Functional replacement of fission yeast γ-tubulin small complex proteins Alp4 and Alp6 by human GCP2 and GCP3

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Summary
Microtubule-organizing centers such as the γ-tubulin ring complex (γ-TuRC) act as a template for polarized growth and regulation of microtubules that are essential for diverse cellular structures and processes in eukaryotes. New structural models of the budding yeast γ-tubulin small complex (γ-TuSC) of the γ-TuRC combined with functional studies done in multiple eukaryotes are revealing the first mechanistic clues into control of microtubule nucleation and organization. Cross-species studies of human and budding yeast γ-TuSC proteins in fission yeast revealed conserved and divergent structural and functional features of the γ-TuSC. We show genetically that GCP3/Spc98 function is fully conserved with Alp6 across species but that functional differences exist between GCP2/Spc97 and Alp4. By further analysis of human γ-TuSC proteins, we found that GCP3 assembles normally into the >2000 kDa fission yeast γ-TuRC and that the GCP3 gene replaces fission yeast alp6. Interestingly, human GCP2 replaces the essential alp4 gene but is unable to rescue a normally recessive G1 defect of the alp4-1891 allele that results in loss of γ-TuRC from poles in subsequent cell cycles. Biochemically, GCP2 incorporation into fission yeast γ-TuRC is limited in the presence of Alp4; instead, the bulk of GCP2 fractionates as smaller complexes. By generating a functional Alp4-GCP2 chimeric protein we determined that the GCP2 N-terminal domain limits its ability to fully displace or compete with Alp4 during γ-TuRC assembly. Our findings have broad importance for understanding the essential domains of γ-TuSC proteins in the γ-TuRC mechanism.

Key words: MTOC, Spc97, Spc98, γ-TuSC, γ-TuRC

Introduction
The microtubule-organizing center (MTOC; Pickett-Heaps, 1969) in eukaryotes provides the nucleating machinery for regulating microtubule attachment and dynamics and establishes microtubule polarity (Heidemann and McIntosh, 1980). It is a major component of the centrosome in animal cells that provides spatiotemporal control for establishment of specialized microtubule networks necessary for a range of essential functions. These include intracellular microtubule organization for vesicle trafficking, cell polarity, cell crawling and chromosome segregation. Specialized structures such as cilia and flagella also depend on MTOC function of basal bodies. An understanding of the structure and mechanism of the MTOC is therefore crucial to such fundamental cell processes as genomic stability, neural development and function, formation of the immunological synapse in cytotoxic T cell lymphocytes and cell migration.

The mechanism of microtubule nucleation is proposed to occur via a γ-tubulin ring complex (γ-TuRC; Moritz et al., 2000). This complex in humans contains at least nine conserved proteins (Choi et al., 2010) including components of the γ-tubulin small complex (γ-TuSC). The γ-TuSC is the immediate template for growing microtubule ends and is composed of multimers of three conserved proteins (Wiese and Zheng, 2006) that in human are γ-tubulin, GCP2 and GCP3 (Oakley and Oakley, 1989; Murphy et al., 1998). Crystal structures and structural models of several MTOC components are now available from multiple eukaryotes that help to provide structural information for deciphering underlying MTOC mechanisms. These include human γ-tubulin (Aldaz et al., 2005; Paluh et al., 2000), the budding yeast γ-tubulin small complex (Kollman et al., 2008) and the human γ-TuRC protein GCP4 (Guillet et al., 2011). Of these proteins, only γ-tubulin, first identified in Aspergillus nidulans, has been demonstrated to function across species in most eukaryotes (Steams et al., 1991; Horio and Oakley, 1994). The ability of other components of the γ-TuSC or γ-TuRC to complement function across species is untested; however, conserved or distinct mechanisms and functional domains are expected. Such mixed component analysis has been informative for understanding how the structure of γ-tubulin relates to its functions impacting the microtubule cytoskeleton (Steams et al., 1991; Horio and Oakley, 1994; Hendrickson et al., 2001; Burns, 1995).

To understand the essential functional domains of γ-TuSC proteins in the γ-TuRC mechanism we used cross-species
analysis to identify inter-specific differences. We expressed human GCP2/GCP3 and budding yeast SPC97/SPC98 genes in fission yeast and tested their ability to functionally complement the corresponding alp4/alph6 genes. By further analysis of human GCP2 and GCP3, we found that GCP3 protein assembles into the full γ-TuRC, and that the gene complements the conditional temperature-sensitive allele alp6-719 and functions in the absence of alph6 by gene replacement. Interestingly, the human GCP2 gene replaces alph4 but is unable to complement a recessive alp4-1891 temperature-sensitive allele. Although GCP2 localizes to spindle poles in the presence of Alp4, its assembly into the γ-TuRC is limited. By generation of an Alp4-GCP2 chimeric protein, we demonstrate that the N-terminal domain of GCP2 limits its ability to fully displace Alp4 in γ-TuRC assembly. Species specificity of budding yeast Spc97 was also observed in genetic assays but was not further explored in this study. Our findings confirm that a conserved mechanism exists for GCP3/Spc98/Alp6 across species. Further, our analysis reveals that the N-terminal domains of GCP2/Alp4 proteins exhibit different assembly capabilities across eukaryotes and that this role includes maintaining γ-TuRC at the spindle pole body through successive generations (Vardy and Toda, 2000).

Results

Human-to-yeast cross-species function of GCP3/Alp6 and GCP2/Alp4 revealed by genetic analysis in fission yeast

Fission yeast is an excellent model organism for analysis of conserved cell cycle mechanisms regulating assembly and function of microtubule cytoskeleton arrays. Human γ-tubulin, GTB1, was previously shown to functionally replace γ-tubulin in fission yeast and has been useful to evaluate γ-tubulin structure and cellular roles (Stearns et al., 1991; Horio and Oakley, 1994; Hendrickson et al., 2001). To genetically test functional compatibility of the remaining γ-TuSC proteins we expressed cDNAs of human GCP2 and GCP3 in fission yeast using multicopy and single-copy integrated plasmids and assessed their ability to complement the respective recessive alleles alp4-1891 and alp6-719 for recovery of temperature-sensitive growth (Vardy and Toda, 2000) (supplementary material Table S1 for strains). By serial-dilution growth assays with human genes, empty vector and wild-type alp4/alph6 gene controls (Fig. 1A), we found that human GCP3 complements alph6-719 growth at the restrictive temperature of 36°C. Human GCP2 is unable to complement alp4-1891 temperature-sensitive growth. However, partial function of GCP2 is suggested by its ability to suppress the phenotype of alph6-719, similar to wild-type alph4. It was previously shown that multi-copy alph4 rescues the alph6-719 allele whereas multi-copy alph6 does not suppress the alp4-1891 allele (Vardy and Toda, 2000). The corresponding γ-TuSC genes from Saccharomyces cerevisiae SPC97/SPC98 yielded similar findings to GCP2/GCP3 in our gene complementation/suppression assays. To confirm that GCP3 is capable of fully replacing Alp6 function in fission yeast, we integrated GCP3 cDNA at the alph6 locus, replacing the fission yeast coding region. Viable integrants were isolated by restored growth of alph6-719 at 36°C and correct replacement was confirmed by colony PCR of chromosomal DNA (Fig. 1B). Although GCP2 cannot complement temperature-sensitive growth of the mutant allele alp4-1891, it does replace the essential alph4 gene. This was confirmed by tetrad analysis of the heterozygous diploid strain.

Fig. 1. The GCP3/Spc98/Alp6 family displays full cross-species functional conservation. Cross-species genetic analysis of human and budding yeast γ-TuSC proteins in S. pombe. (A) Serial-dilution growth assays. Complementation within or suppression across γ-TuSC families using fission yeast mutant alleles alp4-1891 and alp6-719 assayed with integrated human GCP2 and GCP3 or budding yeast SPC97 and SPC98 genes. Representative assay. n=2–5. Cells were grown in liquid PM AUH supplemented medium to logarithmic phase and spotted onto PM AUH plates. (B) GCP3 integration at the alph6 locus by homologous recombination. On top is a cartoon of the recombinant PCR fragment used to target the alph6 locus for gene replacement. Primer sites for colony PCR are shown. Temperature sensitivity is lost when GCP3 replaces alph6-719 and restores full function. Strains are streaked on PM AUHL supplemented medium. Integration of GCP3 at the alph6 locus was confirmed by colony PCR with primers internal and external to the coding region at the 3’ end. Asterisk in lane 1 indicates PCR on a wild-type strain (JP32-1) that produces no band. Lane 2 is a positive GCP3 integrant (JP32-2). (C) Tetrad analysis of diploid strain SP826 carrying one copy of alph4 and one copy of GCP2/HA (strain JPZO45) at 25°C and 36°C. Cartoon diagrams are shown where white is alph4+ and black is GCP2/HA+. n=43 tetrads. (D) Lanes 1 and 2 in the upper gel are integrant confirmation by colony PCR of GCP2 (1) and alph4 (2), respectively. Lanes 1 and 2 were run on the same gel and then cropped and positioned as shown. Below is a western analysis of diploid alph4+/alph4+ (3) and alph4+/GCP2:GCP2-HA (4), respectively, probed with anti-HA and anti-γ-tubulin primary antibody.
alp4+/alp4Δ:GCP2-HA (Fig. 1C; strain JPZO45). We note that meiotic differences might exist between human GCP2 and S. pombe alp4 because 29% (at 25°C; n=7, 2/7) and 17% (at 36°C; n=6, 1/6) of tetrads produced outgrowth of only three of four colonies on YES medium plates (Fig. 1C). We also observed that GCP2* tetrads tended to be smaller than alp4* colonies at 36°C. Tetrads were restreaked and Geneticin resistance was used to identify alp4*:GCP2-HA colonies following tetrad analysis. After restreaking, changes in growth between alp4*:GCP2-HA and alp4* did not persist, indicating that GCP2 cannot germinate as quickly after dissection at 36°C. Colony PCR was performed to confirm one copy of alp4* and one copy of GCP2–HA in the diploid strain (Fig. 1D). These findings indicate that although the GCP3/Spc98/Alp6 family of γ-TuRC proteins are conserved across species as functional homologs, the GCP2/Spc97/Alp4 family proteins have some functional cross-species limitations in fission yeast.

Human GCP2 localizes to S. pombe mitotic MTOCs but does not rescue the γ-TuRC replication defect of alp4-1891

We examined localization of GFP–GCP2 during the cell cycle in fission yeast strains alp4-HA or alp4-1891 (Fig. 2). GCP2 localizes to the spindle pole body MTOC in interphase and mitosis and to the equatorial MTOC in late anaphase (Fig. 2A). The S. pombe strain carrying the recessive alp4-1891 mutant allele does not retain γ-tubulin at spindle poles during successive cell cycles (Vardy and Toda, 2000). To test whether human GCP2 can complement this defect we synchronized cells by hydroxyurea (HU) arrest and release, and analyzed GFP–GCP2 retention at mitotic spindle pole MTOCs in the alp4-1891 mutant and wild-type strains (Fig. 2B). Six hours after HU release, we observed in the alp4-1891 strain that GCP2 is lost from spindle poles in successive cell cycles at 36°C. GCP2 is retained at poles at the permissive temperature of 25°C in this strain and at both temperatures in the alp4-HA wild-type strain. GFP–GCP2 was observed at poles in 96% of cells viewed (where n=100 cell minimum at both temperatures). These findings indicate that although GCP2 localizes to spindle poles and equatorial MTOCs, it is unable to rescue the alp4-1891 defect and become established long-term in γ-TuRC complexes at poles during subsequent cell cycles at the restrictive temperature.

Overproduced human GCP2 affects spindle stability and microtubule arrays in fission yeast

To investigate the effect of GCP2 on fission yeast microtubule arrays, we expressed GCP2 using the prep90Δ:FLAGHis vector in wild-type strain YY105 (strain JPZO44). YY105 carries an α-tubulin–GFP fusion protein for visualizing microtubules (GFP–Atb2; Fig. 3A,B). In live cells at room temperature, the integrity of interphase microtubule arrays is altered (Hagan, 1998) (Fig. 3A, top). In addition, we observed the persistence of spindle microtubules in anaphase as indicated by a bent, elongated spindle pushing against the cell cortex (Fig. 3A, middle, bottom; Fig. 3B for statistics). A time-lapse comparative analysis of anaphase B duration before spindle breakdown (Fig. 3C,D) revealed that on average, cells with integrated prep90Δ:FLAGHis/GCP2 had anaphase spindles that persisted for 196±3 seconds compared with the wild type. These persistent spindles were maintained through early formation of eMTOC microtubule arrays. Next, we performed immunocytochemistry on alp4-1891 cells carrying prep81/GCP2 at 36°C. GCP2 does not rescue the bundled interphase arrays previously shown to be characteristic of alp4-1891 mutants (Fig. 3E) (Vardy and Toda, 2000). Our findings indicate that as for Alp4 (Vardy and Toda, 2000), GCP2 overexpression alters interphase and spindle microtubule arrays in fission yeast, but in a manner that is distinct from the Alp4 phenotypes previously demonstrated.

GCP2 cannot effectively displace Alp4 for full assembly into the high molecular mass γ-TuRC

To determine whether human GCP2 and GCP3 assemble into the >2000 kDa γ-TuRC complex, we separated whole-cell extracts of strains carrying tagged human and fission yeast γ-TuSC proteins by fast protein liquid chromatography (FPLC) over a Superose 6 10/300 GL column as described (Fig. 4) (Vardy and Toda, 2000). FPLC fractions were analyzed by western blotting for the presence of γ-tubulin and tagged proteins Alp4-HA (strain LV15), Alp6-HA (strain LV16), GCP3-HA–His (strain JP32-2) or GCP2-HA (strain JPZO46), respectively (Fig. 4B). Integrated prep90Δ:FLAGHis/GCP2 was also tested in an alp4* background (Fig. 4C; strain JPZO44). The FLAG–His tag does not disrupt Alp4 function in our genetic assays (not shown). Human GCP3–HA–His and GCP2–HA in alp6* and alp4* backgrounds, respectively, fractionate similar to fission yeast Alp4, Alp6 and γ-tubulin γ-TuSC proteins (Fig. 4B). This is illustrated by the
Fig. 3. Overproduced human GCP2 stabilizes microtubule arrays in fission yeast mitosis. (A) Live-cell fluorescence at room temperature. Microtubules are visualized with an α-tubulin fusion GFP–Atb2. Images from top to bottom are cell-cycle phases of interphase, mitosis and mitotic exit formation of equatorial microtubule arrays. Overexpression of GCP2 (strain JPZO44) generates abnormal microtubule arrays versus wild-type (YY105) cells. Persistent anaphase B spindles versus wild type are also observed. Cell-cycle stages were monitored by nuclear morphology using Hoechst 3342 staining. (B) Histograms showing number of phenotypes observed versus number of cells viewed for the three cell cycle stages shown in A; YY105 wild type versus GCP2 overexpression. Dashed lines are number of cells viewed, and orange, blue and white indicate interphase, mitosis, and eMTCC formation, respectively. The number of cells displaying mutant phenotypes over the number of cells viewed is 72/102 for interphase (73%), 105/111 for mitosis (95%), and 84/84 for mitotic exit and eMTCC arrays (100%). All phenotypes are 100% for YY105. (C) Single plane time-lapse images of wild-type strain YY105. Time-course is started (t=0 seconds) as spindle microtubules reach cell ends and monitored until spindle breakdown that initiates centrally as indicated by the arrow. Images were acquired at 30-second intervals. (D) Repeat of C with integrated GCP2 expressed from the strong nmt promoter, prep90× (strain JPZO44). n=7 full time-course series taken for both C and D. On average, cells expressing prep90×FLAGHis/GCP2 had a spindle that persisted for 196±3 seconds longer than in the wild type. (E) Abnormally long interphase microtubules that are characteristic of the alp4–1891 mutant are not altered in the presence of GCP2 (strain JPZO7; Vardy and Toda, 2000). Cells were fixed with methanol and stained with TAT1 antibody to visualize microtubules. This phenotype is observed in 100% of interphase cells 4 hours after HU release at 36°C. n=27. Cells were grown in liquid PM AUH or AUHL supplemented medium. Scale bars: 5 μm.

Fig. 4. Biochemical analysis of γ-TuRC components. (A) Molecular mass distribution showing a general schematic of fractionation profiles based on western analysis. This trace is absorbance units versus fraction number obtained by Fast Protein Liquid Chromatography (FPLC). High molecular mass γ-TuRC peaks occur in fractions 17 and 18. Molecular size markers at 2000 kDa and 669 kDa are shown. (B) Western profiles of whole-cell extracts fractionated by FPLC. Whole-cell extracts were prepared from cells expressing integrated Alp4-HA (strain LV15), Alp6-HA (strain LV16), GCP3-HA-His (JP32-2) or GCP2-HA (JPZO46) and equalized to 30 mg/ml, collected in 0.5 ml fractions from a Superose 6 Column. The γ-tubulin profile shown is from the Alp4-HA strain, done in parallel. GCP3-HA-His was probed with anti-HA. (C) Western of whole cell extracts fractionated by FPLC. Profiles of cells containing integrated prep90×FLAGHis/GCP2 (strain JPZO44), as analyzed in Fig. 3. Tagged constructs were probed with anti-FLAG and the γ-tubulin profile is from the prep90×FLAGHis/GCP2 strain, done in parallel. GCP2 high molecular mass fractions are shifted right, indicating limited assembly into the high molecular mass γ-TuRC. Rather, GCP2 fractionates primarily as smaller complexes. The FLAG-His tag does not disrupt Alp4 function in our growth assays (not shown).
general schematic in Fig. 4A. Interestingly, we found that GCP2 has only limited assembly into the full >2000 kDa γ-TuRC in an alp4- background (strain JPZ044). Instead, the bulk of GCP2 is found in two types of smaller complexes: the first ranging from 1100 to 1700 kDa (Fig. 4C), as well as in smaller complexes of 450–850 kDa (not shown). The nature of these intermediate complexes is unclear but could be informative in future analyses of stepwise assembly of γ-TuSC into γ-TuRC. These data indicate that GCP2 is not optimized to displace or outcompete Alp4 during γ-TuRC assembly, and that this inability probably underlies GCP2 failure to complement alp4-1891 growth at 36°C.

GCP2 competition with Alp4 is limited by its N-terminal sequence in fission yeast

It is proposed that the C-terminal domains of Alp4 and Alp6 interact with γ-tubulin in the γ-TuSC (Kollman et al., 2008) through unique sites on γ-tubulin (Paluh et al., 2000). These interactions are expected to be conserved across species because human γ-tubulin functions in S. pombe (Horio and Oakley, 1994). The alp4-1891 mutation lies within its N-terminal domain (Tange et al., 2004). To test whether inability of GCP2 to fully function in S. pombe resides only with sequence differences in its N-terminal domain or in other regions, we generated multiple Alp4–GCP2 chimeric proteins (Figs 5,6). Attempts to achieve a functional chimeric protein based solely on sequence identities alone were unsuccessful. By using the GCP4 crystal structure (Guillet et al., 2011), which is proposed to be similar to that of GCP2/GCP3, as well as multiple sequence alignments of GCP4 with GCP2/GCP3 and Alp4/Alp6 we were able to generate a functional Alp4-GCP2 chimeric protein (Fig. 5A,B). This chimeric protein retains the N-terminal domain of Alp4 and the γ-tubulin-binding C-terminal domain of GCP2, fused between globular domains. The Alp4-GCP2 chimera complements alp4-1891 conditional growth in serial-dilution growth assays (Fig. 5C). Our ability to use GCP4 as a structural template to generate a functional chimera is consistent with the underlying structural relationship proposed between the GCP4 family and γ-TuSC proteins (Guillet et al., 2011). Our findings here demonstrate that differences between the N-terminal domains of GCP2 and Alp4 are sufficient to prevent full GCP2 assembly into γ-TuRC and function in fission yeast with alp4- backgrounds.

Discussion

The broad importance of the MTOC in establishing polarized microtubule arrays for chromosome segregation, vesicle trafficking, cellular organization, and basal bodies of cilia and flagella makes it of high interest to define conserved mechanisms. Fission yeast is an excellent model organism for analysis of conserved microtubule cytoskeleton mechanisms and cross-species studies of MTOC structure and function. Here we demonstrate that human GCP2 and GCP3 gene replace alp4 and alp6, respectively, in S. pombe. Budding yeast SPC97/SPC98 γ-TuSC genes were also examined in brief and yielded similar growth patterns to GCP2/GCP3 in our gene complementation and suppression assays. Further, we demonstrate by biochemical fractionation that GCP2 is incapable of displacing the bulk of Alp4 protein in γ-TuRC and that this inability is due to differences in its N-terminal. That limited GCP2 assembly into γ-TuRC occurs in alp4- backgrounds is indicated biochemically and by functional changes to microtubule arrays in vivo. The

functional and non-functional chimeric Alp4–GCP2 proteins we have generated (Figs 5,6) provide new tools to hone in on the N-terminal segments that are crucial for γ-TuSC and γ-TuRC assembly and function.

The structure of the γ-TuSC and its ability to self-assemble into a ring in vitro (Kollman et al., 2010) is proposed to require a conformational change to GCP3 (Kollman et al., 2008). This action occurs on multiple γ-TuSCs and closes the V-shaped structure into a tight ring template for growth of 13 protofilament microtubules. That GCP3 is most conserved in function across species might reflect the importance of this proposed conformational change. The functional roles of GCP2/Sp97/
Alp4 and GCP3/Spc98/Alp6 are predicted to have both overlapping and distinct elements. Although the proteins are proposed to be similar, genetic studies in budding yeast reveal functional and structural differences (Nguyen et al., 1998; Geissler et al., 1996; Knop et al., 1997; Vinh et al., 2002; Pereira et al., 1998; Erlemann et al., 2012). This includes replicative roles in spindle pole body duplication, attachment to γ-TuRC proteins and nuclear localization signals to direct the γ-TuSC into the nucleus. Our studies indicate that additional distinctions might exist for the GCP2/Spc97/Alp4 family that limit shared function, even amongst yeasts. Although GCP2 does not fully complement the alp4-1891 allele in S. pombe, it retains the ability to complete some of the required tasks, such as properly localizing to spindle poles and eMTOCs and not impeding spindle microtubule nucleation or formation of equatorial microtubule arrays. As with overexpression of alp4, GCP2 overexpression alters microtubule arrays. In fission yeast, the temperature-sensitive growth phenotype of the alp6-719 allele is suppressed by overexpression of Alp4 (Vardy and Toda, 2000). GCP2 overexpression is also able to rescue the alp6-719 mutation. The localization of this mutation has not yet been determined and the functional relationship between Alp4/Alp6 or GCP2/GCP3 components of γ-TuSC remains unclear.

The inability of GCP2 to fully displace Alp4 in γ-TuRC as indicated in biochemical purification of this complex probably underlies its inability to complement the recessive alp4-1891 allele. The biochemical data indicate that it is the N-terminus of GCP2/Alp4 that directs key assembly steps into the larger multimeric γ-TuSC and γ-TuRC. How GCP2 interacts with γ-tubulin in comparison to Alp4 might also affect its ability to exchange within the complex or be maintained there. The alp4-1891 mutation that lies in the N-terminal domain of Alp4 is suppressed by a mutation in γ-tubulin, gtb1-PL302 (Paluh et al., 2000; Tange et al., 2004) that has been shown to result in enhanced binding of Alp4 to γ-tubulin (Rodriguez et al., 2008). This means that changes to the N-terminus of Alp4/GCP2 proteins can affect the distal γ-tubulin interaction by the C-terminus of these proteins. The relatively low exchange of γ-tubulin at spindle pole bodies in budding yeast (Erlemann et al., 2012) means that the kinetics of the GCP2–γ-tubulin or Alp4–γ-tubulin interaction could play essential roles in stability of the γ-TuSC (Olmsted et al., 2013).

Our difficulty in generating functional chimeric Alp4–GCP2 proteins (one in five attempts successful) reveals the limitations of structure or sequence analysis alone (Fig. 6). The crystal structure of GCP4 provided a suitable model for generating a
successful chimeric Alp4-GCP2 protein. The non-functional chimeric proteins are useful tools to evaluate close helical interactions and will continue to be informative even once crystal data for GCP2 becomes available. Further investigation is needed to determine which additional γ-TuRC proteins are interacting with this N-terminal region. The immediate likely candidate is fission yeast Pcp1, human CDSRAP2 or budding yeast Spc110. Studies in budding yeast suggest a shared relationship between Spe97 and Spc98 in interactions with Spc110 that might not be equivalent (Nguyen et al., 1998; Knop et al., 1997). Cross-species work such as this is vital for elucidating conserved γ-TuRC mechanisms and functional differences in this MTOC between species.

Materials and Methods

Yeast strains, growth and serial-dilution assays

Rich YES/YES and PM minimal fission yeast media with supplemented 75 μg/ml histidine, leucine or uracil and 100 μg/ml adenine were used for liquid culture in addition to culture on plates. Procedures for yeast genetic manipulations are as described (Moreno et al., 1991). Yeast transformations were performed using the Ez-YES Transformation Solution (MP Biomedicals, Santa Ana, CA) with the pREP81 or pREP90-plasmid to leu prototrophy (Huang et al., 2001; Forsburg, 1993). Cells were grown in 10 ml AUH medium at the permissive temperature to logarithmic phase serial-dilution assays. Cell numbers were obtained by hemocytometer, and equalized before spotting on triplicate plates at increasing dilution for growth at 25°C, 30°C and 36°C. Complementation and suppression analysis with cross-species alleles and controls used mutant alleles alp4-1891 and alp6-719 (Varudy and Toda, 2000). All assays were performed at least twice, but on average three times. Minimal supplemented plates with control cells were imaged at 2-5 days after spotting. Strain YY105 was used to visualize α-tubulin-GFP-tagged microtubules in vivo. GCP2 was overexpressed on a multi-copy plasmid by the low-strength nmt promoter (pREP81) or integrated and expressed by the strong nmt promoter (pREP90). For a complete list of strains and plasmids used in this study, refer to supplementary material Table S1.

Bioinformatics and structural analysis

The following resources were used for obtaining sequences and for other bioinformatic analysis: NCBI GenBank and Blastp (GCP2, Q9BSJ2; GCP4, 4757365), Welcome Trust Sanger Institute (Alp4, Alp6 sequence and intron positions), Protein Databank RCSB-PDB (GCP4, 3RIP), PyMol molecular visualization software V 1.5, Jalview V 2.8 and Clustal Omega multiple sequence alignment and analysis software. Four nonfunctional Alp4-GCP2 chimera were generated (Fig. 6): Chimera 1, Alp4(1-238)–GCP2(334–902); Chimera 2, GCP2(1–336)–Alp4(242–540)–GCP2(614–902); Chimera 3, GCP2(1–336)–Alp4(242–566)–GCP2(614–902); Chimera 4, GCP2(1–336)–Alp4(242–784). One functional chimera was generated (Fig. 5): Alp4(1–418)–GCP2(504–902).

Cloning and alp4/alp6 gene replacement

Cloning and chimera generation of fission yeast, budding yeast and human γ-TuRC genes were performed using high-fidelity PCR with oligonucleotides with tailored restriction sites or regions of homology (supplementary material Table S2; Integrated DNA technologies; Roche High Fidelity PCR Master, Roche, Indianapolis, IN). Integration was carried out in pREP vectors using homologous recombination at the autonomous replication site. All constructs were sequenced (Northwoods DNA, Solway, MN; Center for Functional Genomics, University at Albany SUNY, Albany, NY). The integration of the GCP1 gene at the alp6 locus was achieved using a modular PCR-based gene-targeting approach with long tracts of flanking homology, as previously described (Kravchuk and Wahls, 1999). Wild-type chromosomal DNA was extracted using phenol–chloroform, and we generated two 500 bp genomic fragments (upstream (5′) and downstream (3′) of the alp6 open reading frame. These fragments contained homology to a central GCP3 CDNA. A mixture of this fragment along with the left and right flanking regions and an excess of the outermost primers was used in a fourth PCR reaction (Epiconcept MasterAmp Extra-Long PCR Kit, Epiconcept, Madison, WI). This fragment was amplified to 20 μg/ml and purified (Quagen, Valencia, CA). 20 μl of 0.5 μg/μl purified DNA was used for transformation into the alp6-719 mutant strain. Loss of temperature sensitivity was used to screen, and integration was verified by colony PCR. The same colony PCR carried out on the alp6-719 strain alone generated no fragment (Fig. 1B). A second PCR technique was used to replace alp4 with GCP2 at the alp6 locus. Two fragments were generated for double transformation of the S. pombe diploid strain SP826 (supplementary material Table S1). Fragment 1 contained 132 nt genomic sequence upstream of the alp4 open reading frame (ORF), the GCP2 ORF, a 2×HA tag and 75 nt of genomic GCP2 3′ genomic sequence. Fragment 2 contained homology to the 2×HA tag, 75 nt of GCP2 3′ genomic sequence, the kanMX6 Genetic resistance ORF with TEF promoter/terminator and 135 nt genomic sequence downstream of the alp4 STOP. These fragments were combined in vivo at the alp4 locus by homologous recombination using alp4 flanking regions and the central GCP2 3′ homology. SP826 was transformed with 20 μl of water containing 10 μg of each fragment and stable integrants selected by Geneticin resistance on AUH-supplemented plates. Colony PCR was used to verify one copy of alp4 and one copy of GCP2 in SP826 (Fig. 1C).

Western analysis

Whole-cell extracts were prepared using bead beating (Mini-Beadbeater-16, Biospec, Bartlesville, OK) in Buffer A (20 mM Tris-HCl, pH 7.5, 20% glycerol, 0.1 mM EDTA, 5 mM ATP, 100 μM GTP) as described (Varudy and Toda, 2000) with protease inhibitors (1 mM PMSF, 5 mM phenanthroline, 50 μM leupeptin, 2 mM pepstatin, 175 nM aprotinin, 1 mM benzamidine and 200 μM Pefabloc). Multiple centrifugations at 17,000 g ranging from 1 to 30 minutes were used to clarify cell extracts before column loading. Protein concentrations were determined by nanodrop and diluted to ~30 μg/ml before loading onto a Superose 6 10/300 GL column (GE HealthSciences, Pittsburgh, PA). Half-milliliter fractions were collected and stored on ice for immediate analysis. Peak fractions were determined using absorbance units and FPLC analysis of molecular size standards Blue Dextran (2000 kDa), Thyroglobulin (669 kDa), Apoferritin (443 kDa) and BSA (66 kDa). Antibodies for western analysis were: primary mouse anti-γ-tubulin monoclonal 1:10,000 (Sigma-Aldrich, St Louis, MO); primary mouse anti-HA monoclonal 1:500 (Covance, Princeton, NJ), primary rabbit anti-HA epitope tag 1:10,000 (Rockland, Gilbertsville, PA), primary rabbit anti-FLAG 30:10,000 (Sigma-Aldrich), goat anti-rabbit IgG HRP conjugate 1:10,000 (Millipore, Billerica, MA) and goat anti-mouse IgG HRP conjugate 1:10,000 (Novagen, Billerica, MA).

Fluorescence microscopy and immunocytochemistry

Live-cell fluorescence microscopy of GFP-fusion proteins was performed using a Zeiss Observer.Z1 inverted microscope with 63× Plan-Fluaromat 1.4 NA oil and 100× oil 1.45 PlanFLUAR DIC objectives. Live cells remained unfixed and were analyzed at room temperature. Images were acquired using Hamamatsu ORCA ER CCD camera with Zeiss Axiovision Rel 4.8 acquisition software. 10-image 0.2 μm Z-stack images were obtained. Data were compiled using Adobe Photoshop (Adobe Systems) and Microsoft PowerPoint (Microsoft Corporation) software. Per cell minimum per phenotype. Using methanol fixation, we were able to preserve GFP-GCP2 signal to analyze retention or loss of the γ-TuRC from spindle poles at the restrictive temperature over multiple cell cycle progressions as described (Varudy and Toda, 2000). Microtubules were stained with primary TAT antibody (Woods et al., 1989) (1:25), followed by secondary anti-mouse Alexa Fluor 488 IgG (1:50; Invitrogen, Grand Island, NY). Cells were imaged immediately using the Zeiss Observer.Z1 system.

Synchronous culture

Cells were synchronized by hydroxyurea (HU) block and release. HU was added to logarithmically growing cultures in baffled flasks (11 mM final concentration) and cells were harvested for 4 hours at room temperature. Two wash steps were done by centrifugation at low speeds before resuspension in PM AUH liquid medium. Cultures were then shifted to 36°C or room temperature. 1 ml aliquots were taken at multiple time points from 30 to 400 minutes and cells were fixed immediately in methanol at ~20°C for immunocytochemistry.

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Author contributions

T.D.R. developed the methodology for purifying the γ-TuRC, performed genetic and biochemical experiments and contributed to the writing and figures of this study; Z.T.O. designed and performed genetic and cell biological experiments and contributed to the writing and figures of this study; C.N.B. made GFP-fusion and chimeric constructs and performed serial dilution growth assays; A.M.W. assisted in strain construction and gene cloning; L.S. and L.O.C. cloned original cDNAs and performed serial-dilution growth assays; J.L.P. conceived and designed experiments and contributed to the writing of this study.

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Human γ-TuSC in S. pombe 4413