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Fission yeast MOZART1/Mzt1 is an essential γ-tubulin complex component required for complex recruitment to the microtubule organizing center, but not its assembly

Hirohisa Masuda, Risa Mori, Masashi Yukawa, and Takashi Toda

ABSTRACT γ-Tubulin plays a universal role in microtubule nucleation from microtubule organizing centers (MTOCs) such as the animal centrosome and fungal spindle pole body (SPB). γ-Tubulin functions as a multiprotein complex called the γ-tubulin complex (γ-TuC), consisting of GCP1–6 (GCP1 is γ-tubulin). In fungi and flies, it has been shown that GCP1–3 are core components, as they are indispensable for γ-TuC complex assembly and cell division, whereas the other three GCPs are not. Recently a novel conserved component, MOZART1, was identified in humans and plants, but its precise functions remain to be determined. In this paper, we characterize the fission yeast homologue Mzt1, showing that it is essential for cell viability. Mzt1 is present in approximately equal stoichiometry with Alp4/GCP2 and localizes to all the MTOCs, including the SPB and interphase and equatorial MTOCs. Temperature-sensitive mzt1 mutants display varying degrees of compromised microtubule organization, exhibiting multiple defects during both interphase and mitosis. Mzt1 is required for γ-TuC recruitment, but not sufficient to localize to the SPB, which depends on γ-TuC integrity. Intriguingly, the core γ-TuC assembles in the absence of Mzt1. Mzt1 therefore plays a unique role within the γ-TuC components in attachment of this complex to the major MTOC site.

INTRODUCTION Microtubules are noncovalent cytoskeletal polymers composed of α- and β-tubulin heterodimers. These heterodimers assemble into linear protofilaments in a GTP-dependent manner, and 13 protofilaments in turn associate laterally to form a 24-nm-wide hollow cylinder. The longitudinal orientation and distinct biophysical properties of α- and β-tubulins give rise to an intrinsic polarity of microtubules, with β-tubulin facing the fast-growing yet dynamic plus end and α-tubulin the less dynamic minus end (Desai and Mitchison, 1997; Nogales, 2000; Jiang and Akhmanova, 2011). The microtubule cytoskeleton plays a myriad of roles inside the cell, in a variety of physiological processes, including cell motility and polarity, cell signaling, organelle positioning, and chromosome segregation. This functional diversification mirrors different microtubule morphologies in various cell types, such as neurons, cilia, and flagella. Even in a single dividing cell, microtubules undergo dynamic structural alterations (e.g., interphase microtubules and bipolar mitotic spindles) in a spatiotemporal manner during the cell cycle.

The key event in microtubule assembly is an initial nucleation. Unlike under certain in vitro situations, in vivo microtubules nucleate from specific sites called the microtubule organizing centers (MTOCs; Pickett-Heaps, 1969; Brinkley, 1985; Luders and Stearns, 2007), where the microtubule minus end is embedded. The MTOC includes the animal centrosome (Boveri, 2008) and the fungi equivalent, the spindle pole body (SPB). Furthermore, in line with the
organizational diversities of microtubules, there are numerous other MTOCs besides the centrosome and SPB within cells, among them the basal body for axoneme microtubules, the chromosome for mitotic spindles, and the cell cortex for cortical microtubules (Meng et al., 2008; Mishra et al., 2010; Li et al., 2012). Plant cells do not contain a centrosome equivalent but instead nucleate microtubules from acenstorimal MTOCs, including discrete sites on microtubules and the cell cortex (Murata et al., 2005; Ehrrhardt and Shaw, 2006). Despite these structural and spatial variations, most if not all of the microtubules emanate from a universal nucleator called the γ-tubulin complex (γ-TuC; Job et al., 2003; Wiese and Zheng, 2006; Luders and Stearns, 2007).

γ-Tubulin is highly conserved across eukaryotes as a ubiquitous component of the centrosome and SPB (Oakley and Oakley, 1989; Oakley et al., 1990; Horio et al., 1991; Stearns et al., 1991; Stearns and Kirschner, 1994; Zheng et al., 1991; Joshi et al., 1992; Moritz et al., 1995; Sobel and Snyder, 1995; Marshall et al., 1996; Spang et al., 1996). γ-Tubulin does not function on its own, but instead in vivo, it is composed of a large, open, ring structure (~2 MDa and ~32S) via formation of a multiprotein complex with several other nontubulin proteins (referred to as the γ-tubulin ring complex or γ-TuRC; Zheng et al., 1995). These nontubulin complex components are generically designated GCP2-6 (GCP1 is γ-tubulin; Knop et al., 1997; Martin et al., 1998; Murphy et al., 1998; Tassin et al., 1998; Fava et al., 1999; Gunawardane et al., 2000b). Unlike most other eukaryotes, budding yeast contains only three GCPs that mostly form a smaller complex ~300 kDa and 11.3S in size: Tub4/GCP1, Spc97/GCP2, and Spc98/GCP3 (Geissler et al., 1991; Spang et al., 1996; Knop et al., 1997), in a molecular ratio of 2:1:1, respectively (Knop et al., 1997; Vinh et al., 2002). Interestingly, in higher eukaryotes, under high-salt conditions the γ-TuC sedimented with a similar small size (~280 kDa and ~10S) that consists solely of GCP1, GCP2, and GCP3 (referred to as the γ-tubulin small complex or γ-TuSC; Moritz et al., 1998; Oegema et al., 1999; Gunawardane et al., 2000b; Zhang et al., 2000). It was further shown that multiple γ-TuSCs were associated with GCP4, GCP5, and GCP6, which formed γ-TuRCs that possessed more potent microtubule nucleation activity than γ-TuSCs alone (Oegema et al., 1999). In this study, we generally refer to the γ-tubulin complex in the yeast, unless its composition and size are deliberately distinguished as TuSC or TuRC.

Amino acid sequence comparisons of GCP2-6 showed that these proteins share two conserved regions in their C-terminal parts, called GRIP (γ-tubulin ring complex protein motifs/domains 1 and 2 (Gunawardane et al., 2000a; Teixido-Travesa et al., 2012). The precise molecular function of the GRIP domains has remained enigmatic for some time; however, it has recently become clear that these two domains form virtually identical conformations responsible for direct binding to γ-tubulin (Guillet et al., 2011). This notion has provoked novel insights into the γ-TuRC architecture and the molecular mechanism controlling microtubule nucleation. The previously proposed model in which GCP4, GCP5, and GCP6 were posited to have a capping role at the base of the γ-TuRC distal to γ-tubulin (Moritz and Agard, 2001) has been replaced with a new model in which these three GCPs bind γ-tubulin directly, therefore being incorporated into the core ring structure (Guillet et al., 2011; Kollman et al., 2011).

The previous proposed model has persistently been questioned with regard to two crucial points. First, what is the mechanism by which microtubules nucleate from the γ-TuC? Second, how does the γ-TuC attach to MTOCs such as the centrosome and SPB? GCP1-6 have proved to be insufficient for answering both of these critical questions (Masuda et al., 1992; Masuda and Shibata, 1996; Aldaz et al., 2005; Kollman et al., 2010, 2011), and therefore three other γ-TuC-interacting proteins, NEDD1/GCP-WD/GCP7, MOZART2/GCP8, and MOZART1/GIP1/GCP9, have appeared to be of particular interest (Gunawardane et al., 2003; Haren et al., 2006; Luders et al., 2006; Hutchins et al., 2010; Teixido-Travesa et al., 2010; Nakamura et al., 2012; Pinyol et al., 2013). Among the three proteins, only MOZART1 is highly conserved in a broad range of eukaryotes, including fungi and plants (albeit not in budding yeast; Janski et al., 2008, 2012; Hutchins et al., 2010; Nakamura et al., 2012; see Table 1), raising the possibility that this conserved protein is involved in the fundamental functions of the γ-TuC at the MTOCs.

Genetically tractable fission yeast is an ideal system to study the functions of the γ-TuC. In this yeast, GCP1-6 are all conserved (GCP1/Gtb1/Tug1, GCP2/Alp4, GCP3/Alp6, GCP4/Gfh1, GCP5/Mod21, and GCP6/Alp16; Horio et al., 1991; Stearns et al., 1991; Vardy and Toda, 2000; Fujita et al., 2002; Venkatram et al., 2004; Anders et al., 2006; Table 1). Gtb1/Tug1, Alp4, and Alp6 are essential for cell viability, while the other three GCPs are dispensable, as in Aspergillus nidulans and Drosophila melanogaster (Verellet et al., 2006; Xiong and Oakley, 2009). Of equal importance is the fact that human γ-tubulin is functional; the human counterpart is capable of rescuing otherwise lethal fission yeast γ-tubulin deletion mutants (Horio and Oakley, 1994). Furthermore, microtubules nucleate from multiple MTOCs besides the SPB similar to animal cells (Sawin and Tran, 2006), and the individual MTOCs are specifically activated in a

### Table 1: Comparison of γ-TuC proteins in different species.

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<tr>
<th>H. sapiens</th>
<th>X. laevis</th>
<th>D. melanogaster</th>
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<td>γTub23C, CD</td>
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<td>MIPA</td>
<td>Gtb1/Tug1</td>
<td>Tub4</td>
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<tr>
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<td>GCPF</td>
<td>Alp16</td>
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<tr>
<td>MOZART1</td>
<td>MOZART1</td>
<td>MOZART1</td>
<td>GIP1a, b</td>
<td>MZTA</td>
<td>Mzt1</td>
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In humans, MOZART2A and MOZART2B were identified as new components of the γ-TuC (Hutchins et al., 2010); however, these homologues are found only in the deuterostome lineage. NEDD1/GCP-WD interacts with the γ-TuC, yet appears not to be an integral component of the γ-TuC (Choi et al., 2010; Nakamura et al., 2012) and is not conserved in fungi. These two classes of proteins are therefore excluded from this table.

GIP1a and GIP1b (Nakamura et al., 2012) are also called GIP2 and GIP1, respectively (Janski et al., 2012).
cell cycle–dependent manner (Masuda et al., 1992; Hagan and Petersen, 2000; Sawin and Tran, 2006). Intriguingly, MOZART1 is conserved in fission yeast (Hutchins et al., 2010; Table 1). In this work, we present the functional characterization of MOZART1 (called Mzt1). We have unveiled that Mzt1 plays a unique role among the γ-TuC components and is specifically required for the recruitment of the γ-TuC to the SPB, although it is dispensable for the assembly of the core γ-TuC. The conserved mechanisms underlying recruitment and attachment of the γ-TuC and microtubule nucleation are discussed.

RESULTS
The mzt1+ gene is essential for cell viability
The amino acid sequence translated from the genome sequence corresponding to the mzt1+ open reading frame (ORF) predicted that the gene could encode two types of products, which consist of 64 and 97 amino acid residues, depending on the assignment of the initiator methionine (Supplemental Figure S1A). In this study, we defined the shorter ORF as the gene coding for Mzt1 (Figure 1A). This assignment was based on the following three criteria. First, a recent report of its transcripts proposed that the second methionine of Mzt1 is the initiator (Bitton et al., 2011). Second, the sequences of the Mzt1 homologues found in three other closely related fission yeast species—Schizosaccharomyces cryophilus, Schizosaccharomyces japonicus, and Schizosaccharomyces octosporus, show that Mzt1 in these species is composed of 64, 65, and 64 residues, respectively (Bechhoefer and Rhind, 2012; see Figure S1A). Finally, we found that the shorter Mzt1 protein localized to the SPB/MTOC (see Figures 2 and 3) more efficiently than the longer version (Figure S1, B and C).

We first asked whether the mzt1+ gene plays an essential role in cell viability. To explore this possibility, we deleted one copy of the mzt1+ gene in diploids with the PCR-mediated gene replacement method using the nourseothricin-dihydrogen sulfate (clonNAT)-resistant gene as a selectable marker (Bähler et al., 1998; Sato et al., 2005). clonNAT-resistant transformants were subjected to sporulation, and tetrad dissection was performed. Of the 20 asci dissected, we obtained two viable and two nonviable spores per ascus (Figure 1B, left), in which viable colonies displayed clonNAT-sensitive phenotypes (representing wild-type mzt1+), indicating that mzt1+ is essential for cell viability. Microscopic observation of nonviable cells showed that the spores containing the mzt1-deleted locus could germinate and undergo several rounds of cell division, but cells were subsequently arrested with elongated, branched morphologies (Figure 1B, right). This result shows that the mzt1+ gene plays an essential role in successive cell division.

Mzt1 is a component of the γ-TuC
Next we addressed whether Mzt1 is a component of the γ-TuC. To this end, we tagged green fluorescent protein (GFP) into Mzt1’s N-terminus prior to the initiator methionine (see Materials and Methods), enabling the GFP-Mzt1 protein to be produced under the control of the endogenous promoter. GFP tagging did not interfere with Mzt1 function, as a strain producing GFP-Mzt1 divided normally, comparable with a nontagged parental strain. Using this tagged construct, we created a strain that contained GFP-Mzt1 and Alp4-HA (GCP2/Spc97 homologue; Vardy and Toda, 2000). Pull down with a GFP-binding protein (GBP) showed that Mzt1 interacts with both Alp4 and γ-tubulin (Figure 1C). The pull down was then repeated with cells that instead contained GFP-Mzt1 and Alp6-Myc (GCP3/Spc98; Vardy and Toda, 2000). Again, GFP-Mzt1 pulled down Alp6-Myc and γ-tubulin (Figure 1D). Thus Mzt1 is a novel essential component of the γ-TuC in fission yeast.

Mzt1 localizes to the SPB throughout the cell cycle and to the equatorial MTOC during the postanaphase period and is present in approximately equal stoichiometry with Alp4/GCP2
Previous work showed that the γ-TuC localizes to several MTOCs within the cell in a cell cycle–dependent manner (Figure 2A). These
MTOCs include the SPB (during both interphase and mitosis), the equatorial MTOC (eMTOC) during the postanaphase period, and the interphase MTOC (iMTOC) that consists of distinct dots along the cytoplasmic microtubules or on the nuclear membrane (Horie et al., 1991; Drummond and Cross, 2000; Vardy and Toda, 2000; Tran et al., 2001; Fujita et al., 2002; Sawin et al., 2004; Venkatram et al., 2004; Janson et al., 2005; Zimmerman and Chang, 2005; Hoog et al., 2007). To examine the precise cellular localization of Mzt1, we constructed two kinds of strains; one contained GFP-Mzt1, mCherry-tagged Atb2 (α2-tubulin, mCh-Atb2), and mRFP-tagged Sid4 (constitutive SPB component, Sid4-mRFP) (Toda et al., 1984; Chang and Gould, 2000), while the other contained GFP-Mzt1 and mCherry-tagged Alp4 (mCh-Alp4). Observation of the fluorescent signals showed that Mzt1 localized to the SPB throughout the cell cycle, to the eMTOC upon mitotic exit, and, importantly, colocalization was seen with Alp4 (Figure 2, B and C; the position corresponding to the eMTOCs is marked with arrows).

For gaining insight into the stoichiometry between Mzt1 and Alp4 in the cell, the fluorescence intensities of each GFP-tagged protein detected at the SPB that was produced from individual native promoters were quantified during the cell cycle. As shown in Figure 2D, the signal intensities of these two proteins were almost equivalent during both interphase and mitosis. We also performed quantitative immunoblotting of GFP-Mzt1 and GFP-Alp4 in whole-cell extracts. Again it was evident that these two proteins are present in roughly equal quantities (a molecular ratio between Alp4 and Mzt1 is 1:1.1, Figure 2E). These data show that Mzt1 and Alp4 exist with similar stoichiometry both in soluble cell extracts and at the SPB in the cell.

Mzt1 also localizes to the interphase MTOCs
In plant cells, it was reported that a subpopulation of the γ-TuC, such as those existing on mitotic microtubules, contains MOZART1/GIP1, while the other population, such as that localizing to the cortical sites, does not (Nakamura et al., 2012). This suggests that the composition of the γ-TuC may vary within individual MTOCs, depending on the presence or absence of this component. Having seen colocalization of Mzt1 with Alp4 at the SPB and eMTOC, we further observed whether Mzt1 localizes to the other MTOCs, namely the discrete dots along cytoplasmic microtubules or on the nuclear membrane. (B) Mzt1 localizes to the SPB and the eMTOC. Cells containing GFP-Mzt1 (top row) and mCh-Atb2 (MTs) and Sid4-mRFP (SPBs) (middle row) were analyzed; snapshots of interphase (I), mitotic (M), and telophase cells (M-to-I) taken from time-lapse live images are shown. Merged images are presented on the bottom (green for Mzt1 and red for MTs and SPBs). (C) Mzt1 colocalizes with Alp4/GCP2 to the SPB and the eMTOC. The same procedure was followed as in (B), except that cells containing GFP-Mzt1 (top row) and mCh-Alp4 (middle row) were used. The position of the eMTOCs is indicated with arrows. Scale bar: 10 μm. (D) Quantification of fluorescence intensities of GFP-Mzt1 and GFP-Alp4 located at the SPBs during the cell cycle. GFP signals were quantified during both interphase and each stage of mitosis (premetaphase, Pro-Meta; anaphase, Ana; telophase, Telo). (E) Quantification of Mzt1 and Alp4 protein levels in whole-cell extracts. Two strains containing GFP-Mzt1 or GFP-Alp4 and a control nontagged strain were grown at 27°C, and protein extracts were prepared. Immunoblotting was performed with anti-GFP and anti-γ-tubulin antibodies. Relative amounts of GFP-Mzt1 and GFP-Alp4 were normalized using those of γ-tubulin as a control. The relative value of GFP-Alp4 is assigned as 1.0, and that of GFP-Mzt1 is 1.1.
As shown in Figure 3B, the Mzt1 signals observed not at the SPB localization correspond to the iMTOCs (Bratman and Chang, 2007). It was previously shown that remnant microtubule sites upon microtubule depolymerization from their plus ends. It was previously shown that remnant microtubules (marked with arrowheads that correspond to microtubule overlapping zones (arrowheads at 1.5-, 2-, and 4.5-min time points) or branching points where two microtubules merged (arrows at 0.5- and 3-min time points). (B and C) Mzt1 and Alp4 localize to the interphase microtubule nucleation sites upon microtubule depolymerization. Cells containing mCh-Atb2 (MTs) plus Sid4-mRFP (SPBs) and GFP-Mzt1 (B) or GFP-Alp4 (C) were treated with the microtubule-depolymerizing drug TBZ (100 μg/ml) at time 0, and live imaging was subsequently performed. As microtubules depolymerized, localization of Mzt1 and Alp4 to the non-SPB sites on remnant microtubules (marked with arrowheads that correspond to the iMTOCs; Bratman and Chang, 2007) became evident. Scale bars: 10 μm.

To confirm that these non-SPB punctate dots correspond to the iMTOCs, we treated cells with the microtubule-depolymerizing drug thiabendazole (TBZ), and Mzt1 localization was followed as the microtubules depolymerized from their plus ends. It was previously shown that remnant microtubule sites upon microtubule depolymerization correspond to the iMTOCs (Bratman and Chang, 2007). As shown in Figure 3B, the Mzt1 signals observed not at the SPB appeared as dots localizing to the remnant microtubules and often around the nucleus (Figure 3B, arrowheads). Similarly, Alp4 localized to these remnant microtubule sites (Figure 3C, arrowheads). Taking all of these results into consideration, we conclude that, like Alp4, Mzt1 is a constitutive component of the γ-TuC that localizes to all the MTOCs throughout the cell cycle.

Temperature-sensitive mzt1 mutants display interphase microtubule defects at the permissive temperature

To examine the roles of Mzt1 in cell division and microtubule assembly, we constructed several temperature-sensitive (ts) mutants of mzt1. For this purpose, multiple amino acid residues conserved across various species were selected and mutated individually to other random amino acids by PCR-based, site-directed mutagenesis (see Figure 1A for amino acid residues mutagenized, and Materials and Methods and Supplemental Figure S2 for experimental procedures). Figure 4A shows a serial dilution spot assay of four temperature-sensitive mutants isolated (mzt1-E10P, mzt1-D24Q, mzt1-T27W, and mzt1-E42P). Note that similar to previously isolated mutants of γ-TuC components (Vardy and Toda, 2000; Fujita et al., 2002), mzt1 mutants were hypersensitive to TBZ (Figure 4B).

Close inspection of the microtubules and SPBs (mCh-Atb2 and Sid4-mRFP) in the mzt1-T27W mutant showed that mutant cells appeared to contain a reduced number of interphase microtubule bundles, even when grown at the permissive temperature (27°C; Figure 5, A and B). Quantification showed that the number of bundles was indeed significantly reduced: ~1.8 bundles per mutant compared with 4.5 per wild-type cell (Figure 5C). Similar interphase microtubule defects were also observed in the mzt1-E10P or mzt1-D24Q mutants. The appearance of these abnormal interphase microtubules is reminiscent of mutants of the γ-TuC components (Faulh et al., 2000; Vardy and Toda, 2000; Hendrickson et al., 2001; Fujita et al., 2002; Sawin et al., 2004; Venkatram et al., 2004; Zimmerman and Chang, 2005; Anders et al., 2006; Masuda et al., 2006a). Measurement of GFP-Alp4 signals at the interphase SPB in these mzt1 mutant cells showed that the fluorescence signals were already substantially reduced at the permissive temperature, with ~95% reduction at interphase SPBs.
in all three mzt1 mutant alleles (Figure 5D). Also of note was that we were unable to visualize the iMTOC or eMTOC in these mutants (Figure 5B). We envisage that the reduced γ-TuC levels at the MTOCs during interphase leads to the appearance of interphase microtubule defects at this temperature. Furthermore, like other γ-TuC mutants (Paluh et al., 2000; Vardy and Toda, 2000; Fujita et al., 2002; Sawin et al., 2004; Venkatram et al., 2004; Zimmerman and Chang, 2005; Masuda et al., 2006a), mzt1-T27W cells showed bent morphology, even at the permissive temperature (Figure 5B, right), and this defect became more exaggerated at the restrictive temperature (see Figure 7, B and C, later in the paper).

**Mzt1 is required for bipolar mitotic spindle microtubules and chromosome segregation**

Observation of mitotic mzt1-T27W cells incubated at 36°C revealed characteristic defects in spindle microtubule formation and chromosome segregation (designated types I and II, Figure 6, A–D). Of mitotic cells, 47% (n = 30) displayed a significant delay in SPB separation and spindle elongation during early mitosis (Figure 6B, type I). For comparison, a cell exhibiting normal mitotic progression is shown in Figure 6A). In this type of cells, the two SPBs failed to separate toward the cell tips for a duration of more than 20 min, during which they often became spatially closer again. This phenotype, called SPB convergence or spindle collapse, is reminiscent of failure in bipolar spindle formation (Hauf et al., 2007; Hsu and Toda, 2011). Indeed, the spindle microtubules that normally connect the two separating SPBs were dim, with only the two SPB dots clearly visible. In addition, during this period, the chromosomes (CFP-tagged histone H3, Hht1-CFP) never segregated (Figure 6B, top row), indicating this cell underwent mitotic delay with unattached kinetochores. This indicates that Mzt1 is required to form stable spindle microtubules. Presumably, this defect is directly ascribable to the role of Mzt1 in nucleation of mitotic spindle microtubules from the SPB.

In the second category (20%, n = 30, type II), lagging, chromosome-like phenotypes were observed during mid- to late mitosis (Figure 6C, top row, evident at 12 min). Unlike the previous type I cells (Figure 6B), apparently normal spindle microtubules were formed and elongated (middle row). We envision that spindle–kinetochore interaction at the plus end of spindle microtubules is somehow compromised in type II mitotic cells, leading to chromosome segregation defects. The mzt1-E10P mutant also displayed similar phenotypes, although the percentage of types I and II varied from that of mzt1-T27W cells (14% type I and 53% type II; Figure 6D). Collectively Mzt1 is necessary for the proper assembly of both interphase and mitotic spindle microtubules that is essential for accurate chromosome segregation.

**Mzt1 is required for localization of the γ-TuC to the mitotic SPB, and the quantity of the remaining γ-TuC accounts for the varied phenotypic outcomes**

Previous results suggested that Mzt1 may play dual roles in microtubule organization during mitosis: nucleation from the SPB (type I) and kinetochore-microtubule interaction at the plus end (type II). Although spindle microtubules were formed and elongated, it is possible that microtubule organization in type II cells is in fact somewhat defective. To address why and how these two different phenotypes arose, we quantified the fluorescence intensities of Alp4 (GFP-Alp4) and spindle microtubules (mCh-Atb2) in wild-type and each type of mzt1-T27W cells under time-lapse microscopy. At 1.5 h after temperature shift up from 27°C to 36°C, late G2 cells (cells longer than 13 μm) were chosen under the microscope, and subsequent mitotic progression was filmed.

Live images of representative cells of wild type (Figure 7A), type I (Figure 7B), and type II (Figure 7C) are shown (note that 0 min is defined by the time point when SPBs started to separate, which is a landmark for mitotic entry). At each time point, fluorescence intensities of Alp4 (GFP-Alp4) and spindle microtubules (mCh-Atb2) in wild-type and each type of mzt1-T27W cells under time-lapse microscopy. At 1.5 h after temperature shift up from 27°C to 36°C, late G2 cells (cells longer than 13 μm) were chosen under the microscope, and subsequent mitotic progression was filmed.
Mzt1 is an essential, stoichiometric component of the γ-TuC, which localizes to all the MTOCs. To distinguish between these two possibilities, we performed a pull-down assay in mzt1-T27W and mzt1-D24Q mutant cells that contained GFP-Alp4. If the latter scenario was true, interaction between Alp4 and γ-tubulin would be abolished at 36°C. As shown in Figure 8A, it was found that Alp4 was still able to form a complex with γ-tubulin, and indeed, quantification of the amount of γ-tubulin pulled down with GFP-Alp4 was not lessened in either of the mzt1 mutants incubated at the restrictive temperature (Figure 8B). From this result, we favor the notion that Mzt1 is not required for assembly of the γ-TuC, but instead it is specifically needed for the γ-TuC to be recruited and/or tethered to the SPB.

**DISCUSSION**

In the current study, we show that fission yeast Mzt1 is an essential, stoichiometric component of the γ-TuC, which localizes to all the MTOCs throughout the cell cycle. We further show that Mzt1 is required for γ-TuC localization to the SPB, although not for the assembly of the core γ-TuC. Hence, Mzt1 plays a hitherto unknown unique role among the γ-TuC core components, in that it is specifically required for the recruitment and/or attachment of this complex to the major MTOC site.

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**FIGURE 6:** Two different mitotic defects appear in the mzt1 mutant. (A–C) Mitotic phenotypes of mzt1 ts mutants. Time-lapse live images of mzt1-T27W cells corresponding to each representative phenotype are presented; mitotic cells displaying normal spindle formation and elongation and chromosome segregation (A), type I cells displaying unstable spindle microtubules (B), and type II cells exhibiting the lagging, chromosome-like phenotype with apparently normal morphologies of microtubules (C). mzt1-T27W mutant cells containing Hht1-CFP (histone H3, chromosomes, top) and mCh-Atb2 (MTs) and Sid4-mRFP (SPBs) (middle) were grown at 27°C; this was followed by a temperature shift up to 36°C. Merged images are presented on the bottom (green for chromosomes and red for MTs and SPBs). Cells were imaged starting from 2 h later. Scale bar: 10 μm. (D) Classification and quantification of mitotic phenotypes. The data of two mzt1 mutant alleles (mzt1-E10P and -T27W) are shown, type I cells exhibit unstable spindle microtubules, whereas type II cells show the lagging, chromosome-like phenotype.

Results obtained from this experiment indicated that the amount of Alp4 (the γ-TuC) at the mitotic SPBs, which is also proportional to microtubule intensity, correlates with the phenotypic appearance of mzt1-T27W cells (Figure 7G). Importantly, this analysis also demonstrated that Mzt1 is critical for the recruitment and/or retention of the core γ-TuC to the mitotic MTOC. We conclude that Mzt1 is required for the attachment of the γ-TuC to the MTOCs.

**Mzt1 is dispensable for the formation of the core γ-TuC**

Results thus far show that Mzt1 is a constitutive component of the γ-TuC that plays a crucial role in the recruitment and/or retention of the γ-TuC to the MTOCs. We next asked in which step Mzt1 plays a decisive role in γ-TuC function. Two scenarios immediately arise, although they are not mutually exclusive. In one scenario, Mzt1 is a recruiting factor of the γ-TuC to the MTOCs. In the other scenario, Mzt1 is required for complex formation or maintaining the integrity of the γ-TuC, a prerequisite for γ-TuC localization to interphase and mitosis by 83–93% upon 2-h incubation at the restrictive temperature (Figure 9C). It is noted that the degree of reduction was almost indistinguishable between Mzt1 and Alp4. Previous work showed that this mutation leads to the disappearance of Alp4 from the SPB when incubated at the restrictive temperature (Venkatram et al., 2004). The alp6-719 mutants containing GFP-Mzt1 or GFP-Alp4 were each constructed (with mCh-Atb2 and Sid4-mRFP), and the fluorescence intensities of Mzt1 and Alp4 were quantified upon incubation at 36°C. As shown in Figure 9, A and B, it was evident that the intensities of both GFP-Alp4 and GFP-Mzt1 declined noticeably upon 1-h incubation and were further reduced after 2-h incubation.

Quantification of GFP signal intensities confirmed that Alp4 and Mzt1 levels at the SPB were substantially reduced during both interphase and mitosis by 83–93% upon 2-h incubation at the restrictive temperature (Figure 9C). It is noted that the degree of reduction was almost indistinguishable between Mzt1 and Alp4. This result demonstrated that Mzt1 localization to the SPB requires structural integrity of the core γ-TuC. Taking these results together, we consider that, in fission yeast, only the core γ-TuC consisting of Alp4, Alp6, Mzt1, and γ-tubulin can localize to the SPB.
The structural composition of the $\gamma$-TuC within individual MTOCs

In plants and possibly some animal cells, the component composition of the $\gamma$-TuC is not uniform and may vary within the individual MTOCs (Kollman et al., 2011; Nakamura et al., 2012). Our results presented in this study support the notion that, instead, in fission yeast, four essential proteins, Mzt1/MOZRT1, Alp4/GCP2, Alp6/GCP3, and $\gamma$-tubulin, are constitutive components of all the MTOCs, including the SPB, eMTOC, and iMTOC (Figures 2 and 3). However, we could not rule out the possibility that the composition of the $\gamma$-TuC might in fact vary due to the heterogeneity of the other non-essential components (Gfh1/GCP4, Mod21/GCP5, and Alp16/GCP6), which is a proposal that has been previously discussed (Choi et al., 2010; Kollman et al., 2011).

Lower abundances of these three GCPs compared with the core components (−0.1; Fujita et al., 2002) render this investigation technically difficult. In terms of protein quantities, the $\gamma$-TuC contains approximately equal amounts of Mzt1 and Alp4/GCP2 (Figure 2). This substantiates the notion that Mzt1 is a constitutive, stoichiometric component of the $\gamma$-TuC.

Phenotypic variations arising from defects in Mzt1 and $\gamma$-TuC integrity

Characterization of the mzt1 ts mutants indicated that Mzt1 is required for recruitment/attachment of the $\gamma$-TuC to the MTOCs. Furthermore, the quantitative level of $\gamma$-TuC retained at these sites in the mutants appears to be a critical determinant for the varied phenotypes that emerge. During interphase, we have observed a significant reduction in the cytoplasmic microtubule number in the mzt1 ts mutants that is accompanied by the substantial decrease of the $\gamma$-TuC from the SPB, iMTOC, and eMTOC (Figure 5). The residual $\gamma$-TuC at the MTOCs may be responsible for low-level nucleation of cytoplasmic microtubules. On the other hand, two different phenotypes arose during mitosis (Figure 6). Type I mutant cells that retained $\sim$10% of $\gamma$-TuC levels at the SPB could not form stable bipolar spindle microtubules, resulting in arrest during mitosis at the restrictive temperature. Type II cells that contained $\sim$20% of $\gamma$-TuC levels at the SPB proceeded through mitosis, retained $\sim$20% of $\gamma$-TuC almost completely from the SPB, and proceeded through mitosis (Figure 7). Our previous work has shown that alp4-1891 mutants that lost the $\gamma$-TuC almost completely from the mitotic SPB exhibited the emergence of monopolar spindles (Vardy and Toda, 2000; Vardy et al., 2002), although, interestingly, a
modest population of alp4-1891 cells gave rise to the type I phenotype (Masuda et al., 2006b). By contrast, if -30% of Alp4 was retained, cells managed to undergo normal mitosis, despite cytoplasmic microtubule defects (Figures 5 and 7).

Given the appearance of these different phenotypes, we propose that, during both interphase and mitosis, the γ-TuC regulates multiple aspects of microtubule organization and functions ranging from microtubule nucleation, assembly of proper mitotic spindles, and attachment of the plus end of spindle microtubules to the kinetochores. The emergence of the lagging, chromosome-like phenotype with apparently normal morphologies of spindle microtubules is intriguing; this type of mitotic defect was not reported in other γ-TuC components (Vardy and Toda, 2000; Vardy et al., 2002; Hendrickson et al., 2001; Masuda et al., 2006b), except for one particular mutant allele of γ-tubulin (gtb1-PL302) during mitosis (Paluh et al., 2000) and meiosis (Tange et al., 2004). As the densities of spindle microtubules in this type of cells decrease (Figure 7), failure to maintain proper attachment of the kinetochores to spindle microtubules could be attributed to the reduced number of microtubule plus ends. An alternative possibility, although not mutually exclusive, is that the γ-TuC, in particular Mzt1, may play a direct or indirect role in the regulation of microtubule dynamics at the plus end, which is an issue that remains unresolved in the field (Zimmerman and Chang, 2005; Bouissou et al., 2009; Anders and Sawin, 2011; Tanaka et al., 2012).

**Roles of fission yeast MOZART1 in γ-TuC structure and function**

The requirement of Mzt1 for γ-TuC localization to the SPB raised an intriguing possibility that Mzt1 may play a specific role in the attachment of the γ-TuC to this MTOC site independent of interaction with the other γ-TuC components (Figure 7). However, subsequent experiments using alp6-719 ts mutants (Vardy and Toda, 2000; Venkatram et al., 2004) clearly indicated that Mzt1 (and Alp4) localization to the SPB was substantially impaired in this mutant (Figure 9). Accordingly, we deem Mzt1 to be capable of attaching to the SPB only when it forms a stable γ-TuC with other components (Figure 10A). By contrast, Alp4/GCP2 still interacts normally with γ-tubulin in the absence of Mzt1 function (Figure 8), indicating that Mzt1 plays no major roles in the formation of a core γ-TuC structure (Figure 10, B and C). It is of note that human MOZART1 and plant GIP1 also play a critical role in γ-tubulin localization to the MTOCs (Hutchins et al., 2010; Janski et al., 2012), suggesting that the molecular role of MOZART/Mzt1 in the recruitment of the γ-TuC to the MTOC is conserved through evolution.

It is known that two long coiled-coil SPB components, Mto1 and Pcp1, act as specific attachment factors for interphase cytoplasmic MTOCs (Mto1) and mitotic nuclear SPBs (Pcp1) (Sawin et al., 2004; Venkatram et al., 2004; Zimmerman and Chang, 2005; Fong et al., 2010). Mto1 and Pcp1 belong to a conserved centrosomal/SPB protein family, the members of which include human CDK5RAP2, CG-NAP, and pericentrin; fly Centrosomin; and budding yeast Spc110, which are all known to play a crucial role in γ-TuC attachment to the centrosome/SPB (Takahashi et al., 2002; Zimmerman et al., 2004; Sawin et al., 2004; Zhang and Megraw, 2007; Fong et al., 2008; Choi et al., 2010). Although these proteins physically interact with the γ-TuC, they are not regarded as integral γ-TuC components and localize to the centrosome/SPB independent of the γ-TuC. It is shown that a conserved motif found within these members, called CM1/CNN1/γ-TuNA, is responsible for binding to the γ-TuC (Zhang and Megraw, 2007; Samejima et al., 2008; Barr et al., 2010; Choi et al., 2010). However, the precise molecular details regarding the interaction between CM1 and the γ-TuC remain to be determined. It is possible that Mzt1 is spatially exposed toward the outer interface when incorporated into the γ-TuC, thereby being capable of interacting as a bridge with the CM1 motif of these SPB/centrosomal proteins.

It has been suggested that γ-TuC attachment to the MTOCs alone may not suffice for its ability to nucleate microtubules; these two events may rely on different mechanisms, yet they are regulated coordinate (Kollman et al., 2010). It was recently further proposed that, for the γ-TuC to promote microtubule nucleation, a specific conformational alteration within GCP3 (i.e., the straightening of an internal hinge) needs to be induced (Guillet et al., 2011). The currently proposed structural model of either γ-TuSC or γ-TuRC does not include MOZART1 (Kollman et al., 2011). Given that the MOZART1 orthologues, unlike the other GCPs, do not contain the γ-tubulin-binding GRIP 1 and 2 domains, that plant MOZART1/GIP1 directly binds GCP3 (Janski et al., 2008, 2012; Nakamura et al., 2012); and finally, that Mzt1 and Alp4/GCP2 are present in roughly equal quantities (Figure 2), it would be tempting to speculate that direct stoichiometric binding of MOZART1 to GCP3 results in allosteric changes within this GCP, which not only leads to the recruitment of the γ-TuC to the SPB but also is involved in promotion of microtubule nucleation, possibly via interaction with Mto1/Pcp1 attachment factors.

**MATERIALS AND METHODS**

**Strains, media, and genetic methods**

All fission yeast strains used throughout this study are listed and described in Supplemental Table S1. Fission yeast cells were grown and maintained in standard conditions as described by Moreno et al. (1991). Serial-dilution spot tests were carried out by spotting
A novel γ-tubulin complex protein

Isolation of temperature-sensitive mzt1 mutants

The detailed experimental scheme for isolating mzt1 ts mutants is summarized in Supplemental Figure S2. The PCR primers used are listed in Supplemental Table S2. Briefly, the template DNA for mutagenizing PCR containing the mzt1 upstream region of 640 base pairs, the mzt1 gene, the hphR cassette, and the downstream region of 330 base pairs (URS-mzt1-hphR-DRS; Figure S2A) was constructed by fusion PCR, using PCR products amplified from Schizosaccharomyces pombe genomic DNA and pCR2.1-hph (Sato et al., 2005). Primers for site-directed mutagenesis were designed to replace the target amino acids randomly with others (Figure S2B). The following conserved residues, E10, D24, E42, E50, and T27L28S29 (Figure 1A), were specifically targeted for mutagenesis. The DNA fragments containing the mutagenized mzt1 gene and the hphR cassette were obtained by fusion PCR (Figure S2C). The S. pombe cells (HR1758, Table S1) were transformed at 27°C with the DNA fragments on plates containing YE5S rich media and hygromycin B (0.3 mg/ml). The colonies showing temperature-sensitive growth at 36ºC were isolated, and the mutations on the targeted amino acids were confirmed by nucleotide sequencing of the mzt1 gene (Figure S2D).

Fluorescence microscopy, time-lapse live-cell imaging, and quantification

Fluorescence microscopy images were obtained using the DeltaVision microscope system (Applied Precision, Seattle, WA) with a cooled charge-coupled device camera CoolSNAP.HQ.

10-fold serial dilutions from cells at a concentration of $2 \times 10^7$ cells/ml onto plates containing either rich YESS media or rich media with added phloxine B (7.5 μg/ml). The plates were incubated at the necessary temperature within the range of 27 to 36°C.

Nucleic acids preparation and manipulation

Enzymes were used as recommended by the suppliers (New England Biolabs, Ipswich, MA; and Takara Bio, Europe, Saint-Germain-en-Laye, France).

Gene disruption and the N-terminal and C-terminal epitope tagging

A PCR-based gene-targeting method (Bähler et al., 1998) was used for complete gene disruption and epitope tagging (GFP, 3HA, mCherry) in the C- or N-terminus under the endogenous promoter. The mzt1 gene was deleted in diploid cells with the clonNAT-resistance marker gene (natR; Sato et al., 2005).

For N-terminal GFP tagging of Mzt1, the hphR cassette from pCR2.1-hph (Sato et al., 2005) was first integrated into the position at 100 base pairs upstream of the mzt1 ORF. The resultant strain (HR2296) grew normally, indicating that insertion of the cassette did not affect Mzt1 function. Using the genomic DNA isolated from this strain and a GFP-encoding vector DNA as templates, we used PCR to construct the DNA fragment containing the hphR cassette, its 500–base pair upstream region, the 99–base pair 5′-upstream of GFP-mzt1 (mzt1 promoter region), GFP-mzt1, and the 500–base pair 3′-downstream, and this fused PCR product (hphR-GFP-mzt1) was used for transformation to replace the authentic mzt1 gene.

For construction of strains carrying GFP-Alp4 and mCherry-Alp4, the promoter region of alp4 was PCR-amplified and inserted at the BamHI site between a selection marker kanR and GFP of pCSS25 (a gift of Yuji Chikashige, Advanced ICT Research Institute, Kobe, Japan) or mCherry of pCSS25-mCh (constructed by replacing GFP of pCSS25 with mCherry). The alp4 gene was replaced with kanR-GFP-alp4 or kanR-mCherry-alp4, using the vectors as templates for PCR-based gene targeting.
Alp4/GCP2, Alp6/GCP3, and Mzt1/MOZART1) are required for MTOCs; instead, all the four formed. Mzt1, on its own, is not sufficient to localize to the SPB/MTOCs. (C) In the absence of Alp6 function, γ-tubulin attachment to the MTOCs is still possible of assembling; however, this complex is unable to be recruited to the SPB/MTOCs. Second, Mzt1 is required for the recruitment of the γ-TuRC to the MTOCs. For simplicity, nonessential γ-TuC components (Gfh1/GCP4, Mod21/GCP5, and Alp16/GCP6) are omitted from this figure. Of note is that it is currently unknown as to how Mzt1 interacts with the core γ-TuC and the SPB/MTOCs; however, plant MOZRT1 is shown to directly bind to GCP3 (Janski et al., 2008, 2012; Nakamura et al., 2012). (B) In the absence of Mzt1 function (mzt1 mutants), the core γ-TuC is still capable of assembling; however, this complex is unable to be recruited to the SPB/MTOCs. (C) In the absence of Alp6 function (alp6-719 mutant), on the other hand, the core γ-TuC fails to be formed. Mzt1, on its own, is not sufficient to localize to the SPB/MTOCs; instead, all the four γ-TuC components (γ-tubulin/GCP1, Alp4/GCP2, Alp6/GCP3, and Mzt1/MOZART1) are required for complex recruitment to the SPB/MTOCs.

FIGURE 10: A proposed model for Mzt1 function. (A) In wild-type cells (WT), Mzt1 forms a stable, stoichiometric complex with the other core components (γ-tubulin/GCP1, Alp4/GCP2, and Alp6/GCP3) of the γ-TuC. It is essential for cell viability and γ-TuC attachment to the SPB/MTOCs. With regard to Mzt1’s molecular functions, we envision the following two, albeit not mutually exclusive, scenarios. First, Mzt1 facilitates γ-TuRC formation, which leads to the recruitment/attachment of this complex to the MTOCs. Second, Mzt1 is required for the recruitment of the γ-TuC and/or γ-TuRC to the MTOCs. For simplicity, nonessential γ-TuC components (Gfh1/GCP4, Mod21/GCP5, and Alp16/GCP6) are omitted from this figure. Of note is that it is currently unknown as to how Mzt1 interacts with the core γ-TuC and the SPB/MTOCs; however, plant MOZRT1 is shown to directly bind to GCP3 (Janski et al., 2008, 2012; Nakamura et al., 2012). (B) In the absence of Mzt1 function (mzt1 mutants), the core γ-TuC is still capable of assembling; however, this complex is unable to be recruited to the SPB/MTOCs. (C) In the absence of Alp6 function (alp6-719 mutant), on the other hand, the core γ-TuC fails to be formed. Mzt1, on its own, is not sufficient to localize to the SPB/MTOCs; instead, all the four γ-TuC components (γ-tubulin/GCP1, Alp4/GCP2, Alp6/GCP3, and Mzt1/MOZART1) are required for complex recruitment to the SPB/MTOCs.


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