exclusion chromatography, then sucrose gradient centrifugation |
| Würtz et al., 2021[58]  | Recombinant, insect cells (Sf21/HI5) | γ-tubulin, MZT1, GCP2, GCP3, GCP4, GCP5, GCP6, β-actin | 2xFLAG-TEV-GCP5 γ-tubulin-Myc-His6 | Anti-FLAG Affinity Gel | 3xFLAG peptide | –                           |

| Summary of the basic approaches for purification of native or recombinant vertebrate γ-TuRC |

Recently, a high-resolution cryo-EM structure of the γ-TuSC from the yeast Candida albicans was determined (Figure 3A), enabling direct comparison to the vertebrate γ-TuSC (Figure 3B) and thus providing
FIGURE 3  Evolutionary divergence of interfaces in human and C. albicans γ-TuSC. (A) Overall structure of the C. albicans γ-TuSC (EMD-11835, PDB-7ANZ).[51] Coloring as in Figure 2A. The zoomed view focuses on the extended interface in surface representation. The insertions forming the extended interface are indicated. Coloring as in Figure 2A. (B) Overall structure of the human γ-TuSC as observed in the context of the fully assembled γ-TuRC (EMD-21073, PDB-6V6S).[42] Coloring as in Figure 2A. (C) Electrostatic interactions between γ-tubulins associated with GCP2 and GCP3 in the human γ-TuSC. The γ-tubulin copy associated with GCP2 is shown in surface representation colored according to coulombic potential (blue-positive, white-neutral, red-negative). The γ-tubulin copy associated with GCP3 is shown in atomic model representation with positively charged residues forming the interface indicated in blue.

insights into evolutionary conservation of the architecture and regulation of γ-tubulin complexes.[51] Surprisingly, the intermolecular interfaces stabilizing the γ-TuSC units are not entirely conserved between C. albicans and vertebrates. In both systems, lateral interactions between the GRIP1 domains of GCP2 and GCP3 mediate association of the two γ-TuSC spokes, but the predominantly hydrophobic nature of the interface in C. albicans is replaced by mostly electrostatic interactions in vertebrates.[51] In C. albicans, lateral interactions between the spokes are extended by an additional interface formed by species-specific insertions in the GRIP2 domains of GCP2 and GCP3 and the N-terminus of γ-tubulin (Figure 3A). These GRIP2-specific interactions are absent in the vertebrate γ-TuSC units. However, complementary
challenged patches on vertebrate γ-tubulin mediate electrostatic interactions between the two γ-tubulin copies of the vertebrate γ-TuSC unit (Figure 3C) and may thereby provide a stabilizing effect similar to the extended GRIP2/γ-tubulin insertion interface in C. albicans.[51]

From an evolutionary perspective, systematic analysis of GCP2, GCP3 and γ-tubulin primary sequences indicates that the residues required for electrostatic interactions of the two γ-tubulin molecules are conserved in all organisms coding for the full set of γ-TuRC-specific GCPs (Table 1), while the protein insertion sites required for the extended interface as observed in C. albicans are specific to organisms exclusively coding for γ-TuSC components (with or without MZT1), i.e., Saccharomyces. Thus, the overall architecture of the γ-TuSC units seems to be strictly dependent on whether or not they are predicted to fulfill their function in the context of the larger γ-TuRC. It is therefore highly probable that genomic deletion of the GCP4-6 coding regions in Saccharomyces during evolution has gone hand in hand with reorganization of the interfaces that stabilize the γ-TuSC units.[51]

As outlined above, the vertebrate γ-TuRC requires structural alterations to serve as an optimal structural template for microtubule nucleation. This is also the case for the yeast γ-TuSC, which adopts an “open” conformation in the inactive γ-TuSC oligomer helix where every 2nd γ-tubulin molecule is positioned away from the cylindrical geometry of a microtubule. Rearrangement of GRIP2 domains along with the associated γ-tubulins results in a “closed”, more symmetrical and more active conformation of the γ-TuSC helix in vitro. This conformational change may be triggered in vivo by phosphorylation of γ-TuSC subunits and contribute to cell cycle-dependent changes in microtubule nucleation activity of the yeast SPB.[52–54]

SYSTEMS FOR RECOMBINANT EXPRESSION OF THE γ-TuRC

While expression of yeast γ-TuSCs in insect cells has been reported, recombinant expression of human γ-TuRC components was limited to GCP4 and γ-tubulin, and reconstitution of the full complex has failed for a long time.[55–57] However, more recent attempts relying on the γ-TuRC components identified by combined mass-spectrometry and cryo-EM analyses were successful and recombinant expression of the γ-TuRC-encoding genes using the MultiBac or biBGac systems in insect cells has been reported in three independent articles (Table 2).

Würzt et al. expressed the γ-TuRC subunits γ-tubulin, actin, GCP2-6 and MZT1 and isolated structurally intact and microtubule nucleation-competent γ-TuRCs using a gentle and fast single step affinity purification against a double FLAG tag on GCP5, constituting the minimal system yielding functional γ-TuRC to date.[58] Successful reconstitution of the γ-TuRC in absence of MZT2 indicates that the function of the latter is not required for assembly of the γ-TuRC, consistent with its peripheral position in the complex (Figure 2D).[43] In addition to the core γ-TuRC subunits used by Würzt et al., Wieczorek et al. also co-expressed MZT2 and NEDD1 and obtained structurally intact γ-TuRCs after several purification steps.[59] Notably, omitting the luminal bridge components MZT1 and actin from the expression system, Wieczorek et al. were able to capture an eight-spoke γ-TuRC subcomplex that surprisingly had a similar microtubule nucleation activity to the fully assembled γ-TuRC in an in vitro microtubule nucleation assay. It will be interesting to test whether such a γ-TuRC fragment is also active in microtubule nucleation in vivo or can even entirely replace the function of the γ-TuRC in cells.

In the most extensive study, Zimmermann et al. characterized the cryo-EM structure and assembly mechanism of the recombinant γ-TuRC and showed that the recombinant γ-TuRC recapitulates the structural organization of the native γ-TuRC isolated from frog extracts or human tissue culture cells.[60] Remarkably, even though Zimmermann et al. did not co-express actin in their recombinant system, the purified γ-TuRC contained actin as part of the luminal bridge, indicating that insect cell actin can replace human actin in the recombinant human γ-TuRC. Moreover, the authors showed that co-expression of human GCP2, GCP3 and γ-tubulin together with both MZT1 and MZT2 allows assembly of a stable human γ-TuSC unit that has a tendency to self-interact and form small γ-TuSC oligomers. Although MZT2 is not essential for the assembly of the γ-TuRC[58], its co-expression enhanced the yield of soluble γ-TuSC units in this recombinant system, suggesting that MZT2 stabilizes the N-terminal region of GCP2, which it forms stable structural modules with. Whether MZT2 retains such a stabilizing function during and after the assembly of the γ-TuRC in human cells remains to be investigated.

THE RUVBL1-RUVBL2 AAA+-ATPase ASSISTS IN γ-TuRC ASSEMBLY

The central aspect of the study by Zimmermann et al. is the discovery of the RUVBL1-RUVBL2 AAA+-ATPase (Figure 4A) as a cofactor for efficient assembly of the γ-TuRC, both for recombinant expression systems as well as native biogenesis of the γ-TuRC in mammalian cells.[60] The authors showed that RUVBL1-RUVBL2 associates with the human γ-TuSC in vitro and when co-expressed with recombinant γ-TuSC in insect cells. However, co-expression was not required for formation of stable γ-TuSC units, suggesting that the AAA+-ATPase is rather involved in follow-up steps of γ-TuRC assembly. This is consistent with negative stain EM analysis, which indicates binding of the heterohexameric RUVBL1-RUVBL2 ATPase to the open ends of self-assembled γ-TuSC oligomers.[60]

While Zimmermann et al. identified RUVBL1-RUVBL2 activity to be important for efficient assembly of human γ-TuRC, the detailed mechanism remains unclear. RUVBL1 and RUVBL2 are highly conserved AAA+-ATPases with diverse functions ranging from chromatin remodeling and nonsense-mediated mRNA decay to the assembly of kinases (for example mTORC1, ATM, ATR), RNA polymerases and small nucleolar ribonucleoprotein complexes.[61] The RUVBL1-RUVBL2 ring exposes two interfaces, an AAA-face that harbors the ATPase activity and a D1I-face that binds different co-factors and interacting partners that are required for the diverse functions of RUVBL1-RUVBL2 (Figure 4A). For instance, recruitment of the additional subunits RPAP3
Assembly of the γ-TuRC supported by RUVBL1-RUVBL2. (A) Bottom view (left) and side view (right) of a composite model of the human heterohexameric RUVBL1-RUVBL2 complex, generated by aligning atomic models for human RUVBL1 (light green, PDB-2C9O)
[71] and RUVBL2 (dark green, PDB-6H7X)
[72] onto the crystal structure of the RUVBL1-RUVBL2 heterohexamer with truncated DII domains (PDB-2XSZ).
[73] (B) Possible mechanism for the assembly of the γ-TuRC, involving potentially RUVBL1-RUVBL2-mediated steps. (top left) GCP4 and GCP5 associate with GCP4 and GCP6 to form a stable subcomplex, where the GCP6-MZT1 module and potentially actin are likely flexibly associated (semi-transparent). (top right) Three γ-TuSC units composed of GCP2 and GCP3 are recruited, likely assisted by RUVBL1-RUVBL2. The luminal bridge is presumably formed during this step. (bottom left) Finally, the two peripheral γ-TuSC units are incorporated at positions 1/2 and 13/14 with potential involvement of RUVBL1-RUVBL2, completing assembly of the γ-TuRC (bottom right). Coloring as indicated in the top right. Spoke numbering as in Figure 2A.

(Tah1 in yeast) and PIH1D1 to the RUVBL1-RUVBL2 AAA+ ATPase forms the R2TP core complex, which can bind additional molecular chaperones (Hsp70 and Hsp90).
[62–65] In mammals, the R2TP core complex can further associate with the prefoldin subunits PFDN1 and PFDN6, as well as additional prefoldin-like proteins to form the R2TP/prefoldin-like complex. This complex was observed to be crucial for the assembly of RNA polymerase II.
[66] Yet, even in the absence of aforementioned binding partners, the RUVBL1-RUVBL2 complex can form stable complexes with INO80 and SWR1.
[67] In insect cells, co-expression of only RUVBL1-RUVBL2 was indeed sufficient to strongly elevate recombinant γ-TuRC assembly, but it cannot be ruled out that the conserved RUVBL1-RUVBL2 co-factors are directly recruited.
from insect cells. Thus, it remains to be investigated whether RPAP3, Pih1D1, Hsp90 and the prefoldin subunits also play a role in γ-TuRC assembly.

Based on salt fragmentation analysis, it was proposed that the γ-TuRC assemblies from a stable core of GCP4, GCP5 and GCP6 subunits, which interacts with MZT1, actin and three γ-TuSC units to form a luminal bridge-stabilized γ-TuRC subcomplex consisting of 10 spokes. Addition of one γ-TuSC unit on both ends of the spiral then completes γ-TuRC assembly (Figure 4B). Because high-resolution structural data of RUVBL1-RUVBL2 bound to γ-TuRC subcomplexes are not available, it is presently unclear which of these steps are supported by RUVBL1-RUVBL2 activity and how γ-TuSC addition is terminated after assembly of the 14-spoke γ-TuRC.

It is also unclear at what stage during the assembly process MZT1 and MZT2 are incorporated into the γ-TuRC. One likely possibility is that MZT1 and MZT2 already bind to the N-termini of GCP3 and GCP2, respectively, at the stage of γ-TuSC assembly, as suggested by γ-TuSc reconstitution experiments. In this scenario, MZT1 may directly contribute to the formation of the luminal bridge during γ-TuSC recruitment to the GCP4-6 core. This model, however, raises the question of why only two MZT1-GCP3 modules are resolved in the recent cryo-EM reconstructions of the γ-TuRC, even though five copies of GCP3 are present. A likely scenario is that all GCP3 copies (and potentially also GCP5) are associated with MZT1, but the remaining modules are not stably bound to the γ-TuRC core and therefore too flexible to be visualized in an averaging-based approach like cryo-EM. Alternatively, only specific copies of GCP3 might be stably associated with MZT1, depending on their position and molecular surrounding in the γ-TuRC, while MZT1 may dissociate from other GCP3 copies after incorporation into the γ-TuRC.

CONCLUSIONS

High-resolution cryo-EM structures of γ-tubulin complexes from different biological systems along with successful recombinant expression of the full human γ-TuRC have provided detailed insights into the mechanism of γ-TuRC assembly and microtubule nucleation. The combination of these approaches, together with targeted modification and engineering of the γ-TuRC, will allow dissection of the role of actin in the γ-TuRC, the function of the long insertion domain in GCP6, the interaction with recruitment and binding factors such as NEDD1, CDK5RAP2 and XMAP215/ch-TOG, the molecular role of the NME7 subunit, and the impact of mutations in γ-TuRC genes that are associated with the developmental disorder microcephaly on the structure and function of the γ-TuRC.

ACKNOWLEDGMENTS

The authors thank Sebastian Eustermann (EMBL, Heidelberg) and Annett Neuner (ZMBH, Heidelberg) for critical reading of the manuscript. This work was supported by grants of the Deutsche Forschungsgemeinschaft (DFG) to E. Schiebel (DFG Schi 295/4-4) and to S. Pfeffer (DFG PF 963/1-4).

REFERENCES

1. Mitchison, T. (1993). Localization of an exchangeable GTP binding site at the plus end of microtubules. Science, 261(5124), 1044–1047.
2. Jonasson, E. M., Mauro, A. J., Li, C., Labuz, E. C., Mahserejian, S. M., Scripture, J. P., Gregorietti, I. V., Alber, M., & Goodson, H. V. (2020). Behaviors of individual microtubules and microtubule populations relative to critical concentrations: dynamic instability occurs when critical concentrations are driven apart by nucleotide hydrolysis. Molecular Biology of the Cell, 31(7), 589–618. https://doi.org/10.1091/mbc.E19-02-0101
3. Olmsted, J. B., & Borisy, G. G. (1993). Microtubules. Annual Review of Biochemistry, 42, 507–540. https://doi.org/10.1146/annurev.bi.42.070173.002451
4. Kollman, J. M., Merdes, A., Mourey, L., & Agard, D. A. (2011). Microtubule nucleation by γ-tubulin complexes. Reviews of Molecular Cell Biology, 12(11), 709–721. https://doi.org/10.1038/nrm3209
5. Wade, R. H., Chrétien, D., & Job, D. (1990). Characterization of microtubule protofilament numbers – How does the surface lattice accommodate? Journal of Molecular Biology, 212(4), 775–786. https://doi.org/10.1016/0022-2836(90)90236-F
6. Sui, H., & Downing, K. H. (2010). Structural basis of interprotofilament interaction and lateral deformation of microtubules. Structure (London, England), 18(8), 1022–1031. https://doi.org/10.1016/j.str.2010.05.010
7. Chaaban, S., & Brouhard, G. J. (2017). A microtubule bestiary: structural diversity in tubulin polymers. Molecular Biology of the Cell, 28(22), 2924–2931. https://doi.org/10.1091/mbc.E16-05-0271
8. Kollman, J. M., Polka, J. K., Zelter, A., Davis, T. N., & Agard, D. A. (2010). Microtubule nucleating gamma-TuSC assembles structures with 13-fold microtubule-like symmetry. Nature, 466(7308), 879–882. https://doi.org/10.1038/nature09207
9. Keating, T. J., & Borisy, G. G. (2000). Immunostuctural evidence for the template mechanism of microtubule nucleation. Nature Cell Biology, 2(6), 352–357. https://doi.org/10.1038/scib05045
10. Wiese, C., & Zheng, Y. (2000). A new function for the γ-tubulin ring complex as a microtubule minus-end cap. Nature Cell Biology, 2(2), 132–137. https://doi.org/10.1038/nceb0295
11. Knop, M. (1997). The spindle pole body component Spc97p interacts with the gamma-tubulin of Saccharomyces cerevisiae and functions in microtubule organization and spindle pole body duplication. Embo Journal, 16(7), 1550–1564. https://doi.org/10.1093/emboj/16.7.1550
12. Kollman, J. M., Greenberg, C. H., Li, S., Moritz, M., Zelter, A., Fong, K. K., Fernandez, J. J., Sali, A., Kilmartin, J., Davis, T. N., & Agard, D. A. (2015). Ring closure activates yeast gammaTuRC for species-specific microtubule nucleation. Nature Structural & Molecular Biology, 22(2), 132–137. https://doi.org/10.1038/nsmb.2953
13. Xiong, Y., & Oakley, B. R. (2009). In vivo analysis of the functions of gamma-tubulin-complex proteins. Journal of Cell Science, 122(22), 4218–4227. https://doi.org/10.1242/jcs.059196
14. Anders, A., Lourenço, P. C. C., & Sawin, K. E. (2006). Noncore components of the fission yeast gamma-tubulin complex. Molecular Biology of the Cell, 17(12), 5075–5093. https://doi.org/10.1091/mbc.E05-11-1009
18. Masuda, H., Mori, R., Yukawa, M., & Toda, T. (2013). Fission yeast MOZART1/Mzt1 is an essential gamma-tubulin complex component required for complex recruitment to the microtubule organizing center, but not its assembly. *Molecular Biology of the Cell*, 24(18), 2894–2906. https://doi.org/10.1091/mbc.e13-05-0235

19. Dhani, D. K., Govt, B. T., George, G. M., Rogerson, D. T., Bitton, D. A., Miller, C. J., Schwaie, J. W. R., & Tanaka, K. (2013). Mzt1/Tam4, a fission yeast MOZART1 homologue, is an essential component of the gamma-tubulin complex and directly interacts with GCP3(Alp6). *Molecular Biology of the Cell*, 24(21), 3337–3349. https://doi.org/10.1091/mbc.e13-05-0253

20. Masuda, H., & Toda, T. (2016). Synergistic role of fission yeast Alp16GCP6 and Mzt1MOZART1 in gamma-tubulin complex recruitment to mitotic spindle pole bodies and spindle assembly. *Molecular Biology of the Cell*, 27(11), 1753–1763. https://doi.org/10.1091/mbc.E15-08-0577

21. Tovey, C. A., Tubman, C. E., Hamrud, E., Zhu, Z., Dyas, A. E., Butterfield, A. N., Fyfe, A., Johnson, E., & Conduit, P. T. (2018). gamma-TuRC heterogeneity revealed by analysis of Mozart1. *Current Biology*, 28(14), 2314–2323.e6. https://doi.org/10.1016/j.cub.2018.05.044

22. Gomez-Ferreria, M. A., Bashkurov, M., Helbig, A. O., Larsen, B., Pawson, T., Gongas, A.-C., & Pelletier, L. (2012). Novel NEDD1 phosphorylation sites regulate gamma-tubulin binding and mitotic spindle assembly. *Journal of Cell Science*, 125(16), 3745–3751. https://doi.org/10.1242/jcs.105130

23. Bond, J., Roberts, E., Springell, K., Lizarraga, S., Scott, S., Higgins, J., Hampshire, D. J., Morrison, E. E., Leal, G. F., Silva, E. O., Costa, S. M. R., Baralle, D., Raponi, M., Barbani, G., Rashid, Y., Jafari, H., Bennett, C., Corry, P., Walsh, C. A., & Woods, C. G. (2005). A centrosomal mechanism involving CDK5RAP2 and CENPJ controls brain size. *Nature Genetics*, 37(4), 353–355. https://doi.org/10.1038/ng1539

24. Lüders, J., Patel, U. K., & Stearns, T. (2006). GCP-WD is a gamma-tubulin targeting factor required for centrosomal and chromatin-mediated microtubule nucleation. *Nature Cell Biology*, 8(2), 137–147. https://doi.org/10.1038/ncb1349

25. Muroyama, A., Seldin, L., & Lechler, T. (2016). Divergent regulation of functionally distinct gamma-tubulin complexes during differentiation. *Journal of Cell Biology*, 213(6), 679–692. https://doi.org/10.1083/jcb.201601099

26. Haren, L., Remy, M.-Hélè, Bazin, I., Callebaut, I., Wright, M., & Merdes, A. (2006). NEDD1-dependent recruitment of the gamma-tubulin ring complex to the centrosome is necessary for centriole duplication and spindle assembly. *Journal of Cell Biology*, 172(4), 505–515. https://doi.org/10.1083/jcb.200510028

27. Choi, Y.-K., Liu, P., Sze, S. K., Dai, C., & Qi, R. Z. (2010). CDK5RAP2 stimulates microtubule nucleation by the gamma-tubulin ring complex. *Journal of Cell Biology*, 191(6), 1089–1095. https://doi.org/10.1083/jcb.201007030
58. Würtz, M., Böhler, A., Neuner, A., Zupa, E., Rohland, L., Liu, P., Vermeulen, B. J. A., Pfeffer, S., Eustermann, S., & Schiebel, E. (2021). Reconstitution of the recombinant human γ-tubulin ring complex. Open Biology, https://doi.org/10.1098/rsob.200325.

59. Wieczorek, M., Ti, S.-C., Urnauvicus, L., Molloy, K. R., Aber, A., Chait, B. T., & Kapoor, T. M. (2021). Biochemical reconstitutions reveal principles of human γTuRC assembly and function. Journal of Cell Biology, 220.e202009146.

60. Zimmermann, F., Serna, M., Ezquerra, A., Fernandez-Leirio, R., Llorca, O., & Luders, J. (2020). Assembly of the asymmetric human gamma-tubulin ring complex by RUVBL1-RUVBL2 AAA ATPase. Science Advances, 6(51), eabe0894. https://doi.org/10.1126/sciadv.eabe0894

61. Dauden, M. I., López-Perrote, A., & Llorca, O. (2021). RUVBL1-RUVBL2 AAA-ATPase: a versatile scaffold for multiple complexes and functions. Current Opinion in Structural Biology, 67, 78–85.

62. Pal, M., Morgan, M., Phelps, S. E. L., Roe, S. M., Parry-Morris, S., Downs, J. A., Polier, S., Pearl, L. H., & Predromou, C. (2014). Structural basis for phosphorylation-dependent recruitment of Tel2 to Hsp90 by Pih1. Structure (London, England), 22(6), 805–818. https://doi.org/10.1016/j.str.2014.04.001

63. Hořejší, Z., Stach, L., Flower, T. G., Joshi, D., Flynn, H., Skehel, J. M., O’relly, N. J., Ogodowicz, R. W., Smerdon, S. J., & Boulton, S. J. (2014). Phosphorylation-dependent P1H1D1 interactions define substrate specificity of the R2TP cochaperone complex. Cell Reports, 7(1), 19–26. https://doi.org/10.1016/j.celrep.2014.03.013

64. Von Morgen, P., Burdova, K., Flower, T. G., O’reilly, N. J., Boulton, S. J., Smerdon, S. J., Macreuk, L. & Hořejší, Z. (2017). MRE11 stability is regulated by CK2-dependent interaction with R2TP complex. Oncogene, 36(34), 4943–4950. https://doi.org/10.1038/onc.2017.99

65. Vaughan, C. K. (2014). Hsp90 Picks PikhTs via R2TP and Tel2. Structure (London, England), 22(6), 799–800. https://doi.org/10.1016/j.str.2014.05.012

66. Boulon, S., Pradet-Balade, B., Verheggen, C., Molle, D., Boireau, S., Georgieva, M., Azzag, K., Robert, M.-C., Ahmad, Y., Neel, H., Lamond, A. I., & Bertrand, E. (2010). HSP90 and Its R2TP/Prefoldin-like co-chaperone are involved in the cytoplasmic assembly of RNA polymerase II. Molecular Cell, 39(6), 912–924. https://doi.org/10.1016/j.molcel.2010.08.023

67. Eustermann, S., Schall, K., Kostrewa, D., Lakomek, K., Strauss, M., Moldt, M., & Hopfner, K.-P. (2018). Structural basis for ATP-dependent chromatin remodelling by the INO80 complex. Nature, 556(7701), 386–390. https://doi.org/10.1038/s41586-018-0029-y

68. Haren, L., Farache, D., Emorine, L., & Merdes, A. (2020). A stable sub-complex between GCP4, GCP5 and GCP6 promotes the assembly of gamma-tubulin ring complexes. Journal of Cell Science, 133(11), jcs244368. https://doi.org/10.1242/jcs244368

69. Scheidecker, S., Etard, C., Haren, L., Stoetzl, C., Hull, S., Arno, G., Plagnol, V., Drunat, S., Passendr, S., Toutain, A., Obrier, C., Koob, M., Geoffroy, V., Marion, V., Strähle, U., Ostergaard, P., Verloes, A., Merdes, A., Moore, A. T., & Dolfus, H. (2015). Mutations in TUBGCP4 alter microtubule organization via the gamma-tubulin ring complex in autosomal-recessive microcephaly with chorioretinopathy. American Journal of Human Genetics, 96(4), 666–674. https://doi.org/10.1016/j.ajhg.2015.02.011

70. Mitani, T., Punetha, J., Akalin, I., Pehlivan, D., Davidszüdi, M., Coban Akdemir, Z., Yilmaz, S., Aylan, E., Hunter, J. V., Hijazi, H., Grochowski, C. M., Jiangiani, S. N., Karaca, E., Fatih, J. M., Iwanowski, P., Gambin, T., Wlasienko, P., Goszczanska-Ciuchta, A., Bekiesinska-Figatowska, M....Gawlik, P. (2019). Bi-allelic pathogenic variants in TUBGCP2 cause microcephaly and lissencephaly spectrum disorders. Journal of Human Genetics, 64(22), 1050–1051. https://doi.org/10.1016/j.jhge.2019.09.017

71. Matias, P. M., Gorynia, S., Donner, P., & Carrondo, M. A. (2006). Crystal structure of the human AAA(+)-protein RuvBL1. Journal of Biological Chemistry, 281(50), 38918–38929. https://doi.org/10.1074/jbc.M605625200

72. Silva, S. T. N., Brito, J. A., Arranz, R., Sorzano, C. O. S., Ebel, C., Doutht, J., Tully, M. D., Carazo, J.-M., Carrascosa, J. L., Matias, P. M., & Bandeiras, T. M. (2018). X-ray structure of full-length human
RuvB-Like 2-mechanistic insights into coupling between ATP binding and mechanical action. Scientific Research, 8, 13726. https://doi.org/10.1038/s41598-018-31997-z

Gorynia, S., Bandeiras, T. M., Pinho, F. G., Mcvey, C. E., Vonrhein, C., Round, A., Svergun, D. I., Donner, P., Matias, P. M., & Carrondo, M. A. (2011). Structural and functional insights into a dodecameric molecular machine – The RuvBL1/RuvBL2 complex. Journal of Structural Biology, 176, 279–291.

How to cite this article: Böhler, A., Vermeulen, B. J.A., Würtz, M., Zupa, E., Pfeffer, S., & Schiebel, E. (2021). The gamma-tubulin ring complex: deciphering the molecular organisation and assembly mechanism of a major vertebrate microtubule nucleator. BioEssays, e2100114. https://doi.org/10.1002/bies.202100114