Role of microtubules and tea1p in establishment and maintenance of fission yeast cell polarity

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Summary

Microtubules and the protein tea1p have important roles in regulating cell polarity in the fission yeast *Schizosaccharomyces pombe*. Here, using combinations of drugs, environmental perturbations and genetic mutants, we demonstrate that once a cell polarity axis is established, microtubules have at best a minor role in maintaining the cortical actin cytoskeleton and the rate and direction of cell growth. In addition, we find that after perturbations that disrupt cell polarity and the cortical actin cytoskeleton, microtubules are not required for re-establishment of polarity per se. However, after such perturbations, the distribution of cytoplasmic microtubules plays an important role in dictating the position of sites of polarity re-establishment. Furthermore, this influence of microtubule distribution on site selection during polarity re-establishment requires the presence of tea1p, suggesting that tea1p is crucial for coupling microtubule distribution to the regulation of cell polarity. Our results suggest a model in which, at the cellular level, two distinct and separable mechanisms contribute to how tea1p regulates site selection during polarity re-establishment. First, tea1p remaining at cell tips after cortical depolarization can serve as a cortical landmark for microtubule-independent site selection; second, tea1p newly targeted to the cell cortex by association with microtubules can promote the formation of polarity axes de novo.

Key words: Fission yeast, *Schizosaccharomyces pombe*, Microtubules, tea1p, Cell polarity

Introduction

Microtubules play a role in cell polarity in a wide variety of cell types (Ahringer, 2003; Drubin and Nelson, 1996; Gundersen, 2002; Schuyler and Pellman, 2001; Small and Kaverina, 2003; Yarm et al., 2001). In the fission yeast *Schizosaccharomyces pombe*, the normally cylindrical cell shape is altered when microtubule function is impaired either by drugs or mutation (Radcliffe et al., 1998; Sawin and Nurse, 1998; Toda et al., 1983). Although it is still not well understood how microtubules affect polarized growth in detail, or how this operates at a molecular level, it is likely that some functions of microtubules in regulating cell polarity may be mediated by the protein tea1p (Chang and Peter, 2003; Mata and Nurse, 1997).

It has been shown that tea1p is normally concentrated at cell tips and is targeted there by association with the plus ends of growing microtubules (Behrens and Nurse, 2002; Mata and Nurse, 1997; Snaith and Sawin, 2003). Cells deleted for tea1p (*tea1*Δ) are viable but display various polarity defects, including the appearance of bent cells (approximately 30% of cells) at 25°C and both bent cells and branched cells (approximately 10-20%) after a temperature shift to 36°C. *tea1*Δ mutants also show defects in NETO (new-end take-off), which is the transition from monopolar to bipolar growth extension (Mitchison and Nurse, 1985). Several proteins have been identified that might interact functionally with tea1p, including: pom1p, a cortically localized protein kinase involved in cell polarity and cytokinesis (Bahler and Pringle, 1998); bud6/aip3p, an actin-associated protein involved in NETO (Glynn et al., 2001; Jin and Amberg, 2001); tea3p, a tea1p-related protein that contributes to NETO (Arellano et al., 2002); and mod5p, a membrane protein required for anchoring of tea1p to the cell cortex (Snaith and Sawin, 2003).

Previously, we described a system for the induction of branched cells in *S. pombe*. In this system, fission yeast cells are arrested in G1 by the reversible temperature-sensitive *cdc10-129* mutation (MacNeill and Nurse, 1997; Nurse et al., 1976), treated with the microtubule inhibitor thiabendazole (TBZ) and then released from the cell-cycle arrest, in the continued presence of TBZ (Sawin and Nurse, 1998). Under these conditions, typically 35-40% of cells synchronously initiate a completely new polarity axis from the cell middle within two hours after re-entry into the cell cycle, resulting in the formation of branched cells (Fig. 1). We showed that, upon TBZ treatment, the cortical actin cytoskeleton becomes transiently depolarized and subsequently relocalizes from cell tips to cell middles, possibly mediated by cortical polarity proteins such as ral3p/scd2p (Chang et al., 1994; Fukui and Yamamoto, 1988) and the protein kinase pak1p/shk1p/orb2p (Marcus et al., 1995; Ottilie et al., 1995; Sawin et al., 1999; Verde et al., 1998).

Two outstanding questions emerge from these experiments concerning the mechanism by which a cell forms a polarity axis de novo. Do the short microtubules remaining in the middle of cells after TBZ treatment play a role in the initiation of the new axis? If they do, what additional molecules mediate their ability to help recruit or position the cell polarity

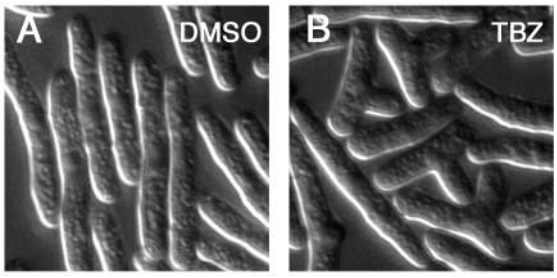


Fig. 1. Assay for cell branching in fission yeast. *cdc10-129* mutant cells are arrested in G1 by a temperature shift, drugs are added, and the temperature is subsequently shifted down to allow cells to re-enter the cell cycle. Cells are shown 2 hours after release from cell-cycle arrest, in the presence of (A) control DMSO or (B) 100 μg/ml TBZ.

machinery? Here we have addressed these questions using a variety of drug treatments, environmental perturbations and genetic backgrounds. These results reveal that, in contrast to previous reports, microtubule disruption in fission yeast does not significantly affect the maintenance of cell polarity. Moreover, although polarity establishment can occur in the absence of microtubules, when microtubules are present, their distribution correlates with the position of polarity establishment sites. This suggests that during or prior to polarity establishment, microtubules communicate with the cell cortex to help position the cell polarity machinery. Finally, we present strong genetic evidence that the protein tea1p is required for this communication from the microtubules to the cell cortex during polarity establishment. On the basis of these results, we present a general model for the microtubule-mediated regulation of cell polarity in fission yeast that can account for a wide range of mutant and/or aberrant morphological phenotypes.

Materials and Methods

Strains, media, drugs

Strains were constructed using conventional methods (Moreno et al., 1991). YE5S medium (Difco Yeast Extract, Becton-Dickinson) was used for branching experiments. For the elutriation experiment in Fig. 4D, Edinburgh Minimal Medium was used, but with sodium glutamate as nitrogen source (5 g/L) instead of ammonium chloride. Induction of ral3-GFP expression from the *nmt1* promoter was as described (Sawin and Nurse, 1998). Stocks of thiabendazole (TBZ; Aldrich) and methyl benzimidazol-2yl carbamate (carbendazim, MBC; Aldrich) were made fresh in DMSO and used at the concentrations specified, with the final DMSO concentration not exceeding 1%. Latrunculin B (LatB; Calbiochem) was used from a 20 mM stock in DMSO, at a final concentration of 200 μM, which we found to be essential for complete depolymerization of the F-actin cytoskeleton, as assayed by our most sensitive methods of staining with Alexa568 Phalloidin (Sawin and Nurse, 1998) (additional data not shown).

Physiological experiments

For physiological experiments, all cultures were grown in shaking water baths. Cell-branching experiments using TBZ in *cdc10-129* backgrounds were performed essentially as described (Sawin and Nurse, 1998), with only minor modifications as required by additional drug additions or wash-outs. Briefly, for TBZ branching experiments, cells were cultured at 25°C, then shifted to 36°C for four hours, and then treated with TBZ or MBC for an additional 30 minutes before shifting back to 25°C in the presence of drug. For addition of TBZ plus MBC, the drugs were added simultaneously. For TBZ-washout, cells were rapidly filtered into fresh, pre-warmed medium 15 minutes after shift-down to 25°C (i.e. 45 minutes after drug addition). For LatB-pulse experiments in *cdc10-129* backgrounds, mutants were grown at 25°C and then shifted to 36°C for four hours, at which time either LatB plus DMSO or LatB plus MBC was added to the culture. After 30 minutes, cells were shifted back to 25°C, and 15 minutes later rapidly filtered into pre-warmed medium containing either DMSO or MBC, as appropriate. The appearance of branched cells was always scored relative to the time of shift-down to 25°C.

Return-to-growth experiments with *tea2-1* and *tea1*Δ mutants were as described (Snaith and Sawin, 2003), except that stationary-phase cells at 25°C were diluted 1:70 into fresh medium at 25°C. Branched cells were scored three hours after dilution.

Temperature shifts of exponentially growing cultures were performed by transfer of culture flasks from one water bath to another, and branched cells scored 2 hours after shift.

In all assays of cell branching, 300 cells were scored for each time point and/or condition.

Microscopy

Immunofluorescence microscopy using TAT1 anti-tubulin antibodies (gift of Prof. K. Gull, University of Oxford) was performed as described (Sawin and Nurse, 1998; Snaith and Sawin, 2003). Stained cells were imaged by laser scanning confocal microscopy (Leica) under constant conditions. Fig. 2 shows maximum projections of Z-stacks, to emphasize the degree of microtubule disruption. Fig. 9 shows average projections of Z-stacks, all taken under the same conditions, to allow comparison of the relative tea1p signal on microtubules and at cell tips in wild-type cells and mutants.

Rhodamine-Phalloidin staining of actin and ral3-GFP localization were done exactly as described (Sawin and Nurse, 1998).

Results

Two microtubule drugs, TBZ and MBC, have different effects on cell polarity

Even at relatively high doses (100 μg/ml; 500 μM), TBZ does not produce a complete disruption of interphase microtubules in fission yeast (Sawin and Nurse, 1998) (see also Fig. 2). In order to determine whether residual microtubules might be important for establishing the new polarity axis during branch formation, we tested whether a different microtubule drug, methyl benzimidazol-2yl carbamate (carbendazim, MBC), might be more effective in depolymerizing microtubules. As seen by anti-tubulin immunofluorescence, at doses above 15-20 μg/ml (80-106 μM), MBC rapidly caused a near-complete disruption of cytoplasmic microtubules, with only a few small tubulin-staining spots (typically 1-3 per cell) remaining in the vicinity of the nucleus, the brightest of these probably being the spindle pole body (Fig. 2D,E). MBC treatment had no discernible effect on microtubules in mutant strains carrying the benzimidazole-resistant *nda3-TB101* mutation in beta-tubulin (Fig. 2H,I) (Sawin and Nurse, 1998; Umesono et al., 1983; Yamamoto, 1980).

We then assayed branch formation in block-and-release experiments with *cdc10-129* cells treated with TBZ versus MBC. Whereas TBZ-treated cells formed branches in a threshold-dependent manner, very few cells formed branches when treated with a wide range of MBC concentrations,

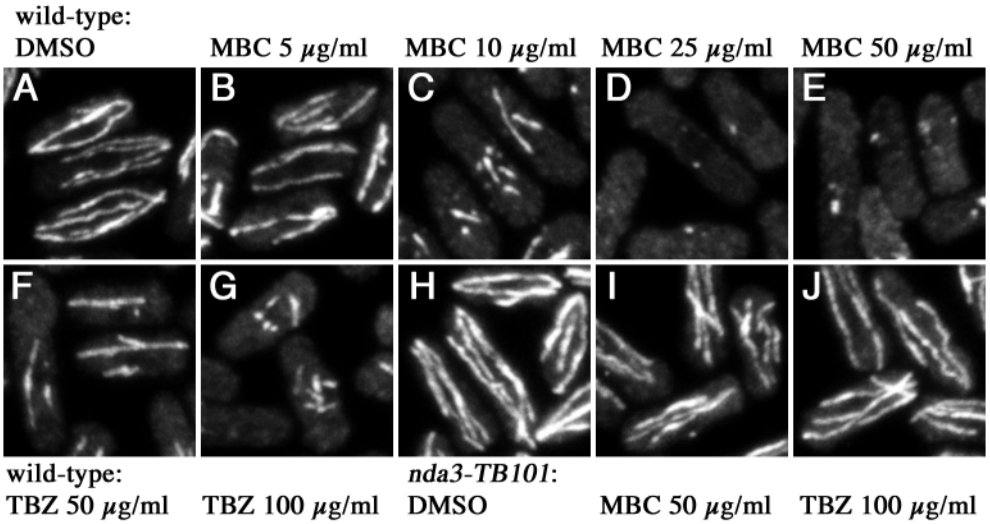


Fig. 2. MBC is a more potent microtubule inhibitor than TBZ. Anti-tubulin immunofluorescence images of wild-type cells (A-G) treated with (A) DMSO, (B) 5 μg/ml MBC, (C) 10 μg/ml MBC, (D) 25 μg/ml MBC, (E) 50 μg/ml MBC, (F) 50 μg/ml TBZ or (G) 100 μg/ml TBZ; and benzimidazole-resistant *nda3-TB101* beta-tubulin mutant cells (H-J) treated with (H) DMSO, (I) 50 μg/ml MBC or (J) 100 μg/ml TBZ. Similar microtubule distributions were seen at both 10 and 90 minutes of drug treatment (not shown).

including concentrations that mimicked the microtubule disruption seen in TBZ-treated cells (Fig. 3). (Branched cells were seen in MBC treatment only at late times, when cells were very long, which is also seen in prolonged MBC treatment of G2-arrested *cdc25-22* mutant cells; K.E.S., unpublished.) Because TBZ treatment transiently disorganizes the polarized actin cytoskeleton at cell tips for 90-120 minutes (Sawin and Nurse, 1998), one possible explanation for the observed differences in branching was that the ability of TBZ to depolarize the actin cytoskeleton, which may be important for branching, results not from microtubule disruption but from the drug affecting an additional, non-tubulin target(s) in the cell. We therefore compared the effects of TBZ and MBC treatment on different markers for cell polarity.

In contrast to cells treated with TBZ, cells treated with MBC did not show any disorganization of the actin cytoskeleton (Fig. 4A,E,F). Similarly, although we previously showed that TBZ treatment transiently delocalizes a ral3-GFP fusion protein from cell tips (Sawin and Nurse, 1998), MBC had no effect on ral3-GFP distribution over time (Fig. 4B,G,H). A combined treatment of both TBZ and MBC also delocalized actin and ral3-GFP (Fig. 4A,B), indicating that the lack of depolarization seen in MBC-treated cells was not due to the specific inhibition of depolarisation by MBC.

Given these striking differences, we also measured rates of cell elongation during the different drug treatments. Whereas TBZ treatment led to a transient arrest in elongation that correlated roughly with the period of actin and ral3-GFP disruption (Sawin and Nurse, 1998), microtubule disruption by MBC had no measurable effect on cell elongation as compared with DMSO-treated controls (Fig. 4C). To confirm that this conclusion is also applicable to wild-type cells, we repeated measurements of cell growth rates using both elutriated cells from asynchronous exponentially growing cultures and also cells recovering from nitrogen starvation. In all cases, we found that TBZ treatment arrested cell elongation, yet MBC treatment did not (Fig. 4C-D; additional data not shown).

Two main conclusions emerge from these experiments. First, in contrast to previous reports (Radcliffe et al., 1998; Sawin and Nurse, 1998; Toda et al., 1983), microtubules do not appear to play a significant role in the maintenance of cell polarity or

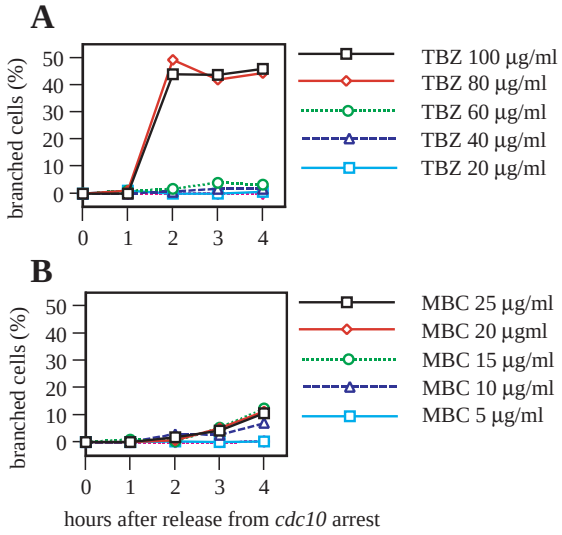
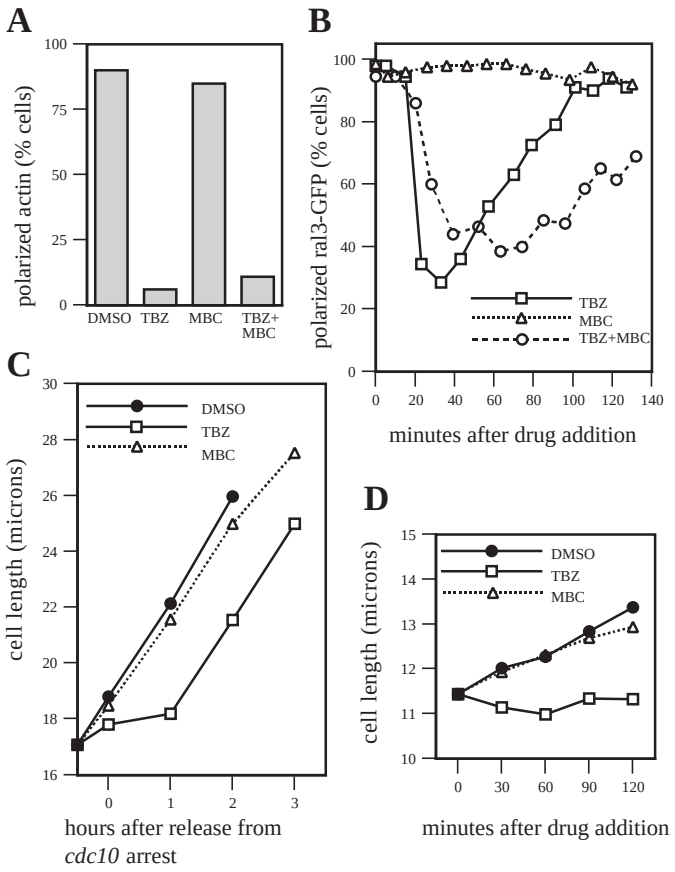


Fig. 3. TBZ but not MBC causes high levels of cell branching. The frequency of cell branching was measured in temperature-shift block-and-release experiments, using *cdc10-129* mutants (see Fig. 1) and different concentrations of (A) TBZ or (B) MBC.

a polarized actin cytoskeleton in fission yeast. Second, the effects of TBZ on cortical cell polarity are likely to be due to effects on an additional target, distinct from tubulin, and thus MBC should be considered a much more specific reagent for microtubule inhibition in fission yeast.

Branch formation depends on the presence of short microtubules

With these results in mind we then wanted to test whether, once a transient depolarization of the cortical actin cytoskeleton cells has occurred, the remaining microtubule distribution is important in establishing the new polarity axis that is created during cell branching. We used TBZ to depolarize the cortical actin cytoskeleton but then either: (1) further depolymerized microtubules with MBC treatment; or (2) washed out TBZ,



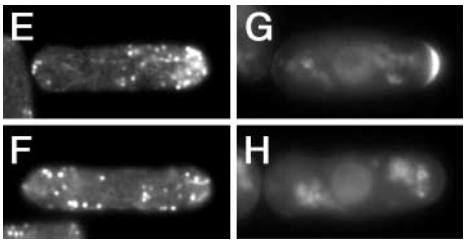


Fig. 4. TBZ but not MBC causes transient depolarization of the cortical actin cytoskeleton and arrest of cell elongation. (A) Percentage of *cdc10-129* arrested cells with a polarized actin cytoskeleton 45 minutes after addition of DMSO, TBZ, MBC or TBZ plus MBC. (B) Time-course showing the percentage of *cdc10-129* arrested cells with polarized ral3-GFP, after addition of TBZ, MBC or TBZ plus MBC. (C) Mean cell length versus time in a *cdc10-129* block-and-release experiment, using cells treated either with DMSO, TBZ or MBC. (D) Mean cell length versus time in wild-type elutriated cells treated either with DMSO, TBZ or MBC. (E-H) Images of *cdc10-129* cells showing Rhodamine-Phalloidin staining of (E) polarized actin and (F) depolarized actin, and live-cell fluorescence of (G) polarized ral3-GFP and (H) depolarized ral3-GFP.

which restores cytoplasmic microtubules to normal lengths within a few minutes (data not shown); or (3) performed the experiment in an *nda3-TB101* mutant background, in which the microtubules are not significantly altered upon TBZ treatment (Fig. 2J), although a brief depolarization of the actin cytoskeleton is nonetheless observed (Sawin and Nurse, 1998). When both TBZ and MBC were added to cells, branch formation was strongly suppressed relative to TBZ addition alone (Fig. 5A), although cells nevertheless recover polarity and resume growth (see Fig. 4B; additional data not shown). Branching was also suppressed when TBZ was washed out of the culture medium (Fig. 5C), and also in the *nda3-TB101* mutant background (Fig. 5E).

These results indicate that, after transient cortical depolarization, the short microtubules caused by TBZ treatment are crucial for the ectopic polarity re-establishment that leads to branching. We interpret this to mean that, after depolarization, microtubules might convey a signal to the cortex that helps determine the position of polarity establishment sites during the recovery of the cortical actin cytoskeleton. According to this view, when microtubules are short (i.e. TBZ treatment), a significant number of cells reposition the cell polarity machinery to an ectopic site, in the middle of the cell. By contrast, when microtubules are long (TBZ wash-out, or *nda3-TB101* mutant), they would mediate polarity re-establishment back at the pre-existing cell tips. Finally, when microtubules are more strongly disrupted, there would be no microtubule-based signal for positioning polarity (re)establishment, but in this case there may be residual cortical landmarks left at the pre-existing cell tips that are able to re-recruit the polarity machinery (see Discussion for further details).

The results of these experiments also suggest that the formation of branches might involve two separable processes: first, some form of depolarization of the cortical actin cytoskeleton and, second, the presence of short microtubules that somehow target the polarity machinery to ectopic cortical sites. Because the mode of action of TBZ is not entirely clear, we therefore examined the effects on cell polarity of other treatments that either depolarize the actin cytoskeleton or produce short microtubules (or both) in the absence of TBZ.

Mutants in the kinesin-related protein tea2p have short interphase microtubules and form branches at a high frequency in the very first cell cycle after being grown to stationary phase and then returned to fresh medium (Browning et al., 2000; Verde et al., 1995). In such return-to-growth experiments, we found that MBC strongly reduced the frequency of cell branching in *tea2-1* mutants, but not in drug-resistant *tea2-1 nda3-TB101* mutants, in which short microtubules persisted (Fig. 6; additional data not shown). Thus, in *tea2-1* mutants recovering from stationary phase, as in TBZ-treated *cdc10-129* cells, short microtubules are necessary for ectopic axis formation, whereas a more complete microtubule disruption allows cells to re-establish polarity at pre-existing cell tips.

tea2-1 mutants normally form branches only in return-to-growth experiments, and not during exponential growth (Browning et al., 2000). However, we found that if we pulsed *cdc10-129 tea2-1* mutants with high doses of the actin inhibitor latrunculin B (LatB) to depolymerize F-actin completely during block-and-release experiments (Rupes et al., 1999), a very high frequency of branched cells could be observed (80-90% of cells), much more than is seen in experiments involving depolarization by TBZ (Fig. 7A; compare with Fig. 5). Strikingly, this branching was almost completely inhibited by microtubule disruption with MBC, confirming our finding that the short microtubules in *tea2-1* mutants are essential for the formation of an ectopic polarity axis. However, these MBC-

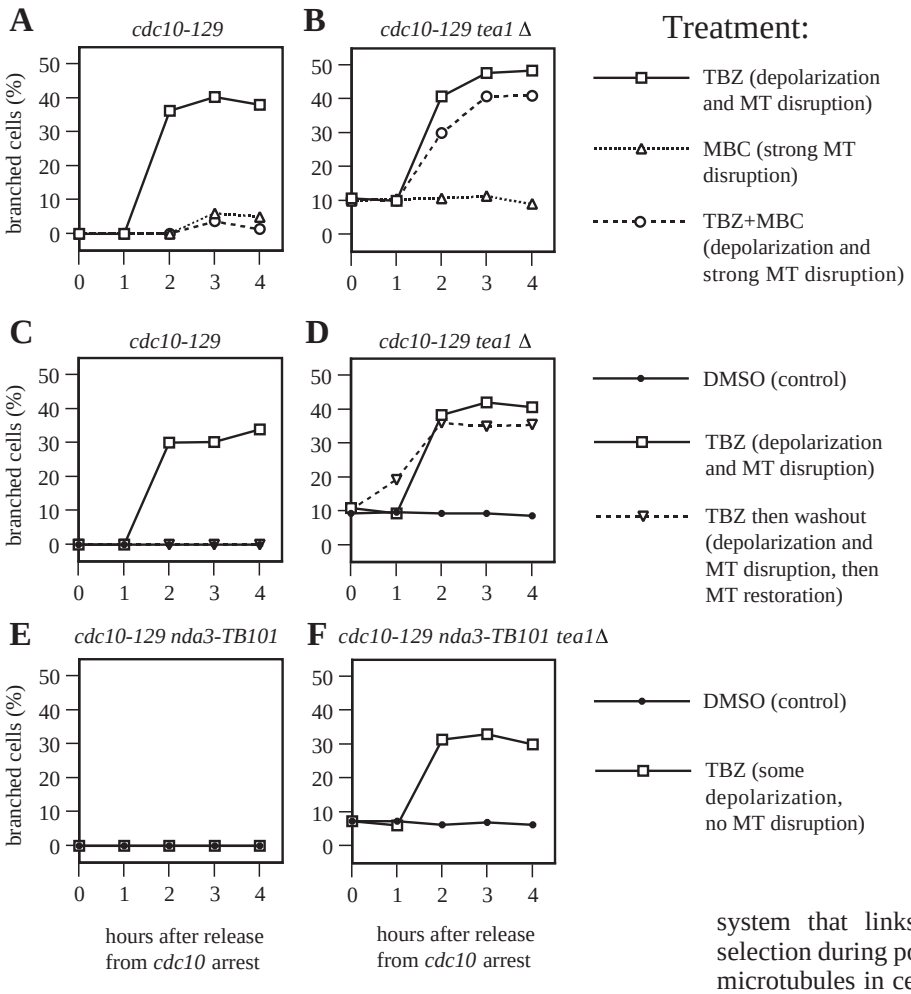


Fig. 5. In *tea1+* cells, the formation of branches after TBZ-induced cell depolarization requires the presence of short microtubules but, in *tea1*Δ cells, formation of branches after TBZ treatment is independent of microtubule distribution. The frequency of cell branching was measured in G1 block-and-release experiments using *cdc10-129* mutants that were either *tea1+* (A,C,E) or *tea1*Δ (B,D,F). The genotype of each strain is indicated in each panel (A-F). In one set of experiments (A,B), cells were treated with either TBZ or MBC, or with TBZ plus MBC. In a second set (C,D), cells were treated with DMSO or TBZ, or with TBZ followed by wash-out of the drug after release from the G1 arrest. In a third set, a benzimidazole-resistant beta-tubulin mutant background (*nda3-TB101*) was used, and cells were treated with either DMSO or TBZ. The baseline frequency of branched cells is higher in *tea1*Δ mutants because they branch in response to the temperature shift required for the *cdc10-129* arrest.

treated cells did continue to elongate (data not shown), further supporting the observation that microtubules are not essential for polarity re-establishment at pre-existing cell tips after depolarization or for cell growth in general. In control experiments, MBC did not lower the branching frequency in *cdc10-129 nda3-TB101 tea2-1* mutants, which have MBC-resistant microtubules (Fig. 7B).

In *tea1*Δ mutants, microtubule distribution is not correlated with the position of polarity establishment sites

Collectively, the results of the above experiments suggest that microtubules in fission yeast are not required for the maintenance of polarized growth or for polarity establishment per se but are primarily important in positioning sites of polarity establishment after depolarization. How might microtubules be linked to the cortical actin cytoskeleton or the polarity establishment machinery? The protein tea1p is known to be transported to cell tips by association with the plus-ends of growing microtubules (Behrens and Nurse, 2002; Mata and Nurse, 1997; Snaith and Sawin, 2003), and thus is a good candidate for a molecule linking microtubule distribution to growth site selection during polarity re-establishment. However, one of the salient features of *tea1*Δ mutants is that they form branches after a variety of environmental perturbations (Behrens and Nurse, 2002; Mata and Nurse, 1997; Sawin and Nurse, 1998; Snaith and Sawin, 2003). Thus, tea1p is clearly not absolutely required for branches to form. To investigate whether tea1p is part of the system that links microtubule distribution to growth site selection during polarity re-establishment, we tested the role of microtubules in cell branching in *tea1*Δ mutant backgrounds.

We first confirmed our previous result (Sawin and Nurse, 1998) that *cdc10-129 tea1*Δ cells treated with TBZ in block-and-release experiments form branches to the same extent and with the same kinetics as *cdc10-129* single mutants (Fig. 5A,B). In addition, we found that *cdc10-129 tea1*Δ double mutants treated with MBC alone did not show any increased branching above the baseline frequency caused by the temperature shift required for the *cdc10* cell-cycle arrest (Fig. 5B). However, the combination of TBZ plus MBC did not significantly reduce the frequency of branching in *cdc10-129 tea1*Δ double mutants relative to that observed with TBZ alone, in contrast to the inhibition of branch formation observed in *cdc10-129* mutants (Fig. 5A,B). Similarly, when TBZ was washed out of *cdc10-129 tea1*Δ cells and the microtubules were allowed to recover, branching still occurred at a similar frequency to that seen without the TBZ wash-out (Fig. 5E). We also found that, unlike *cdc10-129 nda3-TB101* double mutants, *cdc10-129 nda3-TB101 tea1*Δ triple mutants treated with TBZ formed branches to approximately the same extent as *cdc10-129* single mutants (Fig. 5F), despite the fact that their microtubules were not significantly altered as compared with non-drugged cells. These results suggest that the cellular mechanisms by which cells establish branches appear to be different between *tea1+* and *tea1*Δ cells, the former being microtubule dependent and the latter microtubule independent. Furthermore, even when microtubules are not disrupted, *tea1*Δ mutants have difficulty re-finding pre-existing cell tips.

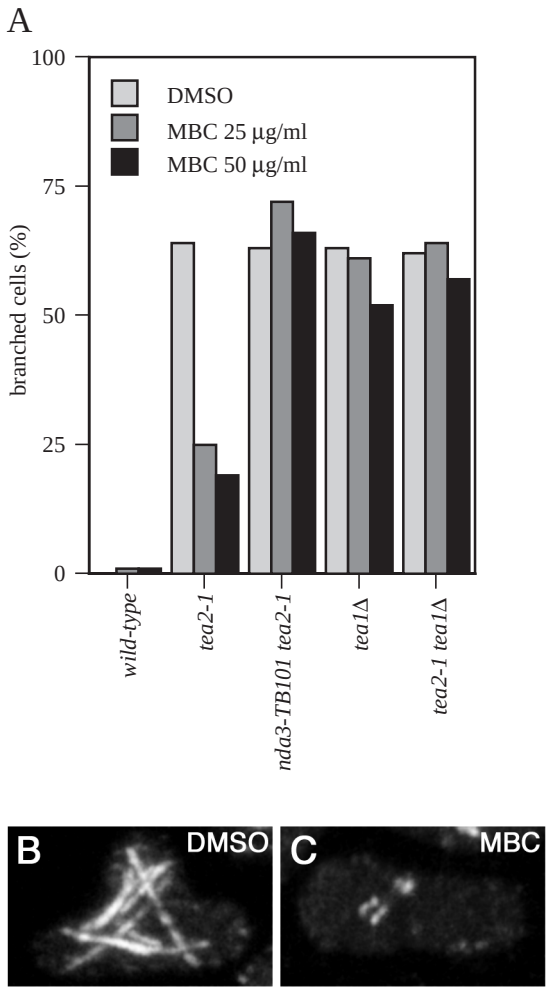


Fig. 6. Microtubule disruption during return-to-growth of *tea2-1* cells reduces the frequency of branching in *tea1+* but not in *tea1*Δ genetic backgrounds. (A) The percentage of branched cells in the indicated wild-type and mutant strains is shown, 3 hours after dilution of stationary cultures into fresh medium containing either DMSO, 25 μg/ml MBC or 50 μg/ml MBC. (B,C) *tea2-1* mutants 2 hours after dilution into either (B) DMSO or (C) 25 μg/ml MBC.

We also tested whether branching of *tea1*Δ cells was independent of microtubule distribution in return-to-growth experiments involving *tea2-1* mutants. In both *tea1*Δ single mutants and *tea2-1 tea1*Δ double mutants, MBC treatment did not significantly reduce the frequency of branched cells (Fig. 6).

We then tested whether the absence of tea1p affects microtubule-dependent branching induced by the actin inhibitor LatB. In block-and-release experiments using LatB pulses, *cdc10-129 tea1*Δ *tea2-1* triple mutants formed branches to the same extent as *cdc10-129 tea2-1* double mutants but, unlike the double mutants, the triple mutants were only slightly affected by MBC treatment (Fig. 7C). Interestingly, we also found a very high frequency of branching after a LatB pulse in block-and-release experiments involving *cdc10-129 tea1*Δ double mutants, where microtubules were not perturbed (Fig. 7D). As in our other experiments using different methods to depolarize cells, these results indicate that, after cortical actin disruption, site selection for polarity re-establishment in *tea1*Δ mutants is independent of the microtubule distribution.

The formation of branches can be promoted by tea1p

Our experiments thus far demonstrate that short microtubules are crucial for the formation of ectopic polarity axes only when tea1p is present. In conjunction with microtubule-dependent cortical targeting of tea1p, these results support the notion that, in *tea1+* cells, tea1p itself plays a direct role in linking microtubules to ectopic polarity-establishment site selection. However, because *tea1*Δ cells form branches after depolarization regardless of the microtubule distribution, and the frequencies of branching observed in *tea1+* and *tea1*Δ strains with short microtubules in our experiments are quantitatively similar, these experiments can provide at best only indirect evidence that tea1p acts positively to promote microtubule-dependent branching in *tea1+* cells (see Discussion). To support this view we therefore sought to establish experimental conditions in

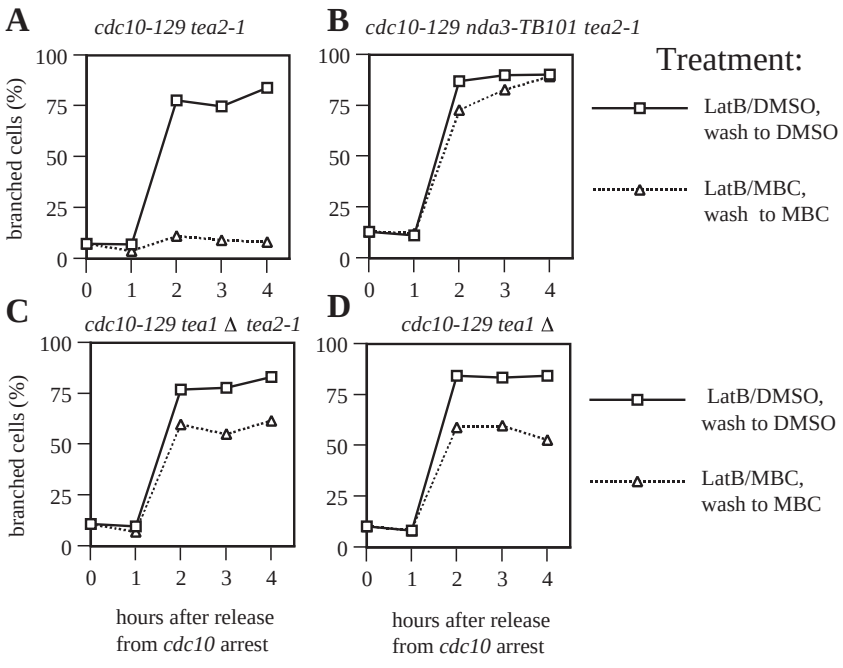


Fig. 7. Transient depolymerization of the actin cytoskeleton by latrunculin B (LatB) triggers microtubule-dependent branching in *cdc10-129 tea2-1* mutants in a *tea1+* background but microtubule-independent branching in a *tea1*Δ background. Percent branched cells in *cdc10-129* block-and-release experiments after either a LatB pulse followed by wash-out or a LatB plus MBC pulse followed by wash-out into MBC. (A) *cdc10-129 tea2-1*, (B) *cdc10-129 nda3-TB101 tea2-1*, (C) *cdc10-129 tea1*Δ *tea2-1*, (D) *cdc10-129 tea1*Δ.

which ectopic targeting of tea1p after a relatively mild depolarization of the cortical actin cytoskeleton might cause *tea1+* cells to branch at higher frequencies than *tea1*Δ cells.

During normal exponential growth, neither *tea1*Δ nor *tea2-1* mutants show a high frequency of branching, but both of these strains can form branched cells at low frequencies after a temperature shift (Browning et al., 2000; Mata and Nurse, 1997). In these mutant strains, branching may be caused in part by a partial depolarization of the cortical actin cytoskeleton induced by the temperature shift (K.S., unpublished), and, based on our results thus far, we would argue that, in this instance, branching in *tea2-1* mutants (but not in *tea1*Δ mutants) might involve ectopic cortical targeting of tea1p by short microtubules. We therefore reasoned that, if a *tea2-1* mutant were able to target tea1p to ectopic cortical sites more efficiently, it might be more likely to form branches after a temperature shift. Recently we identified a novel non-essential gene, *mod5+*, which is required for the anchoring of tea1p to cell tips after it is deposited there by growing microtubules (Snaith and Sawin, 2003). In *mod5*Δ mutants, microtubules are normal, but tea1p fails to accumulate to high levels at cell tips and thus is enriched in the cytoplasm relative to *mod5+* cells, in which tea1p is sequestered at cell tips. We therefore tested whether *mod5*Δ *tea2-1* double mutants would form more branches than *tea2-1* single mutants after a temperature shift during asynchronous exponential growth, and whether this was dependent on tea1p.

Interestingly, whereas *mod5*Δ single mutants did not show any branching in this type of experiment, after a temperature shift the branching frequency in *mod5*Δ *tea2-1* double mutants was over fourfold higher than that seen in *tea2-1* single mutants (Fig. 8A). By contrast, the frequency of branches seen in *mod5*Δ *tea2-1 tea1*Δ triple mutants was no higher than the 'default' level seen in *tea2-1* or *tea1*Δ single mutants alone, suggesting that, in this context, tea1p contributes positively to cell branching. To confirm that these results were not unique to the *tea2-1* mutants, we also used deletion mutants of the CLIP-170-like protein *tip1+* to generate short microtubules (Brunner and Nurse, 2000); *mod5*Δ *tip1*Δ double mutants showed a synergy in branching similar to that seen in *mod5*Δ *tea2-1* double mutants, whereas branching was reduced in *mod5*Δ *tip1*Δ *tea1*Δ triple mutants (Fig. 8A). This demonstrates formally that, under otherwise identical conditions, the presence of tea1p in cells can actually promote ectopic polarity establishment.

A positive role for tea1p in these branching experiments was also supported by anti-tea1p immunofluorescent staining of cells just prior to branching. In *mod5*Δ *tea2-1* mutants, patches of tea1p were found localized to microtubules, but non-uniformly, such that the microtubule-associated tea1p was generally concentrated in the middle of the cell (Fig. 9E,F). By contrast, microtubule-associated tea1p was much lower in the less-frequently-branching *tea2-1* single mutants, and in many of these cells small amounts of tea1p could be observed at cell tips (Fig. 9B,C), which was also seen faintly in *mod5*Δ *tea2-1* double mutants as the branch formed (Fig. 9H). Similar results were also obtained comparing *mod5Δ tip1Δ* mutants with *tip1Δ* single mutants (data not shown).

We also found that tea1p can act to promote high-frequency branch formation in *cdc10-129* block-and-release experiments, under conditions where microtubules are short and a depolarization is relatively mild. Although *nda3-TB101* cells appear to be resistant to the microtubule-depolymerizing effects of TBZ (Figs 2J, 5E), they do show a partial depolarization in response to TBZ treatment (Sawin and Nurse, 1998) (see also Fig. 5F). We therefore treated *cdc10-129 nda3-TB101 tea2-1* triple mutants and *cdc10-129 nda3-TB101 tea1*Δ *tea2-1* quadruple mutants with a TBZ pulse, followed by wash-out of the drug. (The *nda3-TB101* mutation prevents the TBZ from having a strong effect on the short microtubule distribution characteristic of *tea2-1* mutants.) Under these conditions, branches formed at a very high frequency in the triple mutants, but not in the *tea1*Δ quadruple mutants (Fig. 8B). Collectively, these results indicate that tea1p plays an active role in promoting the formation of new polarity axes, and it is not just the absence of tea1p that leads to cell branching.

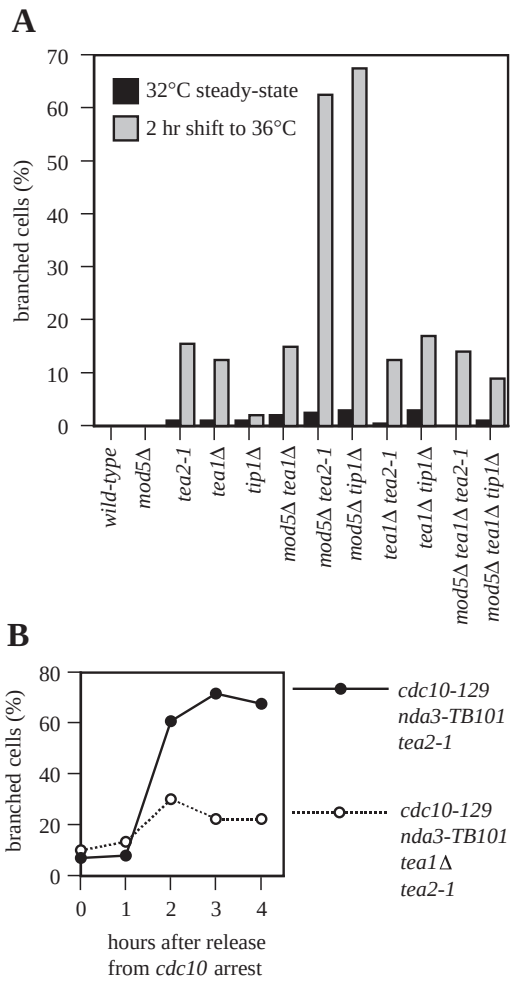


Fig. 8. Tea1p is required for a high frequency of branching after a mild depolarization of the cortical actin cytoskseleton. (A) Temperature shift in *mod5*Δ *tea2-1* double mutants produces a synergy in cell branching that depends on tea1p. The percentage of branched cells in the indicated strains is shown 2 hours after shift-up from 32°C to 36°C. (B) Percentage of branched cells in a *cdc10-129* block-and-release experiment, using a TBZ wash-out (Fig. 5) and either *cdc10-129 nda3-TB101 tea2-1* triple mutants or *cdc10-129 nda3-TB101 tea1*Δ *tea2-1* quadruple mutants.

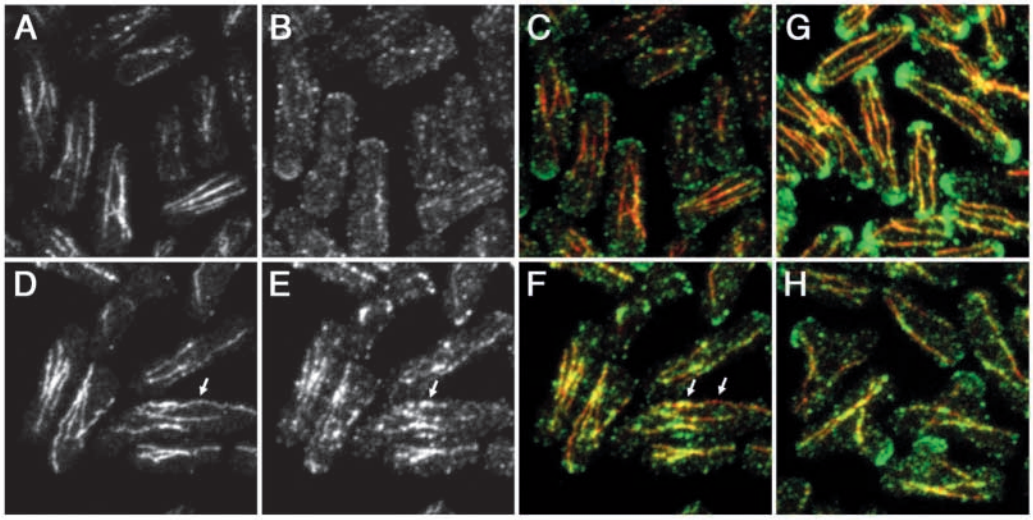


Fig. 9. Tea1p shows a non-uniform association with microtubules in *mod5*Δ *tea2-1* mutants before cell branching. Anti-tubulin (A,D) and anti-tea1p (B,E) immunofluorescence, and merged images (C,F) in *tea2-1* (A-C) and *mod5*Δ *tea2-1* (D-F) mutants 60 minutes after temperature shift to 36°C. Note that, in *mod5*Δ *tea2-1* cells, tea1p often appears more concentrated on microtubules towards the cell middle (yellow in merged panel F), relative to the total microtubule signal (arrows in D-F). Also shown are merged images of branching *mod5*Δ *tea2-1* mutants 90 minutes after temperature shift (H), as well as wild-type cells without a temperature shift (G), to show the relatively low tea1p signal in these mutants as compared with wild-type cells.

Discussion

Previous work involving drug studies (Sawin and Nurse, 1998) and, in particular, temperature-sensitive mutant strains (Radcliffe et al., 1998; Toda et al., 1983) has suggested that microtubules play an important role in fission yeast polarity, but in general the specifics of that role have remained poorly defined. Our main findings are:

(1) Microtubules are not required for maintenance of a growth axis that is already polarized. We have shown that MBC treatment leads to a profound depolymerization of microtubules in fission yeast but has no effect on polarized growth or on the localization of cell-polarity markers such as actin or ral3p, whereas TBZ does. Although TBZ is more commonly used than MBC as a microtubule inhibitor in fission yeast, these previously unknown side-effects of TBZ are sufficiently strong for us to consider it to be unsuitable as a general microtubule inhibitor for physiological studies, unless a specific cortical depolarization is also required. The effects of MBC on cell growth have also been investigated by others (Tran et al., 2000). TBZ and MBC are both benzimidazole derivatives and are structurally similar (Umesono et al., 1983), but the reason for their different effects on cell polarity is not yet known.

(2) Microtubules are not required for establishment of cell polarity per se but can signal to the cortex to determine the position of polarity (re)establishment. It has been proposed that landmarks at cell tips might provide a cortical ‘memory’ that is important for the antipodal positioning of growth sites in fission yeast (Chang and Peter, 2003; Hayles and Nurse, 2001; Mata and Nurse, 1998), and thus cell branching could be considered to be a consequence of the loss of such landmarks. However, it is not possible to argue that depolarized *tea1+* cells with short microtubules form branches solely because they have lost a landmark from cell tips because, when we disrupt microtubules further with MBC, we see a strong suppression of branching and a restoration of growth at cell tips (Figs 5A, 6, 7A).

(3) Tea1p is involved in signalling from microtubules to the cell cortex during polarity establishment and also plays a role in cortical ‘memory’ at cell tips. In *tea1*Δ cells, unlike *tea1+* cells, there is no correlation between microtubule distribution and the position of polarity re-establishment, and in certain types of experiments with *tea2-1* and *tip1*Δ mutants, *tea1*Δ cells branch at a much lower frequency than their isogenic *tea1+* counterparts. Previous observations that tea1p is targeted to cell tips by association with the plus-ends of growing microtubules (Behrens and Nurse, 2002; Mata and Nurse, 1997; Snaith and Sawin, 2003) are consistent with the idea that tea1p might link microtubules to the actin-rich cortex (Glynn et al., 2001), but until now this has not been supported by more functional data. Our genetic experiments not only support the view that tea1p links microtubule distribution to site selection during polarity establishment but also go further to suggest that tea1p is required for such a linkage, and that there is probably no major parallel functional pathway by which such a connection could occur. Thus, we propose that, in *tea1*Δ cells, the microtubule cytoskeleton and the cell polarity establishment machinery cannot ‘talk’ to each other.

However, at the same time, the role of tea1p in polarity-establishment site selection must extend beyond that of being a coupling factor between microtubules and the cell polarity establishment machinery. The initial description of *tea1+* showed that *tea1*Δ cells form branches at a low frequency after a temperature shift (Mata and Nurse, 1997). Moreover, in our experiments we found that after depolarization combined with strong microtubule disruption (in which microtubules would not be able to signal to the cortex via any means), *tea1+* cells are able to re-find pre-existing cell tips efficiently, whereas *tea1*Δ cells are not (Figs 5-7). This suggests that tea1p is also required for some form of cortical landmark at cell tips that allows cells to re-establish polarity at these positions, in a microtubule-independent fashion. Because tea1p is normally found at cell tips, the simplest interpretation of these results is that tea1p is itself an integral component of this cortical memory system. We have recently shown that, although levels of tea1p at cell tips are reduced by microtubule disruption, a significant fraction remains (approximately 50%) (Snaith and Sawin, 2003), and it is this fraction that we propose might act as a landmark for polarity re-establishment.

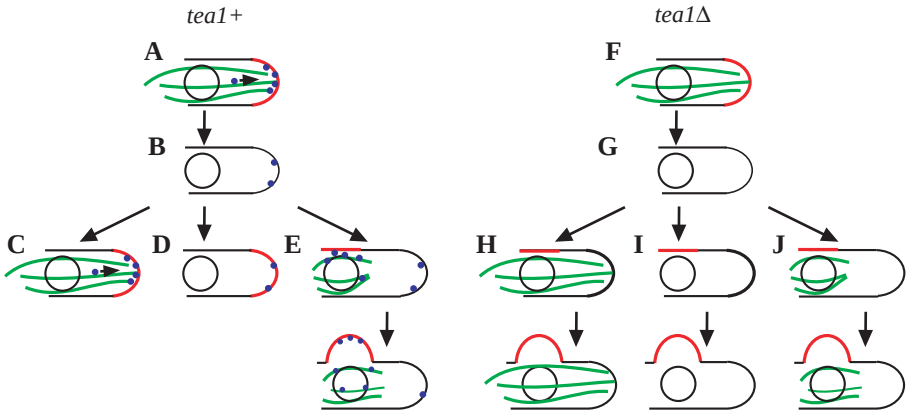


Fig. 10. Model for regulation of cell polarity establishment by microtubules and tea1p (see text for additional details). Patterns of polarity establishment are shown for *tea1+* cells (A-E) and *tea1*Δ mutants (F-J). During steady-state growth in *tea1+* cells (A), a growth zone, including polarized cortical actin (red), is at cell tips. tea1p (blue) is targeted to cell tips by association with the plus ends of growing microtubules (green). (B) Representation of a generic state of microtubule depolymerization in conjunction with depolarization of the cortical actin cytoskeleton, which is achieved alternatively by TBZ treatment (which simultaneously affects microtubules), by growth to an extended stationary phase, or by depolymerization of actin with LatB. Under these conditions, some tea1p can remain at cell tips to provide residual cortical landmark cues for polarity re-establishment. During polarity re-establishment (C-E), cells with normal-length microtubules can re-target tea1p back to cell tips (C), whereas cells with short microtubules (e.g. after TBZ treatment, or *tea2-1* mutants) target tea1p ectopically to the cell middle (E). This targeting of tea1p helps to set up the new polarity axis. If microtubules are strongly disrupted and thus unable to direct tea1p to the cortex, the residual tea1p-dependent landmarks direct polarity re-establishment back to cell tips (D). In *tea1*Δ mutants, microtubules are normal and actin is polarized at cell tips during steady-state growth (F), but the absence of tea1p means that, upon cell depolarization and microtubule depolymerization, cortical landmarks for polarity establishment are not available (G). In addition, because microtubule signalling to the cortex depends on tea1p, *tea1*Δ mutants squander the opportunity to retarget the polarity machinery back to cell tips through a microtubule-based mechanism. As a result, polarity is re-established either randomly or following additional unknown cues (H,I,J).

Suggesting that tea1p has two ‘roles’ in polarity re-establishment – on the one hand, linking microtubules to polarity-establishment site selection, and, on the other hand, acting as a cortical landmark – might seem superficially to imply two distinct molecular functions for tea1p, but there is currently no evidence for this. Rather, we favour the view that in both cases tea1p is performing the same function at the molecular level, that is, helping to recruit the growth polarity machinery (Glynn et al., 2001), although in the two cases the prior history of the tea1p molecules involved may be different.

Model for polarity axis determination

On the basis of our results (and subject to the caveats expressed below), we propose a relatively simple model for microtubule-mediated regulation of cell polarity in fission yeast that can account for a range of morphological phenotypes seen in our work and the work of others (Fig. 10). Inherent in the model, and consistent with all of our findings, is the notion that cell branching is essentially polarity establishment in the wrong place, and therefore subject to the same general controls as polarity establishment in the right place.

The main points of the model are as follows: (1) in wild-type cells, tea1p is continuously targeted to cell tips by microtubules but plays a minimal role in maintaining cell polarity during most of unperturbed vegetative growth (Fig. 10A). (2) If the cortical actin cytoskeleton at cell tips is depolarized transiently (Fig. 10B), tea1p targeting by microtubules helps recruit the polarity machinery to sites at the cortex for polarity re-establishment, and tea1p remaining at cell tips independently contributes to cortical landmarks that can also be used in re-establishment. In cells with normal-length microtubules, sites of microtubule-targeted tea1p deposition are coincidentally at the pre-existing cell tips (Fig. 10C), but in cells with abnormally short microtubules, sites of tea1p accumulation may be in the middle of the cell (Fig. 10E) (see also Behrens and Nurse, 2002; Browning et al., 2000). The targeting of tea1p to an ectopic site can result in a competition for polarity-establishment site selection between this new site and pre-existing cell tips (which may retain some polarity landmarks, including tea1p), and the new ectopic site often wins. By contrast, in cells without microtubules, there is no competition, and weak landmarks at cell tips might be capable of re-recruiting the polarity machinery, even in the absence of microtubules (Fig. 10D). (3) In *tea1*Δ mutants, cortical memory is absent, and/or landmarks are poorly retained at pre-existing cell tips upon depolarization (Fig. 10G); in addition, microtubules can no longer influence polarity-establishment site selection. A site for polarity establishment is chosen either at random or perhaps in response to some cryptic cues that remain to be identified (Fig. 10H,I,J). (4) Finally, after polarity is established at a particular cortical site, and microtubules have fulfilled their role in polarity establishment, the growth polarity machinery functions more-or-less independently of the microtubule distribution.

Caveats to the model

There are several caveats to our conclusions that must be emphasized to avoid overinterpreting our results. First, although our work indicates that overall polarized growth persists after microtubule disruption in fission yeast, we have not addressed whether microtubules make more-minor contributions to maintaining rates or direction of growth. Cell branching is a major polarity change and easily subject to a quantitative analysis, whereas small deviations from wild-type growth (e.g. slightly bent cells) are not so easily quantitated. Given that tea1p and other proteins are constantly delivered to cell tips via microtubules, throughout the cell cycle (Chang and Peter, 2003), it is possible that microtubules contribute to more subtle aspects of the maintenance of polarized growth. In addition, our own data reveal that a very low percentage of cells

can form branches after MBC treatment alone, in the absence of any distinct depolarization stimulus (see, for example, Fig. 3). However, at the same time, an additional explanation for the constant microtubule-based targeting of proteins to cell tips would be that their delivery provides a buffer to help the cell withstand small depolarizing perturbations; that is, after a brief depolarizing stimulus, a kinetically rapid microtubule-based mechanism that immediately enriched tea1p back to cell tips could enable the cell to re-establish polarity back in the right place much more efficiently and accurately. According to this view, the constant targeting of tea1p to cell tips in wild-type cells would represent a back-up mechanism waiting to be put to use in such cases. In light of this, it is interesting to note that exponentially growing cultures of *tea1*Δ cells have a significant population of bent cells (approximately 30% of total cells) with a distinct 'kink', in which the direction of growth at the tips has clearly changed but then remained constant [for representative images, see Mata and Nurse (Mata and Nurse, 1997)]. This could be the consequence of a relatively minor or partial depolarization that does not completely dismantle the polarity apparatus at the cell tips but is nevertheless not properly corrected in *tea1*Δ cells during 're-establishment' (see also Sawin, 1999).

A second caveat to our conclusions is that we have not addressed whether microtubules contribute directly to NETO, the transition from monopolar to bipolar growth that normally occurs in the wild-type cell cycle (Mitchison and Nurse, 1985). *tea1*Δ mutants are defective in this important polarity transition, and thus it is possible that microtubules also play a role here; alternatively, given that tea1p can remain and even slowly accumulate at cell tips after microtubule disruption (Mata and Nurse, 1997; Sawin and Nurse, 1998; Snaith and Sawin, 2003), it is also possible that the proximal mechanisms by which tea1p contributes to bipolar growth transitions may be microtubule independent. This merits further investigation. As different monopolar growth mutants can display different patterns of growth polarity defects (Arellano et al., 2002; Feierbach and Chang, 2001; Glynn et al., 2001; Kim et al., 2003), it is clear that the controls regulating transitions to bipolar growth are likely to be complex, and it is possible that proteins such as tea1p, although not required for polarity establishment in the most generic sense, might be directly involved in the polarity establishment events that convert a non-growing cell tip into a growing one.

A final caveat concerns the mechanism by which tea1p acts to promote branch formation. Our genetic experiments (Figs 8, 9) strongly suggest that, in *tea1+* cells with short microtubules (including TBZ-treated cells), tea1p plays an important positive role in branching. The simplest explanation for this would be that, in such cells, microtubule-based targeting of tea1p to the middle cortex occurs in the same manner as it normally would to cell tips in wild-type cells. However, we note that the *tea2-1* and *tip1*Δ mutants used in these experiments do not have uniformly short microtubules that would target tea1p to a discrete cortical site (Fig. 9) (see also Brunner and Nurse, 2000; Browning et al., 2000). In addition, it has been suggested that mutants in the tea2p kinesin-like protein might have defects in the transport of tea1p to cell tips on microtubules (Browning et al., 2003), although this may still be controversial (Behrens and Nurse, 2002). In our experiments with *tea2-1* and *tip1*Δ mutants, we observed tea1p often enriched along microtubules in the middle of cells, prior to branching (Fig. 9). Although this is consistent with tea1p playing an active role in branching, it does not demonstrate a microtubule-based tea1p-targeting mechanism to the cell middle analogous to what occurs at normal wild-type cell tips. We would therefore speculate that, in *tea2-1* and *tip1*Δ mutants, a high local concentration of microtubule-associated tea1p in the cell middle might act as a local source for diffusion of tea1p to the nearby middle cortex, and this could provide a parallel mechanism for localizing tea1p to the cortex, in addition to microtubule-based vectorial targeting.

Using the model to understand mechanism

Within our model it is important to recognize that, depending on the specific case, the dominant feature governing polarity-establishment site selection could be either ectopically targeted tea1p or cortical landmark tea1p. For example, we recently showed that *mod5*Δ mutants, which are defective in anchoring tea1p at cell tips, form branches in return-to-growth experiments at high frequency only when treated with MBC to depolymerize microtubules (Snaith and Sawin, 2003). By contrast, we have shown here that *tea2-1* mutants in the same type of experiment branch at high frequency only when not treated with MBC, and *tea1*Δ mutants branch at high frequency both in the presence and in the absence of MBC (Fig. 6) (Snaith and Sawin, 2003). Our model explains this diversity of phenotypes: in the case of *mod5*Δ mutants, tea1p is not well-anchored to the cortex and thus might not be present in sufficient amounts or in the correct form to serve as a cortical landmark for polarity establishment at pre-existing cell tips. However, when microtubules in *mod5*Δ mutants are intact, polarity is established at pre-existing cell tips by newly targeted tea1p. By contrast, when MBC is added to *mod5*Δ mutants, virtually no tea1p is found at cell tips (Snaith and Sawin, 2003) and this situation truly phenocopies *tea1*Δ, and thus high-frequency branching occurs (see below). The situation is different in *tea2-1* mutants, which are not defective in tea1p anchoring but have short microtubules. Although pre-existing cortical landmarks of tea1p, vestiges of previous cell growth, might be present at cell tips in *tea2-1* mutants in return-to-growth experiments, these landmarks are in competition with tea1p recruited to cell middles from the short microtubules, and thus branching ensues. When microtubules are disrupted, the cortical landmark tea1p is no longer in competition with ectopically targeted tea1p and can function to re-establish polarity at cell tips. Finally, in *tea1*Δ mutants, there can be neither pre-existing tea1p cortical landmarks nor ectoptic targeting of tea1p, and in this case we propose that the choice of site for polarity establishment is either random or based on some cryptic cues that are otherwise ignored by the cell.

The signalling aspect of tea1p in polarity axis determination is also especially apparent in the experiment of Fig. 8A. Here we imagine that the shift from 32°C to 36°C might cause only a very mild depolarization or disruption of the cortical actin cytoskeleton at cell tips, such that cells may be just 'on the edge' of depolarizing, and whether an individual cell actually needs to 're-establish' polarity could be a probabilistic event. Thus, branching occurs in a relatively low number of *tea1*Δ cells (10-15%, as compared with over 80% after LatB treatment; compare Fig. 7 with Fig. 8A). In *tea2-1* cells, most

tea1p and other cortical landmarks already at cell tips would probably remain there but, in those cells in which polarity was compromised, mobile tea1p would be recruited to cell middles, and thus some branching would occur. (Without the perturbation of the temperature shift, *tea2-1* mutants would not branch because polarity maintenance is essentially microtubule independent.) According to our model, branching in *tea2-1* mutants depends on ectopic positioning of tea1p, so in a *tea1*Δ *tea2-1* double mutant it would be no higher than in either single mutant. In a *mod5*Δ single mutant, in which tea1p is poorly anchored at the cortex, any tea1p lost from cell tips would get targeted back to cell tips by microtubules. But in a *mod5*Δ *tea2-1* double mutant, even though the depolarization might be relatively minor, with some cortical landmarks remaining to restore polarity back to pre-existing cell tips, the fact that there is a higher free cytoplasmic population of tea1p, perhaps in combination with an increased free pool of other polarity proteins (i.e. as a consequence of poor tea1p anchoring), would enable the short *tea2-1* mutant microtubules to position more-significant amounts of tea1p to the cell middles, which then successfully compete against pre-existing cell tips for the polarity establishment machinery. However, in the *mod5*Δ *tea1*Δ *tea2-1* triple mutant, no such targeting of tea1p can occur.

Conclusions

In conjunction with quantitative assays for cell branching, the different experimental conditions described here provide insight into the mechanisms by which microtubules regulate a major cell-polarity transition in fission yeast. Within this context, further experiments with other mutants should help to illuminate the function of additional gene products and to position them in functional pathways or networks. For example, the protein kinase pom1p is dependent on tea1p for its localization to cell tips and is thought to be an effector protein of tea1p (Bahler and Pringle, 1998); repeating the experiments of Fig. 5 with *cdc10-129 pom1*Δ mutants, we have found that, like tea1p, pom1p is also required for microtubule signalling to the cortex during polarity establishment (K.S., unpublished). The application of these methods to novel genes and/or mutations should be equally illuminating.

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