Fission yeast Alp14 is a dose-dependent plus end–tracking microtubule polymerase

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ABSTRACT XMAP215/Dis1 proteins are conserved tubulin-binding TOG-domain proteins that regulate microtubule (MT) plus-end dynamics. Here we show that Alp14, a XMAP215 orthologue in fission yeast, Schizosaccharomyces pombe, has properties of a MT polymerase. In vivo, Alp14 localizes to growing MT plus ends in a manner independent of Mal3 (EB1). alp14-null mutants display short interphase MTs with twofold slower assembly rate and frequent pauses. Alp14 is a homodimer that binds a single tubulin dimer. In vitro, purified Alp14 molecules track growing MT plus ends and accelerate MT assembly threefold. TOG-domain mutants demonstrate that tubulin binding is critical for function and plus end localization. Overexpression of Alp14 or only its TOG domains causes complete MT loss in vivo, and high Alp14 concentration inhibits MT assembly in vitro. These inhibitory effects may arise from Alp14 sequestration of tubulin and effects on the MT. Our studies suggest that Alp14 regulates the polymerization state of tubulin by cycling between a tubulin dimer–bound cytoplasmic state and a MT polymerase state that promotes rapid MT assembly.

INTRODUCTION Microtubules (MTs) are dynamic polymers of αβ-tubulin dimers that play diverse functions in processes such as cell division, intracellular transport, and cellular morphogenesis. Many of the dynamic behaviors of MTs are regulated by proteins at the MT plus end (Akhmanova and Steinmetz, 2008). Although many such proteins have now been identified, in most cases, little is known about how these proteins regulate the MT at a mechanistic level. The conserved XMAP215/Dis1 family of proteins is emerging as key regulators of MT assembly. Many proteins in this family localize to MT plus ends and to sites such as kinetochores and centrosomes and play essential roles in regulating MT dynamics for chromosome segregation. In vitro studies suggest that the recombinant XMAP215 (Xenopus orthologue) is a processive MT polymerase that accelerates the rate of MT assembly while bound at the growing MT plus ends (Brouhard et al., 2008). XMAP215 proteins also have inhibitory effects on MTs under in vitro conditions where soluble tubulin is absent, although the in vivo relevance of this activity remains to be established (Shirasu-Hiza et al., 2003; van Breugel et al., 2003; Brouhard et al., 2008). Important questions remain about the molecular mechanisms of how the XMAP215/Dis1 family localize to growing MT plus ends and function to promote MT assembly.

A key feature of XMAP215/Dis1 family is their ability to bind to soluble αβ-tubulin dimers through conserved tumor overexpressed...
gene (TOG) domains at their N-termini (Al-Bassam et al., 2007; Al-Bassam and Chang, 2011; Slep and Vale, 2007). TOG domains form paddle-like structures composed of helical HEAT repeats and bind to a αβ-tubulin heterodimer in solution using conserved intra-HEAT repeat loops (Al-Bassam et al., 2007). The budding yeast orthologue Stu2p is a homodimer that contains two sets of two TOG domains, whereas mammalian and fly orthologues are monomers and contain five TOG domains. Stu2p (dimer) and XMAP215 (monomer) bind a single tubulin dimer, and electron microscopy studies suggest that the TOG domains wrap around a tubulin dimer to form a globular complex (Al-Bassam et al., 2006; Brouhard et al., 2008). TOG domains are critical for XMAP215 MT polymerase activity (Al-Bassam et al., 2006; Brouhard et al., 2008; Currie et al., 2011). Point mutations inactivating XMAP215 TOG domains suggest that a decrease in the affinity of molecules for tubulin dimer correlate with a decrease in the ability to promote MT assembly in vitro (Widlund et al., 2011). How these TOG domains function in MT polymerase and plus end tracking activities, however, is still poorly understood. It is debated, for instance, whether TOG domains bind to tubulins in the MT lattice or only to soluble tubulin dimers, and whether TOG domains target these proteins for MT plus end tracking. The in vivo relevance of the in vitro observations of XMAP215 to other family members remains to be established.

The fission yeast Schizosaccharomyces pombe is a genetically tractable model organism useful for studying conserved aspects of MT regulation (Sawin and Tran, 2006; Bratman and Chang, 2008). Interphase MTs are organized in three to five bundles oriented along the long axis of these rod-shaped cells. The relatively small number of MTs makes it possible to track and quantitatively measure the dynamics of the MT plus ends in vivo. S. pombe contains two XMAP215/Dis1 orthologues: Dis1 and Alp14. Null mutants of each single gene are viable, but the double mutant is lethal (Garcia et al., 2001). Previous studies on these genes focused largely on their mitotic functions, especially at the kinetochore (Nabeshima et al., 1995; Garcia et al., 2001; Hsu and Toda, 2011). Alp14 interacts with Alp7, an orthologue of transforming acidic coiled-coil proteins, and its nuclear localization is cell cycle regulated by the Ran GTPase system (Sato et al., 2004; Sato and Toda, 2007). A recent study suggests that not all proteins in this family act the same: Dis1 localizes to regions of MT bundling in the spindle midzone and interphase arrays and may contribute to MT bundling and not to interphase MT dynamics (Roque et al., 2010).

Here we study the molecular activities of S. pombe Alp14 using in vivo and in vitro approaches. In vivo, Alp14 localizes to growing MT plus ends, and null mutants of alp14 exhibit short MTs with decreased MT assembly rates and frequent MT pauses. In vitro total internal reflection fluorescence (TIRF) microscopy studies show that recombinant Alp14 tracks MT plus ends and accelerates assembly by twofold to threefold. Mutational analyses show that the N-terminal TOG domains, which bind to tubulin dimer, and the C-terminal region, which binds to the MT lattice, all contribute to MT plus end localization and polymerase activity. In the course of our studies, we discovered that Alp14 activities are highly dose dependent both in vivo and in vitro. Mild (threefold) overexpression of Alp14 or even of just its TOG domains causes dramatic MT loss in vivo, whereas increasing Alp14 concentration in vitro decreases efficiency of its MT assembly activity. Our findings suggest that the balance between Alp14 and free tubulin concentrations is critical for regulation of MT assembly.

RESULTS

Alp14 tracks MT plus ends in vivo independently of other plus end–tracking proteins.

We examined Alp14 localization in living fission yeast cells using a functional Alp14–green fluorescent protein (GFP) fusion that is expressed at near endogenous levels from the alp14 chromosomal locus (Sato et al., 2004). During mitosis, Alp14-GFP localized to multiple dots on the mitotic spindle and spindle pole bodies, as seen previously (Nakaseko et al., 2001; Sato et al., 2004; Figure 1A). In interphase cells, Alp14-GFP localized to cytoplasmic dots. These moved in a linear manner, mostly from the cell center toward the cell tips (see also Nakaseko et al., 2001; Sato et al., 2004). Dual imaging of Alp14-GFP with red fluorescent protein (RFP)-labeled tubulin (Snuth et al., 2010) confirmed that these Alp14 dots track with growing MT plus ends (Figure 1C and Supplemental Movie S1). Alp14 was also sometimes detected at a very low level on the MT lattice. We did not detect any Alp14 accumulation on the plus ends of shrinking MTs. Multiple Alp14 dots within each MT bundle may represent MT plus ends growing within each MT bundle. Alp14-GFP dots largely colocalized with other MT plus end–tracking proteins (termed +TIPs) Mal3 (EB1) or Tip1 (CLIP-170; Figure 1B; Beinhauer et al., 1997; Busch and Brunner, 2004).

Next we tested whether Alp14 localization at growing MT plus ends is dependent on other +TIP proteins. EB proteins are believed

![FIGURE 1: Alp14 tracks MT plus ends in S. pombe cells. (A) Images of fission yeast cells expressing Alp14-GFP fusion protein (FC1907). Alp14 is seen as cytoplasmic dots aligned in linear tracks in interphase cells. In mitotic cells, it is present in bright dots on the mitotic spindle. (B) Alp14 colocalization with other +TIP proteins. Images of cells (FC2347, FC2336) coexpressing Alp14-RFP with Mal3-GFP (EB1) or Tip1-GFP (CLIP-170). (C) Images of cells expressing Alp14-GFP and mRFP-Atb2 (tubulin) in wild-type (FC2493), tea2∆ (FC2471), and mal3∆ (FC2472) backgrounds. Kymographs were derived from images of MT bundles marked by arrowheads. Streaks of Alp14-GFP show plus end tracking of Alp14-GFP in these strains. Scale bar, 5 μm. Timescale bar, 60 s.](image-url)
to recruit many +TIPs to MT plus ends, including the Msps (Drosophila XMAP215 orthologue; Honnappa et al., 2009; Akhmanova and Steinmetz, 2011; Currie et al., 2011). In S. pombe, Mal3 (EB1) may load the kinesin Tea2 onto the MT, which in turn transports Tip1 (CLIP-170) to the MT plus end (Busch et al., 2004; Bieling et al., 2007). We found that Alp14-GFP still localized at MT plus ends in mal3Δ (EB1) and tea2Δ (kinesin) mutant cells, even though the MTs themselves were abnormally short (Figure 1C and Supplemental Movie S2). Thus Alp14 may not be strictly dependent on these other +TIPs for MT plus end tracking.

**alp14Δ** mutants exhibit decreased MT assembly rate and increased frequency of pauses

To test the role of Alp14 on MT dynamics in vivo, we characterized **alp14Δ**-null mutant cells. As shown previously, these cells were viable but grew slowly and formed abnormally bent cell morphologies, similar to other mutants with defective MTs (Figure 2A; Nakaseko et al., 2001; Sawin and Tran, 2006).

To observe the dynamics of MTs, we imaged wild-type and **alp14Δ** cells carrying a GFP-tubulin construct driven by the SV40 promoter (Bratman and Chang, 2007; Snaith et al., 2010) using time-lapse spinning disk confocal microscopy. The number of MT bundles was reduced roughly twofold in **alp14Δ** cells (one to two MT bundles per cell) compared with wild-type cells (three to five bundles per cell; Figure 2B; Tran et al., 2001). MTs were abnormally short on average and exhibited premature catastrophes before reaching the cell tips. The dynamics of MTs were analyzed using kymographs to measure changes in MT length relative to speckle-like fiduciary marks in the MT lattice (Figure 2C; Tran et al., 2001; Janson et al., 2007). This analysis revealed an average twofold decrease in MT assembly rates in **alp14Δ** cells relative to wild-type cells (Figure 2D). MT assembly rates were confirmed by using Mal3-GFP as a marker for growing MT plus ends (Figure 2F). We also found a twofold decrease in MT disassembly rates in **alp14Δ** cells. MTs spent about half as much time in a state of assembly, without a change in the time in disassembly, as compared to wild-type MTs. Of note, MTs spent a significant amount of time in a paused state, which we defined as rate of MT length change <0.5 μm/min (Figure 2E); MTs spent 47% of time in pausing but not at cell tips and an additional 20% of time in pausing at cell tips. This pause phenotype is similar to effects seen with knockdown of the Drosophila orthologue Msps (Brittle and Ohkura, 2005;
Currie et al., 2011). Close examination of the kymographs showed that MT assembly profiles were sometimes jagged, suggestive of short periods of MT assembly punctuated by frequent pauses or catastrophe-rescue transitions (Figure 2C). This may be similar to nanoscale excursions seen with MT ends in vitro (Scheik et al., 2007; Gardner et al., 2011). Thus these analyses show that alp14Δ mutants have a significant defect in persistent, rapid MT assembly.

We tested whether Alp14 affects MT dynamics through effects on the localization of other +TIPs. Some other +TIP mutants, such as mal3Δ (EB1), tip1Δ (CLIP-170), or tea2Δ (kinesin) mutants, also exhibit short MTs (Beinhouzer et al., 1997; Browning et al., 2000; Brunner and Nurse, 2000). We found, however, that Mal3, Tip1, and Tea1 still localize properly to the growing MT plus ends in alp14Δ mutants (Supplemental Figure S1, C–E). Thus the MT plus end localization of Alp14 and these other +TIPs are independent of each other.

**Estimating Alp14 concentration and number of molecules at MT plus ends**

We sought to measure the concentration of Alp14 molecules in the cell. We derived estimates by comparing the fluorescence intensity of cells expressing Alp14-GFP to cells expressing GFP fusion proteins that had been counted previously (Wu and Pollard, 2005; Joglekar et al., 2008; Coffman et al., 2011). We found that Alp14-GFP in wild-type cells, which is expressed from its endogenous promoter as the only Alp14 in the cell, is ∼0.6 μM (∼10,000 molecules/cell), as it has a similar concentration as the myosin II regulatory light chain (Rlc1, estimated at 9600 molecules/cell; Wu and Pollard, 2005; Supplemental Figure S2A). We regard these estimates with caution, especially as they depend on the accuracy of the standards used.

Total tubulin dimer concentration in S. pombe has been estimated to be 3–5 μM, with roughly one-third polymerized, based on quantitative fluorescence imaging and biochemical approaches (P. Tran, personal communication). Thus these initial estimates indicate that the concentration of Alp14 dimer (see later discussion) is ∼10% of tubulin concentration in wild-type cells.

We counted the number of Alp14 molecules on each growing MT plus end by quantifying the fluorescence of individual MT-associated dots. We restricted our analysis to well-isolated, motile Alp14-GFP dots near cell tips. We assumed that the large majority of these associated dots. We restricted our analysis to well-isolated, motile Alp14-GFP dots. We derived estimates by comparing the fluorescence intensity of Alp14 with those of standard proteins are used for comparison (Supplemental Figure S1). The MT plus end localization of Alp14 and these other +TIPs are independent of each other.

Alp14 stimulates MT assembly in vitro

Next we examined the molecular activities of Alp14 in vitro. We purified recombinant full-length Alp14 by using baculovirus expression in insect cells. Recombinant Alp14 is a single species on size exclusion chromatography with a large apparent molecular weight. The large apparent molecular weight of Alp14 suggests that it has an elongated conformation (Figure 5 and Supplemental Figure S3). Alp14 formed stable complexes with soluble tubulin dimer, with a 2:1 binding stoichiometry, similar to Saccharomyces cerevisiae Stu2 protein (Al-Bassam et al., 2006). Using sedimentation equilibrium analytical ultracentrifugation, we showed that Alp14 is a homodimer and that Alp14–tubulin complexes contain a single tubulin dimer per Alp14 dimer, as measured by mass differences between Alp14–tubulin complex and Alp14 protein (Supplemental Figure S3).

We analyzed Alp14 activity on dynamic MTs using an assay based upon total internal reflection fluorescence (TIRF) microscopy (Figure 3; Brouhard et al., 2008; Al-Bassam et al., 2010). Biotin- and Texas red–labeled, GMPCCP-stabilized MT seeds were attached onto fresh silanized glass surface via anti-biotin antibodies. Dynamic MTs were polymerized from the MT seeds in the presence of 6 μM tubulin dimer (5.4 μM tubulin plus 0.6 μM Alexa 488–labeled tubulin). This relatively low concentration of tubulin in our TIRF assays is near its physiological concentration in fission yeast (P. Tran, personal communication). Using TIRF microscopy, we imaged the dynamics of individual MTs grown from MT seeds. Parameters of MT dynamics (assembly and disassembly rates, maximal MT length, and catastrophe frequency) were measured in 40–110 individual MTs at each Alp14 concentration, and average values for each parameter were determined by fitting Gaussian distributions to raw data histograms (Supplemental Table S1 and Supplemental Figure S4).

Moderate concentrations of recombinant Alp14 accelerated MT assembly rates to a maximal of threefold, from 0.45 to 1.2 μm/min at 100 nM Alp14 (Figure 3, Supplemental Table S2, and Supplemental Figure S4). Maximal MT length also increased to a maximal of twofold at 100 nM Alp14. However, at this concentration range, Alp14 did not affect MT catastrophe frequency or promote MT rescues. The average MT assembly periods did not increase with the addition of Alp14. Increasing Alp14 concentration to 100 nM accelerated MT disassembly rate by −30%, from 35 to 45 μm/min. The average MT disassembly period increased up to twofold with increasing Alp14 concentration, most likely because MTs are generally longer.

At higher concentrations (200–1000 nM), rather than accelerate MT assembly rates further, Alp14 promoted MT assembly less effectively (Figure 3, Supplemental Table S1, and Supplemental Figure S4). Dynamic MTs were extremely short and grew very slowly. Assembly rates were similar to or slightly slower than those observed in assays with tubulin alone. In other conditions, high concentrations of Alp14 completely inhibited MT assembly (Figure 3H; see Materials and Methods).

We considered whether the inhibitory effects of Alp14 at high concentrations are caused by free Alp14 sequestering tubulin dimers. To test this, we asked whether adding higher concentrations of tubulin would ameliorate these inhibitory effects. As expected, MT assembly rates were higher at 8 and 10 μM tubulin as compared with 6 μM (Walker et al., 1988). At 200 nM, Alp14 increased MT assembly rates by roughly twofold at each tubulin concentration (Figure 3H and Supplemental Figure S2). However, higher Alp14 concentrations (250–500 nM) inhibited MT assembly at these tubulin concentrations, although the inhibitory effects were somewhat blunted at higher tubulin concentrations. We then used these data to calculate tubulin dimer association rates (Brouhard et al., 2008). In the absence of Alp14 the tubulin dimer association rate was 4.0 μM/min (Figure 3I), similar to what was previously reported (Brouhard et al., 2008). At 200 nM Alp14, the association rate increased to 6.4 μM/min, whereas at 300 nM Alp14, tubulin association rate was less efficient (4.8 μM/min) and similar to that in the absence of Alp14 (Figure 3I). The effects of Alp14 and tubulin concentration on MT assembly did not correlate in a stoichiometric
FIGURE 3: Alp14 is moderate MT polymerase at low concentrations in vitro. (A) Scheme for TIRF microscopy assay used to study MT dynamics. Surface-attached anti-biotin antibodies (blue) specifically bind biotin-labeled, GMPCPP-stabilized MT seeds (red). The MT seeds near the surface nucleate Alexa Fluor 488–labeled (green) dynamic MT from 6 μM soluble tubulin dimers in the presence of GTP. TIRF illumination by 564- and 488-nm lasers. Image adapted from Al-Bassam et al. (2010). (B) TIRF image of growing MTs. Seeds (red) initiate the assembly of dynamic MTs (green) only at their plus ends (+). In any of the experiments, no assembly occurred at the minus ends (−) in the presence of 6 μM tubulin dimer concentration. (C) Kymographs showing the assembly and disassembly of single dynamic MTs. At 6 μM tubulin, dynamic MTs show slow assembly, rapid disassembly, and frequent catastrophes. At 20–100 nM Alp14, MT assembly rate is incrementally increased, as seen in the slopes of dynamic MT kymographs (broken arrows). The MT assembly rate (at 300–1000 nM) becomes slower, indicating that Alp14 promotes inefficient MT polymerase at the higher concentrations. (D) Effect of Alp14 on the dynamic MT assembly rate. Right, Alp14 accelerates MT assembly by threefold at low (0–100 nM) concentration. At higher concentrations, MT assembly is dramatically decreased. Each point (see also Supplemental Table 1) represents the mean of a Gaussian fit to a distribution of a large number of assembly events measured for each Alp14 concentration (distributions shown in Supplemental Figure S4). (E) Increasing Alp14 concentration causes a moderate increase in MT disassembly rates (Supplemental Table 1). Right, Alp14 increases MT disassembly rate moderately by 25% at the low concentration. Each point (Supplemental Table 1) represents an average Gaussian
manner. For instance, increasing tubulin concentration by 2 μM did not suppress inhibitory effects of increasing Alp14p by 50 nM (a 40-fold difference). Thus these data are not consistent with a simple Alp14 tubulin-sequestering mechanism and suggest that more complex mechanism(s)—for instance, effects at the MT plus end—might underlie these inhibitory effects.

**Alp14 tracks growing MT plus ends in vitro**

Next we imaged the localization of fluorescent Alp14 molecules in vitro. We purified recombinant Alp14 fused to a C-terminal eGFP (Alp14-GFP) from insect cells using baculovirus expression. We added 25 nM purified recombinant Alp14-GFP to dynamic MTs grown from 6 μM tubulin in which the seed and dynamic portion of MTs were labeled with different concentrations of Texas red–labeled tubulin, leading to intensely labeled MT seeds and weakly labeled dynamic MTs (Figure 4). We observed Alp14-GFP tracking many of the growing MT plus ends. The intensity of Alp14-GFP signal, however, varied among different growing MTs even in the same field. We observed Alp14-GFP dots suddenly appearing at a MT plus end, suggesting that Alp14 binding to MT plus ends may be cooperative. Alp14-GFP molecules also associated with the MT lattice at a lower level and formed dim particles that moved along the lattice in diffusive-type movement; we observed Alp14-GFP particles on the lattice apparently merging with the more intense Alp14 dots at MT plus ends (Figure 4, C, arrow, and D, arrowheads). During catastrophe events, in contrast to Mal3 (EB1), which disappears from the plus end well before MT shrinkage (Busch and Brunner, 2004; Maurer et al., 2012), Alp14 remained associated with MT plus ends until MT shortening ensued and then disappeared from shrinking plus ends (Figure 4, C and D). The variability in Alp14-GFP binding allowed us to test whether Alp14 promotes MT assembly locally. The total distribution of all dynamic MT assembly events with Alp14-GFP had a very similar distribution to the MT assembly rate observed with 20 nM untagged Alp14. However, MTs plus ends with Alp14-GFP signal at plus ends grew at roughly twofold-higher MT assembly rate than MT plus end without Alp14-GFP throughout their assembly period (Figure 4B). There was no effect on other MT dynamic parameters observed (Figure 4E, Supplemental Figure S5, and Supplemental Table S1). These data show that the localization of Alp14-GFP molecules promotes MT plus end assembly locally.

**Alp14 activity requires TOG domain–tubulin binding**

Alp14 has two conserved N-terminal TOG domains, TOG1 and TOG2. To test the function of these TOG domains, we characterized an alp14 mutant protein (Alp14-TOG1,2) in which TOG1 and TOG2 tubulin-binding sites were inactivated by mutating conserved residues at predicted TOG–tubulin-binding interface loops (W23A, R109A in TOG 1 domain; W300A, K381A in TOG2 domain; Al-Bassam et al., 2007). Size exclusion chromatography showed that recombinant Alp14-TOG1,2 had a similar apparent molecular weight as wild-type Alp14, but it did not bind tubulin dimer and did not shift the tubulin into a higher–molecular weight complex (Figure 5A). In vitro, the addition of Alp14-TOG1,2 (20–100 nM) did not accelerate MT assembly rate or increase the average MT length distribution (Figure 5B). Increasing Alp14-TOG1,2 concentration did not increase MT disassembly rate. These results suggest the Alp14 MT polymerase activity requires TOG–tubulin dimer binding.

We next examined the function of the Alp14 TOG domains in vivo. We expressed fragments or full-length alp14 mutants as mCherry fusions in an alp14Δ background and assayed for their ability to rescue the alp14Δ phenotype by time-lapse imaging of GFP-labeled MTs (Figure 6). Because the effects of these alp14 constructs might be highly dosage dependent, we assayed for rescue in a broad range of expression levels and induction times (see later discussion). Expression of a wild-type alp14* gene rescued the MT phenotype of alp14Δ mutants, as determined by the appearance of a wild type–like interphase MT cytoskeleton. Cells with mCherry-Alp14 exhibited significantly more MT bundles/cell than a vector control. The mCherry-Alp14 construct localized to MT plus ends as well as the MT lattice; the lattice staining of this construct was more apparent than in the GFP fusion, possibly because of slight differences in expression levels. An N-terminal Alp14 fragment (Alp141-509, TOG domains only) did not rescue and localized only diffusely in the cytoplasm. A C-terminal Alp14 fragment (Alp14510-805, deletion of TOG domains) did not rescue either but localized to the MT lattice. No specific MT plus end accumulation was seen with the C-terminal Alp14 fragment, although it was difficult to rule out that some portion of these proteins localized to the plus ends in addition to the MT lattice. Alp14-TOG2 and Alp14-TOG12 mutants showed no rescue, whereas the Alp14-TOG1 mutant showed a partial but significant rescue. The Alp14-TOG1 and TOG2 mutants localized to MT plus ends and lattice, whereas the double Alp14-TOG1,2 mutant exhibited only weak MT lattice localization. These data indicate that the TOG domains are necessary but not sufficient to provide Alp14 function and MT plus end localization and that the Alp14 C-terminus is necessary and sufficient for MT lattice association.
FIGURE 4: Alp14-GFP tracks growing MT plus ends in vitro. (A) Schematic of the TIRF microscopy assay used for imaging MT dynamics and for simultaneous imaging of dynamic MTs and Alp14-GFP localization. Anti-biotin antibodies (blue) bind biotin- and Texas red–labeled, GMPCPP-polymerized MT seeds (red) near the silanized glass surface. The densely labeled Texas red MT seeds nucleate dynamic MT assembly from 6 μM tubulin dimers, less densely labeled with Texas red, in the presence of GTP and 25 nM Alp14-GFP (see Materials and Methods). Image adapted from Al-Bassam et al. (2010). (B) Raw TIRF image of dynamic MTs and Alp14-GFP. Top, dynamic MTs growing at plus ends of MT seeds. Note that MT seeds have a high fluorescence intensity compared with the dynamic portion of the MTs, which are less intensely labeled with Texas red. Middle, Alp14-GFP. Bottom, overlay showing Alp14-GFP at MT plus ends; note that only some MTs display plus end localization. (C) Montage of Alp14-GFP tracking a growing MT plus end. In successive (10 s/frame), the MT plus end assembles while Alp14-GFP molecules track MT plus ends (broken arrow). Weaker GFP signals attributed to single Alp14-GFP molecules (white arrowheads) diffuse along MT lattices and may accumulate at the plus end. Plus end-tracking Alp14-GFP dissociates and disappears upon MT catastrophe and disassembly (white broken arrow). (D) Kymographs of Alp14-tracking dynamic MT plus ends. Left kymograph, Alp14 tracks a growing MT plus end and then dissociates upon activation of MT catastrophe. As MT plus end assembly reinitiates, Alp14 signal reappears midway through the MT assembly event, and then disappears with MT catastrophe. Middle, MT growing with faint Alp14 signal at the growing MT plus end (top, arrowhead), which then suddenly becomes more intense (lower, arrowhead) and disappears upon the occurrence of MT catastrophes. Right kymograph, three successive MT assembly events from a single MT seed. In the first and third MT assembly events, little or no Alp14-GFP tracking localization is observed at MT plus ends (broken arrow). The second event shows MT plus ends with strong Alp14-GFP localization (white arrowheads). Note that Alp14-GFP molecules bind along the MT seed and diffuse along the lattice over time (arrowhead). (E) Twofold acceleration in MT assembly rate correlates with Alp14-GFP tracking along plus end MTs. Top, histogram and a Gaussian fit (blue) of all MT assembly events observed with 25 nM Alp14-GFP, showing an intermediate, half-maximal MT assembly rate similar to the distribution observed at 20 nM Alp14 (Supplemental Figure S5). Middle, histogram distribution (green bars) and Gaussian fitting (green line) of Alp14-GFP tracking MT assembly events have a high MT assembly rate. Bottom, histogram distribution (red bars) and Gaussian fitting (red line) of MT assembly events without Alp14 tracking shows lower MT assembly rates. The full MT dynamic parameters for each sets of events are shown in Supplemental Figure S5.
Overexpression of Alp14 leads to TOG-dependent loss of microtubules

We noted in these rescue experiments that some alp14Δ cells overexpressing Alp14 exhibited an unusual phenotype of complete MT loss. We thus overexpressed a functional mCherry-Alp14 fusion from a thiamine-regulatable nmt41 promoter on a multicopy plasmid in alp14Δ cells expressing GFP-tubulin. On induction of Alp14 expression, many of the cells lost all GFP-marked MTs (Figure 7A, cell 1). Immunofluorescence staining with anti-tubulin antibody confirmed that these cells, indeed, lacked MTs (Supplemental Figure S6). This complete loss of MTs was notable, as it is a stronger phenotype than treatment with MT-inhibitory drugs (e.g., MBC), which leaves a few stable stubs, or any known null mutants of any single MT-regulatory protein (Tran et al., 2001). Other cells in the population exhibited short MTs or MTs of normal length.

To test whether variability in Alp14 expression levels is responsible for the variable MT phenotypes, we measured the fluorescence intensity of mCherry-Alp14 (or mutant versions of Alp14) in each cell. Cells exhibited highly variable levels (>10-fold variability) of mCherry fluorescence, which is likely to be due to the variable copy number of the plasmids in different cells, as well as variability in protein expression. As a general trend, cells with high levels of Alp14 expression lacked MTs (Figure 7A, cell 1). Intermediate levels were associated with short MTs (Figure 7A, cell 2), and low levels were associated with normal MTs (Figure 7A, cell 3). However, there was some overlap—for instance, between the Alp14 levels giving short or no MTs. Quantitative estimates based on fluorescence intensity measurements of individual cells suggested that, generally, levels of Alp14-mCherry of >2 μM (greater than threefold of wild-type levels) corresponded to a MT-loss phenotype, whereas levels of 1–2 μM (around twofold) corresponded to a short-MT phenotype (Figure 7C).

We tested whether this MT-loss phenotype was dependent on the TOG domains. The MT-loss phenotype was not seen when Alp14 TOG1,2 mutant protein or the C-terminal fragment was overexpressed (Figure 7, B and C). Overexpression of Alp14-TOG1 or Alp14-TOG2 still led to MT loss. Expression of just the TOG domains was equally effective as the full-length Alp14 protein. Expression of the Alp14 C-terminal domain or the Alp14-TOG1,2 mutant produced cells with short MTs similar to that of an alp14Δ mutant; it is possible that these proteins confer a dominant-negative effect by dimerizing or competing with the wild-type Alp14 protein. Expression of these constructs in an alp14Δ background led to very similar effects. Thus the ability of Alp14 to inhibit MTs is dependent on its ability to bind to tubulin dimers.

We tested the possibility that Alp14 causes MT depolymerization by activating a MT depolymerase, the kinesin-8 heterodimer Klp5/Klp6 (West et al., 2001; Grissom et al., 2009). Interactions of XMAP215 orthologues with kinesin-8 as well as the kinesin-13 (MCAK) have been found in other cell types (Tournebize et al., 2000; Cassimeris and Morabito, 2004; Gandhi et al., 2011). In fission yeast, alp14Δ is synthetically lethal with klp5Δ or klp6Δ (Garcia et al., 2002). Overexpression of Alp14 in a klp5Δ background, in which the klp5/6 heterodimer is inactive, still led to MT loss (Supplemental Figure S7). Thus the inhibitory effect of Alp14 is not dependent on Klp5/Klp6 kinesin-8.

These data suggest that as in vitro, Alp14 overexpression also causes MT inhibitory effects in vivo. Our in vivo results are consistent with a tubulin sequestration mechanism, as the overexpression of Alp14 to 2 μM would bind ~25% of the total tubulin in the cell (assuming total tubulin concentration is 4 μM). However, it is possible that more complex mechanism(s) also contribute, as suggested by our in vitro data.
**FIGURE 6:** Effect of Alp14 domains on alp14 complementation and localization in vivo. (A) mCherry-Alp4 fusion constructs were expressed from an nmt42 promoter (medium strength) on multicopy plasmids in alp14Δ GFP-tubulin fission yeast cells (FC2484-FC2489). Cells were shifted to inducing conditions (media without thiamine) and examined by time-lapse imaging at multiple time points from 16 to 24 h. Cells with low but detectable level of mCherry proteins were assayed. Table shows a summary of results. Red bars show location of point mutations in the TOG domains. Mutations are: W23A, R109A in TOG1; W300A, K381A in TOG2; Alp14 1-509 in N-term; Alp14 510-809 in C-term. (B) Images of representative fields of alp14Δ cells expressing the indicated construct. mCherry images show protein localization to MT lattice, MT plus ends, and/or cytoplasm. MT images show phenotypes in which interphase cells with an alp14Δ-like phenotype (not rescued) have a small number of dim bundles, whereas cells with a wild type–like phenotype (rescued) have robust MT bundles. Scale bar, 5 μm. (C) Numbers of interphase MT bundles/cell were counted as a measure of Alp14 rescue by these Alp14 constructs (n > 30). These were consistent with other indications of Alp14 activity, including fluorescence intensity of GFP-tubulin in MT bundles and MT dynamics. * p < 0.01, *** p < 0.0001 on t-test compared to empty vector.

**DISCUSSION**

**Alp14 is a conserved MT polymerase**

We show here that S. pombe Alp14 is a MT polymerase that localizes to the MT plus end and accelerates MT assembly rate two to threefold. In vivo, Alp14 localizes to growing plus ends of interphase MTs, and alp14 null mutants exhibit abnormally short interphase MTs with twofold decreased assembly and disassembly rates and increased frequency of pausing. We reconstituted activities of Alp14 on dynamic MTs in vitro: purified Alp14 molecules track growing MT plus ends, increase their assembly rate by about threefold, and increase MT disassembly rate slightly (by 30%), without affecting catastrophe or rescue frequencies. These general properties are consistent with those reported for some other XMAP215/Dis1 family members, such as XMAP215 (Gard et al., 2004; Brouhard et al., 2008). Although some orthologues have been studied in vitro and others in vivo, a strength of this work is that we show that the activities of Alp14 in vitro are generally quantitatively consistent with its effects in vivo. Together, these studies provide strong evidence that Alp14 acts as a MT polymerase that promotes faster MT assembly and inhibits MT pausing in the cell. In addition, our quantitative studies show that Alp14 activities are highly dose dependent and exhibit inhibitory effects at higher concentrations both in vitro
Tracking growing MT plus ends

Alp14 and its orthologues from the XMAP215/Dis1 family define a subclass of MT plus end–tracking proteins distinct from EB-dependent proteins. The majority of known plus end–tracking proteins are believed to be dependent on EB proteins for their localization to growing MT plus ends; EB proteins likely recruit these diverse proteins to the plus end by binding a Ser-X-Gly-Pro sequence (Honnappa et al., 2009; Akhmanova and Steinmetz, 2011). EB proteins have been found to be necessary for the Drosophila orthologue Msps for MT plus end localization in vivo (Currie et al., 2011). These in vivo assays are, however, complicated by the fact that depletion of the EB may inhibit Msps localization indirectly by affecting MT dynamics. In contrast, fission yeast Alp14 does not require other +TIP proteins such as Mal3 (EB1) for its plus end localization in vivo, and indeed purified Alp14 molecules track growing plus ends in vitro, as seen also with purified XMAP215 (Brouhard et al., 2008). EB proteins may recognize a conformation of polymerized tubulin regulated by nucleotide binding state (Maurer et al., 2011, 2012). XMAP215 has a much slower residence time at the MT than EB1 proteins in vitro, suggesting that they may use different mechanisms for plus end localization (Bieling et al., 2007; Brouhard et al., 2008). It will be interesting to determine whether XMAP215/Alp14 proteins and EB proteins recognize similar or different structural features of a growing MT plus end. In this study we roughly estimate that ~100 Alp14 molecules are present on the MT plus end, higher than the predicted ≤13 molecules (Brouhard et al., 2008), suggesting that only a small fraction of the Alp14 at plus ends may be actively functioning at any point.

TOG domains and tubulin binding

Our biochemical analysis demonstrates that Alp14 is a homodimer that binds a single tubulin dimer via its TOG domains, much like the budding yeast orthologue Stu2 with which Alp14 shares 20% of its overall sequence identity (Al-Bassam et al., 2006). The binding of tubulin by the TOG domains is critical for Alp14 protein function, as with other TOG-domain proteins (Al-Bassam et al., 2007, 2010; Al-Bassam and Chang, 2011). How TOG domains regulate tubulin dynamics at the MT end, however, remains unclear and controversial (Slep and Vale, 2007; Al-Bassam and Chang, 2011). TOG domains may, for instance, release their bound tubulin dimers into the MT lattice for polymerization; alternatively, the soluble tubulin dimer clutched by multiple TOG domains may not be released but act

and in vivo, suggesting that Alp14 has additional, more complex roles in regulating the polymerization state of tubulin (see later discussion).
to transiently stabilize the MT lattice or other tubulin polymerization at the plus end (Brouhard et al., 2008; Al-Bassam and Chang, 2011). In another model TOG domains bind and present tubulin oligomers to a growing MT plus end (Kerssemakers et al., 2006); this, however, is not consistent with the observed saturation stoichiometry of tubulin to XMAP215, Alp14, or Stu2 proteins in solution (Al-Bassam et al., 2006; Brouhard et al., 2008; Widlund et al., 2011). As TOG domains contribute to plus end localization, they may also bind to tubulin in exposed protofilaments at the growing plus end. One possibility is that the two TOG1,2 domains not only clutch a free tubulin dimer, but also bind to an exposed tubulin dimer at the growing lattice to position the Alp14 complex on the growing MT end. Comparison of observations with fission yeast XMAP215 and CLASP orthologues (Alp14 and Cls1) makes it clear that TOG domains from these two families are functionally different; for instance, overexpression of the Cls1 TOG domains hyperstabilizes MTs (Bratman and Chang, 2007), whereas overexpression of Alp14 TOG domains destabilizes MTs in vivo. TOG domains may therefore dictate the different activities of these proteins.

Our mutational analysis of the Alp14 TOG domains begins to reveal functional differences between its two TOG domains. Inactive mutations in the tubulin-binding loops of the TOG1 domain had only mild effects on MT polymerase activity, whereas mutations in TOG2 domain or in both TOG domains produced a null-like phenotype. In overexpression assays, the single TOG1 and TOG2 mutants still retained ability to depolymerize MTs, whereas the Alp14-TOG1,2 mutant was inactive, suggesting that the single TOG domains can have some activity by themselves. In contrast in Stu2, TOG1 but not TOG2 is sufficient for tubulin binding and is critical for activity in vivo (Al-Bassam et al., 2006). Sequence differences in the tubulin-binding loops in the TOG domains among XMAP215/Dis family members may account for some functional differences between family members (Slep, 2010; Al-Bassam and Chang, 2011).

The C-terminal domain of Alp14 is necessary and sufficient to bind to the MT lattice. In analogy to Stu2, a Ser-Lys-rich (SK-rich) domain in this region following the TOG domains (Alp14 residues 520–673) is likely to be responsible for this lattice-binding activity (Al-Bassam et al., 2006; Al-Bassam and Chang, 2011). Multiple lattice-binding domains have been found in Drosophila MspS in some linker regions between TOG domains (Currie et al., 2011). A recent study of the Xenopus orthologue XMAP215 shows that a minimal protein consisting of TOG domains and an MT-binding domain from another protein is capable of MT end tracking and polymerase activity, suggesting that the only critical function of this C-terminal domain is in MT lattice binding (Widlund et al., 2011). Alp14 (and orthologues) may initially bind and diffuse on the MT lattice through its C-terminal domain and then accumulate in a cooperative manner at the MT plus end through TOG-dependent interactions with the growing MT plus end (Brouhard et al., 2008).

Dose-dependent inhibitory effects of Alp14

One puzzling aspect of several XMAP215/Dis1 proteins is that under certain conditions, they act as inhibitors of MT formation. In vivo, a threefold increase in Alp14 leads to complete loss of MTs. In vitro, similarly high concentrations of Alp14 cause a decreased MT assembly rate and decreased Alp14 MT polymerase activity. These inhibitory effects are reminiscent of previous findings showing that XMAP215 and Stu2 proteins depolymerize or inhibit MT assembly in vitro when assayed in the absence of soluble tubulin dimer, using nonhydrolyzable GTP analogue, GMPCPP-stabilized MTs (Shirasu-Higa et al., 2003; van Breugel et al., 2003; Brouhard et al., 2008). Brouhard et al. (2008) found that purified XMAP215 associates with shrinking MT plus ends and promotes MT disassembly in the absence of tubulin. However, high concentrations of XMAP215 do not inhibit MT assembly. It has been proposed that in the absence of tubulin, XMAP215 catalyzes MT assembly in reverse, to actively promote disassembly (Brouhard et al., 2008). In this work we observe a strong inhibition of MT assembly when Alp14 concentration is increased. Thus inhibitory effects are seen not only in the absence of soluble tubulin, but also when the ratio of Alp14 to tubulin is high.

One way that Alp14 inhibits MT assembly may be through tubulin sequestration. In vivo, overexpression of Alp14 inhibits MTs at concentrations of ~2 μM, which would be predicted to sequester a significant amount of free tubulin in the cell. This activity is dependent on TOG-tubulin binding activity, and the TOG-domain fragment alone, which does not accumulate on MTs, is sufficient. Genetic analyses suggest that Alp14 does not depolymerize MTs using the Klp5/6 (kinasin-8) depolymerase. Purified Alp14 also has an inhibitory activity in vitro, although at concentrations (>250 μM) well below free tubulin concentrations. We show that simply adding more tubulin does not ameliorate this inhibitory effect, suggesting that more complex inhibitory mechanisms besides tubulin binding and sequestration might be in play. Thus these effects of increasing Alp14 concentration are likely to affect the equilibria of one or more of the multiple steps of Alp14 interacting with the MT. For instance, it is possible that high concentrations cause too many Alp14 molecules to accumulate at the growing MT ends and somehow interfere with MT assembly. The sharp increase in inhibitory activity (from 200 to 250 μM Alp14) is suggestive of a cooperative response. Alp14 could also inhibit MTs by having weak MT depolymerase activity. Consistent with this, we also observe a small twofold decrease in MT disassembly rates in alp14A cells in vivo and a corresponding but smaller (30%) increase in disassembly rates in vitro. In contrast to XMAP215 and MspS, however, we do not detect Alp14 on the ends of shrinking MTs either in vivo or in vitro.

In conclusion, our studies reveal complex roles of Alp14 in regulating the tubulin dimer–to–MT polymer equilibrium. It might not only promote MT assembly but also contribute to regulation of MT disassembly, as well as binding to a significant pool of free tubulin dimer. The highly dose-dependent effects we observed suggest that Alp14 activity and expression may be carefully regulated in the cell. Further study of Alp14 and how it cycles between its multiple states on and off the MT will provide a framework for understanding novel aspects of MT regulation.

MATERIALS AND METHODS

Yeast strains and growth

S. pombe strains used in this study are listed in Supplemental Table S3. Standard methods for media and genetic manipulation were used (see Pombenet at http://www.bcf.usc.edu/~forsburg/references.html). Deletion strains and strains with integrated tags or promoters were constructed by PCR and homologous recombination. For induction of Alp14 expression from the nmt42 promoter, cells were first grown in liquid culture in the presence of thiamine and then washed three times and grown for 16–24 h at 30°C in minimal media lacking thiamine. For induction of mRFP-Atb2 (α-tubulin 2; pSB62), cells were prepared as described, but for 22–24 h incubation without thiamine.

Plasmids and oligonucleotides used in this study are listed in Supplemental Tables S4 and S5. For mutagenesis of alp14, QuickChange Site-Directed Mutagenesis Kit (Strategene, Santa Clara, CA) was used. alp14 wild-type and various mutant constructs were cloned into pREP42X-mCherry backbone plasmid (PSB67) at SalI/Xhol and Xmnl restriction enzyme sites.
Protein expression and purification

Constructs of full-length S. pombe alp14, alp14-GFP, alp14 TOG,1,2 (residues 1–809) genes were cloned by ligation-independent cloning into a modified baculovirus expression vector, in-frame with an N-terminal histidine (His)–maltose-binding protein tag (MBP) flanked by a TEV protease cleavage site to improve protein expression and solubility. Protein expression was carried out by infecting 2 l of Hi5 insect cells at a density of 1 × 10⁶ cells/ml with the amplified baculoviruses. After 60–72 h, insect cells were harvested and resuspended in lysis buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], 300 mM KCl, 10 mM imidazole, pH 7.0) and lysed with a Dounce homogenizer. lysates were clarified by centrifugation at 60,000 × g for 40 min. His-MBP–tagged fusion proteins were isolated by metal affinity chromatography (NTA-agarose; Qiagen, Valencia, CA) and eluted using a 10–400 mM imidazole linear gradient in lysis buffer. Amylose resin (New England BioLabs, Ipswich, MA) was used to purify the proteins using MBP affinity and was eluted with 20 mM Maltose. The N-terminal His-MBP tags were cleaved overnight with 5 U/ml TEV protease and 1 mM dithiothreitol. Alp14, Alp14-GFP, or TOG12-Alp14 constructs were dialyzed against binding buffer (50 mM HEPES, 100 mM KCl, 5 mM β-mercaptoethanol, pH 7.0), loaded onto a MonoS anion exchange column, and eluted using a gradient of 100–700 mM KCl. Alp14–, Alp14-GFP–, or Alp14-TOG1,2–containing fractions were concentrated and loaded onto a Superdex 200 gel filtration column (16/60) equilibrated with binding buffer containing 400 mM KCl and eluted in 1-ml fractions.

Hydrodynamic and mass analyses

Tubulin dimers were purified from bovine brains using the standard approaches (Mitchison and Kirschner, 1984). For size exclusion chromatography, Alp14 and TOG12-Alp14 constructs were allowed to form complexes with various amounts of tubulin dimer for 5 min and were loaded onto a 10/5 Superdex 200 column pre-equilibrated at 4°C with 25 mM HEPES, 200 mM KCl, and 1 mM ethylene glycol tetraacetic acid, pH 7.0, and eluted in 0.5 ml fractions. Fractions were evaluated by SDS–PAGE. Apparent molecular masses and Stokes radii of the Alp14 alone and in complex with tubulin dimer were determined by calibrating the size exclusion column with protein standards (Bio-Rad, Hercules, CA). Stokes radii for different samples were calculated as averages of the Porath and Laurent–Killander Stokes radii, which matched closely. For sedimentation equilibrium analytical ultracentrifugation experiments, purified fractions of Alp14 and Alp14-tubulin complexes were loaded in an Optima XLA (Beckman Coulter, Brea, CA) analytical ultracentrifuge as previously described (Al-Bassam et al., 2006).

In vitro microtubule assays

Total internal reflection microscopy studies of Alp14 with dynamic MTs in vitro were based on a modified version of the approach described by Al-Bassam et al. (2010). Tubulin dimers were labeled with succinimidyl ester or tetrafluoroisopropyl esters of biotin, Alexa Fluor 488, or Texas Red esters (Invitrogen, Carlsbad, CA) and recyled as described in Mitchison and Kirschner (1984). MT seeds were polymerized with 2 mM GMPCPP from a 1.8 mg/ml mixture of Texas red–labeled, biotin-labeled, and unlabeled tubulin dimers in a 4:2:1 ratio at 37°C for 2 h. Glass coverslips were cleaned and silanized as described (Brouhard et al., 2006). Flow cells were constructed as previously described (Al-Bassam et al., 2010). Fully sealed flow cells were mounted onto a Nikon TIRF microscope with a 60× Nikon TIRF lens (Nikon, Melville, NY) warmed to 35°C by a temperature collar. MTs were imaged in the evanescent wave by 488- and 568-nm laser excitation. Dual-emission data were collected in 2.1-s intervals for 18.4 min (1125 s) with a side-entry iXon EM charge-coupled device (CCD; Andor, South Windsor, CT) with each channel projected onto split fields. MT assembly experiments in Figure 3, H and I, were performed using porcine instead of the bovine brain tubulin used for the other experiments. Some subtle differences in the degree of MT growth at high Alp14 concentrations (250–1000 nM) may be due to differences in tubulin or Alp14 protein preparations but did not affect our general conclusions.

Image analysis and measurement of MT dynamics

Image stacks were analyzed using the EMBL-ImageJ software (National Institutes of Health, Bethesda, MD). Briefly, raw image stacks were photobleach corrected by normalization of total image intensity to the average intensity of the first image and scaling up the value of bleached images accordingly. Kymographs were used to measure parameters of MT dynamics. Frequencies of MT catastrophe were measured for individual dynamic MTs from each MT seed by dividing the number of events with the duration of MT assembly (for catastrophe). Raw distributions for assembly rates, disassembly rates, catastrophe frequency, and rescue frequencies were fit by single or multiple Gaussian functions to determine the average and error values for each parameter using the program Origin (OriginLab, Northampton, MA) and are shown in Supplemental Figures S4 and S5.

For live cell imaging, fusion yeast cells were imaged on 2% YE agar pads under a coverslip. Microscopy was performed with a spinning disk confocal microscope system (PerkinElmer, Waltham, MA; Nikon; Solamere, Salt Lake City, UT) with an electron-multiplying CCD camera (Hamamatsu, Hamamatsu, Japan). Images were acquired and analyzed with ImageJ and OpenLab software (Imprisvion, PerkinElmer). Kymographs were constructed with the Volume Slicing tool in OpenLab. Dynamic parameters were determined largely from kymographs using MT speckled patterns as fiducary marks to account for movement of the MT bundle. To determine the number of Alp14 molecules in a cell, we compared florescence intensities of cells expressing mCherry-Alp14 with those expressing Rlc1-mCherry or Arc5-mCherry at endogenous levels, as described. Ratios of the intensities of single dots and cell-wide signal were used to estimate the cytoplasmic pool versus particular forms of Alp14 molecules.

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