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Tea4p Links Microtubule Plus Ends with the Formin For3p in the Establishment of Cell Polarity

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Summary

Microtubules regulate actin-based processes such as cell migration and cytokinesis, but molecular mechanisms are not understood. In the fission yeast *Schizosaccharomyces pombe*, microtubule plus ends regulate cell polarity in part by transporting the kelch repeat protein tea1p to cell ends. Here, we identify tea4p, a SH3 domain protein that binds directly to tea1p. Like tea1p, tea4p localizes to growing microtubule plus ends and to cortical sites at cell ends, and it is necessary for the establishment of bipolar growth. Tea4p binds directly to and recruits the formin for3p, which nucleates actin cable assembly. During "new end take off" (NETO), formation of a protein complex that includes tea1p, tea4p, and for3p is necessary and sufficient for the establishment of cell polarity and localized actin assembly at new cell ends. Our results suggest a molecular mechanism for how microtubule plus ends regulate the spatial distribution of actin assembly.

Introduction

Interactions between the actin and microtubule (MT) cytoskeletons are critical for cell morphogenesis and contribute to diverse cellular processes such as cell migration, axon guidance, spindle orientation, and cytokinesis (Rodriguez et al., 2003). MTs have been proposed to contribute to the spatial distribution and remodelling of actin structures. For example, dynamic MTs play instructive roles in actin-dependent growth cone turning during axon guidance (Buck and Zheng, 2002; Dent and Gertler, 2003), in actin-remodeling at adhesion sites (Small and Kaverina, 2003), and in positioning the contractile ring in cytokinesis (Maddox and Oegema, 2003). However, molecular mechanisms of how MTs regulate the actin cytoskeleton remain poorly understood.

In the fission yeast *Schizosaccharomyces pombe*, MTs and actin have distinct roles in the control of cell polarity. *S. pombe* cells are rod shaped and grow in a polarized manner from the cell ends. Immediately after cell division, the daughter cells initially grow in a monopolar manner from the cell end that preexisted before cell division (the old end). After a point in G2 phase, cells initiate growth from the "new end" (the end created by cell division) in a process known as New End Take Off (NETO), so that they grow in a bipolar mode until mitosis (Mitchison and Nurse, 1985). F-actin is organized at sites of cell growth as actin patches, which may function in endocytosis, and actin cables, which function as tracks for the delivery of secretory vesicles and other cargos to growing cell tips. During NETO, these actin structures are reorganized from a monopolar distribution to a bipolar distribution (Marks et al., 1986). Transient depolymerization of actin by Latrunculin A treatment can trigger NETO, suggesting that reorganization of actin or actin-dependent factors contributes to the initiation of polarized growth at the new end (Rupes et al., 1999).

Formins are a conserved family of actin nucleators that have functions in assembly of diverse actin structures (Wallar and Alberts, 2003). The formin-homology (FH) 2 domain nucleates actin filaments and binds to the growing barbed end of actin filaments (Li and Higgs, 2003; Pruyne et al., 2002; Sagot et al., 2002). Actin nucleation activity is regulated by the actin monomer binding proteins profilin and bud6p, which bind to the proline-rich FH1 domain and the C terminus of formins, respectively (Feierbach et al., 2004; Kovar et al., 2003; Li and Higgs, 2003; Moseley et al., 2004; Pruyne et al., 2002; Sagot et al., 2002). The less conserved N-terminal FH3 domain serves to target formins to specific locations in vivo, but generally its molecular function(s) are poorly understood (Kato et al., 2001; Petersen et al., 1998). *S. pombe* has three formins (for3p, cdc12p, and fus1p), which each nucleate a distinct actin structure (Chang et al., 1997; Feierbach and Chang, 2001; Nakano et al., 2002; Petersen et al., 1995). The formin for3p is responsible for the assembly of actin cables in interphase cells and localizes as dots at cell tips (but not MT plus ends) (Feierbach and Chang, 2001; Nakano et al., 2002).

MTs regulate the positioning of growth sites in fission yeast, as mutations that disrupt the biogenesis or the dynamic properties of MTs often result in curved or branched cell shapes (Umesono et al., 1983). During interphase, cytoplasmic MTs are organized from the nuclear envelope in antiparallel bundles that generally orient along the length of the cell. MT plus ends grow toward both cell tips and contact the cortex for about 100 s before shrinking back to the nucleus (Drummond and Cross, 2000; Tran et al., 2001). The characterization of the kelch repeat protein tea1p promises to reveal the molecular mechanisms of how MTs regulate cell polarity. *tea1* mutants were initially identified in genetic screens as mutants with curved or branched cell shapes (Mata and Nurse, 1997; Snell and Nurse, 1994; Verde et al., 1995). Localization studies revealed that tea1p is transported by riding on the growing plus ends of interphase MTs and deposited selectively at cell ends (Behrens and Nurse, 2002; Feierbach et al., 2004; Mata and Nurse, 1997). Tip1p (a CLIP170-like protein)

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and tea2p (a Kip2p-like kinesin) are responsible for concentrating tea1p to the plus ends of MTs (Browning et al., 2003; Brunner and Nurse, 2000), while a prenylated protein, mod5p, may be required to tether tea1p at the cortex (Snaith and Sawin, 2003). Tea1p plays a critical function in the initiation of the second site of growth; the most penetrant phenotype of *tea1*Δ mutants is a failure to initiate growth at a naive cell tip, so that these cells only grow in a monopolar manner (Mata and Nurse, 1997; Verde et al., 1995). Consistent with this growth pattern, actin structures and the formin for3p localize only to the one growing cell tip in *tea1*Δ cells (Feierbach et al., 2004).

A key question remains: how does tea1p regulate polarized growth? Recently, tea1p was found to reside in large-molecular-weight protein complexes with the formin for3p, bud6p, and the CLIP170 tip1p (Feierbach et al., 2004; Glynn et al., 2001), raising the possibility that tea1p somehow regulates formins in the context of these large complexes. However, the functional significance and the molecular bases of these interactions were not known. Here, in a search for tea1p-interacting proteins, we identify a new protein, tea4p, which binds directly to both tea1p and for3p. Tea4p is a MT plus end factor that is deposited with tea1p at the cortex, where it interacts with the formin to regulate cell polarity. Our results suggest that tea4p facilitates the formation of a tea1p-tea4p-for3p complex, which recruits the formin to the new end in order to initiate polarized cell growth and actin assembly for NETO. These studies thus elucidate a mechanism by which MT plus ends can regulate actin assembly at specific sites in the cell.

Results

Identification of Tea4p as a Tea1p-Interacting Protein

We isolated tea1p-interacting proteins by affinity purification of tea1-TAP (Tandem Affinity Purification tag) and identified them by using tandem mass spectrometry (Figure 1A). Known tea1p-interacting proteins, including for3p and tea3p, were found (Arellano et al., 2002; Feierbach et al., 2004). One of the most abundant proteins was a previously uncharacterized SH3 domain-containing protein (SPBC1706.01) that we named tea4p. tea1p, tea3p, and tea4p were found with 80%, 39%, and 24% peptide coverage, respectively (Figure 1B). The closest homolog of tea4p in *S. cerevisiae* is Bud14p (Blast2 e value = $2e^{-07}$), a protein involved in bipolar bud site selection (Cullen and Sprague, 2002; Ni and Snyder, 2001).

We confirmed the interaction between tea1p and tea4p by using coimmunoprecipitation and two-hybrid approaches. We introduced epitope tags on chromosomal copies of tea1p and tea4p and determined that they were functional by cell morphology. Tea1p-HA and tea4p-GFP coimmunoprecipitated in yeast extracts with either an anti-HA or an anti-GFP antibody (Figure 1E). Tea1p and tea4p also showed a strong interaction in the two-hybrid system (Figures 1C and 1D), which allowed us to map the regions of the proteins important for these interactions. The C-terminal half of tea1p was necessary and sufficient to bind tea4p (Figure 1C), while the C-terminal third of tea4p mediated the interaction with tea1p (Figure 1D). Both of these regions contain regions of predicted coiled-coils, raising the possibility that coiled-coil interactions mediate the tea1p-tea4p interaction.

We then showed that tea1p and tea4p bind directly by using bacterially expressed 6His-tagged fragments in in vitro assays. The C-terminal half of tea4p specifically bound to MBP-tea1C, but not to MBP alone (Figure 1F). We also noted that tea1C can dimerize through its coiled-coil region. As in the two-hybrid system, tea4N did not bind to tea1C (Figure 1F). Thus, tea4p binds tea1p directly.

Tea4p Colocalizes with Tea1p at MT Plus Ends and Cell Tips

To examine the localization of tea4p, we imaged cells with functional tea4p-GFP or tea4p-YFP fusions expressed under the endogenous promoter. Tea4p-GFP localized to cell ends and to the plus ends of growing MTs (Figure 2A). Similar to tea1p, tea4p-GFP dots appeared to be deposited at the cell cortex when a growing MT reached the cell end (Movie S1; see the Supplemental Data available with this article online). Tea4p-YFP colocalized precisely with tea1p-CFP both at cell tips and at MT plus ends (Figure 2B).

Tea4p localization was strictly dependent on tea1p. In *tea1*Δ cells, tea4p-GFP was diffuse in the cytoplasm (Figure 2C). The expression levels of tea4p-GFP were, however, not affected (Figure 2D). Tea1p with a truncation of the tea4p-interacting region (tea1Δ200) was deficient in tea4p localization (Figure 2C). While this tea1p mutant protein associates with MT plus ends, it is deficient in cell polarity regulation (Behrens and Nurse, 2002). The cell tip localization of tea4p depends on the same factors necessary for tea1p localization: in *tip1*Δ and *tea2*Δ, tea4p dots were still associated with MTs, but only low amounts were detected at cell tips; in *mod5*Δ cells, tea4p dots were present at the end of MTs, but they failed to be anchored efficiently at the cell tips (Figure 2C and data not shown). Like tea1p, tea4p localization was independent of tea3p and for3p (data not shown; Arellano et al., 2002; Feierbach et al., 2004). Thus, tea1p functions to localize tea4p.

An interaction between tea1p and the CLIP170 tip1p has been shown to concentrate tea1p at the MT plus end (Feierbach et al., 2004). The kelch repeats at the N terminus of tea1p were necessary and sufficient for this interaction (Figure 2E). As this tea1p region is distinct from the C-terminal tea4p-interacting region, tip1p, tea1p, and tea4p may associate in a ternary complex. Consistent with this model, tip1p coimmunoprecipitated with tea4p from *tea1*$^{+}$ yeast extracts, but not from *tea1*Δ yeast extracts (Figure 2F). In contrast, the association of tea1p with tip1p was *tea4* independent (Figure 2G). Therefore, tea1p functions to link tip1p and tea4p in a common complex.

Tea4p Regulates Cell Polarity

*tea4*Δ cells were fully viable, grew at wild-type rates, and displayed morphological defects similar to *tea1*Δ mutants. In contrast to the wild-type rods, *tea4*Δ cells were curved or T shaped and often divided slightly off-center (Figures 3A and 3F). In addition, these cells grew

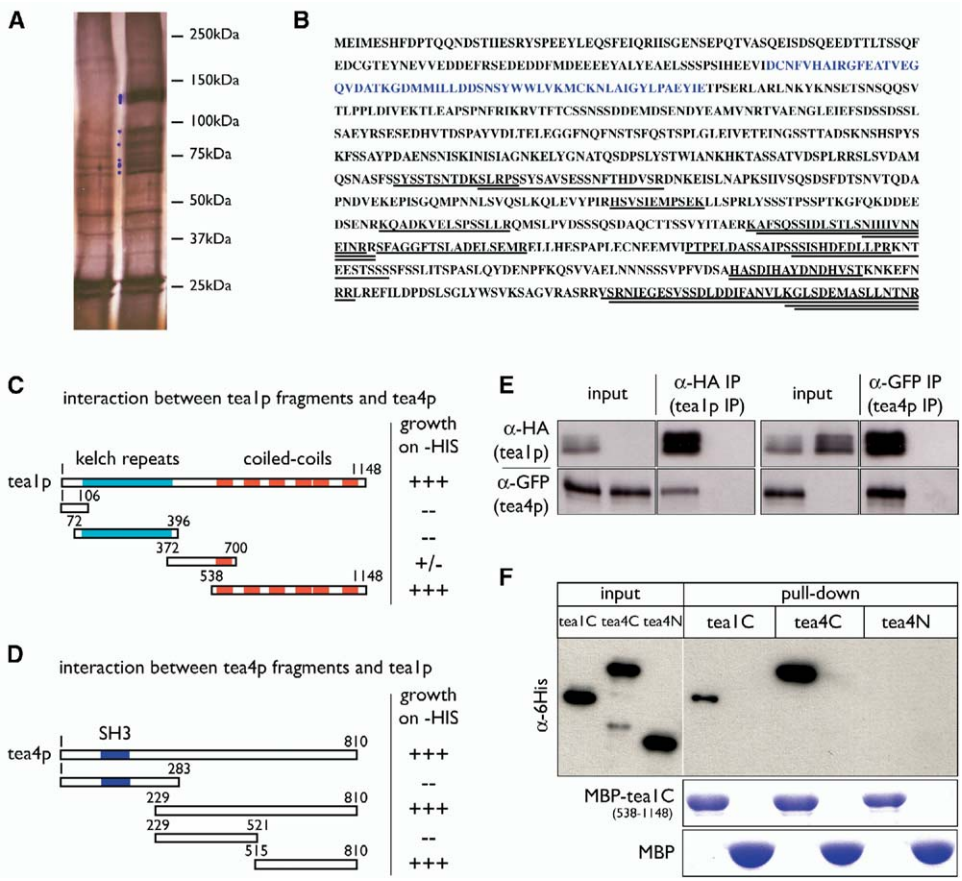

Figure 1. Tea4p Binds Tea1p Directly

(A) Silver-stained SDS-PAGE of IgG immunoprecipitates from extracts expressing endogenous untagged (left) or TAP-tagged tea1p (right). Blue dots mark major protein bands present in the tea1p-TAP immunoprecipitate not present in the control lane.

(B) Predicted protein sequence of tea4p. Peptides identified by tandem mass spectrometry are underlined, and the SH3 domain is highlighted in blue.

(C) Two-hybrid interaction between pGAD-tea4 (full-length) and pGBD-tea1 fragments.

(D) Two-hybrid interaction between pGBD-tea1 (full-length) and pGAD-tea4 fragments.

(E) Coimmunoprecipitation of endogenous tea1p-HA and tea4p-GFP from yeast extracts. The coimmunoprecipitation was performed in both directions (left: anti-tea1p-HA, right: anti tea4p-GFP).

(F) Tea4p binds to tea1p in vitro. Binding of bacterially expressed proteins was assayed by affinity column. 6His-tea1C (aa 538–1148) and 6His-tea4C (aa 229–810), but not 6His-tea4N (aa 1–283), bind to MBP-tea1C, but not MBP alone. 6His-tagged protein fragments were detected by Western blotting with an anti-6His antibody. Coommassie staining of MBP fusions indicates equal loading.

in a monopolar manner through interphase and failed to initiate bipolar growth (>75%, n = 264 septated cells; Figures 3B and 3E).

We assayed for actin organization by staining cells with Alexafluor-phalloidin and also by imaging a type-V myosin. In wild-type bipolar cells, actin patches and cables are present at both cell ends. To examine the origin of actin filaments, we imaged myo52p, a type-V myosin, which moves toward the barbed ends of actin filaments to the sites where actin cables are assembled (Motegi et al., 2001; Win et al., 2001). Accordingly, myo52p-GFP accumulates at growing cell ends, showing a monopolar distribution in pre-NETO cells and a bipolar distribution in post-NETO cells (Figure S1 and data not shown) (Motegi et al., 2001; Win et al., 2001).

*tea4*Δ mutants showed a defect in actin organization, in that they organized actin patches and actin cables at one end of the cell only (Figure 3C). This cell end corresponded to the growing end in these monopolar cells. Consistent with this actin organization, myo52p-GFP cytoplasmic dots moved toward and accumulated at this end (Figure S1 and data not shown). Actin organization at the cell division site was not affected. We also did not detect any significant interphase MT defects in *tea4*Δ mutants: interphase MTs displayed normal organization, polymerization, and catastrophe rates (Figure 3D and data not shown). Therefore, tea4p may regulate the spatial distribution of actin, but not more global aspects of cytoskeletal dynamics.

To compare *tea4*Δ cells with other monopolar mutants (Glynn et al., 2001; Niccoli et al., 2003), we examined patterns of cell growth by time-lapse microscopy (Figures 3F and 3G). *tea4*Δ mutants grew like *tea1*Δ mutants. The large majority of *tea4*Δ daughter cells (90%,

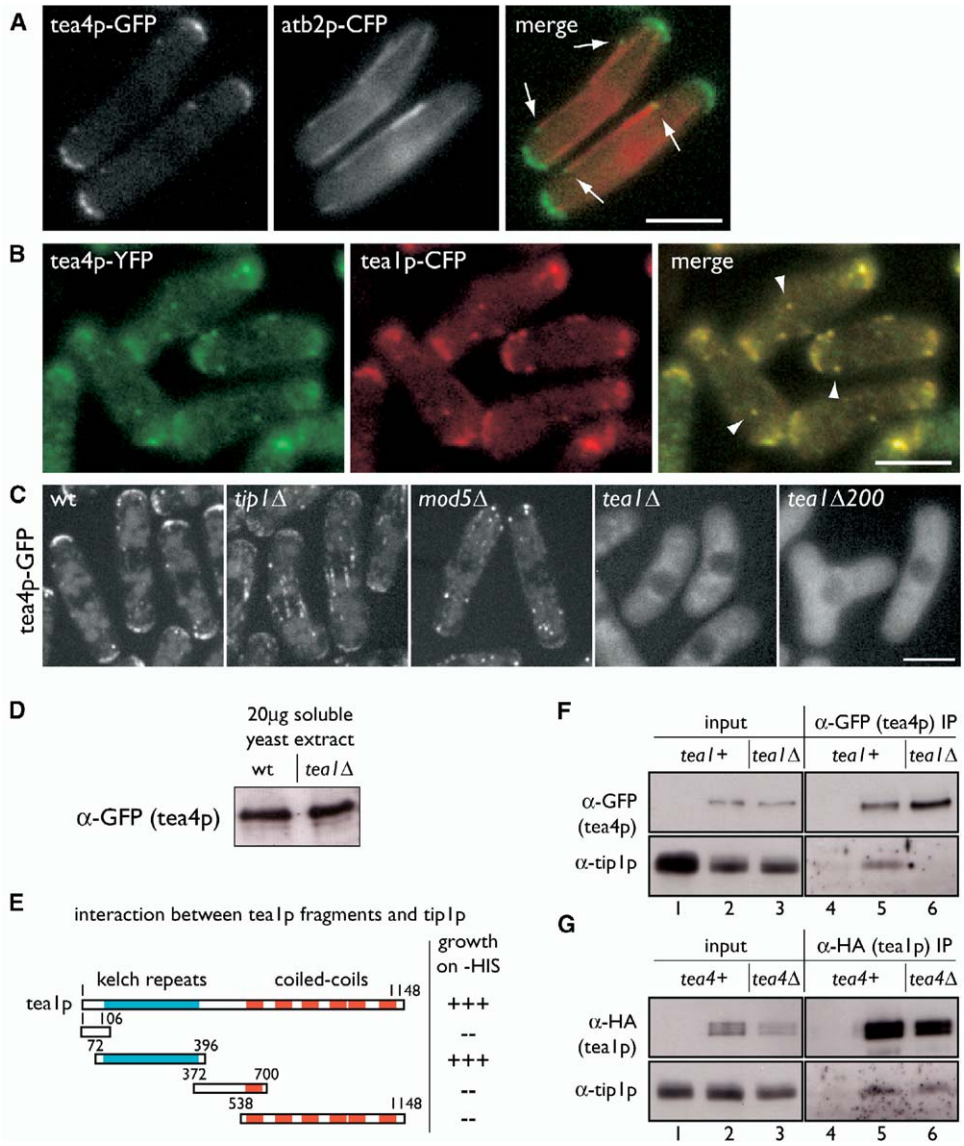

Figure 2. Tea1p Links Tea4p to MT Plus Ends and Cell Tips

(A) Tea4p-GFP localizes to MT plus ends (arrows) and to cell tips. Images of live cells expressing endogenous tea4p-GFP (left; green) and CFP-atb2p (α-tubulin) to label MTs (middle, red).

(B) Tea4p-YFP (green) colocalizes with tea1p-CFP (red) on MT plus ends (arrowheads) and at cell tips. Cells were fixed in methanol for 30 min. Similar observations were made on live cells.

(C) Localization of tea4p-GFP in wild-type and *tip1*Δ, *mod5*Δ, *tea1*Δ, and *tea1*Δ200 mutant live cells, as indicated. Images of wild-type, *tip1*Δ, and *mod5*Δ are maximum intensity projections of multiple confocal sections, while others are single wide-field images. The scale bars indicate 5 μm.

(D) Immunoblot showing expression levels of tea4p-GFP in wild-type and *tea1*Δ cells. 20 μg total proteins was loaded in each lane.

(E) Two-hybrid interaction between pACT-tip1C and pGBD-tea1 fragments.

(F) Tip1p coimmunoprecipitates with tea4p in a *tea1*-dependent manner. Extracts from yeast expressing endogenous untagged tea4p (lanes 1 and 4) or GFP-tagged tea4p (lanes 2 and 5), or from *tea1*Δ cells expressing GFP-tagged tea4p (lanes 3 and 6), were immunoprecipitated with anti-GFP antibodies and immunoblotted with anti-GFP and anti-tip1p antibodies.

(G) The association of tip1p with tea1p is *tea4* independent. Extracts from yeast expressing endogenous untagged (lanes 1 and 4) or HA-tagged (lanes 2 and 5) tea1p, or *tea4*Δ extracts expressing HA-tea1p (lanes 3 and 6), were immunoprecipitated with anti-HA antibodies and immunoblotted with anti-HA and anti-tip1p antibodies.

n = 90) both grew in the same direction: one of the daughter from its old end, the other from its new end. The direction of growth of the daughters usually followed that of the mother cell (17/18 cells). In addition, *tea1*Δ*tea4*Δ double mutants had the same phenotype as the single mutants (data not shown). These results indicate that tea1p and tea4p function in the same pathway.

Tea4p Binds Directly to the Formin For3p

Next, we studied the molecular mechanism of how tea4p regulates cell polarity. Tea1p has been shown

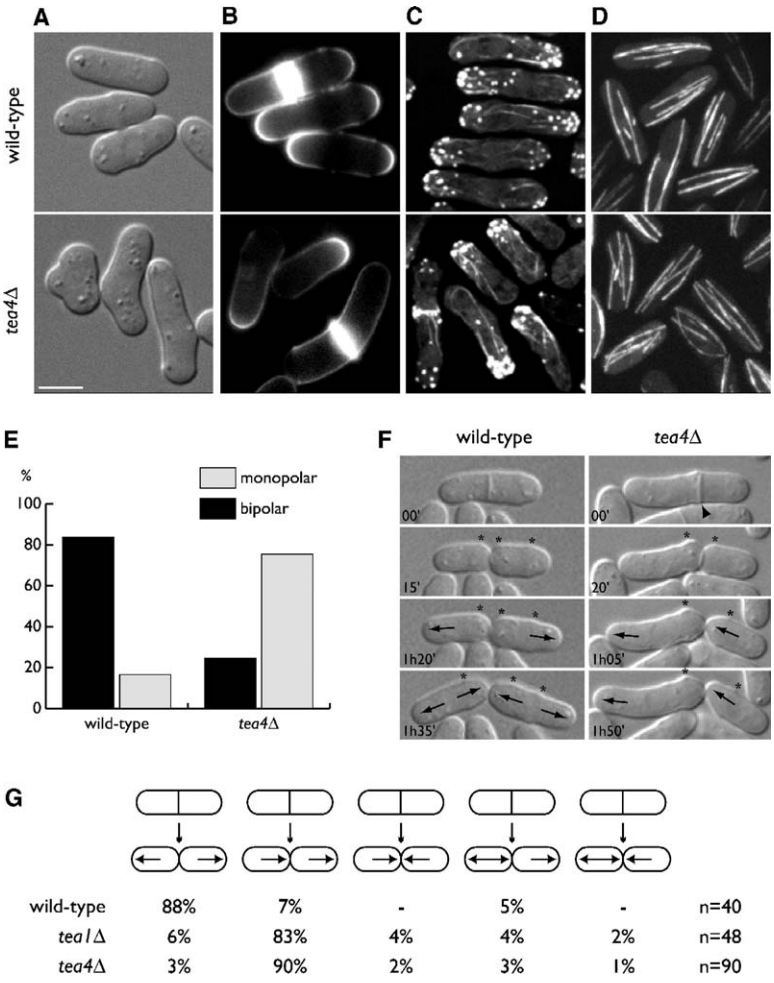

Figure 3. Tea4p Is Required for Bipolar Growth

(A) Differential interference contrast (DIC) images of wild-type (upper panels) and *tea4*Δ cells (lower panels). The scale bar indicates 5 μm.

(B) Cells were stained with calcofluor, a dye which preferentially stains growing regions of the cell wall. Wild-type cells show bipolar growth, while *tea4*Δ cells show monopolar growth.

(C) Alexafluor-phalloidin-stained wild-type and *tea4*Δ cells showing bipolar and monopolar actin organization, respectively.

(D) Wild-type and *tea4*Δ cells expressing GFP-atb2p-GFP to visualize MTs.

(E) Percentages of monopolar and bipolar growth patterns in wild-type (n = 206) and *tea4*Δ cells (n = 264) as examined by calcofluor staining of septated cells.

(F) Time-lapse DIC images of wild-type and *tea4*Δ cells showing different growth patterns. Asterisks denote birth scars. Arrows indicate direction of growth. The arrowhead indicates a slightly off-center septum in *tea4*Δ.

(G) Quantitation of growth patterns of wild-type, *tea1*Δ, and *tea4*Δ cells as measured by time-lapse microscopy.

previously to associate with the formin for3p in large complexes (Feierbach et al., 2004). However, it was not known whether this association is direct and what the function of this interaction is. We tested whether tea4p interacts with for3p. In soluble yeast extracts, for3p-myc coimmunoprecipitated with tea4p-GFP (Figure 4A), indicating that tea4p and for3p are present in the same complex in vivo. Importantly, a bacterially expressed N-terminal fragment of for3p (6His-for3N) was able to bind tea4p in vitro (Figure 4B), showing that this interaction between for3p and tea4p is likely to be direct. In contrast, for3p did not bind to the C-terminal region of tea1p in this assay (Figure 4B), suggesting that the interaction between for3p and tea1p is probably indirect. Furthermore, the interaction between tea1p and for3p in yeast extracts was not present in extracts from *tea4*Δ cells (Figure 4C), showing that tea4p is required for for3p-tea1p interaction. Together, these results show that tea4p directly binds to for3p and tea1p and links these proteins together.

We mapped the interaction domains between for3p and tea4p. In in vitro binding assays, bacterially expressed tea4p interacted with the N-terminal half of for3p, which contains the FH3 domain, but not its C-terminal half, which contains the FH1 and FH2 domains (Figure 4B). Similarly, for3N, but not for3C, interacted with tea4p in two-hybrid assays. Deletion analysis defined amino acids 137–515 in for3p as the minimal sufficient region for interaction with tea4p (Figure 4D). This region overlaps with the predicted FH3 domain of for3p (aa 306–507, as defined by SMART) and the rho binding region (Nakano et al., 2002). This N-terminal region is necessary and sufficient for localization of for3p at cell tips (Nakano et al., 2002), suggesting that tea4p binding to this for3p region may regulate for3p localization.

No specific domain of tea4p could be defined in for3p binding. Neither the tea4p C-terminal region, which mediates the interaction with tea1p, nor the N-terminal half interacted with for3p reproducibly (Figure 4E), suggesting that multiple parts of the protein may be required for strong interaction. What is clear though is that the SH3 domain of tea4p was dispensable for binding, as shown by deletion or specific point mutation in the SH3 domain.

Asymmetric Localization of Polarity Factors in *tea4*Δ Cells

We next tested if tea4p affects the spatial distribution of for3p and tea1p. We first reexamined the localization of for3p in a wild-type background. Although for3p has been previously reported to reside at both ends of wild-type cells (Feierbach and Chang, 2001; Nakano et al., 2002), we found that for3p-YFP was present only at the

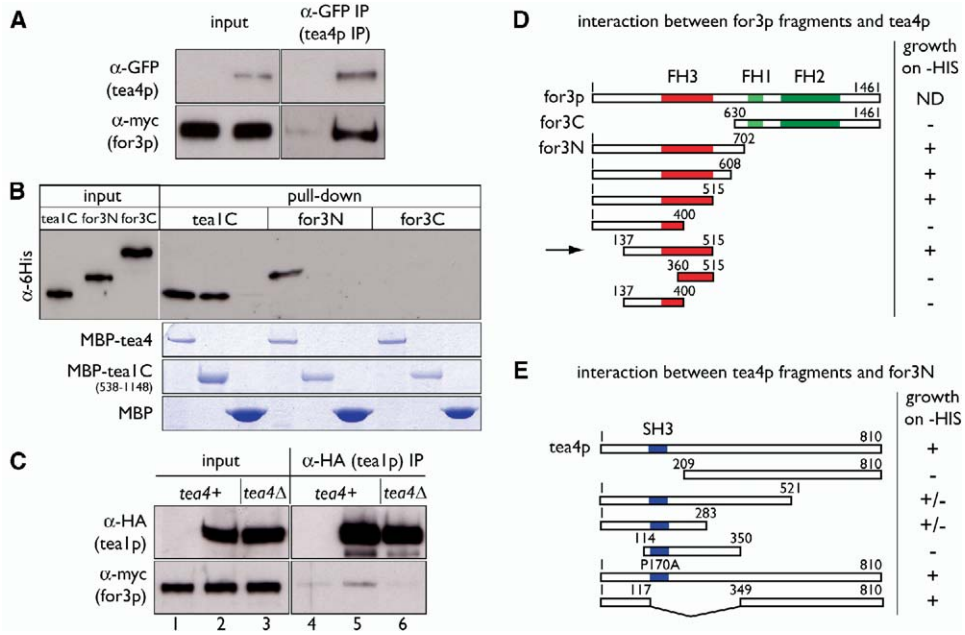

Figure 4. Tea4p Binds For3p Directly and Links Tea1p with For3p

(A) Coimmunoprecipitation of endogenous tagged for3p-myc and tea4p-GFP from yeast extracts.

(B) For3p binds tea4p in vitro. Interactions between bacterially expressed proteins were assayed by affinity columns. Recombinant 6His-for3N (aa 1–702), but not 6His-for3C (aa 630–1461), binds to MBP-tea4, but not MBP-tea1C or MBP alone. Interaction of 6His-tea1C (aa 538–1148) with MBP-tea4 and MBP-tea1C was used as a positive control. 6His-tagged proteins were detected by immunoblotting with anti-6His antibody. Coommassie staining of MBP fusions shows equal loading.

(C) The coimmunoprecipitation of for3p with tea1p depends on *tea4*. For3p-myc extracts expressing endogenous untagged (lanes 1 and 4) or HA-tagged tea1p (lanes 2 and 5), or for3p-myc *tea4*Δ extracts expressing HA-tea1p (lanes 3 and 6), were immunoprecipitated with anti-HA antibodies and immunoblotted with anti-HA and anti-myc antibodies. Quantitation and normalization of the bands show that for3p in the tea1p immunoprecipitate is decreased >5-fold in *tea4*Δ mutant extract (lane 6).

(D) Two-hybrid interaction between pGAD-tea4 (full-length) and pGBD-for3 fragments. The arrow indicates the minimal region of for3p that binds tea4p. ND: not done.

(E) Two-hybrid interaction between pGBD-for3N (aa 1–702) and pGAD-tea4 fragments.

growing end in a significant number of monopolar pre-NETO cells (Figure S2A). In *cdc10* cells that were arrested in G1 phase (pre-NETO) for longer periods of time, for3p was clearly concentrated at the growing old end (for3p was undetectable in >70% of nongrowing ends, n = 146; Figure S2B). These results suggested that for3p may transiently leave the new cell tip after septation. However, the extensive time lapse imaging through the cell cycle needed to test this possibility was not possible with this full-length for3p-YFP construction, because of its dim signal and problems with photobleaching.

To circumvent this problem, we examined a brighter fusion of GFP with an N-terminal fragment of for3p that contains the tea4p binding site. This fragment for3N (1–702) is necessary and sufficient for cell tip targeting (Nakano et al., 2002) and thus can be used to study targeting mechanisms for for3p. In timelapse images, we observed that for3N localized to the region of the septum during cell division (Figure 5A). Upon cell division, for3N began to localize to the old end. Some for3N remained at the new end for a period but then left (in 95% cells, n = 40 cells); thus, prior to NETO, cells exhibited a monopolar for3N distribution. At NETO, for3N then localized again to the new end. Consistent with this, for3N localized to only the one growing end of pre-NETO *cdc10* cells (Figure S2C). These data with the full-length and truncation of for3p indicate that, although some residual for3p is left at the new end immediately after cell division, it then leaves the new end, before being retargeted to the new end at NETO.

This for3p targeting at NETO is dependent on tea4p. In *tea4*Δ cells, full-length endogenous for3p, as well as for3N, localized to only the growing cell end in these monopolar cells, and they were not maintained at the nongrowing ends after cell division (Figures 5B and 5C). For3p and for3N localization patterns to the cell division site were otherwise normal (data not shown). These for3p patterns are consistent with the monopolar distribution of actin and myo52p-GFP in these cells (Figure 3). Thus, tea4p is required for for3p localization at the cell tip, specifically during initiation of bipolar growth.

Tea4p also affected tea1p distribution. In wild-type cells, tea1p accumulates symmetrically at both cell tips, even in pre-NETO monopolar cells (Mata and Nurse, 1997). Surprisingly, tea1p was concentrated at the nongrowing cell tip in *tea4*Δ cells (Figure 5C). This asymmetry was not due to defective transport on MTs, as tea1p-GFP dots moved normally on MT plus ends

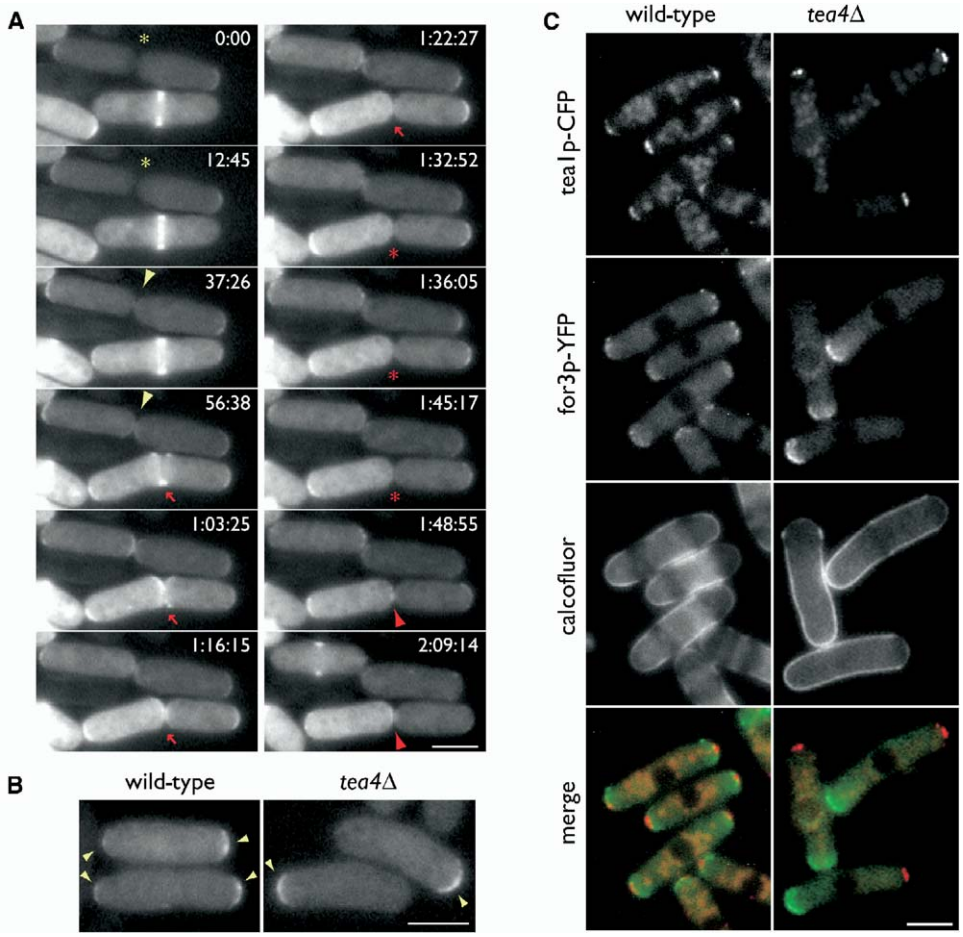

Figure 5. Tea4p Regulates the Localization of For3p and Tea1p

(A) Wild-type cells expressing GFP-forN from the *nmt42* promoter were induced for 16 hr in medium lacking thiamine and then imaged on EMM agar pads at 23°C by time-lapse fluorescence microscopy. GFP-for3N concentrates at the septum during cell division. After cell division, GFP-for3N is initially present at the new cell end (arrows), but then is not maintained and disappears from the new end (asterisks). It then relocalizes to the new end at NETO (arrowheads). Two representative examples (red and yellow) are highlighted. Time is indicated in hr:min:sec.

(B) Localization of GFP-for3N (aa 1–702) in wild-type (left) and *tea4*Δ (right) cells. Arrowheads indicate the localization at both cell ends in wild-type cells and at one cell end in *tea4*Δ cells.

(C) Tea1p-CFP (red), for3p-YFP (green), and calcofluor staining in wild-type and *tea4*Δ cells. In *tea4*Δ cells, for3p localizes to the growing cell end, while tea1p localizes to the nongrowing end. The scale bars indicate 5 μm.

toward both cell tips (Movie S2). However, at the growing cell end, tea1p-GFP dots were not maintained at the cortex after MT shrinkage. This asymmetry was also not due to abnormal for3p distribution or asymmetric actin cable organization, as tea1p-GFP was still largely asymmetric in *tea4*Δ *for3*Δ double mutants (data not shown). Interestingly, other polarity factors associated with tea1p, such as GFP-mod5p, tip1p-YFP, tea2p-GFP, and tea3p-GFP, were also concentrated at the nongrowing ends (Figure S3 and data not shown).

In summary, *tea4*Δ mutants have a novel phenotype in which sets of polarity factors are distinct from each other; actin, formin, and myo52p concentrate at the growing cell tip, and tea1p and its associated factors concentrate at the nongrowing cell tip. Thus, one function of tea4p is to directly or indirectly bring these distinct sets of polarity factors together at cell tips for bipolar growth. As tea1p and tea4p are present at the new end prior to for3p, tea4p may recruit and maintain for3p at the new end for the establishment of cell polarity.

Overexpression of Tea4p Induces Actin Cable Formation

To further explore the role of tea4p in regulating tea1p and for3p, we examined the effects of tea4p overexpression. We expressed tea4p in wild-type cells from an inducible *nmt1* promoter. At a relatively early time point after induction (17 hr after thiamine removal), for3p was delocalized from the cell tips in more than half of the cells (56%, n = 245) and was either diffuse or localized in discreet dots (Figure 6A). In contrast, at this time point, the majority of these cells with delocalized for3p still showed normal localization of tea1p at

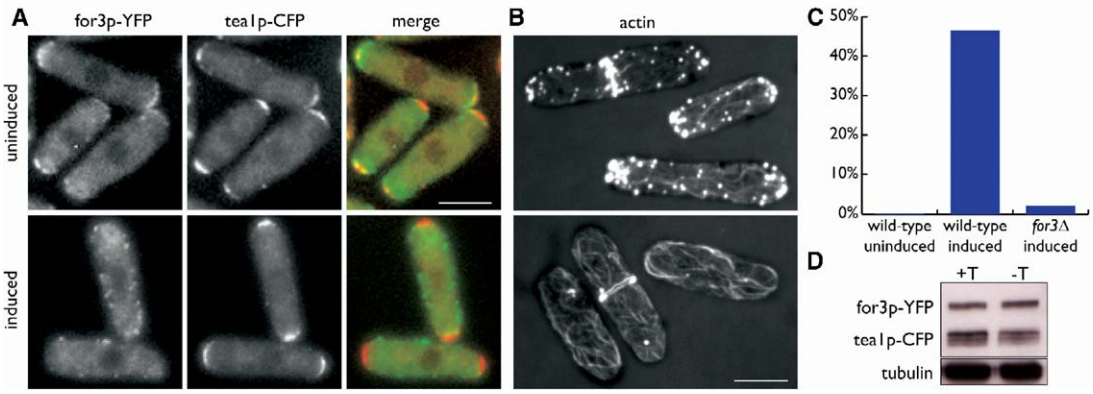

Figure 6. Overexpression of Tea4p Affects For3p Localization and Stimulates the Assembly of Actin Cables

(A) Localization of for3p-YFP (left, green) and tea1p-CFP (middle, red) in wild-type cells transformed with a plasmid expressing *tea4* under the control of the inducible *nmt1* promoter. Top: before induction, for3p-YFP and tea1p-CFP signals overlap at both cell tips. Bottom: after 17 hr induction, for3p-YFP is delocalized around the cortex of the cell, while tea1p-CFP is at cell tips.

(B) Confocal images of Alexafluor-phalloidin-stained cells expressing *nmt1-tea4* before (top) or after (bottom) 17 hr induction. The scale bars indicate 5 μm.

(C) Quantification of the percentage of interphase cells showing exclusively actin cables upon tea4p overexpression as determined by phalloidin staining.

(D) Immunoblot of for3p-YFP and tea1p-CFP in cells expressing *nmt1-tea4*, before (+T) or after 17 hr induction (–T). 20 μg total proteins was loaded in each lane.

both cell tips (57%, n = 137). However, at later time points, overexpression of tea4p also delocalized tea1p, as well as mod5p and tip1p (data not shown). Thus, overexpression of tea4p perturbed the localization of polarity factors and uncoupled the localization of tea1p and for3p.

Actin staining revealed that many of these cells overexpressing tea4p were filled with actin cables (46% of interphase, n = 183; Figures 6B and 6C). Consistent with the delocalization of for3p, these actin cables were disorganized, and myo52p-GFP was delocalized (data not shown). Actin patches were absent or greatly reduced. Prolonged overexpression of tea4p induced cell death, and thus we were unable to assay the consequence of this reorganization on cell growth and morphology. The formation of these actin cables in interphase cells was dependent on for3p, as overexpressing tea4p in *for3*Δ cells resulted in only patches and no cables (≤2% of interphase cells were observed to have cables, n = 349) (Figure 6C). Thus, the loss of actin patches in tea4p-overexpressing cells may be a consequence of competition between the actin cables and patches for limited actin monomer pool. Some *for3*Δ cells overexpressing tea4p still showed the actin ring without patches, and a subset displayed unusual perinuclear actin rings (12%, n = 534; data not shown). One possibility is that another formin such as cdc12p may be responsible for the assembly of these other abnormal actin structures in the absence of for3p. Thus, overexpression of tea4p appears to stimulate formin activity.

We tested several mechanisms to explain the induction of actin cables. First, this effect was not mediated simply by higher levels of for3p, as for3p levels were unchanged upon tea4p overexpression (Figure 6D), and overexpression of full-length for3p did not produce similar effects (data not shown; Nakano et al., 2002). Second, we wondered whether tea4p regulates an auto-inhibitory mechanism seen in other formins, which is mediated by the interaction between the N-terminal and C-terminal regions of the formin. (Wallar and Alberts, 2003). However, for3p lacks a canonical DAD sequence (Higgs and Peterson, 2004), and overexpression of the C-terminal portion of for3p (including the putative DAD region), which is predicted to relieve this auto-inhibition, did not lead to the large accumulation of actin cables (unpublished data). Third, this accumulation of actin cables was also not dependent on tea1p, as similar accumulation of actin cables was found in *tea1*Δ cells overexpressing tea4p. Therefore, the mechanism of how tea4p induces actin assembly is still not clear. However, taken together, these results provide further evidence that tea4p interacts with for3p in vivo, affects its localization, and, at least when overexpressed, can somehow stimulate its activity.

Tea4p Functions to Bridge For3p and Tea1p

These in vivo and in vitro results all indicate that tea4p functions to physically bridge tea1p and for3p. If this bridging function is the primary function of tea4p, we predicted that tea4p might be dispensable in a situation in which this bridge was artificially recreated. We constructed a protein fusion containing tea1p connected by a $(Gly\text{-}Ala)_5$ peptide linker to for3p. Strikingly, this tea1p-for3p fusion restored bipolar growth in *tea4*Δ cells. While *tea4*Δ cells exhibited monopolar for3p localization, *tea4*Δ cells expressing this protein fusion showed endogenous for3p (not only the for3p attached to tea1p) at both cell tips (Figure 7A). These cells were able to assemble actin cables from both cell ends, indicating that for3p was active at both tips; actin patches were also present at both cell ends (Figure 7B). Furthermore, calcofluor staining showed that 50% of the cells were now able to grow in a bipolar manner (n > 200;

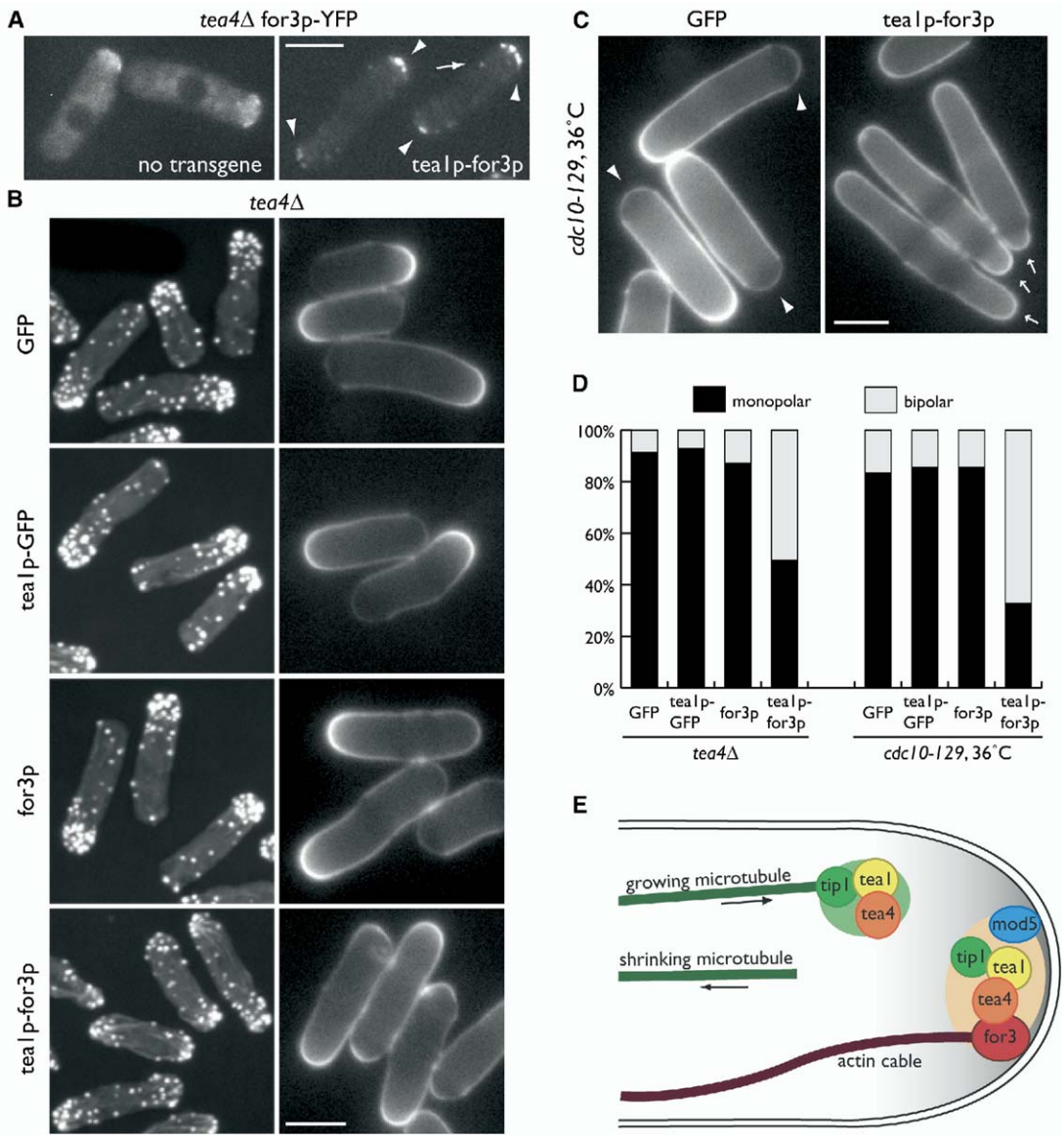

Figure 7. A Tea1p-For3p Fusion Suppresses *tea4⊿* and Triggers NETO

(A) *tea4*Δ for3p-YFP cells carrying empty vector or a tea1p-for3p fusion construct expressed from the weak *nmt81* promoter were grown in medium lacking thiamine for 18 hr and imaged by fluorescence microscopy. Tea1p-for3p targets endogenous for3p-YFP to MTs ends (arrow) and at both cell tips (arrowheads).

(B) *tea4*Δ cells carrying the indicated transgenes expressed as in (A). Cells stained with Alexafluor-phalloidin (left panels) and calcofluor (right panels). Note the bipolar actin distribution and cell growth in tealp-for3p-expressing cells (bottom panels).

(C) *cdc10-129* cells with plasmids expressing the indicated transgenes, under the *nmt81* promoter, were grown in medium lacking thiamine for 16.5 hr at 25°C, shifted to 36°C for 4 hr, and then stained with calcofluor. Arrowheads indicate new ends that are not growing (left panel), while arrows point to new ends that have initiated growth. The scale bars indicate 5 μm.

(D) Quantification of growth patterns in cells described in (B) and (C), as assayed by calcofluor staining.

(E) Model for establishment of cell polarity at the new end. Tip1p (CLIP170) and tea1p transport tea4p on growing MT plus ends to the cell end. These factors are deposited specifically at cell tips when the MT shrinks. During NETO, tea1p-tea4p at the cortex interacts with and recruits for3p (and probably other polarity factors) to form a complex, which assembles actin cables and establishes polarized cell growth.

Figures 7B and 7D). In contrast, expression of functional tea1p or for3p alone (from the same promoter, or from a much stronger *nmt1* promoter), or GFP only, did not induce bipolar actin organization or bipolar growth (Figures 7B and 7D; data not shown). Thus, a major function of tea4p may be to physically link tea1p with for3p.

Tea1-For3p Promotes NETO

We next examined the role of the interaction between tea1p, tea4p and for3p in promoting NETO. We hypothesize that tea4p contributes to NETO by bringing tea1p and for3p together at the new cell end, probably in the context of a larger complex. To test whether formation of a tea1p-for3p complex is sufficient for polarity estab-

lishment, we expressed the tea1p-for3p fusion in *cdc10* cells that are arrested in G1. While *cdc10* mutants are generally monopolar, tea1p-for3p expression induced a significant proportion of these cells to grow in a bipolar manner and exhibit bipolar actin organization (n > 200; Figures 7C and 7D). In contrast, expression of for3p or tea1p alone was not sufficient for inducing bipolar growth under these conditions (Figure 7D). Imaging of endogenous for3p in wild-type cells expressing the tea1p-for3p fusion showed that the formin was transported on the growing MT plus ends and targeted to both cell ends (data not shown). Together, these results suggest that formation of a tea1p-for3p complex at the new cell end is sufficient to initiate cell polarity even in G1-arrested cells.

Discussion

Linking MTs to Actin Assembly

Here, we provide evidence for a molecular mechanism by which MT plus ends regulate actin assembly in a spatially distinct manner. In the study of MT regulation of cell polarity in fission yeast, much research has focused on the characterization of the tea1p protein. In isolating tea1p-interacting proteins, we identified tea4p, which binds to tea1p directly and functions with tea1p in the regulation of cell polarity. Tea4p is targeted to specific sites on the cell cortex by riding on the plus ends of growing MTs. Links from the MT binding protein tip1p (CLIP170) via tea1p to tea4p attach tea4p to these MT ends (Figure 7E). Once at the cell end, tea1p and tea4p function to regulate cell polarization, specifically to promote actin assembly and polarity establishment at the new cell end for NETO during G2 phase. In this process, tea4p acts as a link between the MT plus end and the formin for3p, an actin nucleating factor (Figure 7E). Although an interaction between tea1p and for3p was noted previously (Feierbach et al., 2004), binding studies show that the interaction between these proteins is probably not direct, but is bridged directly by tea4p. The phenotypes of deleting and overexpressing tea4p on for3p distribution and actin cables confirm that tea4p interacts with for3p and regulates its spatial distribution in vivo. Furthermore, the ability of a tea1p-for3p protein fusion to substitute for tea4p further demonstrates that an important function of tea4p is to physically link tea1p and for3p. Thus, these studies elucidate molecular links that connect MT plus ends to actin assembly and suggest a simple mechanism for the regulation of cell polarization by MTs (Figure 7E).

Establishment of a Second Site of Growth

A major unanswered question in fission yeast biology is how a second site of polarized cell growth is established at a naive cell tip during NETO. Studies of cells, such as budding yeast or chemotaxing migrating cells, that have a single polarity axis demonstrate that positive feedback loops can reinforce even slight differences in spatial signals to form a defined polarity axis (Irazoqui et al., 2003; Waterman-Storer and Salmon, 1999; Wedlich-Soldner et al., 2003). A problem in bipolar establishment is how to initiate a new site of polarized growth in the presence of a strong preexisting axis. In *S. pombe*, the first site of growth is established often at the site of previous growth in a tea1p/tea4p-independent manner. In these cells, positive feedback mechanisms may be sufficient to promote the accumulation of for3p at the old end. A key event of NETO is the establishment of actin assembly at a second cell tip. Our results provide some of the first, to our knowledge, molecular insights into this process.

Our studies indicate that the formation of a complex between tea1p, tea4p, and for3p is a critical event for NETO. In *tea4*Δ mutants, in which tea1p and for3p are no longer linked together, bipolar growth cannot be established. The ability of a tea1p-for3p fusion to trigger bipolar growth even in G1-arrested cells demonstrates that complex formation is sufficient for establishing polarity. This fusion may bypass NETO controls by forcing the assembly of a complex between for3p, tea1p, and likely other polarity factors at the new end. Thus, the "seeding" of for3p complexed with tea1p and tea4p at the new cell end may be a key event in initiating actin cable assembly and setting off positive feedback mechanisms that establish and maintain polarized cell growth.

One important function of this complex is to localize and maintain for3p at the new cell end. The tea4p-interacting domain of for3p at the N terminus is necessary and sufficient for the proper localization of for3p, but not for its actin assembly activity. Analyses of the localization patterns of for3p and of this N-terminal domain suggest that for3p must be recruited to the new end for NETO. This recruitment is dependent on tea1p and tea4p; in *tea1*Δ and *tea4*Δ mutants, for3p is not localized to the second cell end. Further evidence that tea4p affects for3p localization is that tea4p overexpression delocalizes for3p. As tea4p is only needed for a specific transition in for3p localization at the new end for NETO, it is likely that other factors are responsible for for3p localization at other sites.

A second function of the complex may be to activate for3p in order to stimulate actin assembly. Evidence for this role comes from the overexpression of tea4p causing an impressive accumulation of actin cables in a for3p-dependent manner. But *tea4* loss-of-function phenotypes suggest that tea4p is not strictly required for for3p activation. for3p is still active at one of the cell tips in *tea4*Δ mutant cells. Further, for3p is activated at the new end by a tea1p-for3p fusion in the absence of tea4p; in this case, the tea1p-for3p fusion might facilitate complex formation with a formin activator (possibly a rho GTPase), or the fusion construct with tea1p may itself partially activate for3p. Thus, although tea4p may have the ability to stimulate formin activity, it could be functionally redundant with other activators, or function indirectly by promoting interactions with other activators.

Finally, there must be additional regulators of NETO responsible for temporal regulation. While tea1p and tea4p are deposited at the cell tips early in the cell cycle, NETO is triggered only later in G2 phase. The cell cycle regulators of NETO are still poorly understood. These NETO controls are bypassed by the tea1p-for3p fusion, suggesting that one ultimate target of these NETO controls may be to stimulate the association between tea4p and for3p. Further molecular studies into

the cell cycle control of tea4p and for3p promise to provide further insights into NETO regulation.

Additional Functions of Tea1p and Tea4p

Tea1p and tea4p have additional function(s) to that of recruiting the formin for3p. The striking asymmetric distribution of cell tip factors in *tea4*Δ cells implies that tea4p is required to anchor tea1p (and many other tea1p-dependent polarity factors) at growing ends. This anchoring function is independent of for3p, as both tea1p and tea4p localize normally at cell tips in *for3*Δ cells (Feierbach et al., 2004) and tea1p-GFP still localizes largely asymmetrically in *tea4*Δ*for3*Δ cells. Therefore, these findings predict that tea4p binds to another factor that anchors it at the cell tip. Tea1p is also likely to play additional roles in cell polarity to that of linking tea4p with tip1p, as a fusion protein between tip1p and tea4p, which targeted tea4p to MT plus ends, did not complement a *tea1*Δ mutant (unpublished data).

Tea1p and tea4p also contribute to cell polarization independently of for3p. Although for3p is strictly required for actin cable assembly, *for3*Δ mutants still grow in a polarized manner and exhibit an interesting mixture of abnormal growth patterns (Feierbach and Chang, 2001). This polarized growth is dependent on tea1p and tea4p, as *for3*Δ*tea4*Δ and *for3*Δ*tea1*Δ double mutants have more severe polarity defects than each single mutant (Feierbach et al., 2004 and data not shown). Thus, these genetic results combined with biochemical results (Feierbach et al., 2004) indicate that these polarity factors function in the context of large multifunctional protein complexes.

Interactions between Actin and MTs in Other Cell Types

The mechanism of regulating a formin by MT plus ends is likely to be relevant for the regulation of cell morphogenesis in other cell types. The full importance of the formins in the assembly of diverse actin structures such as a filopodia, contractile rings, and cell-cell contacts is only beginning to be appreciated in animal cells (Wallar and Alberts, 2003). It is likely that the spatial organization of formin localization and activity provide key contributions to cellular architecture. Although MTs are recognized to be important in many of these actin-based processes, how they regulate actin assembly and formins is still not well defined. Formins have also been implicated at the interface between the actin and MTs in other cell types, but more in the context of MT regulation. In mammalian cells, mDia1 functions with the MT plus end proteins EB1 and APC in the stabilization of MTs (Palazzo et al., 2001; Wen et al., 2004), and, recently, mDia3 was shown to localize to kinetochores and affect chromosome segregation (Yasuda et al., 2004). However, obvious roles of *S. pombe* for3p in regulating MT dynamics or mitosis are not apparent (Feierbach and Chang, 2001).

The possible homologs of tea1p and tea4p have generally not yet been well characterized. In *S. cerevisiae,* the nearest homologs of *S. pombe* tea1p, tea4p, and for3p are Kel1/Kel2p, Bud14p, and Bni1p, respectively. Although association between these factors has not been reported to date, mutant phenotypes and genetic interactions suggest that these proteins also function together to regulate cell polarity (Cullen and Sprague, 2002; Philips and Herskowitz, 1998). In animal cells, the equivalents of tea1p and tea4p are less clear. A mammalian protein with some functional similarity (but low sequence homology) to tea4p is WISH/DIP. Like tea4p, this protein contains a N-terminal SH3 domain protein and interacts with mammalian formins, mDia1 and FHOD1, with an N-terminal region binding to FHOD1 (Satoh and Tominaga, 2001; Westendorf and Koka, 2004). Many kelch proteins regulate cytoskeletal processes in animal cells (Adams et al., 2000). Of note, the kelch repeat protein gigaxonin associates with MTs in neurons (Ding et al., 2002), *Drosophila* Kelch organizes ring canals (Robinson and Cooley, 1997), and Keap1 associates with the mid-body, adherens junctions, and focal adhesions and is required for cytokinesis (Skop et al., 2004; Small and Kaverina, 2003; Velichkova et al., 2002). Further study of these proteins and their possible interactions will be a key part of understanding the molecular principles of cell morphogenesis.

Experimental Procedures

Yeast Strains, Media, and Genetic Methods

S. pombe strains used in this study are listed in Table S1. Standard methods for *S. pombe* media and genetic manipulations were used throughout. Tagged and deletion strains were constructed by using a PCR-based approach (Bähler et al., 1998) and confirmed by analytical PCR. The morphology of a *tea4*Δ strain was also rescued by expression of tea4p-GFP under control of the *nmt81* promoter on a plasmid, confirming that the phenotype was caused by deletion of *tea4* and that tagged *tea4* was functional (data not shown). To compare the phenotypes of *tea1*Δ, *tea4*Δ, and *tea1*Δ*tea4*Δ double mutants, we assayed morphology in log-phase growth and the number of T-shaped cells formed after transient actin depolymerization in response to heat shock (Sawin and Snaith, 2004).

Microscopy

Microscopy was performed by using either a wide-field fluorescence microscope or a spinning disk confocal microscope (Pelham and Chang, 2001), and images were acquired, processed, and analyzed with the OpenLab software (Improvision). Actin stainings were performed as described (Pelham and Chang, 2001) by using AlexaFluor 488-phalloidin (Molecular Probes). MTs were observed in cells transformed with either GFP-tubulin (pDQ105) or CFP-tubulin (pRL72) (Glynn et al., 2001). Cell growth patterns were determined essentially as described (Feierbach and Chang, 2001), except that no Sigmacote was used.

Plasmid Construction and Two-Hybrid Analysis

Plasmids used in this study are listed in Table S2. Tea1p-for3p and tea1p-GFP fusions were linked by a 10 amino acid linker (5xAla-Gly), expressed under the *nmt81* promoter, and were functional, as they rescued the morphology of *tea1*Δ cells. Tea1p-for3p and for3p expressed under the *nmt81* promoter also rescued morphology and actin cable formation in *for3*Δ cells. Two-hybrid interactions were tested on medium lacking histidine in strain AH109 (Clontech). As pGBD-for3N was able to grow on medium lacking histidine when cotransformed with an empty pGAD vector, interactions were assayed on –HIS medium containing 5–10 mM 3′ AT, which suppressed this auto-activation.

Immunoprecipitations

Yeast extracts, immunoprecipitations and tea1p-TAP purification were performed similarly to the methods described by Feierbach et al. (2004) and Gould et al. (2004) with modifications (see Supplemental Data for details of procedures). The unseparated mixture of tea1p-associated proteins was digested with trypsin, and the resulting peptides were analyzed by multidimensional, high-perfor-

mance liquid chromatography-coupled tandem mass-spectrometry and were identified by database searching, as described (MacCoss et al., 2002).

In Vitro Binding Assays

MBP (Maltose Binding Protein), MBP-tea1C, and MBP-tea4 fusions were expressed in *E. coli*, affinity purified on an amylose resin (Clontech), and stored at 4°C on the resin in the presence of sodium azide. 6His-tagged tea1C, tea4N, tea4C, for3N, and for3C were expressed in *E. coli*, and soluble extracts were prepared in B buffer (20 mM Tris [pH 8.0], 20 mM KCl, 130 mM NaCl, 1 mM MgCl2, 2 mM EDTA, and protease inhibitor cocktail) by treatment with lysozyme, sonication, and centrifugation at 14 krpm for 20 min in the SS-34 rotor (Beckman). Soluble extracts were stored at −80°C. In vitro binding assays were performed in micro bio-spin columns (Biorad) by adding equivalent amounts of His-tagged protein extracts (as judged by Western blotting) to MBP fusion-coupled amylose resin and incubation for 2 hr at 4°C. The resin was subsequently washed five times with B buffer and five times with B buffer + 0.05% NP-40. MBP-fusions and associated proteins were eluted in 60 μl 10 mM maltose. After addition of sample buffer, samples were analyzed by SDS-PAGE, Coomassie staining, and Western blotting.

Supplemental Data

Supplemental Data including additional figures and movies (as described in the text) as well as Supplemental Experimental Procedures and tables of yeast strains and plasmids are available at http://www.developmentalcell.com/cgi/content/full/8/4/479/DC1/.

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