

tea1 and the Microtubular Cytoskeleton Are Important for Generating Global Spatial Order within the Fission Yeast Cell

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Summary

Fission yeast cells identify and maintain growing regions exactly opposed at the ends of a cylindrical cell. *tea1* mutants disrupt this organization, producing bent and T-shaped cells. We have cloned *tea1* and shown that *tea1* is located at the cell poles. Microtubules are continuously required to transfer *tea1* to the cell ends, and *tea1* is located at the ends of microtubules growing toward the cell poles. We suggest that *tea1* acts as an end marker, directing the growth machinery to the cell poles. *tea1* is down-regulated in cells treated with pheromone that grow toward a mating partner and no longer maintain their ends exactly opposed. *tea1* may also influence microtubular organization, affecting the maintenance of a single central axis.

Introduction

An important problem for a cell is how it organizes itself in space. This organization requires that different components or points located at a distance within the cell are correctly positioned with respect to each other. The problem is similar in principle to a developmental field establishing positional information during embryogenesis but acts at the level of a single cell. There are many examples of this phenomenon: epithelial cells have opposed apical and basal faces with different properties (Eaton and Simons, 1995); *Drosophila* oocytes contain determinants localized to discrete regions of the cytoplasm (Lehmann, 1995); and bipolar budding yeast cells bud at the opposite end of the cell to the previous bud (Roemer et al., 1996). In the case of the fission yeast *Schizosaccharomyces pombe*, two growing tips located at the ends of the cylindrical cell grow apart in a precisely opposed manner (Snell and Nurse, 1993). This is a simple case of spatial organization in which a cell identifies and maintains domains at its extremities that are directly opposite each other. Two points are said to be antipodal when they are located at the extreme opposite ends of an object, and we shall use this term to describe the organization of tip growth in fission yeast, where intracellular domains are directly opposed to each other at the extremities of the cell along a single central axis.

Immediately after cell division, a newly born fission yeast cell starts growth in a unipolar mode, growing only at the old tip that already existed in the mother cell. After a certain period of the cell cycle, the cell switches to a bipolar mode, with both old and new tips undergoing growth. This transition is called new-end take off, or NETO. In both unipolar and bipolar growth modes, the

cell elongates while keeping its ends precisely opposed. Upon onset of mitosis, all tip growth ceases and resumes again only after cytokinesis and cell division are complete; this occurs at the two old tips, now present in two different cells (Mitchison and Nurse, 1985). The precise opposition of growing tips is maintained throughout vegetative growth but is not found in cells undergoing conjugation, in which the rigid relationship between the tips needs to be uncoupled to allow a growing tip to seek out a cell of opposite mating type (Fukui et al., 1986; Leupold, 1987).

In order to understand how a fission yeast cell can identify its ends and maintain them growing in an antipodal fashion, we have isolated mutants in which the tips do not grow away from each other in a precisely opposed manner (Snell and Nurse, 1994; Verde et al., 1995). When tips are offset by a small angle, bent cells are generated, but when the angle subtended is increased, a new third tip growing out of the side of the cell body is made, producing T-shaped cells. Mutants behaving in this manner define two genes, *tea1* and *tea2* (for tip elongation aberrant), which we have hypothesized may define gene products acting as markers of the extreme opposite ends of the fission yeast cell (Verde et al., 1995). In this paper, we describe the cloning of *tea1* and characterize its encoded protein, *tea1*. The protein is found to be tightly located at the ends of the cell, consistent with a role as an end marker, and the microtubular cytoskeleton is shown to be required to maintain the location of *tea1* at the cell tips. From these results, we propose that *tea1* is constantly transferred to the extremities of the cell by the microtubules, producing a dynamic mechanism whereby the space of the cell is explored, and the antipodal positions of the growing tips are identified and maintained, thus contributing to global spatial order within the cell.

Results

Cloning and Deleting the *tea1* Gene

Mutants in the *tea1* gene are viable, and their bent and T-shaped phenotype can only be identified by microscopic inspection of the cells within individual colonies. It proved impossible to clone *tea1* by complementation because of plasmid loss, so we used positional cloning as an alternative method. *tea1* was mapped to within 1.3 cM of the *nmt1* gene and was located within an ordered cosmid library of the *S. pombe* genome (Hoheisel et al., 1993). Cosmids were selected that spanned the region around the *nmt1* gene and were individually transformed into a *tea1-3* mutant strain. One cosmid completely rescued the mutation, and after subcloning a 4.3 kb fragment from this cosmid was found to contain the rescuing activity.

Sequencing of the 4.3 kb fragment identified a 3441 bp ORF of 1147 amino acids and a molecular mass of 127 kDa (Figure 1A). Analysis of the sequence revealed six internal repeats at the N-terminal half of the protein (amino acids 82–394) belonging to the kelch family (Xue

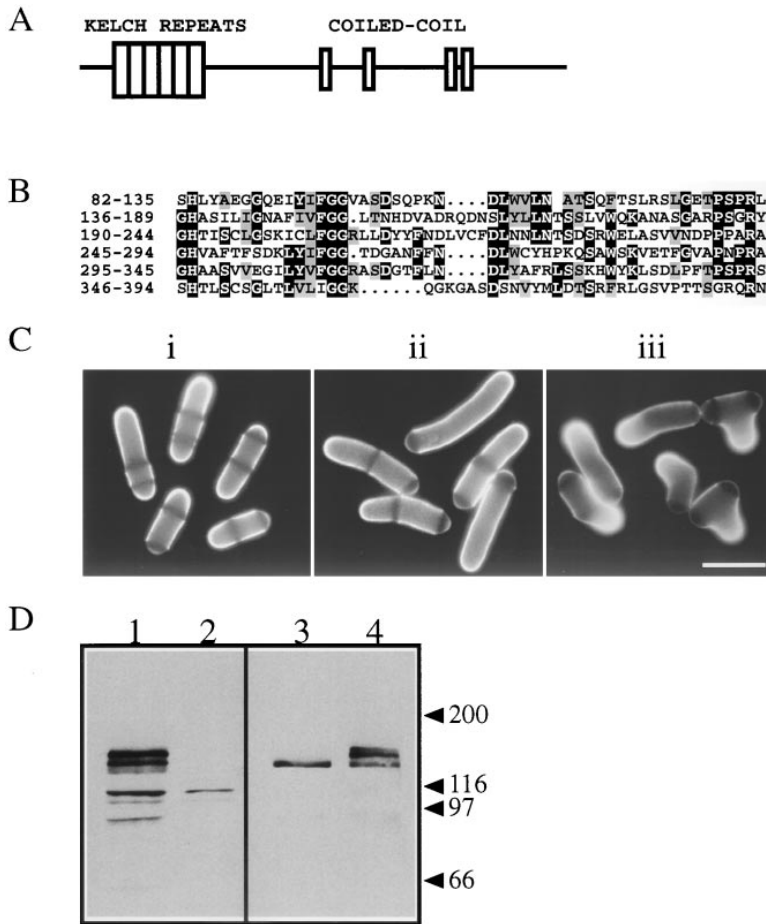


Figure 1. Molecular Analysis and Deletion of the *tea1* Gene

(A) General structure of the predicted *tea1* protein. The position of the six kelch internal repeats is shown by the boxes on the left. Regions predicted to form coiled-coils ($p > 0.85$) are shown by the boxes on the right.

(B) Alignment of the kelch repeats. Identical residues on at least three repeats are shown in closed boxes, and conserved residues in shaded boxes.

(C) Morphological phenotype of a *tea1Δ* strain. All cells are stained with the cell wall-binding compound calcofluor. (i) Wild-type cells grown at 25°C; (ii) *tea1Δ* cells grown at 25°C; (iii) *tea1Δ* cells grown at 25°C and transferred to 36°C for 4 hr.

(D) Characterization of antibodies against *tea1*. Affinity-purified antibodies raised against the C-terminal half of *tea1* were used to probe Western blotted extracts from wild-type cells (lane 1), *tea1Δ* cells (lane 2), *tea1Δ* cells transformed with plasmid pR2 containing the *tea1* gene lacking the first ATG (lane 3), or *tea1Δ* cells transformed with plasmid pR1 containing the whole *tea1* gene (lane 4).

Scale bar = 5 μ m.

and Cooley, 1993; Bork and Doolittle, 1994). An alignment of the repeats is shown in Figure 1B. Comparison of the repeats with proteins in the PIR and Swissprot databases revealed that the most similar repeats were in two hypothetical proteins from *Saccharomyces cerevisiae* (PIR accession numbers S46769 and S57704), in host-cell factor (Wilson et al., 1993), and in actin-fragmin kinase (Eichinger et al., 1996). Kelch repeats are thought to be structural repeats (Bork and Doolittle, 1994) and are present in several proteins known or suspected to bind actin (Xue and Cooley, 1993; Varkey et al., 1995; Way et al., 1995; Eichinger et al., 1996). Several regions of the C-terminus showed a high probability of forming coiled-coil interactions (Figure 1A), as predicted by the Lupas algorithm (Lupas et al., 1991). The two *S. cerevisiae* ORFs that showed homology to *tea1* in the N-terminus also had a similar coiled-coil structure.

We constructed a deletion of the *tea1* gene in a diploid strain by replacing most of the ORF with the *ura4⁺* gene. Sporulation of the resulting diploids produced four viable spores, which grew with normal generation times at 18°C, 25°C, 32°C, and 36°C. Examination of the cells under the microscope revealed that cells deleted for the *tea1* gene had a defect in cell shape similar to the original *tea1* mutants (Snell and Nurse, 1994). At 25°C, about 30% of the cells can be seen to be bent (Figure 1C, [iii]; compare with wild type in [ii]), and at 36°C the phenotype becomes stronger, with 35% of the cells obviously

bending and 20% forming T shapes (Figure 1C, [iii]). Because scoring bent cells is difficult in short, normally growing cells, we produced elongated cells by using a temperature-sensitive *cdc25* mutant. This mutation causes slight elongation at 25°C and blocks the cell in G2 at 36°C, making it very elongated. This allows the bent phenotype to be more easily scored. With these conditions, the phenotype exhibited a high penetrance, with a total of 70% of cells bent at 25°C when cells are only slightly elongated and 95% bent or T shaped after 3 hr at 36°C. We conclude that the *tea1* gene is necessary to maintain the growing tips precisely opposed to each other and so plays a role in maintaining antipodal growth in the fission yeast cell.

Localization of *tea1*

To investigate the intracellular localization of *tea1*, we raised polyclonal antibodies against bacterially produced fragments of the *tea1* protein fused to a 6His tag. After affinity purification, the antibodies against the C-terminal half of the protein recognized two bands of 130 and 135 kDa in extracts from wild-type cells (Figure 1D, lane 1), which were absent in *tea1Δ* extracts (Figure 1D, lane 2). Lower molecular weight bands probably represented degradation products. Affinity-purified antibodies produced against the N-terminal half of the protein did not detect the *tea1* protein in wild-type cells, but bands of 130 and 135 kDa were obtained with this

antibody in extracts derived from cells overexpressing *tea1* (data not shown). The *tea1* ORF has a second ATG at position 135, and a *tea1* genomic clone lacking the first ATG (pR2) was able to rescue a *tea1Δ*, showing that the first 45 amino acids were not essential for the protein function. When the *tea1* protein was examined in a *tea1Δ* strain containing this plasmid, a single band was detected of the same size as the lower band (130 kDa) in wild-type cells (Figure 1D, lane 3). In contrast, extracts from *tea1Δ* cells with a plasmid containing the full ORF (pR1) had both bands (Figure 1D, lane 4). The presence of the two bands could be explained either by alternative use of the first or second ATG of the ORF, given that the predicted size difference between the two products is 4.6 kDa (similar to that observed), or alternatively, by the N-terminus being processed in some manner.

We used the antibodies to determine the intracellular localization of the *tea1* protein by indirect immunofluorescence. The majority of *tea1* was localized to a small region at both poles of wild-type cells with some dots elsewhere in apparently random positions within the cell (Figure 2A). The specificity of the staining was confirmed by the fact that antibodies raised against nonoverlapping regions of the protein produced similar results and by the absence of any signal when *tea1Δ* cells were stained (Figure 2A).

During the early part of the cell cycle, the cell grows in a unipolar manner, while during mitosis neither tip undergoes growth (Mitchison and Nurse, 1985). In order to establish whether *tea1* marks growing tips or just tips, we monitored *tea1* localization in cells at different stages of the cell cycle. Double staining of *tea1* and tubulin was used, allowing cells at certain stages of the cell cycle to be identified (Hagan and Hyams, 1988); during mitosis there is a mitotic spindle, during cytokinesis microtubules form a postanaphase structure, and during interphase cells have cytoplasmic microtubules extending along the cell. It is not known whether the cytoplasmic microtubules observed by immunofluorescence are single microtubules or microtubule bundles. In interphase cells, *tea1* staining was found at both ends of the cells (Figure 2B, [i]). In mitotic cells with an intranuclear spindle (Figure 2B, [ii]), *tea1* remained at the tips, but the intensity of the staining was lower than in interphase cells. In cells just completing division, *tea1* could still be seen at both of the old ends, but was also just visible at the newly formed ends in the region of the septum (Figure 2B, [iii]). In recently separated cells, *tea1* was present at both tips, but the staining was asymmetric (Figure 2B, [iv]), being weaker at the new end that was recognized by its flat shape. This experiment shows that *tea1* is present at both poles of the cells throughout the cell cycle whether they are growing or not.

To confirm that both tips contain *tea1* whether they are growing or not, we blocked cells both early and late in the cell cycle, when growth was either monopolar or bipolar (Mitchison and Nurse, 1985). We used a *cdc10^{ts}* mutant that blocks at late G1, before activation of bipolar growth, and a *cdc25^{ts}* mutant, which blocks entry into mitosis after activation of bipolar growth. After 4 hr at the restrictive temperature of 36°C, *tea1* was present at

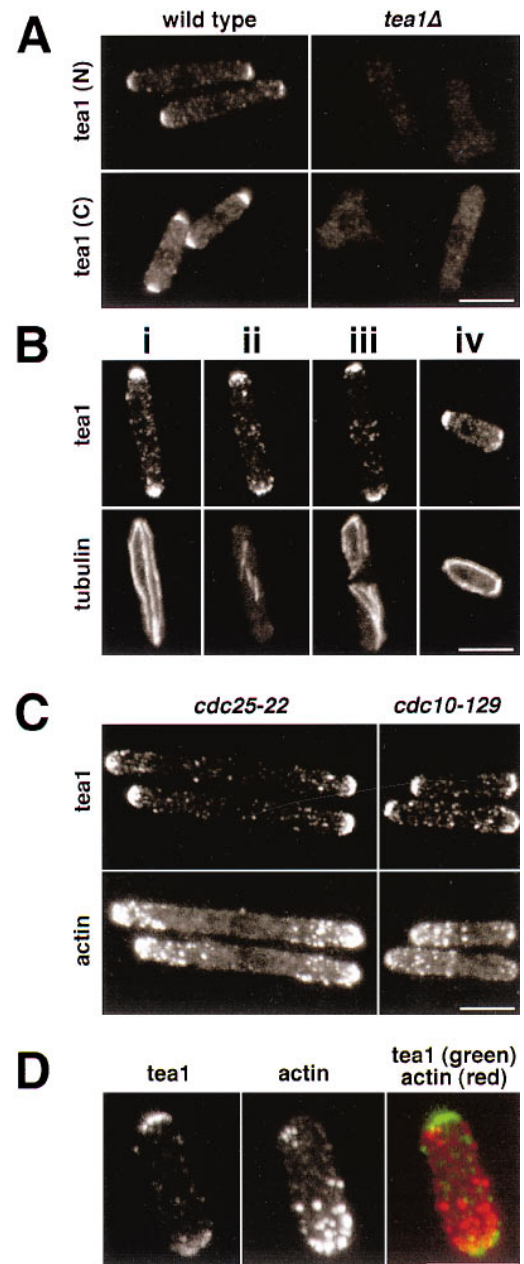


Figure 2. Immunolocalization of *tea1*

(A) Specificity of the staining. Wild type or *tea1Δ* cells were fixed and stained with antibodies raised against the N-terminal half of *tea1* (*tea1* [N]) or the C-terminal half (*tea1* [C]).

(B) *tea1* localization in the cell cycle. Wild-type cells grown at 25°C were fixed and double stained with antibodies against *tea1* (top) or tubulin (lower panels). (i) Interphase; (ii) Mitosis; (iii) Septation; (iv) Early interphase.

(C) *tea1* localization in cell-cycle mutants. *cdc25-22* cells or *cdc10-129* cells were grown at 25°C, transferred to 36°C for 4 hr, fixed, and double stained with antibodies against *tea1* (top) or actin (lower panels).

(D) *tea1* localization in *orb2* mutants. *orb2-34* mutants were grown at 25°C, fixed, and double stained with antibodies against *tea1* or actin. The overlaid stainings are shown on the right, with *tea1* in green and actin in red.

Scale bars = 5 μm.

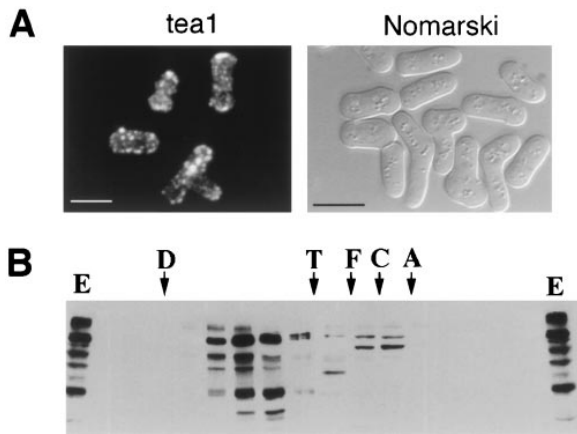


Figure 3. tea1 Is Part of a High Molecular Weight Complex and Effects of tea1 Overexpression

(A) Overexpression of *tea1* causes cell shape defects. Cells carrying an integrated copy of *tea1* driven by the *nmt1* promoter were grown in minimal medium containing thiamine. Thiamine was removed from the medium to induce the promoter. After 16 hr at 36°C, the cells were fixed and stained with antibodies against *tea1* (left panel) or viewed using Nomarski optics (right panel). Scale bars = 5 μm.
 (B) *tea1* is present in a high molecular mass complex. Fission yeast protein extracts were prepared and fractionated by gel filtration as described in Experimental Procedures. The fractions were Western blotted and probed with antibodies against *tea1*. *tea1* is very degraded after the fractionation procedure. The positions of the total extracts (E) and the molecular mass markers are shown: Dextran blue (D, 2000 kDa), thyroglobulin (T, 700 kDa), ferritin (F, 440 kDa), catalase (C, 230 kDa), and albumin (A, 67 kDa).

both poles in both the *cdc25^s* and *cdc10^s* cells (Figure 2C). In these experiments, the cells were also double stained for actin, which is located in growing tips of fission yeast (Marks et al., 1986). As expected, actin was located at both tips in the *cdc25^s* mutant and mostly at a single tip in the *cdc10^s* mutant (Figure 2C), and it was also clear that *tea1* was more tightly concentrated at the tips than actin. This was confirmed with an *orb2^s* mutant that grows only at one end (Verde et al., 1995). In this mutant, *tea1* was located tightly at both ends of the cell, while actin was located in a more diffuse manner at the single growing end (Figure 2D). Although the distribution of *tea1* sometimes appears asymmetric in *orb2* mutants (Figure 2D), there is no correlation between the presence of actin and high or low levels of *tea1* at the same pole.

We conclude that *tea1* acts as an end marker, regardless of whether the ends are actively growing or not. This location is consistent with a role for *tea1* in identifying the poles of the cell and maintaining a single central axis. If *tea1* acted as a focus for orientating the growing tip machinery, then by marking the cell ends *tea1* would restrict growth to the regions necessary to establish and maintain antipodal growth. To investigate this further, *tea1* was expressed under the control of the *nmt1* thiamine-repressible promoter (Maundrell, 1990). After removing thiamine, *tea1* levels increased about 50-fold (data not shown), and *tea1* was located at random positions throughout the cell (Figure 3A, left panel). This caused the formation of bent and T-shaped cells (Figure 3A, right panel). This defect could be due to the mislocalization of *tea1*. Alternatively, high levels of *tea1* could

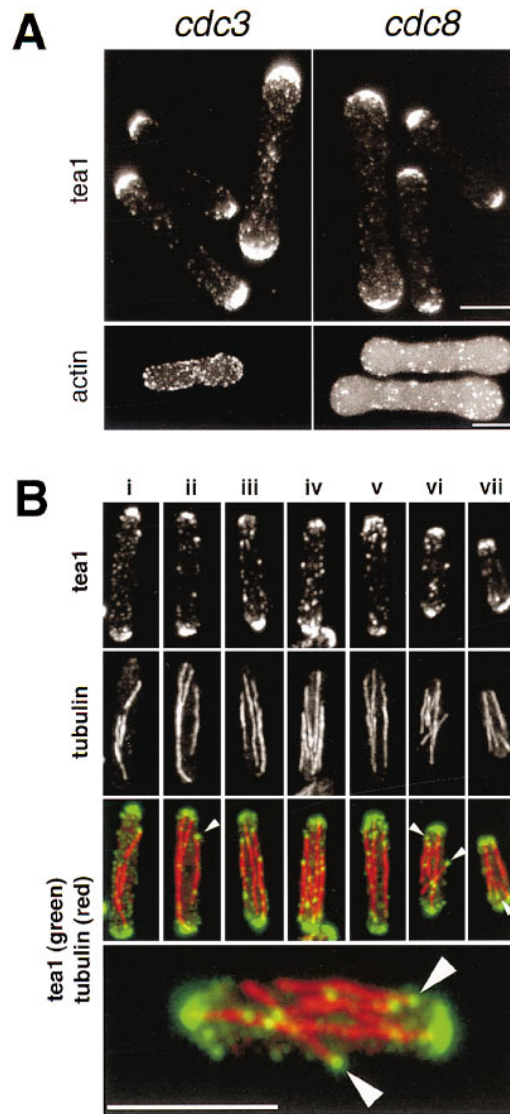


Figure 4. Localization of tea1 and the Cytoskeleton

(A) Localization of *tea1* in mutants in actin-binding proteins. *cdc3-313* (left) and *cdc8-382* (right) mutants were incubated at 36°C for 4 hr, fixed, and stained with anti-*tea1* antibodies (top panels) or with rhodamine-phalloidin to show the actin cytoskeleton (lower panels).
 (B) *tea1* colocalizes with the ends of cytoplasmic microtubules. Wild-type cells were double stained with antibodies against *tea1* (top) and tubulin (middle). An overlay of both stains is shown in the lower panels, with *tea1* in green and tubulin in red. The arrowheads show accumulation of *tea1* at only one end of microtubules. The cell from (vi) is shown at higher magnification. Scale bars = 5 μm.

titrate away other essential proteins. It is likely that *tea1* does interact with other proteins, given that it migrates as part of a high molecular mass complex of around 1000 kDa after gel filtration. (Figure 3B).

Role of the Cytoskeleton in Locating tea1

We next investigated whether the actin or tubulin components of the cytoskeleton were involved in locating *tea1* at cell poles. To test if actin has a such role, we

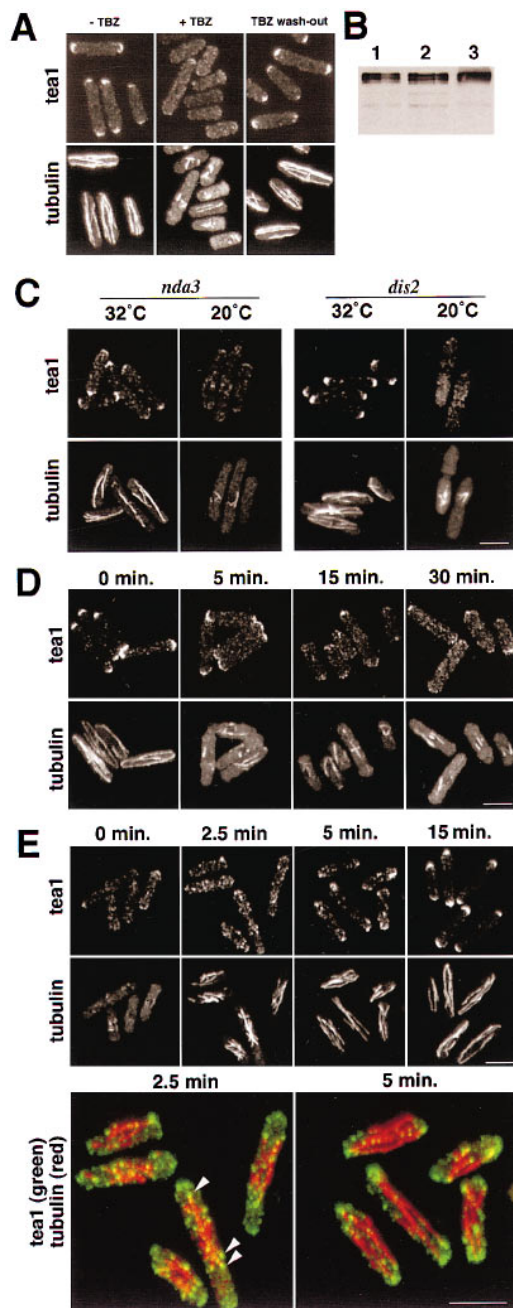


Figure 5. Role of the Microtubular Cytoskeleton in tea1 Localization
(A) Effect of TBZ on the localization of tea1. Wild-type cells were grown at 25°C, incubated with TBZ at a concentration of 100 μ g/ml for 30 min, washed with medium free of TBZ, and incubated at 25°C for a further 10 min. Samples were taken before addition of TBZ (–TBZ), after 30 min in TBZ (+TBZ), and 10 min after the wash (TBZ wash-out). Cells were fixed and immunostained with antibodies against tea1 (top) and tubulin (lower panels).
(B) tea1 levels are not affected by TBZ. TBZ was added to cells growing at 25°C to a concentration of 100 μ g/ml. Cells were collected 0, 15, and 30 min after addition of the drug, processed for Western blotting, and probed with anti-tea1 antibodies.
(C) Localization of tea1 in *nda3* (β -tubulin) and *dis2* mutants. *nda3-KM311* cells (left panels) were grown at 32°C and transferred to 20°C for 1 hr, fixed, and double stained with antibodies against tea1 (top) or tubulin (lower panels). *dis2-1* mutants (right panels) were grown at 32°C, transferred to 20°C for 6 hr, and processed as described for *nda3*.

examined temperature-sensitive mutants in *cdc3* (encoding profilin) and *cdc8* (encoding tropomyosin), which disrupt the actin cytoskeleton and fail to organize actin into the tips at 36°C (Balasubramanian et al., 1992, 1994; Chang et al., 1996). In both mutant strains, tea1 was still correctly located to the ends (Figure 4A, top panels), even though cortical actin patches were delocalized (Figure 4A, lower panels). These observations indicate that a normally organized actin cytoskeleton is not required to locate tea1 at the poles.

Although most of tea1 is found at the cell tips, some tea1 can be seen as dots in random locations throughout the cell. After double staining for tea1 and tubulin, 60% of these dots were found clearly to colocalize with the ends of cytoplasmic microtubules (Figure 4B). In most cases where both ends of a single microtubule could be distinguished, tea1 dots were observed at only one end of the microtubule. The arrowheads mark clear examples (Figure 4B, [ii], [vi], and [vii]).

To examine further the role of microtubules in the intracellular localization of tea1, we depolymerized microtubules using the tubulin-binding drug thiabendazole (TBZ) (Umesono et al., 1983). Before treatment with the drug, tea1 and tubulin showed normal patterns of distribution. Addition of TBZ caused microtubules to become very short, and at the same time tea1 disappeared from the ends of the cell, becoming located randomly throughout the cell (Figure 5A). When TBZ was washed out, microtubules and tea1 returned to their usual location (Figure 5A). Addition of TBZ did not affect tea1 levels (Figure 5B), indicating that TBZ did not change protein stability. To confirm that delocalization of tea1 was caused by disorganization of the microtubules and not by some other nonspecific effect of TBZ, we disrupted microtubules using a cold-sensitive mutant in the *nda3* gene (Umesono et al., 1983; Hiraoka et al., 1984), which encodes β -tubulin (Figure 5C). At the permissive temperature of 32°C, cytoplasmic microtubules in the *nda3-KM311* mutant were similar to those of wild-type cells, although slightly shorter. At this temperature, tea1 was found to be present at both poles of the cells. After 1 hr at the restrictive temperature of 20°C, cytoplasmic microtubules had almost disappeared, and tea1 became completely delocalized. A similar temperature shift in a control wild-type strain did not produce any effect on either microtubules or tea1 distribution (data not shown).

tea1 is reduced in level at cell ends during mitosis, which could be explained by the absence of cytoplasmic microtubules. This explanation predicts that tea1 should

(D) Kinetics of tea1 delocalization after TBZ treatment. TBZ was added to cells growing at 25°C, and cells were fixed after 0, 5, 15, and 30 min and immunostained with anti-tea1 (top) and anti-tubulin antibodies (lower panels).

(E) Kinetics of relocation of tea1 after cold shock. Wild-type cells were incubated on ice for 30 min and transferred to a 25°C water bath. Samples were taken after 0, 2.5, 5, and 15 min at 25°C. Cells were stained with antibodies against tea1 (top) or tubulin (middle). An overlay of the stainings at times 2.5 and 5 min is shown in the lower panels, with tea1 in green and tubulin in red. The arrowheads show clear examples of colocalization of tea1 and microtubules. Scale bars = 5 μ m.

become more delocalized in cells blocked in mitosis without cytoplasmic microtubules. To test this possibility, we used cold-sensitive mutants in *dis2* (Ohkura et al., 1989). At 32°C, the mutants had normal *tea1* and microtubule distributions. After 6 hr at 20°C, cells arrested in mitosis and *tea1* was almost completely delocalized (Figure 5C).

We next followed the kinetics of delocalization of *tea1*, after treatment of the cells with TBZ, and of relocalization of *tea1*, when microtubules were allowed to regrow after previous depolymerization. Cells were treated with TBZ, and the localization of *tea1* and microtubules monitored by indirect immunofluorescence (Figure 5D). Microtubules were already disorganized within 5 min of drug addition; at this point, *tea1* was still largely localized at the poles, although the end staining was reduced and had become more patchy. After 15 min, microtubules were observed to be very short, and *tea1* was almost completely delocalized. To follow the relocalization of *tea1*, we depolymerized microtubules using a cold-shock treatment (Figure 5E). This treatment causes disorganization of microtubules, which is rapidly reversed by transferring the cells into warm medium (J. Hyams, V. Snell, J. Cope, and P. N., unpublished data). Incubation of cells on ice for 30 min depolymerized the microtubules and led to delocalization of *tea1*. After 2.5 min in warm medium, short microtubules became visible in all cells, and microtubules were longer after 5 min and looked completely normal after 15 min. *tea1* was observed as bright dots dispersed throughout the cell after 2.5 min, some staining was detected at cell tips after 5 min, and tip staining was predominant after 15 min. Examination of the overlaid *tea1* and tubulin distributions revealed that 80% of the dispersed *tea1* spots colocalized with the ends of the regrowing microtubules (Figure 5E, lower panels; the arrowheads show clear examples).

We conclude that localization of *tea1* at the ends of the cells is dynamic and can only be maintained for a short time (5–15 min) in the absence of intact microtubules. In addition, *tea1* is found on the tips of the growing microtubules recovering after disruption and becomes located at the ends of the cell when microtubules extend along the whole cell. These results indicate that microtubules transfer *tea1* to the ends of the cell and thus identify microtubules as playing an important role in establishing and maintaining antipodal growth.

Loss of Antipodal Behavior during Conjugation

During conjugation, tip growth is not maintained in an antipodal manner because it is necessary for a tip to grow more flexibly so it can grow toward a cell of opposite mating type (Fukui et al., 1986; Leupold, 1987). Therefore, we examined *tea1* behavior in cells exposed to the pheromone P factor. We used the P factor-responsive strain *sxa2Δcyr1Δh⁻* (Maeda et al., 1990; Imai and Yamamoto, 1992), which is slightly shorter than wild-type cells. Addition of pheromone caused the production of thin and bent mating projections (Figure 6A). The *tea1* levels were found to be reduced 3- to 4-fold by 5 hr after addition of pheromone, as determined by both Western blot (Figure 6B) and immunofluorescence

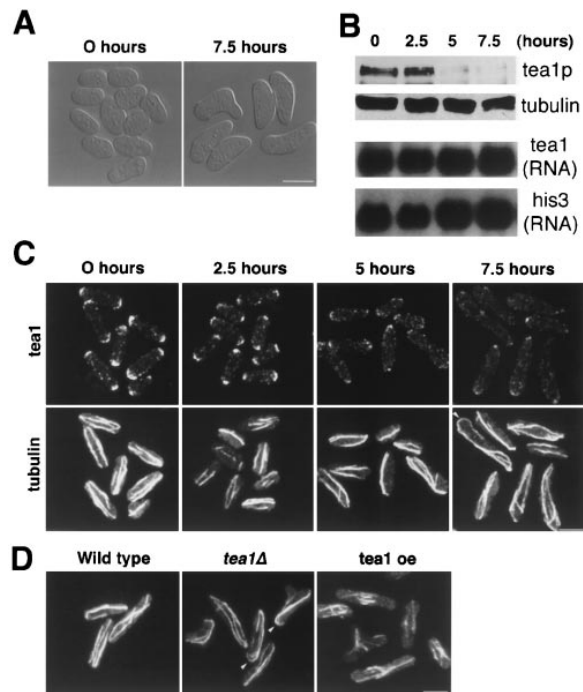


Figure 6. Effects of Pheromone on *tea1* and Effects of *tea1* on Microtubules

(A–C) Effect of pheromone on *tea1*. P factor was added to *sxa2Δ::ura4⁺ cyr1Δ::LEU2 h⁻* cells to a final concentration of 1.6 μg/ml, and the cells were incubated at 25°C. Samples were taken after 0, 2.5, 5, and 7.5 hr in pheromone. (A) Effect of pheromone on fission yeast morphology as seen by Nomarski. (B) Extracts were processed for Western blotting and probed with antibodies against *tea1* and tubulin, or for Northern blotting, and hybridized with *tea1* or *his3* probes. The band corresponding to *tea1* mRNA is not detected in a *tea1Δ* (data not shown). (C) Cells were immunostained with antibodies against *tea1* (top) and tubulin (lower panels).

(D) Effects of *tea1* on microtubules. Wild type (left) or *tea1Δ* cells (middle) were grown at 25°C, shifted to 36°C for 4 hr, fixed, and stained with an antibody against tubulin. Cells overexpressing *tea1* at 36°C for 16 hr were processed similarly (right). The arrowheads in (C) and (D) show abnormal microtubules. Scale bars = 5 μm.

(Figure 6C). In contrast, *tea1* messenger RNA levels were not reduced (Figure 6B), showing that the regulation is posttranscriptional. Thus, in a physiological situation in which the controls that maintain antipodal growth need to be overridden, *tea1* is down-regulated.

Effects of *tea1* on Microtubules

During the course of these experiments, we noted some effects of *tea1* on microtubules (Figure 6D). In wild-type cells, cytoplasmic microtubules run along the main axis of the cell to the poles of the cell, but do not normally extend any further. In *tea1Δ* cells, we found that in about 10%–15% of the cells microtubules curled round the ends of the cell (Figure 6D, arrowheads), a situation that was very rare in wild type-cells (<0.5%). Furthermore, in pheromone-treated cells that have reduced levels of *tea1*, microtubules were also found to curl round the ends of the cells (arrowhead in Figure 6C). Overexpression of wild-type *tea1* also caused defects in cytoplasmic microtubules, which were found to be much

shorter than normal. These results suggest that, as well as being transferred by microtubules, *tea1* may also affect the organization of the microtubular cytoskeleton. *tea1* may have some inhibitory effect on microtubular growth, such that absence of *tea1* allows microtubules to grow around the ends of the cells, while high levels of *tea1* cause microtubules to be shorter than normal. Thus, *tea1* could inhibit microtubular growth in the tips of the cell and thus contribute to the organization of microtubules necessary to maintain a single central axis.

Discussion

In this paper, we have shown that *tea1* plays an important role in the spatial organization of a fission yeast cell. Our major observations and conclusions are as follows: (i) the *tea1* gene is required to maintain the growing tips in an antipodal manner, keeping the two tips growing in a precisely opposed fashion; (ii) *tea1* acts as a tightly localized end marker, regardless of whether the tips are growing; (iii) the microtubular cytoskeleton is required for locating and maintaining *tea1* at the cell tips; (iv) *tea1* level is down-regulated by pheromone; and (v) *tea1* affects the organization of the microtubular cytoskeleton in a way that may contribute to the maintenance of antipodal growth behavior.

When *tea1* is deleted, cells fail to grow with their tips precisely opposed. The tip centers are not located at the ends of a single axis running through the middle of the cell, as is the case in a wild-type cell, but instead the centers become offset at an angle from each other. If the angle is small, a bent cell is produced, but if the angle becomes greater, an extra tip is generated that grows out of the side of the cell body, producing a T-shaped cell. Given that *tea1* is located at the ends, we propose that *tea1* acts as an end marker, defining where the cell-growth zones should be organized and thus the direction of growth. It is important to understand that *tea1* is not required for a growth zone to become organized; it is only required to place the growth zone in the correct place. The components making up the growth machinery can form a growth zone in the absence of *tea1*, but *tea1* is necessary to locate the growth zone in the correct place at the cell tips, presumably by interacting with components making up the growth machinery.

The *tea1* gene is required to maintain antipodal growth throughout the cell cycle and not only when polarity is reestablished after mitosis. This is shown by the behavior of *cdc2^{ts}-tea1^{ts}* double-mutant cells that form some branches and become highly bent even though they fail to enter mitosis. However, if repeated cycles of mitosis are allowed in the absence of cytokinesis using a septation mutant, more branched cells are formed, suggesting that *tea1* is particularly important during polarity reestablishment after mitosis (Verde et al., 1995; see Figure 4).

Microtubules are required for *tea1* to be located at the cellular ends. Within 15 min of disrupting microtubules, *tea1* becomes delocalized throughout the cell. Several experiments support the conclusion that proper *tea1* localization is a consequence of the microtubular cytoskeleton being used for *tea1* transference to cell ends.

First, when microtubules grow back after having been disrupted, *tea1* is initially observed at the ends of the regrowing microtubules. As the microtubules lengthen, *tea1* is moved toward the tips of the cell and becomes deposited there after the microtubules have attained full length. Therefore, the ends of the cell are identified by a dynamic process requiring *tea1* to be transferred to the cell ends by the microtubular cytoskeleton. Second, mutants in genes such as *nda3* (β -tubulin) (Umesono et al., 1983; Hiraoka et al., 1984) and *atb2/ban5* (α_2 -tubulin) (Umesono et al., 1983; Toda et al., 1984; Verde et al., 1995; Yaffe et al., 1996) or treatment with drugs such as TBZ (Umesono et al., 1983), all of which disrupt microtubules, generate bent or T-shaped cells. In very elongated *cdc* cells, antipodal behavior is lost and cells bend and occasionally form branches (J. M. and P. N., unpublished data). In the conditions used in the experiments with the *cdc* mutants in this paper, microtubules extended along the length of the cells, and *tea1* was localized at the poles. However, it is possible that bending and branching in very elongated cells is caused by microtubules failing to reach the cell ends, leading to incorrect deposition of *tea1*. We suggest that in normal cells, the microtubules explore the space of the cell and usually become distributed along the long axis of the cell, finishing in the region of the cell poles. As a consequence, *tea1* is placed at the cellular tips, leading to a proper localization of the growth machinery and then to antipodal growth. With this view, it is *tea1* localized at the cell ends that carries out *tea1* function, by acting as a marker for the growth machinery. However, we cannot exclude the possibility that *tea1* carries out its function by influencing microtubule distribution, which in turn determines the direction of growth. In this view, it is the *tea1* bound to microtubule ends that is active, while the accumulation of *tea1* at the poles of the cells is a by-product of the distribution of microtubules.

Because *tea1* is usually only detected at one end of a microtubule, the transference to the cell poles is likely to be polarized. The polarity of microtubules in a fission yeast cell has not yet been determined, but because after disruption they grow back in all directions from the middle of a cell (J. Hyams, V. Snell, J. Cope, and P. N., unpublished data), it is probable that microtubules of opposite polarity are distributed along the length of the cell. This means that in the tip regions, there will be both (+) and (-) ends, with *tea1* being delivered by only those microtubules of the appropriate polarity. *tea1* could be transferred to cell ends by microtubules in two different ways. First, *tea1* could bind to the microtubules at any point along their length and then be transported toward the end, presumably by a motor protein. If this is correct, then the terminal location of *tea1* in regrowing microtubules implies that the rate of transport must exceed the rate of microtubular growth. Second, *tea1* may become localized at the ends of growing microtubules and be moved toward the cell tips simply by microtubular elongation. Since *tea1* accumulates at the ends of growing microtubules and at the tips of the cell when the microtubules reach the cell end, there must be some mechanism that ensures that *tea1* is transferred from the microtubule end to the cell tip. This could be due to different affinities of *tea1* for the motor or microtubule ends and

some component located in the region of the cell surface, such as the plasma membrane or the cell cortex, or due to a changed affinity of *tea1* for the motor or the microtubule end when the microtubule stops growing. It is also possible that *tea1* is associated with vesicles during microtubule transport, which might facilitate transference to the plasma membrane by membrane fusion.

For *tea1* to be correctly located at the cellular ends, the microtubules must run along the long axis of the cell, finishing at the poles and depositing *tea1* at the tips of the cell. We shall discuss three models that could bring this about. In the first model, some as-yet-unidentified marker is already located at the cellular ends. This marker stabilizes the microtubules such that they run along the long axis of the cell, ensuring that *tea1* is delivered to the ends. Therefore, the localization of this marker gives rise to a distribution of microtubules, such that *tea1* is delivered to the tips, and marks the position of the end to the growth machinery. Growth machinery present in the middle of the dividing cell at the time of septation could be responsible for locating this unknown marker. This creates a cycle of contiguous markers in which the localization of a component provides the spatial information to position the next one. When *tea1* is deleted, the cell loses the connection between this marker and the growth machinery, leading to growth at incorrect places and to the formation of bent and T-shaped cells.

In the second model, microtubules form along the long axis of the cell, finishing at the poles because this is the energetically most favorable mode of microtubular growth. If microtubules are sufficiently stiff that they do not bend easily within the cell, then they will extend throughout the cellular space until they run along the long axis and extend between the two poles of the cell. As a consequence, *tea1* will be delivered to exactly the correct position in the cell to organize antipodal growth. In this view, the growth properties of microtubules result in them searching the space inside the cell, continuously finding the ends of the cell that are then marked by deposition of *tea1*. This model does not rely on a cycle of contiguous markers to identify the position of the ends, but on the ability of microtubules to explore and recognize the geometry of the cell (Kirschner and Mitchison, 1986). The third model is an elaboration of the second, incorporating the effects *tea1* has on microtubular growth. As before, the microtubules search the space of the cell, and their properties ensure that the ends of growing microtubules come into the vicinity of the cellular tips. When *tea1* is overexpressed, the high levels of *tea1* lead to shortened microtubules, while deletion of *tea1* or pheromone addition allows microtubules in some cells to curve around the ends of some cells, growing more than usual. Therefore, an accumulation of *tea1* in a specific region of the cell could reduce or stop microtubular growth, forcing the microtubule ends to remain within that region. This provides a positive feedback whereby microtubule ends that have identified the cellular poles by their growth properties become stabilized in this region by the effect of *tea1*. As a consequence, more *tea1* is delivered, leading to greater stability. The interest of the second and third models is

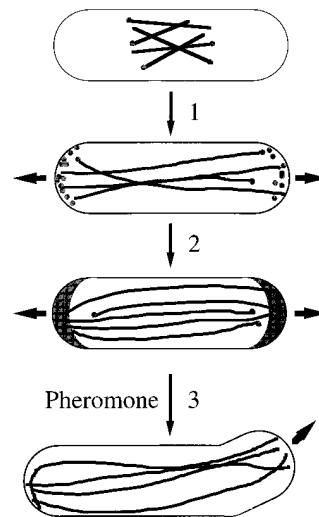


Figure 7. A Model for *tea1* Role in Maintaining Antipodal Growth. Microtubules are represented by dark lines inside of the cell; *tea1* is represented by stippled dots and stippled regions at cell ends. (1) Microtubules explore the cell and find the cell poles as a consequence of their growth properties. (2) *tea1* is transferred by the microtubules to the ends of the cell, where *tea1* acts as an end marker that directs the growth machinery to the correct place, ensuring that ends are kept exactly opposed to each other. (3) When cells are treated with mating pheromone, *tea1* is down-regulated, allowing growth to be directed toward a mating partner.

that they provide some explanation of how spatial order acting over a distance can be generated within a cell.

Immediately after mitosis and cell division, fission yeast cells initiate polarized growth from the old tip, the end of the cell inherited from the mother cell. They then elongate unipolarly until NETO, when they activate growth at the new end, leading to bipolar growth (Mitchison and Nurse, 1985). At mitosis, the cytoplasmic microtubular cytoskeleton is disrupted and reestablished about the time of cell division (Hagan and Hyams, 1988). During mitosis, *tea1* staining at the old cellular tips is somewhat reduced, presumably owing to the transient disappearance of cytoplasmic microtubules, but rapidly increases again after cell division. In the newly born cell, the old tip is marked with *tea1* more heavily than the newly formed tip. This may provide some explanation for why growth in the next cell cycle is usually activated at the old end (Mitchison and Nurse, 1985). A prediction of this model is that cells released from a mitotic block during which *tea1* has been delocalized would randomly select between the old and the new tip as the site for the initiation of growth after mitosis.

The models described above generate spatial order without recourse to external cues. However, when a growing tip is seeking out another cell for mating, the fixed relationship between the two cellular tips must be relaxed, allowing growth toward a mating partner (Fukui et al., 1986; Leupold, 1987). In these circumstances, *tea1* levels are depressed, uncoupling growth-zone positioning from the *tea1* control system. The positioning of the growth zone will respond to the external cues provided by pheromone gradients outside the cell and is no longer subject to the internal organizing mechanism.

Thus, the control allows a shift between order generated from within the cell to order imposed from without. A model of how *tea1* could perform its function is presented in Figure 7.

The localization of *tea1* to the ends of the cell by microtubules in the manner described in models 2 and 3 above would generate positional information within the cell. In other organisms, localized deposition of proteins or RNAs inside the cell by the cytoskeleton is also used as a mechanism to produce positional information. This localization then gives rise to localized growth or to differences in cell fate after division. A well-studied system is the axial budding pattern observed in haploid budding yeast cells, where new buds appear adjacent to the position of old buds. It is thought that the old bud position is first marked by a ring of septins, which is then used to position a cortical tag requiring the *BUD3*, *BUD4*, and *BUD10/AXL2* genes that in turn determines the position of a new bud and septin ring (for review, see Roemer et al., 1996). This cycle of contiguous markers, locally assembling the cortical tag that locates the next cycle of septins, is formally similar to the first model described above. Addition of pheromone changes the axial growth pattern, such that the growth of mating projections occurs toward a mating partner and causes the down-regulation of *BUD4* and *AXL2* (reviewed in Roemer et al., 1996). Thus, the shift in the control of growth from internal cues to external cues could work in a similar way in both yeasts, by down-regulation of genes required for the internal cues. However, this control system differs from the one we have described because it is based on the history of the cell, in the sense that an earlier event localized in space, such as the septin ring-cortical tag, makes a mark at that location that is used by a later event. In fission yeast, the *tea1*-microtubular system is dynamic, with the cellular space being reexplored and reestablished regularly after each cell division. This allows any earlier mistakes to be corrected and also provides an explanation for how location within a cell can be determined across space at a position distant from the original location, which is not easily possible with a historical model.

The *tea1*-microtubular system in fission yeast we have described here can generate a cellular field whereby global spatial order is imparted to a cell. The microtubules explore the space of the cell, identifying the cell ends, and the delivery of *tea1* marks the ends for locating the growth zones and may also stabilize the microtubular pattern to generate a more robust positioning system. Further analysis of this system will be useful to understand the way in which the cytoskeleton and interacting proteins can lead to global cellular organization in fission yeast. This should also be of relevance to understanding related problems in other eukaryotes.

Experimental Procedures

S. pombe Methods

Standard methods were used for growth, transformation, and genetic manipulation of *S. pombe*. All experiments were carried out in EMM2 minimal medium. Plasmids used were pIRT2 and pREP3X (Moreno et al., 1991). Pheromone experiments were carried out as described (Stern and Nurse, 1997).

Mapping of the *tea1* Gene

Genetic mapping was carried out in three steps. First, *tea1* was shown to map to chromosome III by using mitotic haploidization as described (Alfa et al., 1993). Second, the position of *tea1* on the chromosome was determined by long-range mapping, measuring genetic linkage in a *swi5* mutant background (Schmidt, 1993) (*swi5* strains were a gift of H. Schmidt). A *tea1-3 swi5-39 ura4-294 tps14-5 h⁺* strain was constructed and crossed to an *ade5-36 swi5-39 h⁻* strain. Tetrad analysis of this cross showed that *tea1* is localized to the right arm of chromosome III, between the *tps14* and the *ade5* genes. Third, the *tea1* gene was mapped by linkage analysis in a *swi5⁺* background. A *tea1-3 ura4-D18* strain was crossed to mutants in genes that map to this chromosome arm (*tps28-9*, *arg1-230*, and *nmt1::ura4*). Tetrad analysis of the crosses was performed, and *tea1* was found to be closely linked to the *nmt1* gene (1.3 cM, 116 tetrads analyzed).

Tagging and Transformation of Cosmids

Cosmids in the *nmt1* region (provided by E. Maier) were tagged with a randomly inserted transposon Tn1000 containing the *his7⁺* gene (Morgan et al., 1996) (gift of S. Sedgwick), allowing selection of cosmids after transformation. Tagged cosmids were individually transformed into a *tea1-3 his7-366* strain and screened for complementation of the cell-shape defect. Cosmid 25D6 (Hoheisel et al., 1993) completely rescued the mutant phenotype.

Subcloning, Sequencing, and Deletion of *tea1*

Cosmid 25D6 was partially digested with *Sau3A*, and fragments of about 6 kb were gel purified and cloned into the *Bam*HI site of pIRT2. All clones were pooled and transformed into *tea1-3 leu1-32*, and the transformants screened for rescue of the *tea* phenotype. Five complementing plasmids were isolated. Four of them contained a 4.3 kb *Bam*HI fragment. The other one (pR2) contained 3.9 kb of the same 4.3 kb fragment, lacking the first 10 codons of the *tea1* ORF.

Sequencing was carried out using an ALF system from Pharmacia. The sequence was analyzed using the Genetics Computer Group package. Prediction of coiled-coil regions was done using the Lupas algorithm (Lupas et al., 1991).

To delete *tea1*, a 4.3 kb *Bam*HI fragment containing the *tea1* gene and flanking regions was digested with *Hind*III, removing a 2.5 kb fragment containing 72% of the ORF (codons 68–895). This fragment was replaced with the *ura4⁺* gene and transformed into a *ura4-D18* diploid strain. Stable transformants were isolated, and deletion of one copy of *tea1* was confirmed by Southern blotting.

Production of *tea1* Antibodies and *tea1* Analysis

A *Sac*I-*Bgl*III fragment from pR2 containing most of the 5' half of *tea1* and a *Bgl*III-*Bam*HI fragment containing the 3' half were cloned into the bacterial pQE30 expression vector (Quiagen), producing 6His-tagged versions of fragments of *tea1* containing amino acids 10–553 and amino acids 554–1147, respectively. The proteins were purified using *Ni*²⁺-NTP resin under denaturing conditions and subsequently by SDS-PAGE. The gel slices containing the protein with Freund's adjuvant were used to inoculate rabbits. The antibodies were affinity purified from nitrocellulose strips containing the corresponding His-tagged protein as described (Pringle et al., 1991).

Preparation of total boiled protein extracts, Western blot analysis, and detection of antibodies were done as described (Grallert and Nurse, 1996). The antibodies used were affinity-purified anti-*tea1*-N-terminus (1:100 dilution), anti-*tea1*-C-terminus (1:2000), and anti- α -tubulin monoclonal antibody (1:5000; Sigma T-5168). Extracts for gel filtration were prepared by protoplasting cells as described (Grallert and Nurse, 1996), centrifuged at 100,000 \times g for 60 min, and loaded onto a Superose-6 column.

Immunofluorescence Techniques

Cells were fixed by adding formaldehyde to 4% final concentration to the medium for 30 min, or collected by filtration and fixed with -20°C methanol, and processed as described (Alfa et al., 1993). Similar results were obtained with both fixations. Cell stainings are of cells fixed with methanol except for Figure 2D and for the actin

staining in Figure 4A, for which formaldehyde was used. Affinity-purified antibodies against the N-terminal half of tea1 were used at 1:20 dilution, and antibodies against the C-terminal half at 1:60. For immunofluorescence of tubulin, TAT1 monoclonal antibody was used (Woods et al., 1989) (gift of K. Gull) at 1:15 dilution and for actin N350 (Amersham) at 1:150. Fluorescein-linked anti-rabbit (Amersham) at 1:150 dilution and Cy3-linked anti-mouse (Sigma) at 1:2000 were used as secondary antibodies. Rhodamine-phalloidin stainings of actin were done as described (Alfa et al., 1993). Photographs were taken using a Bio-Rad MRC600 confocal microscope and analyzed with Bio-Rad software.

Overexpression of tea1

The *tea1* gene was overexpressed using the thiamine-repressible *nmt1* promoter (Maundrell, 1990). A 4.3 kb fragment BamHI containing *tea1* full ORF was cloned into the BamHI site of pREP3X (Forsburg, 1993).

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**EMBL Nucleotide Sequence Database
Accession Number**

The EMBL Nucleotide Sequence Database accession number for the *tea1* gene sequence described in this paper is Y12709.