Regulation of a formin complex by the microtubule plus end protein tea1p

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The plus ends of microtubules have been speculated to regulate the actin cytoskeleton for the proper positioning of sites of cell polarization and cytokinesis. In the fission yeast *Schizosaccharomyces pombe*, interphase microtubules and the kelch repeat protein tea1p regulate polarized cell growth. Here, we show that tea1p is directly deposited at cell tips by microtubule plus ends. Tea1p associates in large "polarisome" complexes with bud6p and for3p, a formin that assembles actin cables. Tea1p also interacts in a separate complex with the CLIP-170 protein tip1p, a microtubule plus end–binding protein that anchors tea1p to the microtubule plus end. Localization experiments suggest that tea1p and bud6p regulate formin distribution and actin cable assembly. Although single mutants still polarize, *for3Δbud6Δtea1Δ* triple-mutant cells lack polarity, indicating that these proteins contribute overlapping functions in cell polarization. Thus, these experiments begin to elucidate how microtubules contribute to the proper spatial regulation of actin assembly and polarized cell growth.

Introduction

Interactions between the microtubule and actin cytoskeletons contribute to cellular processes such as cell migration, cytokinesis, nerve growth cone guidance, nuclear positioning, and wound healing (Waterman-Storer and Salmon, 1999; Goode et al., 2000; Small et al., 2002). It has been proposed that microtubules contribute to these processes in part by controlling the proper spatial distribution of actin structures. One clear example is in cytokinesis, in which microtubules of the mitotic spindle somehow function to position the site of contractile actin ring assembly at the cortex (Rappaport, 1996; Maddox and Oegema, 2003). In cell migration, microtubules may be required for steering the actin cytoskeleton in the proper direction, for instance in nerve growth cone guidance (Tanaka and Kirschner, 1995; Dent and Gertler, 2003). However, in general, the molecular bases for how microtubules direct the actin cytoskeleton are still poorly understood.

The fission yeast *Schizosaccharomyces pombe* serves as a model cell in which to study microtubule–actin interactions, as microtubules and actin regulate cell polarity and cell shape in these cells (Chang, 2001). Fission yeast are simple rod-shaped cells that grow at cell tips in a regulated manner. After cell division, they initially grow only at the previous cell tips (the old ends), and then later in G2 phase, initiate cell growth at the new ends (Mitchison and Nurse, 1985). Microtubules are organized in linear arrays of anti-parallel bundles so that microtubule plus ends repeatedly touch and shrink at both cell tips (Brunner and Nurse, 2000; Drummond and Cross, 2000; Tran et al., 2001). Disruption of these microtubules causes aberrant cell shapes such as bent or branched (T-shaped) cells (Toda et al., 1983; Sawin and Nurse, 1998).

The analysis of the kelch repeat protein tea1p has begun to elucidate how microtubules may regulate fission yeast cell polarity. *tea1Δ* mutants exhibit abnormal cell shapes much like cells with disrupted microtubules, and grow only from one cell tip (Snell and Nurse, 1994; Verde et al., 1995; Mata and Nurse, 1997). Tea1p is located on the growing plus ends of microtubules and in dots at the cell tip (Mata and Nurse, 1997; Behrens and Nurse, 2002). The localization of tea1p at the microtubule plus end is dependent on the CLIP-170 tip1p and the Kip2-like kinesin tea2p (Browning et al., 2000; Brunner and Nurse, 2000). Indirect observations and time-lapse images of cells with abnormal tea1p dynamics suggest that microtubule plus ends deliver tea1p to the cell tip; when the microtubule shrinks away, tea1p may be released from the microtubule and "docks" at the cell cortex (Mata

and Nurse, 1997; Behrens and Nurse, 2002; unpublished data). For instance, in a *mod5* mutant, tea1p localizes on the microtubule but does not dock at the cell tip (Snaith and Sawin, 2003). However, direct observation of tea1p deposition at the cell tips in wild-type cells has not been definitively shown. As tea1p has strong effects on cell polarity but only subtle effects on microtubule dynamics, tea1p may directly regulate cell polarity and possible actin distribution at the cell tip.

Formins are a conserved family of proteins with roles in cell polarization and cytokinesis (Wallar and Alberts, 2003). Recent reports show that they directly nucleate actin filament assembly in vitro and regulate actin filament elongation while bound to the growing barbed end of actin filaments (Evangelista et al., 2002; Pruyne et al., 2002; Sagot et al., 2002b; Li and Higgs, 2003; Zigmond et al., 2003; Moseley et al., 2004). Formins are responsible for the formation of diverse actin structures including actin cables, contractile rings, filopodia, endosome actin tails, and adherens junctions (Evangelista et al., 2002; Sagot et al., 2002a; Gasman et al., 2003; Peng et al., 2003; Kobielak et al., 2004). The *S. pombe* formin for3p, which is located at cell tips, is required specifically for assembly of actin cables in interphase cells (Feierbach and Chang, 2001; Nakano et al., 2002). These actin cables may contribute to polarized growth by functioning as tracks to guide polarized targeting of secretory vesicles to the growing cell tip (Schott et al., 1999). One likely regulator of for3p is the actin-binding protein bud6p/aip3p (Glynn et al., 2001; Jin and Amberg, 2001). Its budding yeast homologue (Bud6p/Aip3p) is an actin monomer–binding protein that interacts with the formins Bni1p and Bnr1p (Evangelista et al., 1997; Kikyo et al., 1999; Jin and Amberg, 2000, 2001) and acts as a cofactor with profilin to increase actin assembly by Bni1p in vitro (Moseley et al., 2004). *S. pombe* mutants lacking tea1p, for3p, or bud6p have varying defects in cell shape and cell polarity establishment at one or both cell tips (Snell and Nurse, 1994; Feierbach and Chang, 2001; Glynn et al., 2001). We have shown previously that *S. pombe* bud6p interacts with tea1p (Glynn et al., 2001).

Here, we address two questions about tea1p: is tea1p directly deposited by microtubules at the cell tip, and how does tea1p regulate cell polarity and actin cable distribution? We observed directly that tea1p is deposited by plus ends of microtubules. Biochemical analyses show that tea1p associates with for3p, bud6p, and the CLIP-170 tip1p in distinct high molecular weight complexes. Localization experiments suggest that tea1p acts to regulate the localization of formin and actin cables at specific cell tips. These experiments contribute key insights into the molecular mechanisms of tea1p trafficking and function and suggest a model for how microtubule plus ends regulate actin assembly through regulation of a formin.

Results

Microtubule plus ends deposit tea1p at the cell tips

The specific localization of tea1p at cell tips appears to be a critical step in the regulation of cell polarity and shape in fission yeast. Although it has been proposed that microtubules affect spatial organization by depositing tea1p at the cell
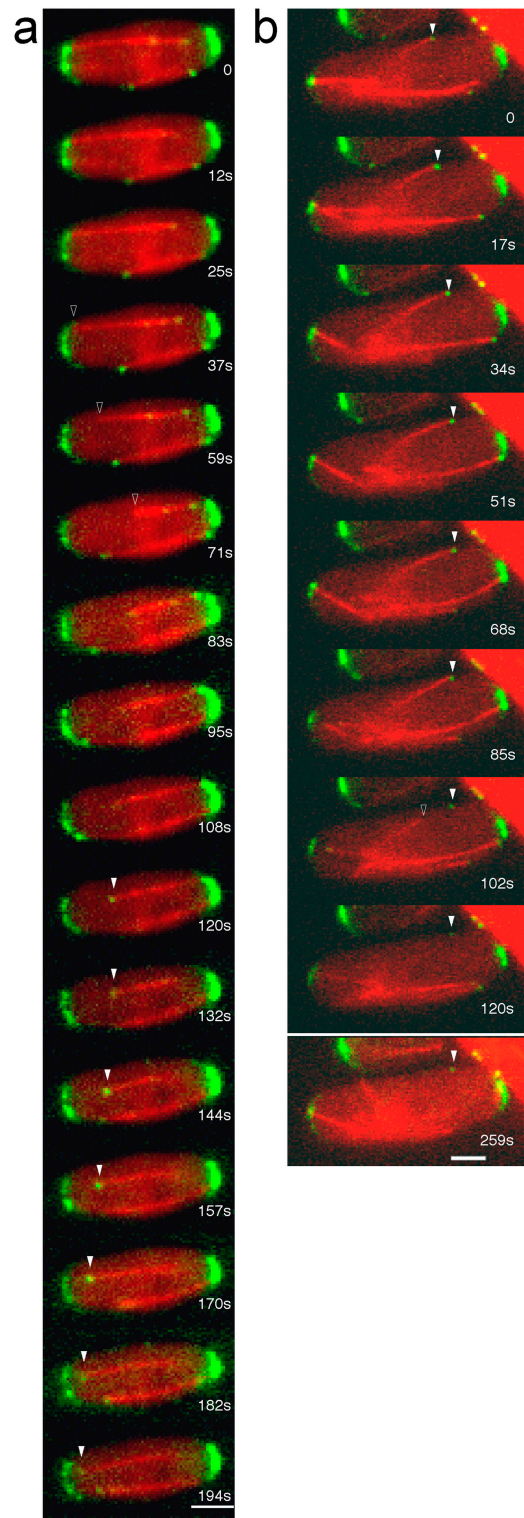

Figure 1. Microtubule plus ends deposit tea1p on the cell surface. (A) Time-lapse images of a wild-type cell expressing tea1p-YFP (green) and CFP-atb2p (α-tubulin, red). Times in seconds are listed. Arrowheads show examples of growing microtubules with tea1p at the plus end; empty arrowheads show a shrinking microtubule without tea1p. (B) Time-lapse images of an *rsp1-1* cell expressing tea1p-YFP (green) and CFP-atb2p (red) grown in liquid minimal media to late log phase at 30°C. Arrowheads show an example of a tea1p dot that is retained at the cell surface after microtubule shrinks. Additional examples are shown in Videos 1–3 (available at http://www.jcb.org/cgi/content/full/jcb.200403090/DC1). Bar, 2.5 μm.

tips, this deposition event has not been observed directly. One alternative model, for instance, is that the mechanisms of localizing tea1p to the microtubule and cell tip are independent. To visualize tea1p, we constructed *S. pombe* strains that express tea1p-YFP and CFP-atb2p (α-tubulin). The tea1p-YFP fusion, which was constructed by integration of a YFP cassette into the *tea1*+ locus at the COOH terminus, was expressed from the endogenous *tea1*+ promoter and was functional. In wild-type cells, time-lapse imaging revealed that tea1p dots moved from the middle of the cell to both cell tips on the growing plus ends of microtubules (Fig. 1 a; Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200403090/DC1; see also Behrens and Nurse, 2002; Snaith and Sawin, 2003). In general, tea1p dots did not move away from the cell tips, except in rare examples where tea1p appeared to be on shrinking microtubule plus ends (<10% cells; Video 2). In addition to microtubule localization, tea1p dots were also present in dense collections at the cell tip. We tried to observe specific dots at the cell tip as they arrived on the microtubule, to see if they are retained after the microtubule shrank away. We did see some suggestive examples, but in general the tea1p dots at the cell tip were too dense to follow the dots in a definitive manner.

To observe the behavior of tea1p in locations where tea1p dots are not as dense, we imaged *rsp1-1* cells, which display abnormal microtubule asters instead of the longitudinal array of microtubule bundles. Rsp1p is a J-domain protein required for the disassembly of the equatorial microtubule-organizing center and colocalizes with microtubule-organizing center components such as the γ-tubulin complex (Zimmerman et al., 2004). Despite the abnormal microtubule organization, *rsp1-1* mutants display normal microtubule plus end dynamics (where microtubules only shrink from cell tips) and generally form normal rod cell shapes. However, under certain conditions (such as 37°C and growth on agar plates), *rsp1-1* cells develop severe cell polarity phenotypes that may be secondary to microtubule organization defects. We imaged tea1p-YFP and atb2p-CFP in *rsp1-1* cells under conditions in which some microtubules touch and shrink from the sides of the cells. In these cells, tea1p dots appeared on the cell sides as well as at cell tips. Because of the low density of tea1p dots at the cell sides, we were able to record clear examples of tea1p deposition (Fig. 1 b; see Video 3 for many more examples in one field; Video available at http://www.jcb.org/cgi/content/full/jcb.200403090/DC1). In each case, tea1p on the growing microtubule plus end contacted the cell surface and persisted after the microtubule shrank away. Some tea1p dots were retained for the length of the movie (>150 s after microtubule catastrophe in Fig. 1 b), whereas others persisted only transiently at the cell surface. These direct observations provide the strongest evidence to date that microtubule plus ends dictate tea1p localization at cell tips by direct deposition.

Tea1p associates with for3p and bud6p in high molecular weight complexes

After being deposited at the cell tips, tea1p may regulate cell polarity by interacting with other polarity factors. We tested whether tea1p associates with the cell polarity factors bud6p and for3p (formin) using four different approaches. Epi-
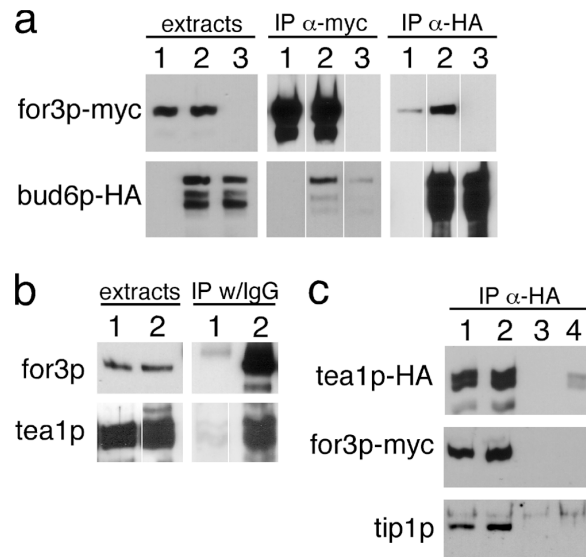

Figure 2. **For3p, bud6p, tea1p, and tip1p coimmunoprecipitate.** (a) For3p-myc and bud6p-HA coimmunoprecipitate. Extracts expressing (1) for3p-myc (BFY19), (2) for3p-myc bud6p-HA (BFY168), or (3) bud6p-HA (FC592) were immunoprecipitated using protein A–Sepharose beads complexed with either α-HA or α-myc antibodies and were Western blotted. (b) For3p-TAP immunoprecipitates tea1p. Extracts expressing (1) for3-myc (BFY19) or (2) for3p-TAP (BFY198) were immunoprecipitated with mouse α-rabbit Dynal magnetic beads complexed with rabbit IgG, and were Western blotted with α-myc antibodies (the HRP anti–rabbit secondary recognizes both the α-myc antibody and the TAP tag) or anti-tea1p antibodies. (c) Tea1p-HA immunoprecipitates for3p-myc and tip1p. Extracts expressing tea1p-HA for3p-myc (BFY190, lanes 1 and 2) or for3p-myc (BFY19, lanes 3 and 4) were immunoprecipitated with sheep α-mouse Dynal magnetic beads complexed with mouse anti-HA antibodies, washed in buffer containing either 250 mM NaCl (lanes 1 and 3) or 150 mM NaCl (lanes 2 and 4; see Materials and methods), and immunoblotted. White lines indicate that intervening lanes have been spliced out.

tope-tagged tea1p, for3p, and bud6p protein fusions were expressed from the endogenous promoters at their chromosomal locus. These tagged proteins were functional, as they supported normal cell polarity in the absence of untagged protein.

First, using these epitope-tagged strains, we tested for coimmunoprecipitation from yeast extracts. For3p coimmunoprecipitated with bud6p-HA (Fig. 2 a). For3p and tea1p also coimmunoprecipitated (Fig. 2, b and c). A coimmunoprecipitation association between tea1p and bud6p was shown previously (Glynn et al., 2001).

Second, we used two-hybrid tests to confirm these interactions (Table I). An NH2-terminal fragment of for3p (aa 1–868), but not a COOH-terminal fragment (aa 429–1461) containing the FH1 and FH2 domains, interacted with tea1p, suggesting that an NH2-terminal region of for3p, separate from the FH1 and FH2 domains, is responsible for tea1p binding. The COOH-terminal region (aa 429–1461) of for3p (but not the NH2-terminal 1–868-aa region) interacted with the COOH-terminal region of bud6p (aa 907–1385); these interaction regions were consistent with those defined for *Saccharomyces cerevisiae* Bni1p and Bud6p (Evangelista et al., 1997; Kikyo et al., 1999). These two-hybrid

Table I. **Two-hybrid assay interactions**

DNA-binding domain	Activation domain	lacZ assay
for3(1–868)	tea1	+
for3(429–1461)	tea1	–
for3(1–868)	bud6(907–1385)	–
for3(429–1461)	bud6(907–1385)	+
for3(1–868)	vector	–[a]
for3(429–1461)	vector	–
vector	tea1	–
vector	bud6(907–1385)	–
tea1	tip1(1–462)	+
tea1	tip1(1–299)	+
tea1	tip1(1–207)	–
tea1	tip1(290–462)	+
tea1	vector-Snf4	–

Numbers shown indicate amino acids.
[a]for3(1–868) construct alone yields a faint blue color in the lacZ assay.

analyses performed with the *S. pombe* proteins in *S. cerevisiae* suggest these interactions are direct, or bridged by a small number of highly conserved proteins. However, as different regions of for3p interacted with tea1p and bud6p, the for3p–tea1p interaction is probably not solely bridged by bud6p.

Third, sucrose gradient fractionation revealed that these proteins in soluble yeast extracts reside solely in multiple large protein complexes (Fig. 3). No monomer fractions of these proteins were detected. Tea1p migrated in three complexes whose peak fractions corresponded to 12, 45, and 75S in size (Fig. 3, a and b; wt panels). Bud6p migrated in complexes of ~20S (the bottom molecular weight bud6p band) and in two larger complexes (45 and 75S) that co-migrated with tea1p. Although tea1p and bud6p were found previously to co-migrate in a small (12–20S) complex (Glynn et al., 2001), we found that in these conditions in which the concentration of detergent is reduced, these proteins migrated in complexes with distinct peaks. For3p co-migrated with bud6p in the 20S complex, and with bud6p and tea1p in the 45 and 65S complexes (although the peak of for3p in the 45S complex, which was reproducible, was sometimes not as pronounced).

Immunoblots of tea1p and bud6p showed multiple forms of these proteins with molecular weights higher than predicted from the amino acid sequence (Fig. 2 and Fig. 3). The mobility shift of tea1p may be due to phosphorylation, possibly by the PAK kinase shk1p/orb2p (Kim et al., 2003; unpublished data). The abundance of these tea1p and bud6p forms varied in the different complexes and in immunoprecipitations (Fig. 2 and Fig. 3). For instance, a high molecular weight form of bud6p was the dominant form in for3p immunoprecipitations (Fig. 2 a) and in complex C and D (Fig. 3; wt panels). Future experiments will be required to probe the nature and significance of these possible post-translational modifications.

Fourth, we examined these complexes in yeast extracts derived from different mutant backgrounds (Fig. 3). Using sucrose gradients, we found that in *for3Δ* extracts, bud6p and tea1p were still in complexes, but they sedimented with smaller S values (Fig. 3 a); bud6p and tea1p migrated together 2–3 fractions smaller in complex D (the 75S com-
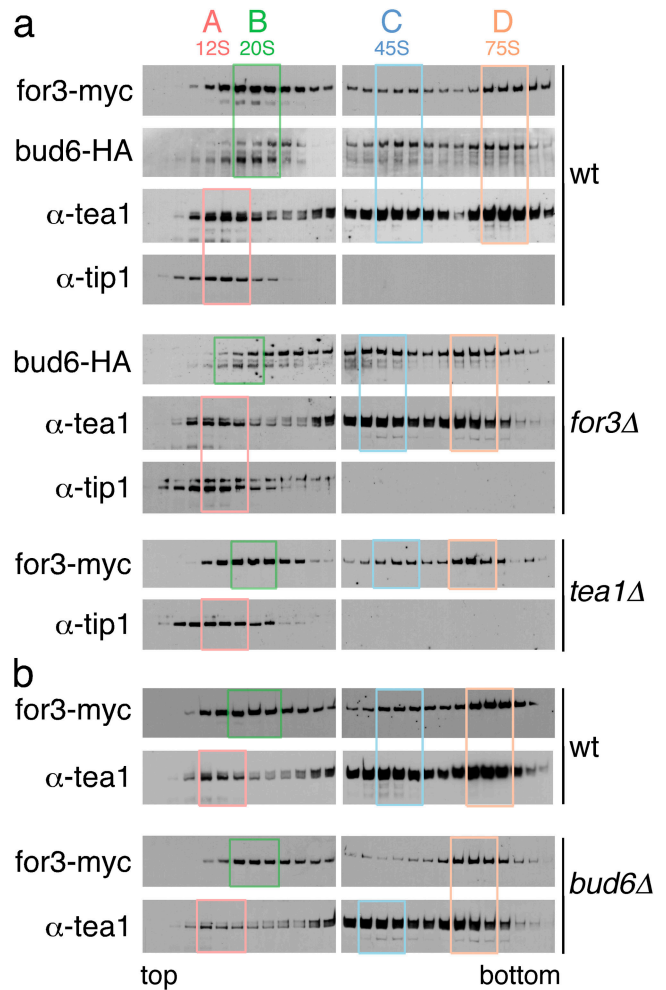

Figure 3. **Tea1p, for3p, bud6p, and tip1p reside in multiple complexes.** (a) Soluble yeast extracts were fractionated on velocity sucrose gradients, and fractions were immunoblotted with appropriate antibodies. Fractions were loaded so the fractions from the top of the gradient (containing smaller complexes) are on the left. Colored boxes mark peaks of proteins in complexes. Four complexes of ~12, 20, 45, and 75S are labeled A, B, C, and D, respectively. Labels on the right denote the genotype of the yeast strain analyzed. a and b represent two representative experiments in which multiple sucrose gradients were prepared and centrifuged in parallel, so that gradients can be directly compared. (a) Fractionation of for3p-myc bud6p-HA (wild-type, BFY168), *for3Δbud6-HA* (BFY186), and *tea1Δfor3-myc* (BFY184) extracts. (b) Fractionation of for3p-myc bud6p-HA and *bud6Δfor3-myc* (BFY192) extracts.

plex), and possibly one fraction smaller in complex C (the 45S complex). In *tea1Δ* extracts, for3p in complex D similarly shifted two fractions smaller, although there was no detectable shift in complex C (Fig. 3 a). In *bud6Δ* extracts, tea1p in complex D shifted together to a smaller complex by 1–2 fractions, and may shift one fraction smaller in complex C. Interestingly, although for3p co-migrated with tea1p in the smaller complex D, it was absent or greatly reduced from complex C (Fig. 3 b). This finding suggests that bud6p may be required to attach for3p to complex C. Because of the large size of these complexes, a lack of changes in sedimentation behavior in mutant extracts does not rule out that these proteins interact in that particular complex. However, the observed changes provide further evidence, especially in
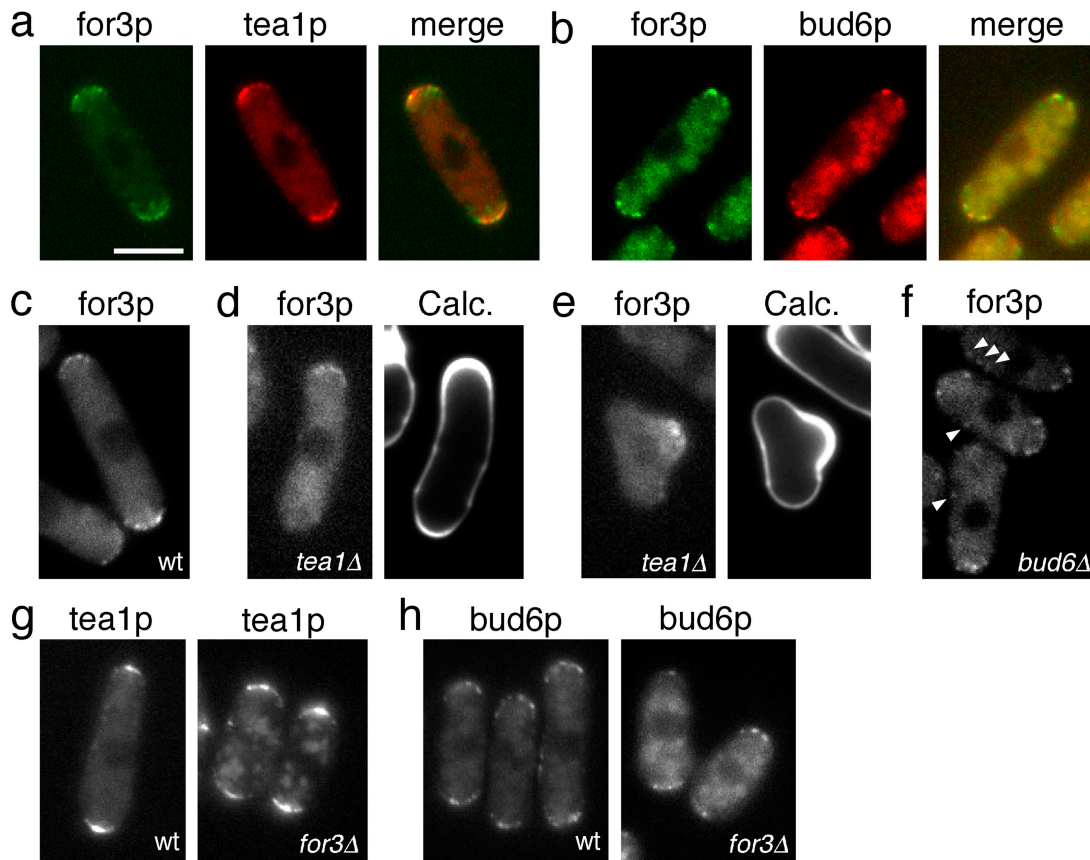
Figure 4. **Localization dependency relationships between for3p, bud6p, and tea1p.** Localization of YFP- or CFP-tagged proteins were imaged by fluorescence microscopy in living wild-type and mutant cells. (a) Localization of for3p-YFP (green) and tea1p-CFP (red; BFY125) in interphase cells. (b) Localization of for3p-YFP (green) and bud6p-CFP (red; BFY112) in interphase cells. (c) Localization of for3p-YFP in wild-type cells (BFY81). (d) Localization of for3p-YFP in a *tea1Δ* cell (BFY122). Calcofluor preferentially stains the active site of cell growth. (e) Localization of for3p-YFP in a T-shaped *tea1Δ* cell. (f) Localization of for3p-YFP in *bud6Δ* cells (BFY120). Arrows show for3p-YFP dots on the sides of cells. (g) Localization of tea1p-YFP in wild-type (FC871) and *for3Δ* cells (BFY191). (h) Localization of bud6p-CFP in wild-type (BFY104) and *for3Δ* cells (BFY148). Bar, 5 μm.

complex D, that these proteins reside together in common protein complexes.

It is not clear whether for3p and bud6p actually interact in the 20S complex B. In contrast to the shifts in the large complexes, there were little or no effects on the migration of complex B in either *for3Δ* or *bud6Δ* extracts. In addition, the lower molecular weight form of bud6p that co-migrated with for3p at 20S was not the dominant form of bud6p that coimmunoprecipitated with for3p. However, we found that the 75S complexes disassembled over time into 20S complexes containing for3p and bud6p, with bud6p primarily in the higher molecular weight forms (unpublished data). Together, the coimmunoprecipitation, two-hybrid, and sucrose gradient data provide strong evidence that tea1p, bud6p, and for3p physically associate.

Tea1p and bud6p regulate for3p localization

To determine how tea1p and bud6p may affect for3p in vivo, we tested first whether tea1p and bud6p regulate for3p localization. These three gene products localize during interphase to multiple dots at both cell tips, even at nongrowing (preNETO) cell tips. These proteins also reside at the cell division site during cell division. During mitosis, tea1p per-

sists at the cell tips, whereas for3p and bud6p leave the cell tip and accumulate more at the cell division plane. Thus, in just-divided cells, tea1p localization precedes for3p and bud6p at the old cell tips. At cell tips in interphase cells, a subset of for3p dots colocalized with tea1p dots, and all the for3p dots colocalized precisely with bud6p dots (Fig. 4, a and b). In addition, tea1p (but not for3p or bud6p) localizes to the plus ends of growing cytoplasmic microtubules (Mata and Nurse, 1997; Feierbach and Chang, 2001; Glynn et al., 2001; Behrens and Nurse, 2002; Nakano et al., 2002).

The distribution of for3p dots at cell tips was dependent on tea1p and bud6p. In *tea1Δ* cells, for3p was generally localized at only one of the tips, and thus was missing from one of the cell tips (Fig. 4 d; 81% cells, *n* = 85 cells). In the *tea1Δ* cells that form abnormal projections from the side of the cell, for3p was located primarily in this abnormal projection and was absent from both old cell tips (Fig. 4 e). Generally, *tea1Δ* cells only grow from one cell tip (or only from the abnormal projection), and Calcofluor staining revealed that for3p was located at the cell tip that was actively growing (Fig. 4, d and e). Tea1p may be required both for the establishment and maintenance of for3p localization. In wild-type cells, for3p is established at the new ends by its localization to the cell divi-

sion ring and septum, whereas the establishment of for3p at the old ends occurs during septation (Feierbach and Chang, 2001). In rod-shaped *tea1Δ* cells, in one of the daughter cells, for3p was established at the new end (end that was previously the septum), but not the old end (the end that did not grow in the previous cell cycle); in the other daughter cell, for3p was initially present at both the old and new ends immediately after cell division, but then was not maintained at the new end (unpublished data). This localization behavior was similar to that of bud6p in *tea1Δ* mutants (Glynn et al., 2001). In interphase *bud6Δ* cells, for3p-YFP dots were generally reduced in number (∼50% fewer), less uniform, and in some cells, delocalized to the sides of the cell (Fig. 4 f). This behavior is consistent with the reduction of for3p from complex C seen in sucrose gradients (Fig. 3 b). However, for3p appeared to localize properly at the cell division site in both *bud6Δ* and *tea1Δ* cells (unpublished data). Thus, bud6p and tea1p are required for the proper spatial distribution of the formin for3p at certain cell tips.

In contrast, tea1p and bud6p localization patterns were largely independent of for3p. In *for3Δ* cells, tea1p-YFP was localized normally at cell tips in the large majority of cells (Fig. 4 g, right; 92% cells, $n = 100$). Bud6p-CFP localized normally in most *for3Δ* cells (65% cells, $n = 35$ cells). Bud6p-CFP was reduced from one or both ends in other cells (Fig. 4 h, right), showing some interdependency between bud6p and for3p. In contrast to the cell tip localization patterns, the localization of for3p, tea1p, and bud6p at the cell division site were all independent of each other (unpublished data). Thus, these data suggest a dependency pathway in which at certain cell tips, tea1p acts to position bud6p, and bud6p positions for3p.

Tea1p and bud6p regulate actin cable distribution

As for3p is thought to nucleate actin cable assembly, we then examined if tea1p and bud6p influence the formation or distribution of actin cables. We fixed and stained cells for F-actin using Alexa Fluor® phalloidin and imaged them using confocal microscopy. In bipolar wild-type cells, actin was localized in patches that were concentrated at two growing ends, and in a network of actin cables that traversed the long axis of the cell (Fig. 5 a, first panel; 95% cells, $n = 35$). *bud6Δ* mutants exhibited normal concentration of actin patches at the growing end, but only faint actin cables. We measured the fluorescence intensity of Alexa Fluor® phalloidin–stained actin cables (normalized to the intensity of actin patches, which were similar in the *bud6Δ* and wild-type strains) and found that individual actin cables in *bud6Δ* mutants were ∼50% less bright than those in wild-type cells (Fig. 5 b). These findings are consistent with the fact that for3p is partially delocalized in a *bud6Δ* mutant and with a recent finding that budding yeast Bud6p stimulates formin activity in vitro (Moseley et al., 2004). As actin cables consist of bundles of actin filaments, there may be fewer actin filaments present in *bud6Δ* actin cables.

Tea1p affected F-actin distribution in a different manner. In rod-shaped *tea1Δ* cells, actin patches were generally more concentrated at one cell tip, consistent with their monopolar growth pattern (Fig. 5 a, third panel; Mata and Nurse, 1997). *tea1Δ* mutants had robust actin cables, as fluorescence inten-
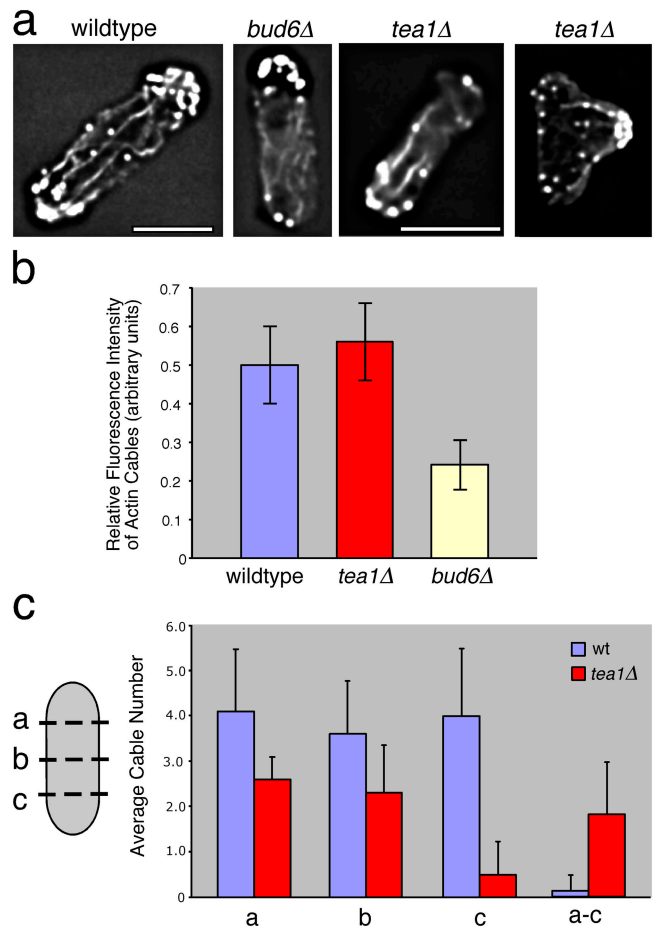

Figure 5. **Tea1p and bud6p affect actin cable organization.** (a) Wild-type (FC421), *bud6Δ* (FC592), and *tea1Δ* (FC691) cells were fixed and stained for F-actin using Alexa Fluor® phalloidin. Single focal planes in the middle of the cell of deconvolved confocal images are shown. Actin cables are the filamentous structures. (b) Fluorescence intensity of Alexa Fluor® phalloidin–stained cables relative to Alexa Fluor® phalloidin–stained actin patches in the same cell. (c) Number of actin cables. In each cell, the number of actin cables in all focal planes was counted at three points corresponding to 25 (a), 50 (b), and 75% (c) of the cell length. In *tea1Δ* cells, (a) was assigned to the growing cell tip and (c) to the nongrowing cell tip. In bipolar wild-type cells, no significant difference was seen at the two tips, and the assignment of (a) and (c) was random. Bars, 5 μm.

sity levels of individual cables were near wild-type levels (Fig. 5 b). To measure the distribution of actin cables, we counted the number of actin cables that cross the cell at three points (25, 50, and 75% of the cell length). Although the numbers of actin cables close to the growing cell tip were similar in *tea1Δ* and wild-type cells, the number of actin cables at the nongrowing cell tip in *tea1Δ* cells was reduced (Fig. 5 c). These distributions of actin cables are consistent with a monopolar distribution of for3p in *tea1Δ* mutants and the bipolar distribution in wild-type cells. Cables in *bud6Δ* mutants were generally difficult to quantify in a definitive manner, as they were so faint. T-shaped *tea1Δ* cells further illustrate how tea1p and for3p regulate the spatial distribution of actin cables. In these cells, actin cables appeared to emanate primarily from the abnormal projections, which contain for3p (Fig. 5 a, fourth panel; Fig. 4 e). Thus, tea1p and bud6p may regu-
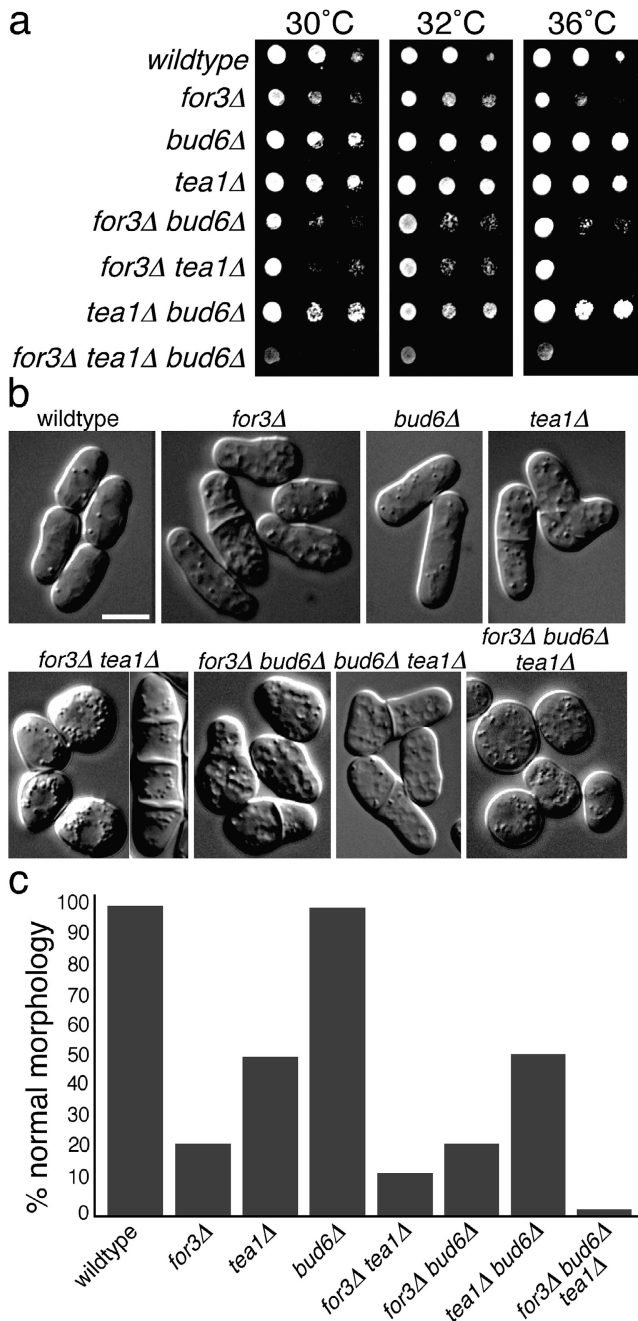
a

b

c

Figure 6. **Synthetic genetic interactions between *for3*⁺, *bud6*⁺, and *tea1*⁺ reveal functions in general cell polarity.** (a) Growth rates. 10-fold serial dilutions of cells with the indicated genotype were spotted onto rich media and grown at 30, 32, and 36°C on agar plates for 4 d. (b) Differential interference contrast images of representative cells with the indicated genotype. (c) Quantitation of shapes of cells of the indicated genotype grown in liquid culture (*n* > 150 cells). Bar, 5 μm.

late cell polarity by affecting formin localization (and perhaps activity) and actin cable organization.

Tea1p, bud6p, and for3p contribute to general cell polarity

To determine if tea1p and bud6p function solely to regulate for3p or whether they have additional functions, we ana-

lyzed double- and triple-mutant combinations (Fig. 6). If these proteins function in a strict linear pathway, we predicted that multiple mutants would have similar phenotypes as single mutants. However, *for3Δtea1Δ* cells grew slower (Fig. 6 a) and had more aberrant morphology than either single mutant, as most cells were ovoid in shape (Fig. 6, b and c). These double mutants did not form T-shaped cells seen in *tea1Δ* cells (Fig. 6 b, compare *tea1Δ* panel to *for3Δtea1Δ* panel), suggesting that for3p is required for cell growth from the sides of cells, as is bud6p (Jin and Amberg, 2001). In addition, 30% of these *for3Δtea1Δ* cells exhibited a different morphology that was not apparent in either single mutant: long cells with multiple septa (Fig. 6 b; *for3Δtea1Δ*, right cell). This phenotype is indicative of a cell–cell separation defect similar to those of septin mutants (Longtine et al., 1996) or exocyst mutants (Wang et al., 2002), and suggests a defect in initiating cell growth at the new ends (Fig. 6 b). *for3Δbud6Δ* double mutants were similar to *for3Δ* single mutants, but were slightly rounder (Fig. 6, b and c), whereas *bud6Δtea1Δ* mutants exhibited no synthetic effects and resembled *tea1Δ* mutants (Glynn et al., 2001). Strikingly, the *for3Δbud6Δtea1Δ* triple-mutant cells were extremely slow growing and formed round or oval cell shapes (Fig. 6). The severe polarization defect in the triple mutant indicates that these proteins do not simply regulate the transitions from monopolar to bipolar growth as previously thought, but work together to organize general polarized growth. These synthetic genetic interactions demonstrate that these genes do not operate in a linear pathway, but may function in parallel pathways or in the context of a common complex (the "polarisome;" see Discussion) that is required for polarized cell growth.

Tea1p interacts with the CLIP-170–like protein tip1p

To find additional proteins that interact with tea1p, we performed a two-hybrid screen and identified a third tea1p-interacting protein: tip1p, a CLIP-170 homologue (Brunner and Nurse, 2000). Protein coimmunoprecipitation from yeast extracts confirmed this two-hybrid interaction (Fig. 2 c). Two-hybrid analyses further showed that a central region in the coiled coil domain of tip1p (aa 207–299) was required for the tea1p interaction, but not its COOH-terminal "cargo-binding" or putative metal-binding domains (aa 299–462; Table I). Interestingly, in a sucrose gradient, tip1p co-migrated with the smallest (12S) tea1p complex that appeared to be distinct from the complexes containing for3p and bud6p (Fig. 3 a, wt). The migration of tip1p changed little (one fraction smaller in some experiments) in a *tea1Δ* mutant, suggesting that other proteins may be the same complex.

Previous papers have shown that tip1p (but not for3p or bud6p) colocalizes with tea1p on the microtubule plus end (Brunner and Nurse, 2000; Niccoli and Nurse, 2002). Tip1p is required for proper localization of tea1p to the microtubule plus ends and cell tips, and *tip1Δ* mutants exhibit similar morphological defects as *tea1Δ* cells (Brunner and Nurse, 2000). We examined the abnormal distribution of tea1p in *tip1Δ* cells using time-lapse microscopy. Dual imaging of tea1p and microtubules showed that tea1p dots were in multiple dots or dashes all along the microtubule, with increased concentration around medial regions of mi-

crotubule overlap (Video 4, available at http://www.jcb.org/cgi/content/full/jcb.200403090/DC1). Many tea1p dots on the microtubules were not motile. Other tea1p dots moved in either a minus end– or plus end–directed manner and were present on growing or shrinking microtubule ends and sites along the microtubule bundles. The large number of tea1p dots and their occasional distribution in lines suggested that they are localized not only at microtubule plus ends, but also along the length of microtubules. A previous report showed that in a *tea1Δ* mutant, tip1p localizes normally at the microtubule plus end, but does not accumulate at the cell end (Brunner and Nurse, 2000). Thus, the interaction between tea1p and tip1p may help tip1p attach tea1p to the plus end of the microtubule, and allow tea1p to retain tip1p at the cell end.

Discussion

Here, we have investigated mechanisms of tea1p localization and function, which reveal a potential pathway for how microtubules regulate actin assembly and cell polarity. Our analyses here, together with previous ones (Brunner and Nurse, 2000; Behrens and Nurse, 2002; Snaith and Sawin, 2003), suggest that tea1p is carried on growing plus end microtubules to the cell tip through an association with the CLIP-170 tip1p (Fig. 7). When the microtubule reaches the cell cortex, tea1p docks at the cell tip and is retained after microtubule shrinkage. At the cell surface, tea1p may regulate cell polarity and actin cable distribution through interaction with the formin for3p and bud6p. Here, we have defined physical and functional interactions between tea1p with tip1p, for3p, and bud6p. Tea1p appears to reside in at least two types of protein complexes: a 12S tea1p–tip1p complex and large (75S and possibly a 45S) tea1p–bud6p–for3p complexes, which may identify different molecular aspects of tea1p in this multi-step process. These analyses suggest how microtubule-based transport and regulation of cell polarity factors function to establish or maintain their cell polarity and cell shape.

Cell polarity and formin regulation

These analyses provide new insights into the regulation of formins and illustrate how the spatial regulation of formins may control the spatial organization of actin. Characterization of the *bud6Δ* mutant suggests that bud6p is required for proper for3p localization and is needed to attach for3p to the 45S complex (complex C). Further, the faint actin cables in *bud6Δ* mutants are consistent with a finding that bud6p stimulates formin actin assembly in vitro (Moseley et al., 2004). As budding yeast bud6p and formin homologues directly interact through their COOH-terminal regions (Kikyo et al., 1999), it is likely that fission yeast bu6p and for3p also interact directly.

Tea1p may somehow regulate the localization of for3p and bud6p at cell tips. In *tea1Δ* mutants, for3p, bud6p, and actin cables are concentrated at only one cell tip, indicating that tea1p is needed for for3p and bud6p localization to the second cell tip. The physical interactions among these proteins suggest that tea1p may directly recruit or stabilize for3p and bud6p to the second cell tip. However, it is also possible that
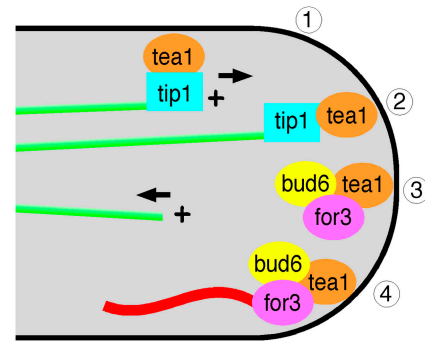

Figure 7. **Model of how microtubule plus ends regulate actin assembly.** Step 1: tea1p is bound to the microtubule plus ends by interaction with the CLIP-170 tip1p, and the growth of microtubules (green) delivers tea1p to the cell tip. Step 2: tea1p leaves the microtubule and docks at the cell surface when the microtubule shrinks back. Step 3: at certain cell tips, tea1p may recruit, stabilize, or otherwise regulate the polarisome complex, which includes bud6p and the formin for3p. Step 4: the complex promotes polarized cell growth, with for3p functioning to assemble actin cables (red) at the cell tip.

tea1p affects these factors in a more indirect manner. Because of photobleaching problems for for3p-YFP, we have been unable use time-lapse microscopy to observe if tea1p dots stimulate the formation of for3p dots at cell tips. We should also stress that for3p and bud6p still localize to one of the cell tips and to the septum in the absence of tea1p, suggesting that additional mechanisms (such as cortical landmarks) contribute to for3p localization and activity.

As for3p, bud6p, and tea1p function in actin regulation and cell polarity, we speculate that large complexes represent polarisome complexes. An analogous polarisome complex in budding yeast has been proposed (Sheu et al., 1998; Pruyne and Bretscher, 2000), but large complexes containing formins have not yet been directly demonstrated in any other organism. The round cell phenotype of the *for3Δ bud6Δ tea1Δ* triple mutant may reflect the phenotype of a nonfunctional polarisome complex. Biochemical analyses of the *S. pombe* complexes suggest that complexes are still mostly intact in single mutants, but may have defects in their regulation or activity. The synthetic genetic effects indicate that these polarisome factors collectively not only regulate actin cable formation, but additional aspects of polarized cell growth, such as secretion. Future identification of additional polarisome complex components will provide further molecular insights into polarisome functions.

CLIP-170s and microtubule plus ends

CLIP-170 is a conserved microtubule plus end–binding protein that regulates microtubule stability (Carvalho et al., 2003). In budding yeast and mammalian cells, CLIP-170s have been found to attach the dynactin complex to the microtubule plus ends (Goodson et al., 2003; Sheeman et al., 2003). However, dynein appears to have only meiotic-specific functions in fission yeast (Yamamoto et al., 1999). In fission yeast, the CLIP-170 tip1p functions to stabilize microtubules when they contact the sides of cells (Brunner and Nurse, 2000). In addition, tip1p also functions to attach

tea1p to the microtubule plus end. In the absence of tip1p, it is interesting that tea1p still associates with microtubules and moves along microtubules in both minus end and plus end directions, suggesting that tea1p also associates with other microtubule-associated proteins, and possibly one or more motor proteins. One candidate motor protein is tea2p, a Kip2-like kinesin that appears to move factors such as tea1p and CLIP-170 to the microtubule plus end (Browning et al., 2000, 2003). Interactions among other microtubule plus end–binding proteins including tea2p and the EB1 homologue mal3p have been found (Browning et al., 2003). Further analyses will test whether the tea1p–tip1p associate complex represents a microtubule plus end complex.

Interactions between microtubules and actin

It is becoming increasingly clear that many cytoskeletal processes, including cell polarization, organelle transport, and cytokinesis, depend on interactions between the microtubule and actin cytoskeletons. Our analyses suggest a simple model for how microtubules may instruct the actin cytoskeleton. Conversely, in some cell types the actin cytoskeleton and/or formins may also function to move or stabilize microtubules (Palazzo et al., 2001; Gundersen, 2002). For instance, in cytokinesis, microtubule plus ends are stabilized at the cortex specifically in the region of the future cell division site (Canman et al., 2003). However, there is little evidence that actin or for3p directly control microtubule dynamics in interphase fission yeast cells (Feierbach and Chang, 2001; unpublished data). Mammalian CLIP-170 has been implicated in microtubule–actin interactions, for instance through interaction with IQGAP protein (Fukata et al., 2002). The animal equivalents to tea1p and bud6p are not yet clear. The formin-interacting regions of yeast Bud6 proteins are similar to a region in Rho kinase (Glynn et al., 2001; Moseley et al., 2004), but the functional significance of this Rho kinase region has not been investigated in animal cells. Of kelch repeat proteins similar to tea1p (Adams et al., 2000), budding yeast Kel1 and Kel2 are required for polarized cell growth (Philips and Herskowitz, 1998), mammalian Keap protein is localized on focal adhesions and zipper junctions (Velichkova et al., 2002), p97 is a Rab effector (Diaz et al., 1997), and *Drosophila* kelch is a component of ring canals (Robinson and Cooley, 1997). Future work will be needed to test how these conserved sets of proteins may function together in the integration of the microtubule and actin cytoskeletal networks in different cell types.

Materials and methods

Yeast strains, media, and genetic methods

S. pombe strains used in this paper are listed in Table S1 (available at http://www.jcb.org/cgi/content/full/jcb.200403090/DC1). Standard methods for *S. pombe* media and genetic manipulations were performed as described previously (Feierbach and Chang, 2001; Glynn et al., 2001). pFA6a-TAP-kanMX was obtained from K. Gould (Vanderbilt University, Nashville, TN; Tasto et al., 2001). Tea1p fusions contained a linker sequence of $(Gly)_6$ between tea1p and the tags. All fusions were made by recombination of PCR-derived constructs into the COOH terminus of the appropriate gene at the chromosomal locus and were functional.

Microscopy

Wide-field and spinning disc confocal microscopy were performed as described previously (Pelham and Chang, 2001). For live-cell imaging, 1–2

µm cell slurry in media was placed under the coverslip, with no agarose pad or sealant. Actin staining was performed as described previously (Pelham and Chang, 2001) using Alexa Fluor® 488 phalloidin (Molecular Probes, Inc.). Acquisition, three-dimensional reconstruction, and re-strained iterative deconvolution were performed using Openlab software (Improvision).

Immunoprecipitations and sucrose gradients

For extract preparation, yeast cells were grown in Edinburgh minimal media with appropriate amino acid supplements, harvested, washed, and re-suspended in an equal volume of CXS buffer (50 mM Hepes, pH 7.5, 20 mM KCl, 1 mM $MgCl_2$, 2 mM EDTA, and protease inhibitor cocktail). The cell slurry was quick-frozen as pellets in liquid nitrogen and ground while frozen into a powder using a mortar and pestle. The resulting powder was thawed and protease inhibitors and 0.1% Triton X-100 were added. The difference in Triton X-100 concentration accounts for the difference in 12–20S complex mobilities seen in Glynn et al. (2001), which used 1% Triton X-100. Velocity sucrose gradients were performed as described previously (Glynn et al., 2001). For Western blotting, we used monoclonal anti-HA antibody HA.11 (Covance), polyclonal anti-myc antibody A-14 (Santa Cruz Biotechnology, Inc.), polyclonal rabbit anti-tea1p antibody (a gift from P. Nurse, Imperial Cancer Research Fund, London, UK), and rabbit polyclonal anti-tip1p antibody (a gift from D. Brunner, EMBL, Heidelberg, Germany). For estimation of complex size, we probed sucrose gradient fractions for ribosomal subunits using anti-ribosomal antibodies (gifts from J. Warner, Einstein College of Medicine, Bronx, NY and L. Pon, Columbia University, New York, NY) and ran gel filtration markers (Bio-Rad Laboratories) in parallel sucrose gradients.

For immunoprecipitations, 50 µl soluble yeast extract was added to 25 µl protein A–Sepharose bead slurry (Sigma-Aldrich; Fig. 2 a), 25 µl Dynal mouse anti–rabbit magnetic bead slurry (Dynal Corp.; Fig. 2 c), or 25 µl Dynal sheep anti–mouse magnetic bead slurry (Dynal Corp.; Fig. 2, b and c). The protein A–Sepharose beads were washed twice in 1× PBS, pH 7.4, and preabsorbed for 2 h with either monoclonal anti-HA antibody HA.11 (Covance) or monoclonal anti-myc antibody 9E10 (Santa Cruz Biotechnology, Inc.). Dynal mouse anti–rabbit beads were washed twice in 1× PBS, pH 7.4, and complexed with 2 µg of 10 mg/ml IgG antibody (Sigma-Aldrich). Dynal sheep anti–mouse beads were washed twice in 1× PBS, pH 7.4, and complexed with 2 µg of 5 mg/ml anti-HA antibody. After a 90-min incubation, all reactions were washed three times with CXS buffer (Fig. 2, a and b) or CXS buffer with 150 or 250 mM NaCl (Fig. 2 c). Dynal beads were collected using a Magnetic Particle Concentrator (Dynal Corp.). Immunoprecipitations were then boiled in 45 µl sample buffer and loaded onto SDS-PAGE gels.

Two-hybrid protein interaction analyses

A fragment of the *bud6*⁺ gene (nt 3361–4801) was inserted into pGAD GH vector (CLONTECH Laboratories, Inc.). The *for3* two-hybrid constructs were gifts from K. Nakano and I. Mabuchi (University of Tokyo, Tokyo, Japan; Nakano et al., 2002). Two-hybrid constructs were transformed into TAT7 (Mat a his3-Δ200 leu2-3,112 trp1-Δ90 ade2-101 gal80 lys::LYS2:: lexAopHIS3 ura3::URA3::lexAop-lacZ). Lac Z expression and/or histidine auxotrophy were scored.

Online supplemental material

Time-lapse movies of tea1p dynamics in wild-type and *rsp1-1* mutants are available at http://www.jcb.org/cgi/content/full/jcb.200403090/DC1.

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