MZT Proteins Form Multi-Faceted Structural Modules in the $\gamma$-Tubulin Ring Complex

Graphical Abstract

Highlights

- Identification and de novo models of MZT1 and MZT2 in the native $\gamma$-TuRC
- MZT1 and MZT2 form structurally mimetic subcomplexes with different GCP-NHDS
- MZT1/GCP3-NHD and MZT1/GCP6-NHD occupy the $\gamma$-TuRC lumenal bridge
- MZT2/GCP2-NHD interacts with the $\gamma$-TuNA peptide on the outer face of the $\gamma$-TuRC

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In Brief

Wieczorek et al. show how the microproteins MZT1 and MZT2 expand binding interfaces across the $\gamma$-TuRC—the cell’s microtubule nucleating machinery—by forming similarly shaped, “modular” subcomplexes with the $\alpha$-helical N-terminal domains of different $\gamma$-tubulin complex proteins (GCPs).
MGT Proteins Form Multi-Faceted Structural Modules in the γ-Tubulin Ring Complex

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SUMMARY

Microtubule organization depends on the γ-tubulin ring complex (γ-TuRC), a ~2.3-MDa nucleation factor comprising an asymmetric assembly of γ-tubulin and GCP2-GCP6. However, it is currently unclear how the γ-TuRC-associated microproteins MZT1 and MZT2 contribute to the structure and regulation of the holocomplex. Here, we report cryo-EM structures of MZT1 and MZT2 in the context of the native human γ-TuRC. MZT1 forms two subcomplexes with the N-terminal α-helical domains of GCP3 or GCP6 (GCP-NHDs) within the γ-TuRC “lumenal bridge.” We determine the X-ray structure of recombinant MZT1/GCP6-NHD and find it is similar to that within the native γ-TuRC. We identify two additional MZT/GCP-NHD-like subcomplexes, one of which is located on the outer face of the γ-TuRC and comprises MZT2 and GCP2-NHD in complex with a centrosomin motif 1 (CM1)-containing peptide. Our data reveal how MZT1 and MZT2 establish multi-faceted, structurally mimetic “modules” that can expand structural and regulatory interfaces in the γ-TuRC.

INTRODUCTION

Microtubules facilitate many fundamental cellular processes, including the trafficking of intracellular components and the faithful segregation of chromosomes (Forth and Kapoor, 2017; Vale, 2003). Microtubules are nucleated by the γ-tubulin ring complex (γ-TuRC), a ~2.3-MDa assembly including the GTPase γ-tubulin, the γ-tubulin complex proteins 2–6 (GCP2-6) (Consolati et al., 2020; Liu et al., 2020; Moritz et al., 2000; Murphy et al., 2001; Stearns and Kirschner, 1994; Wieczorek et al., 2020; Zheng et al., 1995), and the mitotic-spindle organizing proteins associated with a ring of γ-tubulin 1 and 2 (MZT1 and MZT2) (Hutchins et al., 2010).

MZT1 (initially called GIP-1) was discovered in A. thaliana as a GCP3-interacting protein (Janski et al., 2008). MZT1 and MZT2 were subsequently identified in human γ-TuRCs (Hutchins et al., 2010; Teixidó-Travesa et al., 2010), and both proteins have been consistently reported in biochemical analyses of vertebrate γ-TuRCs (Choi et al., 2010; Consolati et al., 2020; Liu et al., 2020; Thawani et al., 2018; Wieczorek et al., 2020). Although MZT1 depletion in cultured cells phenocopies the effects of γ-tubulin RNAi (Hutchins et al., 2010), studies in fission yeast, C. elegans, D. melanogaster, A. nidulans, plant, and human cells have argued that MZTs are not required for γ-TuRC assembly; rather, they interact with GCPs to mediate γ-TuRC localization at specific microtubule organizing centers such as centrosomes (Sallee et al., 2018; Teixidó-Travesa et al., 2010), inner plaques of spindle pole bodies (Gao et al., 2019), basal bodies (Tovey et al., 2018), or pre-existing microtubules (Janski et al., 2012; Masuda et al., 2013; Nakamura et al., 2012). However, exactly how MZTs bind to γ-TuRCs is not clear.

One clue comes from studies of γ-TuRC “attachment factors,” such as the pericentriolar protein CDK5Rap2 (Choi et al., 2010). CDK5Rap2 contains a conserved centrosomin motif 1 (CM1) found in several other γ-TuRC attachment factors, including D. melanogaster centrosomin, S. cerevisiae Spc72, and S. pombe Mto1 (Kollman et al., 2011; Tovey and Conduit, 2018). This motif has also been called the γ-TuRC nucleation activator (γ-TuNA), which in CDK5Rap2 is essential for maintaining the centrosomal localization of γ-TuRCs in cultured human cells (Choi et al., 2010). Importantly, previous microscale thermophoresis and immunoprecipitation studies have suggested a tripartite interaction among the γ-TuNA, MZT proteins, and GCPs (Lin et al., 2016), but the location of such a subcomplex in the γ-TuRC is not known.

Recent structures of native vertebrate γ-TuRCs demonstrated that the complex is built from two asymmetric halves: one containing multiple γ-tubulin small complexes (γ-TuSCs) comprising γ-tubulin, GCP2, and GCP3 located at positions 1–8 of the complex; and another containing an arrangement of GCP4, GCP5, GCP6, and a final γ-TuSC in an altered conformation (positions 9–14) (Consolati et al., 2020; Liu et al., 2020; Wieczorek et al., 2020). One key insight from these structures is that the MTOC is built from two asymmetric halves: one containing multiple γ-tubulin small complexes (γ-TuSCs) comprising γ-tubulin, GCP2, and GCP3 located at positions 1–8 of the complex; and another containing an arrangement of GCP4, GCP5, GCP6, and a final γ-TuSC in an altered conformation (positions 9–14) (Consolati et al., 2020; Liu et al., 2020; Wieczorek et al., 2020).
Figure 1. *De Novo* Structural Model for MZT1 in Complex with GCP3-NHD in the γ-TuRC Lumen

(A) Secondary structure prediction of human MZT1. Top: primary sequence (red, predicted coiled-coil); middle: JNETHMM prediction (Drozdetskiy et al., 2015); bottom: confidence score. α helices H1–H3 are shown as green bars.

(B) Cartoon representation of initial models for MZT1 helices H1–H3. Dotted lines indicate connectivities between α helices.

(C) Surface representation of segmented cryo-EM density submap “A” with α-helical segments and a well-resolved connecting loop indicated.

(D) Two views of a *de novo* molecular model for MZT1 (green cartoon representation).

(E) Example of the fit of MZT1 side chains (stick representation) into the corresponding cryo-EM density (mesh representation).

(F) Secondary structure prediction of human GCP3-NHD. Top: primary sequence (red, predicted coiled-coil); middle: JNETHMM prediction (Drozdetskiy et al., 2015); bottom: confidence score.

(G) Cartoon representation of initial models for GCP3-NHD helices H1–H5. Dotted lines indicate connectivities between α helices.

(H) Surface representation of segmented cryo-EM density submap “B” with α-helical segments and a well-resolved connecting loop indicated.

(I) Two views of a *de novo* molecular model for GCP3-NHD (green cartoon representation).

(J) Example of the fit of GCP3-NHD side chains (stick representation) into the corresponding cryo-EM density (mesh representation).

(K) Cartoon representation of an initial model for MZT1 helices H1–H5 in complex with GCP3-NHD helices H1–H5. The connecting loop is indicated.

(L) Image shows the model of MZT1 helices H1–H5 in complex with GCP3-NHD helices H1–H5. The connecting loop is indicated.

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M2020 (Figure S1A). A substantial and unexpected density—the lumenal bridge—also spans across the inside of the γ-TuRC. The lumenal bridge contains an actin-like protein sandwiched between γ-tubulins at γ-TuRC positions 2 and 3 on one side and a bundle of ~16 α helices on the other. While all three recent cryo-EM γ-TuRC structures report a lumenal bridge, the ~16 α-helical densities are unassigned (Consolati et al., 2020; Liu et al., 2020; Wieczorek et al., 2020) (Figure S1A). In addition, the MZTs have not been assigned to any portion of the γ-TuRC due to their small size (~8 kDa for MZT1 and ~16 kDa for MZT2), a lack of prior structural data, and ambiguities in connecting densities between secondary structure elements in unassigned regions of the complex.

Here, we acquire additional cryo-EM data for the human γ-TuRC, which allowed us to build de novo models of MZTs in the native complex. We identify two copies of MZT1 found in structurally mimetic subcomplexes: one copy associates with the N terminus of GCP3, and the other associates with GCP6. We reconstitute and solve the X-ray crystal structure for one of these subcomplexes, validating our cryo-EM findings. Our models lead to the identification of two additional MZT/GCP-like subcomplexes in the γ-TuRC distal to the lumenal bridge, one of which comprises MZT2, GCP2, and the CM1 of a γ-TuRC attachment factor. Our results reveal that together with N-terminal portions of the GCPs, MZT1 and MZT2 form multi-faceted, structurally mimetic “modules” at diverse locations in the γ-TuRC.

RESULTS

MZT1 Forms a Small α-Helical Bundle in the γ-TuRC Lumenal Bridge

MZT1 is an ~82-residue-long protein conserved across vertebrates (Hutchins et al., 2010; Janski et al., 2012; Nakamura et al., 2012). Secondary structure predictions suggest that MZT1 contains three short, contiguous α helices (Figures 1A and 1B). Helix H3 is also predicted to form a coiled-coil (Figure 1A), suggesting that MZT1 interacts with itself or other α-helical protein domains.

To locate MZT1 in the γ-TuRC, we examined the ~16 unasigned α helices in our previous cryo-EM reconstruction of the γ-TuRC lumenal bridge (domain ii in Figure S1A; Wieczorek et al., 2020). Located adjacent to an actin-like protein (domain i in Figure S1A), domain ii lacks unambiguous connecting densities between the ~16 short α-helical elements (~10–20 residues each), which has precluded their assignment in published γ-TuRC reconstructions (Liu et al., 2020; Wieczorek et al., 2020; Consolati et al., 2020).

To overcome these challenges, we collected additional cryo-EM datasets for γ-TuRCs isolated from HeLa S3 cells and combined the resulting particles with those from our previous work (Wieczorek et al., 2020). We performed particle subtraction and focused 3D refinement to reconstruct the density surrounding the γ-TuRC lumenal bridge and position 13 (see below), as described previously (Figures S1B and S2A–S2C; Table S1; see Method Details) (Wieczorek et al., 2020). The resulting density maps have similar overall resolutions to those in our previous work (lumenal bridge FSC0.143 = 3.6 Å; position 13 FSC0.143 = 4.5 Å; Figure S2A), but, importantly, they exhibit improved local resolution (Figures S2B and S2C), particularly in the previously difficult-to-resolve connecting loops between α helices in lumenal bridge domain ii, which allowed part of this density to be segmented into contiguous polypeptide “submaps” (submaps A and B; Figures 1C and 1H).

Submap A revealed an arrangement of three short α helices (Figure 1C) consistent with secondary structure predictions for MZT1 (Figure 1A); gratifyingly, a de novo MZT1 model could be built and refined into this density (Figure 1D; Data S1; Table S2). The fit between model side chains and the corresponding density further validated MZT1’s identity (Figure 1E; Data S1). The model accounts for ~95% of MZT1’s primary sequence and reveals a three α-helix N-shaped fold (Figure 1D; Data S1). These results provide a model for human MZT1 and localize this small protein to the γ-TuRC lumenal bridge.

MZT1 Intercalates with a GCP3 N-Terminal Five-α-Helix Bundle

MZT1 is surrounded by multiple unassigned α-helical densities also found within domain ii (submap B; Figures 1H and S1A). A strong candidate protein for submap B is the N-terminal portion of one of GCP2, GCP3, GCP5, or GCP6, which were not accounted for in recent γ-TuRC reconstructions (Liu et al., 2020; Wieczorek et al., 2020; Consolati et al., 2020). Secondary structure predictions suggest that these domains adopt a fold of typically five short (~10–15 residues) α helices in a motif we term the N-terminal α-helical domain (GCP-NHD; Figures 1F, 1G, and S3A). GCP3-NHD in particular has been shown to interact with MZT1 in cells and in vitro (Cota et al., 2017; Dhani et al., 2013; Janski et al., 2008, 2012; Leong et al., 2019; Lin et al., 2016; Nakamura et al., 2012; Tovey et al., 2018). Notably, submap B corresponds to a single polypeptide that contains five contiguous α helices and surrounds the MZT1 density (Figure 1H). Gratifyingly, a de novo model for GCP3-NHD could be built and refined into submap B (Figures 1I and 1J; Data S1; Table S2). Additionally, the C-terminal end of the GCP3-NHD model connects to a previously unassigned loop that snakes down the lower (N) domain.
of the GCP2 “stalk” at position 7 towards the bottom of GCP3 at position 6 (Figure S3F). This loop was therefore assigned to GCP3 residues 113–129 (Figures S3G and S3H; Data S1; Table S2) and suggests that the GCP3-NHD in the lumenal bridge could stem from GCP3 at γ-TuRC position 6.

The GCP3-NHD model reveals a W-shaped fold that intercalates with MZT1 in an unusual α-helical dimerization motif (Figure 1K). The eight-α-helical subcomplex formed by MZT1 and GCP3-NHD (hereafter MZT1/GCP3-NHD) resembles a flattened pyramid and comprises 8 out of ~16 α helices in lumenal bridge domain ii (Figures 1K and 2A; hereafter domain ii-A), which are positioned adjacent to GCP3 (position 8), GCP4 (position 9), and GCP5 (position 10) (Figure S1A; Wieczorek et al., 2020). Part of the MZT1/GCP3-NHD interface comprises hydrophobic residues L28, I31, L44, and V48 from helices H1 and H2 of MZT1 and L81, I93, L94, and L97 from helices H4 and H5 of GCP3-NHD (Figure 1L). Together, our data show that MZT1 and GCP3-NHD form a compact, α-helical subcomplex in the γ-TuRC lumenal bridge.

A Second MZT1 and the N Terminus of GCP6 Occupy the Remainder of the Lumenal Bridge

We next examined the ~8 remaining unassigned α helices in lumenal bridge domain ii (hereafter, domain ii-B; Figure 2A), which contact MZT1/GCP3-NHD on one end and the actin-like protein on the other (Figure S1A). Remarkably, our model for MZT1/GCP3-NHD could be rigid-body fitted into the domain ii-B density (Figure 2B), suggesting a second copy of MZT1/GCP3-NHD occupies the lumenal bridge. However, while the side chains of a second MZT1 model fit the domain ii-B density (Figure 2D; Data S1; Table S2), the GCP3-NHD model did not (data not shown).

Instead, we discovered that a de novo model for GCP6-NHD could be built and refined into domain ii-B of the γ-TuRC lumenal bridge (Figures 2C and 2E; Data S1; Table S2). GCP6-NHD also adopts a W-shaped fold that intercalates with the second N-shaped MZT1 to form a subcomplex similar in size, shape, and topology to MZT1/GCP3-NHD (hereafter, MZT1/GCP6-NHD; root-mean-square deviation [RMSD] = 0.7 Å; Figures S3B–S3E). Further, the C-terminal portion of GCP6-NHD connects to the previously identified γ-TuSC-associated α-helix that contacts several γ-TuSCs from positions ~3–6 of the γ-TuRC (Liu et al., 2020; Wieczorek et al., 2020; Consolati et al., 2020). Consistent with secondary structure predictions and our assignment of GCP6-NHD in domain ii-B of the lumenal bridge, a de novo molecular model for GCP6 residues 130–195 could be built and refined into the γ-TuSC-associated α-helix (hereafter, the GCP6 belt; Figures 2C and S3A; Data S1; Table S2). The GCP6 belt interacts with the γ-TuSCs via electrostatic interactions between GCP6 residues E157, E168, and E183 and clusters of basic residues at the lumenal side of the GCP2–GCP3 interfaces (Figures S3I–S3K). Supported by the assignment of the GCP6 belt, our results indicate that a second copy of MZT1 forms a subcomplex with GCP6-NHD that is similar in fold to MZT1/GCP3-NHD.

MZT1/GCP6-NHD interacts with MZT1/GCP3-NHD via GCP3-NHD helices H1 and H2 and GCP6-NHD helices H3 and H4 in a pseudo-C2 symmetry (Figure 2G). This interface contains a mixture of polar (e.g., GCP3-NHD residues Q33 and R37 and GCP6-NHD residues R67, D87, E90, and E94; Figure 2H) and non-polar contacts (e.g., GCP3-NHD F32 and V36 and GCP6-NHD L71 and F75; Figure 2I). The other end of MZT1/GCP6-NHD interacts with the actin-like protein at the barbed-end groove—a common binding site for actin-binding proteins (Pollard, 2016)—via GCP6-NHD helix H2 (Figure 2J). Though the identity of the actin-like protein is not yet established, GCP6-NHD contains at least two bulky hydrophobic residues (F46 and F50) proximal to hydrophobic and aromatic residues found in the barbed-end groove of the docked β-actin crystallographic model (e.g., Y143, I345, and L349; Figure 2K; Rould et al., 2006). Together, our results show that MZT1/GCP6-NHD is sandwiched between an actin-like protein and MZT1/GCP3-NHD to complete the γ-TuRC lumenal bridge (Figure 2L), and that MZT1 forms structurally mimetic subcomplexes with either GCP3-NHD or GCP6-NHD.

A MZT1/GCP6-NHD Subcomplex with a Native Fold Can Be Reconstituted In Vitro

To understand how the MZT1/GCP-NHD subcomplexes behave biochemically, we asked if one of them could be reconstituted. We generated bacterial expression constructs for full-length human MZT1 (recMZT1) and residues 1–119 of human GCP6 (recGCP6-NHD; Figure 3A; see Method Details). Initial purification tests indicated that recMZT1 and recGCP6-NHD are not soluble when expressed individually (data not shown). We therefore tested whether the co-overexpression of these two proteins could facilitate the formation of a stable recMZT1/recGCP6-NHD subcomplex (Figure 3B). Gratifyingly, this co-overexpression strategy yielded soluble recMZT1 and recGCP6-NHD that co-eluted as a single peak over a gel filtration column (Figures 3C and 3D). These data show that recMZT1 and recGCP6-NHD form a biochemically stable complex.

To further characterize purified recMZT1/recGCP6-NHD, we obtained crystals of the complex, which were used to generate a model by X-ray crystallography (3.3 Å; Figure 3E; Table S3; PDB: 6M33; see Method Details). Remarkably, the crystallographic model of recMZT1/recGCP6-NHD revealed a near-identical fold to MZT1/GCP6-NHD cryo-EM model (RMSD = 1.4 Å; Figure 3F). Both models indicate that the interface formed between MZT1 and GCP6-NHD totals ~2,200 Å² and comprises hydrophobic residues in the core of the subcomplex (e.g., V110, L111, and L114; Figures 2F, S3D, and S3E), which have been shown to be important for a GCP6-MZT1 interaction from a previous yeast-two-hybrid analysis (Cota et al., 2017). These results validate our assignment of MZT1/GCP6-NHD in the γ-TuRC lumenal bridge and show that MZT1 and GCP6-NHD can form a stable complex independent of the γ-TuRC.

A Third MZT/GCP-NHD-Like Subcomplex Is Found at the γ-TuRC “Overlap”

The modular nature of the MZT1/GCP-NHD subcomplexes suggested they could occupy other unassigned densities in the γ-TuRC. We focused on the small (~2 × 2 × 3 nm) unassigned density found at the γ-TuRC “overlap” (Figures 3G, 3H, and S1A; Wieczorek et al., 2020). This density is located above γ-tubulin at position 1 and adjacent to GCP3 at position 14.
Figure 2. A Second Copy of MZT1 Complexed with GCP6-NHD Is Found in the γ-TuRC Lumen
(A) Surface representation of domains ii-A and ii-B of the γ-TuRC lumenal bridge density.
(B) Models for MZT1 (green cartoon representation) and GCP3-NHD (pink cartoon representation) in domain ii-A and a rigid-body fit of a second copy of MZT1/ GCP3-NHD (light blue cartoon representation) docked into domain ii-B (transparent surface representation).
(C) De novo molecular model for MZT1 (green cartoon representation) in complex with GCP6-NHD (red cartoon representation). A model for the GCP6 belt is also shown.
(D and E) Examples of the fit of modeled side chains (stick representations) for MZT1 (D) and GCP6-NHD (E) into the corresponding density map (mesh representations).
(F) Zoom-in view highlighting hydrophobic residues (stick representation) that form part of the MZT1/GCP6-NHD interface.
(G) Cartoon representation of MZT1/GCP3-NHD interacting with MZT1/GCP6-NHD within the lumenal bridge. Triangles indicate the pseudo-C1 symmetry formed by the two subcomplexes.
(H and I) Zoom-in views highlighting (H) polar and (I) nonpolar residues that form part of the GCP3-NHD-GCP6-NHD interface.
(J) Cartoon representation of the actin-like protein/GCP6-NHD interface.

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Limited resolution precluded de novo model building and assignment of protein(s) to the overlap density; however, in a recent cryo-EM study of the X. laevis γ-TuRC, some secondary structure elements are resolved in this region (Figure 3I) (Liu et al., 2020). Remarkably, our model for MZT1/GCP3-NHD could be rigid-body fitted into the overlap density of the X. laevis γ-TuRC (Figure 3J; Liu et al., 2020). Helices H1 and H3 of the docked MZT1 model each appear to protrude ~10 Å past the observable density, suggesting the presence of another MZT1-like protein (e.g., MZT2; see below) or that the MZT1 α helices are dynamic. Nonetheless, our docking result shows that the overlap density corresponds to a third MZT1/GCP-NHD-like subcomplex.

MZT2 Adopts a MZT1-Like Fold and Interacts with GCP2 and a Dimeric Fragment of CDK5Rap2 on the Outer Face of the γ-TuRC

Lastly, we turned to the α-helical densities found at the outer face of the γ-TuRC at position 13 (GCP2) and adjacent to position 12 (GCP6; Figures 4A and 4B), previously termed the CC (coiled-coil) and HB (α-helical bundle) (Wieczorek et al., 2020). Remarkably, MZT1/GCP3-NHD could be rigid-body fitted into the HB density (Figure 4C). However, as with the overlap region (Figures 3G–3J), helices H1 and H3 of the docked MZT1 model extend ~10 Å past the experimental density (Figure 4C). Further, a ~15-Å-long α-helical density extends from the C-terminal region of the docked GCP3-NHD model perpendicular to the other

(Figures 3G and 3H). Limited resolution precluded de novo model building and assignment of protein(s) to the overlap density; however, in a recent cryo-EM study of the X. laevis γ-TuRC, some secondary structure elements are resolved in this region (Figure 3I) (Liu et al., 2020). Remarkably, our model for MZT1/GCP3-NHD could be rigid-body fitted into the overlap density of the X. laevis γ-TuRC (Figure 3J; Liu et al., 2020). Helices H1 and H3 of the docked MZT1 model each appear to protrude ~10 Å past the observable density, suggesting the presence of another MZT1-like protein (e.g., MZT2; see below) or that the MZT1 α helices are dynamic. Nonetheless, our docking result shows that the overlap density corresponds to a third MZT1/GCP-NHD-like subcomplex.

See also Table S3.
Figure 4. MZT2/GCP2-NHD Interact with the γ-TuRC Peptide on the Outer Face of the γ-TuRC

(A) Schematic of the γ-TuRC highlighting subunits from positions 12–14 and indicating the viewing angle in (B). Subunits are colored as in Wieczorek et al. (2020).

(B) Surface representation view of EMDB: EMD-21070 (Wieczorek et al., 2020) indicating the location of the CC and HB densities.

(C) Two views of the MZT1/GCP3-NHD model (cartoon representation) rigid-body docked into the HB. Mismatches between the MZT1/GCP3-NHD model and the HB density are indicated (text and arrow).

(D) Cartoon representations of MZT2/GCP2-NHD (dark green and purple cartoon representations) in the HB density (transparent surface).

(E) Cartoon representation of a de novo model for GCP2-NHD. The additional C-terminal α-helix that is not present in GCP3-NHD is indicated.

(F) Model for MZT2/GCP2-NHD (dark green and purple cartoon representations) in the HB density (transparent surface).

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HB α-helices (Figure 4C). This indicated that although the overall fold of the HB resembles the MZT1/GCP3-NHD subcomplex, likely neither MZT1 nor GCP3-NHD occupies the HB.

Instead, we considered MZT2, a small (~16 kDa) γ-TuRC protein that exists as two paralogs in human cells (MZT2A and MZT2B; ~96% identity), for the following reasons: (1) secondary structure predictions indicate that MZT2 likely adopts a contiguous 3–4 α-helical fold (Figures S4A and S4C; see Method Details); (2) after refinement into the HB density, a de novo Cα MZT2 model adopts a three-helix N-shaped fold (Figures 4D, 4F, and S4D; Table S4), suggesting that despite low (~20%) sequence identity between MZT1 and MZT2, there is structural conservation between these two proteins; and (3) MZT2 helices H1 and H3 are ~10 Å shorter than those in MZT1 (Figure 4D), consistent with secondary structure predictions and the shorter length of the HB α-helical densities (Figures 4C, S4A, and S4C). These results collectively argue that MZT2 occupies the MZT1-like part of the HB.

To address the remaining ~6 α-helical HB densities, we examined secondary structure predictions of the GCP-NHDs. In contrast with GCP3-NHD (5 α helices), GCP2-NHD is predicted to contain six contiguous α helices (Figures S3A, S4B, and S4E), suggesting that GCP2-NHD occupies the remaining portion of the HB density. Several lines of evidence support this hypothesis: (1) the HB density directly contacts the GCP2 stalk at position 13 (Wieczorek et al., 2020); (2) an exclusive interaction between MZT2 and GCP2 has been reported in a yeast-two-hybrid analysis (Lin et al., 2016); and (3) contacts between multiple residues in MZT2 helices H1 and H2 and in GCP2-NHD have recently been identified in a cross-linking mass spectrometry analysis of native human γ-TuRCs (Consolati et al., 2020). Motivated by this information, a Cα model for GCP2-NHD that is consistent with secondary structure predictions could be built and refined into the remaining portion of the HB density (Figures 4E and 4F; Table S4; see Method Details). Together, these observations are consistent with a model in which the HB region corresponds to a subcomplex of MZT2 and GCP2-NHD (MZT2/GCP2-NHD) that structurally mimics MZT1/GCP3-NHD.

MZT2/GCP2-NHD (the HB) also associates with an α-helical coiled-coil density (the CC; Figure 4B; Wieczorek et al., 2020). Notably, neither the HB nor CC densities at position 13 are observed in other reported γ-TuRC reconstructions (Consolati et al., 2020; Liu et al., 2020). This is likely due to our employment of the ~50-amino-acid fragment of CDK5Rap2 encompassing a conserved CM1 found in multiple γ-TuRC attachment factors—the so-called γ-TuNA (Choi et al., 2010)—to purify the γ-TuRC.

Secondary structure predictions indicate that ~60% of the γ-TuNA sequence forms an α-helical coiled-coil (Data S1). This suggested that a dimeric coiled-coil of γ-TuNA peptides occupies the CC density.

Taking advantage of the better-resolved side chain densities in the CC than the HB in our updated γ-TuRC density map (Figures S2A and S2C), a de novo atomic model for two copies of the γ-TuNA could be built into the CC (Figure 4G and 4H; Data S1; Table S4; see Method Details). We find that two γ-TuNA peptides form a ~30-residue-long dimeric, parallel, and left-handed coiled-coil (Figure 4G). As expected for an amphipathic coiled-coil, a string of hydrophobic residues line the γ-TuNA-γ-TuNA interface (Figure S4F). The coiled-coil is sandwiched between the MZT2/GCP2-NHD from the bottom and the GCP2 (position 13) C-domain from the top (Figures 4G and 4J). Residues L79 and F75 from γ-TuNA helix A and GCP2 C-domain residues L614, V617, L618, M668, and L762 populate the CC/GCP2 C-domain interface (Figure 4I). In turn, γ-TuNA helix B contacts GCP2-NHD helices H1 and H2 (Figures 4G and S4H). Together, our data show that the γ-TuNA forms a dimeric coiled-coil that associates with MZT2/GCP2-NHD, and this tripartite subcomplex docks onto the outer face of the γ-TuRC.

**DISCUSSION**

In this study, we combine cryo-EM, biochemical reconstitutions, and X-ray crystallography to determine the structure of MZT proteins in the native human γ-TuRC. Our data show that γ-TuRCs contain at least four distinct, structurally mimic subcomplexes, which we name MZT modules: (1) MZT1/GCP3-NHD and (2) MZT1/GCP6-NHD, both of which are found in the luminal bridge; (3) a MZT/GCP-NHD-like module at the overlap density; and (4) MZT2/GCP2-NHD, which is found at position 13 in complex with two copies of the γ-TuNA (Figure 4K).

The MZT modules are built from 3 α helices contributed by a MZT (MZT1 or MZT2) intercalated with 5–6 α helices from a GCP-NHD (GCP2-NHD, GCP3-NHD, or GCP6-NHD; Figure 4K). The interface formed between MZT1 and GCP3/6-NHDs is surprisingly large for such small protein domains (~2,200 Å² total; Figure S3C and S3E) and is mediated by similar hydrophobic interactions between hydrophobic residues in MZT1 (e.g., L28 and L31 in helix H1) and relatively conserved residues in GCP-NHDs (e.g., L93, L94, and L97 in GCP3-NHD helix H5 and V110, L111, and L114 in GCP6-NHD helix H5; Figures 1L and 2F). This suggests that MZT1 can bind to different GCP-NHDs via similar hydrophobic interactions (Cota et al., 2017), explaining the reported lack of MZT1’s specificity for different GCPs.
(Cota et al., 2017; Lin et al., 2016). Despite low (~20%) sequence similarity between MZT1 and MZT2, MZT2/GCP2-NHD forms a similar hydrophobic core in part between MZT2 and helix H5 of GCP2-NHD (Figure S4G). However, previous work suggested that MZT2 only interacts with GCP2 (Lin et al., 2016); this specificity may arise from contacts formed between MZT2 and GCP2-NHD helix H6, which is missing in the other GCP-NHDs (Figures 4E and 4F). It is also worth noting that our MZT2 model does not fit into the “staple” density on the outer face of each γ-TuSc (data not shown), which was recently assigned to MZT2 based on cross-linking mass spectrometry data (Consolati et al., 2020), suggesting the “staple” instead corresponds to an extension of the disordered GCP2 N terminus, as previously proposed (Wieczorek et al., 2020). The MZT modules interact with the γ-TuRC not via the MZTs, but rather through the solvent-exposed surfaces of the GCP-NHD helices H1–H4 (Figures 2H–2K, 3J, and S4H). We note that compared with the GCP N- and C-domains (>30% identity; Murphy et al., 2001; Wieczorek et al., 2020), the GCP-NHDs are less conserved (~10% identity), which may confer functional diversity to different MZT modules. As an example, the GCP6-NHD helix H2 is more structured than in GCP3-NHD, allowing it to point the bulky side chain of the GCP6-NHD-specific Phe50 toward the barbed-end groove of the actin-like protein (Figure 2K). Our results suggest that the solvent-exposed GCP-NHD residues will determine the specificity of a MZT module for its particular binding site within the γ-TuRC.

We also report that the CC density corresponds to the γ-TuNA, a fragment of CDK5RAP2, and one of several γ-TuRC attachment factors required for proper γ-TuRC localization in cells (Kollman et al., 2011). The γ-TuNA forms a dimeric coiled-coil that binds to MZT2/GCP2-NHD at the outer face of GCP2 on the asymmetric half of the γ-TuRC (position 13). Only one γ-TuNA dimer/MZT2/GCP2-NHD subcomplex is resolved at this position of the γ-TuRC, despite the presence of four more copies of GCP2 in the holocomplex (positions 1, 3, 5, and 7; Figures 4K and S1A), suggesting that MZT modules take advantage of the diverse interfaces provided by the asymmetric organization of γ-TuRC components (Wieczorek et al., 2020). The γ-TuNA contacts both the GCP2 C-domain (via γ-TuNA helix A; Figure 4I) and GCP2-NHD helices H1 and H2 in the MZT2/GCP2-NHD module (via γ-TuNA helix B; Figure S4H). γ-TuNA Phe75—a conserved hallmark of CM1 (Lin et al., 2014)—contributes to both of these interfaces (Figures 4I and S4H), potentially explaining why an F75A CDK5Rap2 mutant fails to rescue the loss of centrosomal γ-TuRC localization upon CDK5Rap2 depletion (Choi et al., 2010). Our models provide a template for studying how CM1-containing γ-TuRC attachment factors are recruited to the holocomplex at well-defined locations to establish diverse microtubule networks in cells.

Intriguingly, MZT1 is non-essential in D. melanogaster and is only expressed in testis (Tovey et al., 2018), and MZTs are absent in budding yeast (Kollman et al., 2011). Similarly, MZT2 is missing in plants, D. melanogaster, C. elegans, and fungi but is evolutionarily conserved in the deuterostome lineage of animals (Kollman et al., 2011; Teixidó-Travesa et al., 2010). In an accompanying study, Huang et al. (2020 [this issue of Cell Reports]) also report crystal structures for MZT modules and demonstrate that the MZT1 homolog in S. pombe is dispensable for targeting γ-TuRCs to spindle poles during mitosis but is still essential for cell viability. These observations have led to a model in which γ-TuRCs exist in multiple compositional states, even within the same cell type, allowing cells to tune the γ-TuRC from a microtubule nucleator to a microtubule anchor (Gao et al., 2019; Muroyama et al., 2016; Salee et al., 2018; Tovey et al., 2018). However, three recent, independent structures of vertebrate γ-TuRCs isolated using different affinity handles all display a well-defined luminal bridge (Consolati et al., 2020; Liu et al., 2020; Wieczorek et al., 2020). Our findings demonstrate that in addition to their γ-TuRC-targeting roles in cells (Tovey and Conduit, 2018), MZT proteins constitute a significant portion of the γ-TuRC luminal bridge, a prominent structural feature found in all native γ-TuRCs for which a near-atomic structure has been generated. In cells that lack the MZTs, similar modules may still form via evolutionary modifications of the GCP-NHDs or the presence of other, undiscovered MZT-like microproteins. Alternatively, the luminal bridge—whose precise role in the γ-TuRC is not yet clear—may be dispensable for some γ-TuRC functions (e.g., microtubule nucleation) but essential for others (e.g., microtubule anchoring). Our study lays a structural foundation for studying how MZTs regulate the function of γ-TuRCs with mixed compositions.

STAR METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2020.107791.

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# STAR★METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Anti-γ-tubulin, mouse monoclonal, clone GTU-88 | Millipore-Sigma | Cat# T6557 |
| Bacterial and Virus Strains |        |            |
| DH5α                | ThermoFisher | Cat# 18265017 |
| Rosetta             | Novagen | Cat# 71400 |
| BL21-CodonPlus (DE3)-RIL | Stratagene | Cat# 230245 |
| Biological Samples  |        |            |
| HeLa S3 cytoplasmic extract, gift from Robert Roeder | (Abmayr and Carrozza, 2001) | N/A |
| Purified native γ-tubulin ring complex from HeLa S3 cells | This study | N/A |
| Chemicals, Peptides, and Recombinant Proteins |        |            |
| Recombinant GFP nanobody | This study | N/A |
| Recombinant GFP-γ-TuNA | This study | N/A |
| Guanosine 5′-triphosphate, Disodium salt trihydrate | Jena Bioscience | Cat# NU-1012 |
| Complete EDTA-free Protease Inhibitor Cocktail | Roche | Cat# 11873580001 |
| IPTG                | Gold Biotechnology | Cat# I2481C |
| Dithiothreitol (DTT) | Gold Biotechnology | Cat# DTT25 |
| L-Glutathione reduced | Gold Biotechnology | Cat# GL55500 |
| PreScission protease | In-house | N/A |
| TEV protease        | In-house | N/A |
| Recombinant recMZT1/recGCP6-NHD | This study | N/A |
| Critical Commercial Assays |        |            |
| Hampton crystal screen: SaltRx1 | Hampton Research | Cat#HR2-107 |
| Glutathione Agarose | GE Healthcare Life Sciences | Cat#17075604 |
| CloneAmp HiFi PCR Premix | Takara Bio. | Cat#639298 |
| In-Fusion HD Cloning Plus | Takara Bio. | Cat#638920 |
| Deposited Data      |        |            |
| Focused refinement γ-TuRC density map corresponding to the lumenal bridge | This study | EMD-21984 |
| Focused refinement γ-TuRC density map surrounding positions 12 and 13 | This study | EMD-21985 |
| Structural models of the γ-TuRC lumenal bridge domain ii proteins: MZT1/GCP3-NHD and MZT1/GCP6-NHD (including GCP6 “belt”) | This study | PDB ID 6X0U |
| Structural models of MZT2/GCP2-NHD and the γ-TuNA coiled-coil at position 13 of the γ-TuRC | This study | PDB ID 6X0V |
| Structural model recMZT1/recGCP6-NHD determined by X-ray crystallography | This study | PDB ID 6M33 |
| Oligonucleotides    |        |            |
| Primers for hstGCP6(1-119) F GGGGCCCCCTGGGATCATGGCTAGTATAACCCAGC | This study | N/A |
| Primers for hstGCP6(1-119) R GGGCCGCTCGAGTCTGACTTACAACGGCCAGCTGAAC | This study | N/A |
| Primers for hstMZT1 F TCCAGGGGCCCATATGGCGAGTAGCACCG | This study | N/A |

(Continued on next page)
## RESOURCE AVAILABILITY

### Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Tarun M. Kapoor (kapoor@rockefeller.edu).

### Materials availability
All unique/stable reagents generated in this study are available from the Lead Contact without restriction.

### Data and code availability
The accession numbers for the cryo-EM maps [https://www.ebi.ac.uk/pdbe/emdb/](https://www.ebi.ac.uk/pdbe/emdb/) reported in this paper are: EMDB: EMD-21984, EMD-21985. The accession numbers for the structural models [https://www.rcsb.org/](https://www.rcsb.org/) reported in this paper are: PDB: 6X0U, 6X0V, and 6M33.

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Primers for hsMZT1 R GGTGGTGGTGCTCGAGTCAG CTTGTGATATTTTCAG | This study | N/A |
| Bacterial expression vector for anti-GFP nanobody clone LG-16, gift from Michael Rout | Fridy et al., 2014 | N/A |
| pET His6 Sumo TEV LIC cloning vector (1S), gift from Scott Gardia | Addgene (Unpublished) | pET His6 Sumo TEV LIC cloning vector (1S) Addgene plasmid # 29659; [http://addgene.org/29659; RRID:Addgene_29659](http://addgene.org/29659; RRID:Addgene_29659) |
| pRcCMV Cep215, gift from Erich Nigg | Graser S, Stierhof YD, Nigg EA J Cell Sci. 2007 Dec 15:120(Pt 24):4321-31. Epub 2007 Nov 27. | pRcCMV Cep215 (Nigg CW493) Addgene plasmid # 41152; [http://addgene.org/41152; RRID:Addgene_41152](http://addgene.org/41152; RRID:Addgene_41152) |
| pMW96 – pET expression vector for His6-SUMO-TEV-GFP-PreScission-γ-TuNA | Wieczorek et al., 2020 | N/A |
| Expression plasmid for hsGCP5-NTD | This study | N/A |
| Expression plasmid for hsMzt1 | This study | N/A |
| RELION | Zivanov et al., 2018 | [https://www3.mrc-lmb.cam.ac.uk/reliion/index.php?title=Main_Page](https://www3.mrc-lmb.cam.ac.uk/reliion/index.php?title=Main_Page) |
| EMAN2 | Tang et al., 2007 | [https://blake.bcm.edu/emanwiki/EMAN2](https://blake.bcm.edu/emanwiki/EMAN2) |
| Coot | Emsley et al., 2010 | [https://www2.mrc-lmb.cam.ac.uk/personal/pemsley/coot/](https://www2.mrc-lmb.cam.ac.uk/personal/pemsley/coot/) |
| UCSF Chimera | Pettersen et al., 2004 | [https://www.cgl.ucsf.edu/chimera/](https://www.cgl.ucsf.edu/chimera/) |
| Phenix | Williams et al., 2018 | [https://www.phenix-online.org](https://www.phenix-online.org) |
| PyMol | The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC. | [https://pymol.org/2/](https://pymol.org/2/) |
| Serial EM | Mastronarde, 2005 | [https://bio3d.colorado.edu/SerialEM/](https://bio3d.colorado.edu/SerialEM/) |
| MotionCor2 | Zheng et al., 2017 | [https://emcore.ucsf.edu/ucsf-motioncor2](https://emcore.ucsf.edu/ucsf-motioncor2) |
| CTFFIND4 | Rohou and Grigorieff, 2015 | [https://grigoriefflab.umassmed.edu/ctffind4](https://grigoriefflab.umassmed.edu/ctffind4) |
| HKL2000 | HKL Research, Inc. | [https://www.hkl-xray.com/](https://www.hkl-xray.com/) |
| Hitrap NHS-Activated HP | GE Healthcare | Cat# 17071601 |
| Superdex 75 10/300 GL | GE Healthcare Life Sciences | Cat# GE17517401 |
| HiLoad 16/60 Superdex 75 prep grade | GE Healthcare Life Sciences | Cat# GE17106801 |
| Hitrap Q HP | GE Healthcare Life Sciences | Cat# GE29051325 |

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**Continued**
EXPERIMENTAL MODEL AND SUBJECT DETAILS

HeLa S3 cell cytoplasmic extracts were prepared according to Abmayr and Carrozza (2001) and were a generous gift from Dr. Robert Roeder. Bacterial protein expression constructs were expressed in Escherichia coli BL21(DE3) pRIL or Rosetta cells cultured in LB medium supplemented with appropriate antibiotics at 37°C and at 230 rpm until induction, at which point the temperature was shifted to 16-18°C.

METHOD DETAILS

**Purification of native human γ-TuRCs**

GFP-γ-TuNA and GFP nanobodies constructs were expressed and purified from Escherichia coli BL21(DE3) pRIL cells, as described previously (Wieczorek et al., 2020). Native γ-TuRCs were isolated from HeLa S3 cell cytoplasmic extracts, a kind gift from Dr. Robert Roeder, based on the reported affinity of human γ-TuRCs for CDK5RAP2 (Choi et al., 2010), as described previously (Wieczorek et al., 2020).

**Cryo-electron microscopy of native γ-TuRCs**

2 μL of thawed γ-TuRCs was applied to plasma treated Quantifoil R2/2 300-square-mesh copper grids coated with a continuous carbon film. After 5-10 min the sample was blotted away and replaced with another 2 μL. This was repeated for a total of 5-8 applications. As γ-TuRCase storage buffer contained ~30% (w/v) sucrose, 20 μL of washing buffer (40 mM HEPES-KOH pH 7.5, 150 mM KCl, 1 mM MgCl₂, 0.01% Tween-20 (v/v), 0.1 mM GTP, and 1 mM 2-mercaptoethanol) was used to rinse the grid twice with 1-2 min incubation between each rinse. Finally, 3 μL of washing buffer was applied to the grid. The grid was transferred to a Vitrobot IV (FEI), blotted for 4-5 s at 100% humidity and 4°C, and then plunged into liquid ethane.

Micrographs were recorded on an FEI Titan Krios equipped with either a Gatan K2 Summit (Datasets 1 to 4) or Gatan K3 (Dataset 5; Table S1) detectors using Serial EM automated data collection (Mastronarde, 2005). Images were corrected for beam-induced drift using MotionCor2 (Zheng et al., 2017). CTF parameters for drift corrected micrographs were estimated using CTFFIND4 (Rohou and Grigorieff, 2015). Subsequent processing employed Relion v.3.0 (Zivanov et al., 2018), using a similar processing pipeline described previously in order to clean autopicked particles in Datasets 4 and 5 (Table S1; Wieczorek et al., 2020). The resulting “good” particles from Datasets 4 and 5 were combined with equivalent particles from Datasets 1 to 3 collected in our previous study (Wieczorek et al., 2020).

The combined particles were re-extracted using a pixel size of 1.036 Å (instead of 1.335 Å used in our previous study). Due to computational resource restraints, the box size was kept at 368 pixels, which resulted in some globular density at position 14 being close to the edge of both the box and circular masks. However, as this region could not be well-resolved due to high flexibility anywhere (data not shown), this was not an issue for reconstructing the luminal bridge or position 13 of the γ-TuRC in this study.

The combined, re-extracted particles were subjected to 3D refinement followed by 3D classification, resulting in 744,583 “final” particles selected for the final overall γ-TuRC refinement. Subsequently, particle subtraction followed by rounds of 3D focused classification and refinement were used to generate improved density maps for the luminal bridge and the area surrounding position 13 (Figures S1B and S2), as described previously (Wieczorek et al., 2020).

**Cryo-EM model building**

The overall quality of side chain models generated in this study is summarized in Data S1. Jpred was used for secondary structure predictions (Drozdzetski et al., 2015). LOMETS was used to generate de novo homology models of individual MZT1 and GCP-NHD α helices (Wu and Zhang, 2007). SWISS-MODEL was used to generate template-based homology models for GCP6-NHD (GCP3-NHD as template), MZT2 (MZT1 as template) and GCP2-NHD (GCP3-NHD as template) (Waterhouse et al., 2018). Because of limited side chain information, we could not distinguish between MZT2A and MZT2B paralogs and chose to use MZT2A for simplicity. However, we note that within our modeled region of MZT2A, there is only one conservative amino acid change (G50A; found in the loop between helices H2 and H3) when the primary sequence is aligned to MZT2B (Teixido-Travesa et al., 2010), suggesting that the core MZT-like fold adopted by both proteins is likely identical. An initial model for the γ-TuNA coiled-coil model was generated using CCBUILDER 2.0 (Wood and Woolfson, 2018). All other de novo-built structural elements, as well as real-space refinements, were generated in Coot (Emsley et al., 2010) and refined using PHENIX (Adams et al., 2010; Williams et al., 2018).

**Purification and X-ray crystallography of recMZT1/recGCP6-NHD**

Codon optimized GCP6 residues 1-119 (NM_020461) were amplified and inserted into the pGEX-6p1 expression vector (GE Healthcare) to produce an N-terminal His-tagged protein. Both bacterial expression constructs were co-transformed into Escherichia coli Rosetta strain (Novagen). Protein production was induced by 0.5 mM IPTG at 18°C overnight. The cell pellet was resuspended in a buffer containing 20 mM HEPES (pH 7.4), 150 mM NaCl, and 3 mM DTT, disrupted by French press and clarified by centrifugation at 15,000 g at 4°C for 20 min. Clarified lysate was loaded onto a GST column (GE Healthcare), and GST bound proteins were digested by PreScission protease overnight at 4°C.
PreScission-digested samples were eluted and further purified by HiTrap Q (GE Healthcare). Protein was eluted by a NaCl gradient and then subjected to size exclusion chromatography (HiLoad 16/60 Superdex 75 prep grade Superdex 75 16/60 for crystal production, or Superdex 75 10/300 GL for analytical gel filtration as in Figure 3). The peak fractions were analyzed by SDS-PAGE. Purified protein fractions were collected and concentrated using Amicon Ultra 15 (Millipore) for crystallization.

recMZT1/recGCP6-NHD crystals were grown in a 13 mg/ml protein concentration at 20°C in hanging drops containing 1 µL of the protein and 1 µL of a reservoir solution consisting of 6M ammonium nitrate and 0.1M Tris pH 8.5. Proteins appeared as triclinic crystals (space group P 3221) that contain one molecule per asymmetric unit and diffract to ~3 Å. Data were collected at beamline 13B, 13C and 05A in the National Synchrotron Radiation Research Centre (Taiwan). X-ray intensities were processed using HKL2000 (Otwinowski and Minor, 1997). Phase determination was carried out using data collected from seleno-L-methionine-labeled crystals at selenium peak and inflection wavelengths using PHENIX (Adams et al., 2010). The initial model was built into the electron density map and refined using PHENIX and Coot (Adams et al., 2010; Emsley et al., 2010). The final structure was refined to a resolution of 3.3 Å with an R-factor of 22.2% (Rfree 29.8%), and there were no outliers in the Ramachandran plot. Coordinates and structure factors have been deposited in the Protein Data Bank (Table S3).

Visualization and analysis
Figures were generated in PyMol (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.). Molecular graphics and analyses performed with UCSF Chimera, developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, and by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, with support from NIH P41-GM103311 (Pettersen et al., 2004). Electrostatic potential coloring was performed in PyMol using the “vacuum electrostatics” function in PyMol. Hydrophobic coloring in Figure S4G was achieved using the “color_h.py” script in PyMol (https://www.expasy.org/tools/pscale/Hphob.Eisenberg.html) (Eisenberg et al., 1984). Structural alignments were performed using the “align” command in PyMol using the GCP-NHDs as the alignment reference. Protein-protein contact maps were generated using the COCOMAPS server (Vangone et al., 2011).

QUANTIFICATION AND STATISTICAL ANALYSIS
Resolution estimations of cryo-EM density maps are based on the 0.143 Fourier Shell Correlation (FSC) criterion (Rosenthal and Henderson, 2003). All statistical validation performed on the deposited models (PDB ID 6X0U, 6X0V, and 6M33) was done using the Phenix package (Tables S2–S4) (Williams et al., 2018).
Supplemental Information

MZT Proteins Form Multi-Faceted Structural Modules in the γ-Tubulin Ring Complex

Michal Wieczorek, Tzu-Lun Huang, Linas Urnavicius, Kuo-Chiang Hsia, and Tarun M. Kapoor
Figure S1. Cryo-EM data processing workflow for reconstruction of γ-TuRC density submaps, related to Figures 1, 2, and 4. A) Two views of the overall γ-TuRC density map (surface representation; EMDB: EMD-21074 (Wieczorek et al., 2020)). Unassigned structural features of the γ-TuRC are indicated. Schematic of the γ-TuRC and lumenal bridge domains i and ii are shown on the left. B) Focused 3D classification and refinement procedures used to improve the resolution in the overall γ-TuRC density map.
Figure S2. Cryo-EM reconstruction of the native human γ-TuRC lumenal bridge and region surrounding position 13, related to Figures 1, 2, and 4. A) Gold-standard Fourier shell correlation (FSC) curves of the “lumenal bridge” (blue) and “position 13” (red) γ-TuRC density submaps. The FSC at 0.143 is indicated. B) The “lumenal bridge” γ-TuRC density map analyzed by ResMap (Kucukelbir et al., 2014), showing a resolution distribution ranging from 2 to 8 Å. C) The “position 13” γ-TuRC density map analyzed by ResMap (Kucukelbir et al., 2014), showing a resolution distribution ranging from 3.5 to 7.5 Å.
Figure S3. Details of MZT1/GCP3-NHD and MZT1/GCP6-NHD models and interactions with the γ-TuRC, related to Figures 1 and 3. A) Multiple sequence alignment of human GCP2, GCP3, GCP5 and GCP6. Sequences predicted to form α-helices (red) or β-strands (green) according to the JNETPRED output of Jpred are indicated. The α-helical sequence corresponding to the GCP6 “belt” is labeled. B) Contact map between MZT1 and GCP3-NHD residues for the MZT1/GCP3-NHD subcomplex. Plots in B) and D) were generated using the COCOMAPS server (Vangone et al., 2011). C) Two views in surface representation of GCP3-NHD-proximal MZT1 residues colored in green (top) and the MZT1-proximal GCP3-NHD residues colored in pink (bottom). D) Contact map between MZT1 and GCP6-NHD residues for the MZT1/GCP6-NHD subcomplex. E) Two views in surface representation of GCP6-NHD-proximal MZT1 residues colored in green (top) and the MZT1-proximal GCP6-NHD residues colored in red (bottom). F) Details of the loop extending from MZT1/GCP3-NHD and contacting the rest of the γ-TuRC. View in F) is parallel to the MZT1/GCP3-NHD - GCP2 (pos. 7) & GCP3 (pos. 6) interface highlighting the connecting density observed to stem from the C-terminal portion of GCP3-NHD (transparent surface). Cartoon representations of MZT1/GCP3-NHD, GCP2 (pos. 7) and GCP3 (pos. 6) are shown for reference. G) Rotated view of the interface in F) showing the position of a GCP3-NHD C-terminal loop (stick representation in segmented density (transparent surface)) between GCP2 (pos. 7) and GCP3 (pos. 6) (surface representations). H) Zoomed-in view of the loop in G). I) Details of the GCP6 “belt” and its interaction with the γ-TuRC. I) shows a surface representation of the GCP6 “belt” (red) spanning the luminal face of GCP2/3 from positions 3-6. J) Zoomed-in view of the luminal face of GCP2/3 from positions 3-6, colored according to electrostatic potential. Prominent patches of basic residues are indicated. K) Rotated, zoomed-in view of the GCP6 “belt” colored according to electrostatic potential. Locations of repeating acidic residues are indicated.
Figure S4. Details of the MZT2/GCP2-NHD module and its interaction with the γ-TuNA, related to Figure 4. A)-B) Secondary structure prediction analysis of A) human MZT2A (“MZT2”) and B) GCP2-NHD. Shown are the primary sequence (top), the JNETHMM prediction (middle; (Drozdetskiy et al., 2015)), and the confidence score for the prediction (bottom). α-helical elements (labeled) are shown as colored bars. β-sheet elements are...
shown as colored arrows. Dotted outlines indicate secondary structure elements with low-scoring confidence levels. Asterisk in A) indicates that only residues 1-100 of MZT2A are displayed, as the remaining 58 residues are predicted to be unstructured. C) Schematic of the predicted α-helical organization of MZT2 (green rectangles; not to scale). D) Two views of a MZT2 homology model (dark green cartoon representation) using our MZT1 model as a template. E) Schematic of the predicted α-helical organization of GCP2-NHD (purple rectangles; not to scale). An additional C-terminal α-helix not present in GCP3-NHD is indicated. F) Zoomed-in views of the γ-TuNA-γ-TuNA coiled-coil interface, highlighting hydrophobic contacts between copies A and B (stick representations). G) Left: Cartoon representation of the MZT2/GCP2-NHD model. Helix H5 of GCP2-NHD and helices H1-H3 of MZT2 are indicated. Right: The same model with labeled helices colored according to “hydrophobicity” (see Methods). Helix H6 of GCP2-NHD is omitted for clarity. H) Left: Cartoon representation of the γ-TuNA coiled-coil and MZT2/GCP2-NHD models. Right: Zoomed-in view of the γ-TuNA (helix B, cartoon and stick representation) - GCP2-NHD interface (helices H1 and H2, cartoon representation only as the model does not include side chains). Aromatic residues on γ-TuNA are labeled. Sequences corresponding to GCP2-NHD helices H1 and H2 are shown, with aromatic residues highlighted in red. Note also the high percentage of hydrophobic residues in GCP2-NHD helices H1 and H2.
Table S1. Cryo-EM data collection statistics, related to Figures 1, 2, and 4.

|                      | Dataset 1 | Dataset 2 | Dataset 3 | Dataset 4 | Dataset 5 | Total       |
|----------------------|-----------|-----------|-----------|-----------|-----------|-------------|
| Voltage (kV)         | 300       | 300       | 300       | 300       | 300       | 300         |
| Pixel size (Å/pixel) | 1.335     | 1.036     | 1.036     | 1.036     | 0.86      | 1.036       |
| Exposure time (s)    | 9.9       | 4.2       | 4.2       | 4.2       | 1.2       | 4.2         |
| Total dose (e⁻/Å²)   | 45        | 45        | 45        | 45        | 45        | 45          |
| Number of frames     | 33        | 21        | 21        | 21        | 24        | 21          |
| Micrographs          | 4,783     | 42,408    | 22,658    | 28,206    | 8,596     | 106,651     |
| Autopicked particles | 836,778   | 5,182,081 | 2,967,555 | 3,521,079 | 1,535,783 | 14,043,276 |
| Good particles       | 99,526    | 613,631   | 328,217   | 216,739   | 58,065    | 1,361,178   |
| Final particles      | 2,201     | 333,862   | 202,411   | 164,032   | 42,077    | 744,583     |

Table S2. Model refinement statistics for lumenal bridge domain ii, related to Figures 1-2.

| Model composition |                      | Lumenal bridge domain ii |
|-------------------|----------------------|--------------------------|
|                   |                      | (includes MZT1/GCP3-NHD, MZT1/GCP6-NHD, and GCP6 “belt”) |
|                   |                      |                          |
| Model composition |                      |                          |
| Chains            | 4                    |                          |
| Non-hydrogen atoms| 3091                 |                          |
| Protein residues  | 398                  |                          |
| Ligands (GDP/Mg²⁺)| 0                    |                          |
| Refinement statistics |                 |                          |
| Map               | EMD-21984            |                          |
| Resolution (Å)    | 3.6                  |                          |
| Map sharpening B-factor (Å²) | -84                  |                          |
| d FSC model (0.143) | 3.2                  |                          |
| CC (mask)         | 0.82                 |                          |
| Rms deviations    |                      |                          |
| Bond lengths (Å)  | 0.004                |                          |
| Bond angles (°)   | 0.601                |                          |
| Validation        |                      |                          |
| MolProbity score  | 1.53                 |                          |
| Clashscore, all atoms | 5.26              |                          |
| Rotamer outliers (%) | 0.00              |                          |
| Ramachandran plot |                      |                          |
| Favored           | 96.32                |                          |
| Allowed           | 3.42                 |                          |
| Outliers          | 0.26                 |                          |
| CaBLAM outliers (%) | 3.31               |                          |
| C₈ outliers (%)   | 0.00                 |                          |
Table S3. X-ray data collection and refinement statistics, related to Figure 3.

| Crystals         | recMZT1/recGCP6-NHD (Native) | recMZT1/recGCP6-NHD (Se-Met) |
|------------------|------------------------------|------------------------------|
| Space group      | P 3\_2\_1                    | P 3\_1\_21                   |
| Cell dimensions  |                              |                              |
| a, b, c (Å)      | a = 69.6                     | a = 68.7                     |
|                  | b = 69.6                     | b = 68.7                     |
|                  | c = 119.2                    | c = 116.6                    |
| α, β, γ (Å)      | α = β = 90°                  | α = β = 90°                  |
|                  | γ = 120°                     | γ = 120°                     |
| Data collection  |                              |                              |
| Wavelength (Å)   | 0.99984                      | 0.97915                      |
| Resolution (Å)   | 20.0-3.3                     | 20.0-3.6                     |
| Total reflections| 16021                        | 28703                        |
| Unique reflections| 5211                        | 3894                         |
| R\text{merge} (%)| 5.5 (62.1)                   | 9.0 (77.1)                   |
| CC1/2 (%)        | 99.7 (69.5)                  | 99.8 (84.0)                  |
| <\langle/σ\rangle> (%)| 17.1 (2.0)      | 10.5 (1.8)                   |
| Completedness (%)| 98.2 (98.2)                 | 99.4 (93.8)                  |
| Redundancy       | 3.1                          | 7.4                          |
| Refinement       |                              |                              |
| R\text{work} / R\text{free} (%) | 22.2/29.8                |                              |
| Bond length rmsd (Å) | 0.010                      |                              |
| Bond angle rmsd (°)     | 1.34                        |                              |
| Ramachandran plot (%) | 93.92                      |                              |
| Favored region   | 93.92                       |                              |
| Outlier region   | 0.0                         |                              |
| PDB code         | 6M33                         |                              |

\text{a} Highest resolution shell is shown in parentheses
\text{b} Validated by MolProbity (Chen et al. 2010)
Table S4. Model refinement statistics for CC & HB densities, related to Figure 4.

| Model composition |  |
|-------------------|---|
| Chains            | 4 |
| Non-hydrogen atoms| 1384 |
| Protein residues  | 211 |
| Ligands (GDP/Mg²⁺)| 0 |

| Refinement statistics |  |
|-----------------------|---|
| Map                   | EMD-21985 |
| Resolution (Å)        | 4.5 |
| Map sharpening B-factor (Å²) | -200 |
| d FSC model (0.143)   | 4.1 |
| CC (mask)             | 0.75 |

| Rms deviations |  |
|----------------|---|
| Bond lengths (Å) | 0.002 |
| Bond angles (°)  | 0.341 |

| Validation |  |
|------------|---|
| MolProbity score | 1.91 |
| Clashscore, all atoms | 15.16 |
| Rotamer outliers (%) | 0.00 |

| Ramachandran plot |  |
|-------------------|---|
| Favored           | 96.52 |
| Allowed           | 2.99 |
| Outliers          | 0.50 |
| CaBLAM outliers (%) | 0.52 |
| Cβ outliers (%)   | 0.00 |
Data S1. Cryo-EM model building, assignment and quality assessment summary, related to Figures 1, 2, and 4.

**MZT1** in luminal bridge domain iiA

**Prediction:** MASSGAGAAAAANLNAVRETMDVLLISRIILNTQGLMETLSCVRLCEQGINPEAL

**Model:** MASSGAGAAAANLNAVRETMDVLLISRIILNTQGLMETLSCVRLCEQGINPEAL

SSVIKLKATEALKAENMTS

SSVIKLKATEALKAENMTS

Example density (see also Figure 1E)

Cross-correlation (masked) = **0.83**
GCP3-NHD in luminal bridge domain ii-A

Prediction: MATPDQKSPNVLLQNLCCRLGRSEADVQPFYAVRVIGSNFAPTVRDEFILVAEKIKK
Model: ELIRQRRREDAALFSELHRKLHSQGVLKKNWSILYLSSLSEDPRQPSKVSSYATLFAQ
ELIRQRRREDAALFSELHRKLHSQGVLKKNWSILYLSSLSEDPRQPSKVSSYATLFAQ
ALPRAHSTPPYAPPQTLPSYQDRSAOQSAGSAGVGSSGIISSLGLCALSGBPAPQSL
ALPRAHSTPPYAPPQTLPSYQDRSAOQSAGSAGVGSSGIISSLGLCALSGBPAPQSL

Example density
(see also Figure 1J)

Cross-correlation (masked) = 0.82
**MZT1** in lumenal bridge domain ii-B

Prediction: MASSGAGAAAAANLANVRETMDVLEISRIINTGLDMETLSICVRLCEQGINPEAL

Model: MASSGAGAAAAANLNAVRETMDVLEISRIINTGLDMETLSICVRLCEQGINPEAL

SSVKELKATEALKAENMTS

SSVKELKATEALKAENMTS

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Example density (see also Figure 2D)

Cross-correlation (masked) = 0.75
**GCP6-NHD** in luminal bridge domain ii-B (including GCP6 “belt”)

Prediction: MASTQLDDELCAELLPAAKTSLIQRSVNRKRAKSLKKVAYNLFTNLFDQETQQLQDP
Model: MASTQLDDELCAELLPAAKTSLIQRSVNRKRAKSLKKVAYNLFTNLFDQETQQLQDP

MSKLPAKILMMSTFDLVRGGLSFAQKLRLEELVEEELAAAPCPLLLEVQSVLDLLVQLAGS
MSKLPAKILMMSTFDLVRGGLSFAQKLRLEELVEEELAAAPCPLLLEVQSVLDLLVQLAGS
GPPQVLPRKRDYFLNKHAGVRNVPYSYGDCDDLSSVFEMDVQSLISRRECLCHSMIQETLQ
GPPQVLPRKRDYFLNKHAGVRNVPYSYGDCDDLSSVFEMDVQSLISRRECLCHSMIQETLQ
VMEAAPGTGLPTVGL
VMEAAPGTGLPTVGL

**Example density**
(see also Figure 2E)

**Cross-correlation (masked) = 0.77**
\( \gamma\text{-TuNA} \) coiled-coil in CC density

Predicted coiled-coil

\begin{align*}
\text{Prediction: } & \text{TVSPTRNMDKENQITELKKENFLKLRKYFLEERMQSFHGPTEHIY} \\
\text{Model (A): } & \text{TVSPTRNMDKENQITELKKENFLKLRKYFLEERMQSFHGPTEHIY} \\
\text{Model (B): } & \text{TVSPTRNMDKENQITELKKENFLKLRKYFLEERMQSFHGPTEHIY}
\end{align*}

Example density (see also Figure 4H)

Cross-correlation (masked) = 0.80