# Fission Yeast Cell Morphogenesis: Identification of New Genes and Analysis of Their Role during the Cell Cycle

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***Abstract.*** To identify new genes involved in the control of cell morphogenesis in the fission yeast *Schizosaccharomyces pombe* we have visually screened for temperature-sensitive mutants that show defects in cell morphology. We have isolated and characterized 64 mutants defining 19 independent genes, 10 of which have not been previously described. One class of mutants, defining 12 *orb* genes, become round and show a complete loss of cell polarity. A second class of mutants exhibits branched or bent morphologies. These mutants show defects in either selection of the growth site, defining two *tea* genes, or in the maintenance of growth direction, defining five *ban* genes. Immunofluorescence analysis of these morphological mutants shows defects in the organization of the microtubule and actin cytoskeleton. These defects include shortened, bundled, and asymmetrically localized microtubules and enlarged and mislocalized actin patches. Analysis of the mutant phenotypes has allowed us to order the genes into four groups according to their function during the cell cycle: genes required for the maintenance of cell polarity throughout the cell cycle; genes necessary only for the reestablishment of cell polarity after mitosis and not for maintaining cell polarity once it is established; genes essential for the transition from monopolar to bipolar growth and genes that serve as 'polarity markers'.

MAINTENANCE of cell form and polarity is fundamental to cell function and differentiation. Our understanding of cell morphogenesis is still limited, but a genetic approach has proven to be useful in budding yeast (for review see Chant, 1994) and, more recently, in fission yeast (see Snell and Nurse, 1993; Nurse, 1994). Fission yeast is an attractive system for the study of cell form and polarity because cells are cylindrical in shape and grow in a highly polarized manner. Moreover, cells undergo three morphological transitions closely coupled with progression through the cell cycle. After cytokinesis, the newly divided daughter cells initiate growth in a monopolar fashion. This process is described as old end take off or OETO because cells grow from the cell tip that existed in the previous cell cycle (Streiblova and Wolf, 1972). In early G2, ~0.3 of a cell cycle after daughter cell separation, and after the cell has attained a critical cell length, cells switch to bipolar growth using the new tip formed by cell division (new end take off or NETO)[1] (Mitchison and Nurse, 1985). Bipolar growth continues until ~0.75 of the cell cycle, when tip elongation ceases and mitosis occurs. Changes in the cytoskeleton are correlated with these cell cycle transitions (Marks et al., 1986). Actin patches are concentrated at the old end during the early phases of the cell cycle and then also appear at the new end when bipolar growth is initiated. At mitosis actin disappears from the tips and assembles into an actin ring, located in the middle of the cell and marking the place where septation will occur (Marks and Hyams, 1985). Actin is necessary for cell growth, because disruption of the actin cytoskeleton by cytochalasin D blocks protoplast regeneration and cell-wall deposition (Kobori et al., 1989). The microtubule cytoskeleton also undergoes changes during the cell cycle (Marks et al., 1986; Hagan and Hyams, 1988). Cytoplasmic microtubules, which extend along the main cell axis during interphase, depolymerize at the interphase–mitosis transition when the intranuclear spindle forms. At telophase, cytoplasmic microtubules are transiently nucleated by MTOCs located at the cell equator. Disruption of the microtubule cytoskeleton does not prevent polar growth (Ayscough et al., 1993), but microtubules are likely to have a role in cell polarity because cold-sensitive *nda3* mutants, defective in β-tubulin, and thiabendazole-treated cells, bend and branch (Umesono et al., 1983*a*,*b*; Hiraoka et al., 1984).

Signal transduction pathways are already known to play important roles in the control of fission yeast morphogenesis. Small GTP-binding proteins, identified as polarity genes in budding yeast, are also essential for *Schizosaccharomyces pombe* polarized growth. Homologues of budding yeast polarity genes in fission yeast are *cdc42sp* (*CDC42* homologue) (Fawell et al., 1992; Miller and Johnson, 1994), *cwg2* (*CDC43* homologue) (Díaz et al., 1993), *ral1/scd1* (*CDC24* homologue) (Fukui and Yamamoto, 1988; Chang

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1. *Abbreviation used in this paper*: NETO, new end take off.

et al., 1994), and *ral3/scd2* (*BEM1* homologue) (Fukui and Yamamoto, 1988; Chang et al., 1994), with *cdc42sp* and *ral1/scd1* encoding components of a GTPase cycle. Mutants in these genes have a rounded shape. Mutants in *ras1* are also rounded during vegetative growth and become completely round under conditions of nitrogen starvation (Fukui and Kaziro, 1985; Fukui et al., 1986). Recently *ral1/scd1* and *ral3/scd2* have been shown to interact with *ras1* (Chang et al., 1994), which has been proposed to have a function similar to the *RSR1/BUD1* gene in budding yeast (Bender and Pringle, 1989; Chant and Herskowitz, 1991; Zheng et al., 1995). The protein kinase C pathway is also important for the control of cell shape in *S. pombe* because mutations in *pck2* (a protein kinase C homologue) (Toda et al., 1993) and mutants in the interacting gene *ppe1* (phosphatase 2A homologue) (Shimanuki et al., 1993) are rounded and deformed. Deletion of another kinase, *kin1*, was also shown to alter cell shape (Levin and Bishop, 1990).

It is unclear how these signaling and phosphorylation pathways control the dynamics of cytoskeletal structures and modulate morphological transitions during the cell cycle. To gain further insight into these problems, in this paper we have first identified a more complete set of genes involved in the control of fission yeast cell morphogenesis by isolating temperature-sensitive mutants showing defects in cell morphology. This study has extended considerably an existing collection of mutants identified in our laboratory (Snell and Nurse, 1994). One class of mutants (*orb*) shows a complete loss of polarity and a round form at the restrictive temperature. A second class shows defects in the choice of the growth site (*tea* mutants) or in the maintenance of growth direction (*ban* mutants). Second, we have characterized these mutants with respect to their microtubule and actin cytoskeleton, and have identified various defects in these structures associated with changed cellular morphology. Finally, we have analyzed the role of these genes during the cell cycle, and have grouped them according to their functions at the different morphological transitions.

## Materials and Methods

### Strains and Growth Conditions

The strain used for mutagenesis was *ade6-M210 leu1-32* h−. The strains used for linkage or complementation analysis were TP40-2A h+ *leu1-32 his2 sts5-7* (Toda et al., 1991); *cwg1-2 ura4-D18 leu1-32* h−, *cwg2-1 ura4-D18 leu1-32* h+, *pap1-6 leu1-32* h−, *pap1-8 leu1-32* h−, kindly provided by Dr. A. Duran (Universitad de Salamanca, Salamanca, Spain); JX124 h90 *leu1-32 ura4-D18 ade6-M216 ral1::ura4+*, JX273 h90 *leu1-32 ura4-D18 ade6-M216 ral13::ura4+*, kindly provided by Dr. M. Yamamoto (University of Tokyo, Tokyo, Japan). The strains used for cell-cycle analysis were *cdc2-33 leu1-32* h+ and *cdc11-119* h+.

Cells were cultivated in rich yeast extract (YE) medium or in Edinburgh minimal (EMM) medium with the appropriate supplements (Moreno et al., 1991), as indicated. Cells in liquid cultures had been growing exponentially for at least eight generations, at densities below $10^7$ cells/ml, before observation.

### Mutagenesis and Mutant Isolation

Mutagenesis was performed as described (Moreno et al., 1991) using 300 μg/ml *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine for 15 min. This procedure gave 50% killing. Cells were plated on YEA at 500 surviving cells/plate. Colonies formed after 3–4 d incubation at 25°C. The colonies were replica plated onto fresh YEA plates and then incubated at 35.5°C for 12 h, and visually screened for colonies with aberrantly shaped cells. Isolated strains were further selected, and only penetrant phenotypes were finally pursued. Mutants used for further analysis were outcrossed twice. Mutants displaying ovoid or irregular shapes were discarded. Linkage analysis and all following genetic procedures were conducted as described (Moreno et al., 1991). The mutants displaying the most penetrant phenotype were chosen as representative for each linkage group; however, the various alleles displayed small phenotypic differences. Synthetic lethality interactions were determined by tetrad analysis, analyzing at least 10 complete tetrads and at least one NPD (nonparental ditype). Phenotypes were observed at 25, 32, and 36°C. *Orb8-62 orb9-46* and *orb2-34 orb6-25* double mutants were inviable at 32 and 36°C. *Orb1-9 orb2-34, orb1-9 orb6-25, orb11-59 orb9-46*, and *orb11-59 orb8-62* were inviable at 36°C.

### Plasmids and Transformation Procedures

*CDC42Sp*$^+$ borne on pWH5 was a gift of Dr. D. I. Johnson (University of Vermont, Burlington, VT); *pck1*$^+$, *pck2*$^+$, *ppe1*$^+$, and *pyp1*$^+$ on pDB248 were a gift of Dr. T. Toda (Imperial Cancer Research Fund, London, UK); *kin1*$^+$ on pDB248 was a gift of Dr. D. Levin (Johns Hopkins University, Baltimore, MD); and *ras1*$^+$, *ral1*$^+$, *ral2*$^+$, and *ral3*$^+$ on pDB248′ were a gift of Dr. M. Yamamoto. Cells were transformed by the lithium acetate procedure (Moreno et al., 1991).

### Cytology

Cells were cultured in YE medium at 25°C and then incubated at 36°C for the indicated time. For Calcofluor staining, 1 μl of a Calcofluor solution (1 mg/ml Calcofluor in 50 mM sodium citrate, 100 mM sodium phosphate, pH 6.0) was added to 10 μl cell culture: cells were then photographed immediately. For tubulin and actin staining, cells were fixed in methanol for at least 15 min. Immunofluorescence was performed as described (Hagan and Hyams, 1988). For microtubule staining, the primary antibody used was the monoclonal TAT1, kindly donated by Dr. Keith Gull (University of Manchester, Manchester, UK). For actin staining the primary antibody used was the monoclonal N350 (Amersham Corp., Arlington Heights, IL). In both cases, the secondary antibody was a sheep anti–mouse Cy3-conjugate F(ab′)2 fragment (Sigma Chemical Co., St. Louis, MO). Cells were then immobilized on gelatin-coated coverslips and observed using a microscope (Axioskop; Carl Zeiss, Inc., Thornwood, NY). Cells were photographed using a confocal microscope (600; Bio-Rad Laboratories, Hercules, CA), with a 0.2-μm interval between successive pictures and analyzed using Bio-Rad software. Measures of microtubule length were taken using Bio-Rad software.

## Results

### Genetic Analysis of the orb, tea, and ban Mutants

After mutagenesis, we visually screened 55,000 colonies for temperature-sensitive mutants showing defects in cell morphology. We isolated 64 strains, which identified three mutant classes (Fig. 1), previously described in a more limited screen that yielded 18 strains (Snell and Nurse, 1994). These 64 strains included 51 *orb* (for spherical) mutants that are almost perfectly round and show no polarized growth (Fig. 1 *b*), 8 *tea* (for tip elongation aberrant) mutants that are triangular and misplace the site(s) of growth (Fig. 1 *c*), and 5 *ban* (for banana) mutants that are curved and do not maintain the correct direction of growth (Fig. 1 *d*).

All 51 spherical mutants were backcrossed to a wild-type strain 975h+ and 43 showed 2:2 segregation of the mutant phenotype, indicating that these mutations lie within single genes. Eight mutants were sterile at the permissive temperature. Linkage analysis showed that the 43 round mutants defined 12 *orb* genes (Table I), including five that had been previously described in our laboratory (*orb1-5*; Snell and Nurse, 1993). *Orb1*, *orb2*, *orb6*, *orb7*,

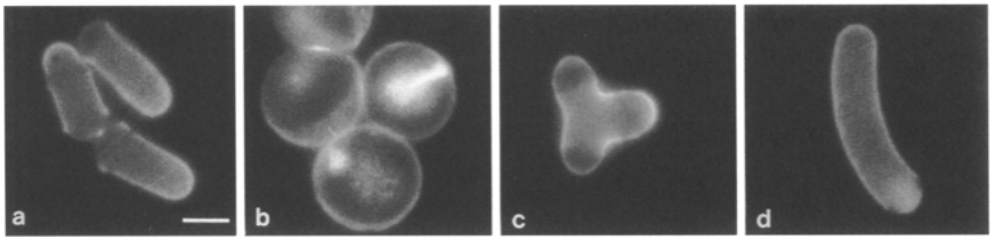


*Figure 1.* The three phenotypic categories of shape mutants stained by Calcofluor. (*a*) Wild-type strain *ade6-M210 leu1-32* h−. (*b*) *orb6-25*. (*c*) *tea1-3*. (*d*) *ban2-92*. Cells were incubated at 36°C for 7 h and then stained with Calcofluor to visualize the cell wall. Bar, 5 mm.

*orb8*, *orb9*, and *orb11* mutants are able to grow at restrictive temperature, in spite of their altered morphology. The double mutants between *orb1*, *orb2*, and *orb6* and between *orb8*, *orb9*, and *orb11* are inviable and unable to form colonies at restrictive temperature, suggesting synthetic lethal interactions between the gene products (Table I; see Materials and Methods).

A number of fission yeast genes are known to generate rounded shapes when mutated (for review see Snell and Nurse, 1993). We checked allelism of representative mutants of the *orb* genes to these genes, *sts5*, *ral1*, *ral3*, *cwg1*, and *cwg2* (see Table I) by linkage and complementation analysis. *Orb7* is allelic to *cwg2* (recombination frequency <0.8%, diploid not complementing) (Díaz et al., 1993). *Orb11* is linked closely to the gene *cwg1*, defined by osmotically sensitive mutants (recombination frequency <1%) (Ribas et al., 1991*a*,*b*) and so far not cloned. *Orb4* is allelic to *sts5*, identified by staurosporine sensitivity and round-shaped mutants (Toda et al., 1991), and suppressed by phosphatases $ppe1^+$ and $pyp1^+$ (Toda, T., personal communication, reported in Snell and Nurse, 1994).

We also tested if any of our mutants could be complemented by plasmids carrying the following genes: $ras1^+$, $ral1^+$, $ral2^+$, $kin1^+$, $pck1^+$, $pck2^+$, $cdc42sp^+$, $ppe1^+$, and $pyp1^+$. The phenotype of five out of the eight sterile round mutants was rescued by multicopy expression of $ral1^+$/$scd1^+$ and one of these sterile mutants was shown to be allelic to *ral1/scd1* by protoplast fusion. These sterile strains have not been studied further. The *orb12* mutants are partially suppressed by $pck1^+$ and $pyp1^+$, as well as by $ras1^+$ (Table I). This genetic analysis indicates that five new *orb* genes, *orb6*, *orb8*, *orb9*, *orb10*, and *orb12*, have been identified in this study. Because *orb4* and *orb7* are allelic to the previously identified genes *sts5* and *cwg2*, respectively, we shall use these earlier names to describe them.

*Table I. orb Genes*

| Gene | Alleles | Synthetic lethality | Close linkage* | Multicopy suppression |
|---|---|---|---|---|
| *orb1* | 6 (3‡) | *orb2, orb6* | | |
| *orb2* | 2 (4‡) | *orb1, orb6* | | |
| *orb3* | 1 (1‡) | | | |
| *orb4* | 12 (1‡) | | *sts5*§ | $pck1^+$, $pyp1^+$ |
| *orb5* | 2 (2‡) | | | |
| *orb6* | 4 | *orb1, orb2* | | |
| *orb7* | 1 | | *cwg2*‖ | |
| *orb8* | 6 | *orb9, orb11* | | |
| *orb9* | 1 | *orb8, orb11* | | |
| *orb10* | 4 | | | |
| *orb11* | 2 | *orb8, orb9* | *cwg1*‖ | |
| *orb12* | 2 | | | $pck1^+$, $pyp1^+$, $ras1^+$ |

*<1 cM.
‡Other alleles identified in a previous screen: see Snell and Nurse, 1994.
§Toda et al., 1991.
‖Ribas et al., 1991; Diaz et al., 1993.

*Tea* mutants mislocalize the site of growth (Fig. 1 *c*), appearing branched and often curved. The *ban* mutants cannot maintain the direction of growth, appearing curved and often elongated (Fig. 1 *d*). All 13 *tea* and *ban* mutants showed 2:2 segregation of the phenotype when backcrossed to wild type and defined six independent genes by linkage analysis (Table II). *Tea1* and *ban1* were identified previously (Snell and Nurse, 1994), while *tea2*, *ban2*, *ban3*, *ban4*, and *ban5* are new genes. Both *tea* genes are well-represented by a number of alleles, but the *ban* genes are identified mostly by single alleles, indicating that the screen for *ban* genes has not been exhaustive.

### *Functional Analysis of the Mutants during the Cell Cycle*

In fission yeast cells, polarized growth is reinitiated after mitosis following reassembly of the actin cytoskeleton at the cell tips. We can expect that the gene functions required to establish cell polarity after cell division may be different from those necessary to maintain polarity of growth throughout the cell cycle. These two classes of genes can be distinguished by blocking cells before mitosis using a cell cycle mutant: if the function of a mutant gene is only required to reestablish polarity after mitosis, then cells will not express the mutant phenotype while arrested in G2. This approach has been used to investigate *orb5* gene function (Snell and Nurse, 1994). For this experiment, we used a mutant allele of the cell-cycle gene *cdc2* (*cdc2-33*), which arrests cells predominantly in G2 (see Fig. 2 *b*) (Nurse et al., 1976). We constructed double mutants with *cdc2-33* and representative mutants of the *orb* and *tea* genes, and then monitored changes in cell shape after shift to the restrictive temperature. We also evaluated the ability of a mutant to polarize after mitosis by constructing double mutants with the *cdc11-119* strain,

*Table II. tea and ban Genes*

| Gene | Alleles | Phenotype |
|---|---|---|
| *tea1* | 6 (2*) | T shaped, curved |
| *tea2* | 2 | T shaped, curved |
| *ban1* | 0 (1*) | curved |
| *ban2* | 1 | curved |
| *ban3* | 1 | curved |
| *ban4* | 2 | curved |
| *ban5* | 1 | curved |

*Other alleles identified in a previous screen: see Snell and Nurse, 1994.

which blocks septation and cytokinesis, while the nuclear cycle continues (Nurse et al., 1976; Marks et al., 1986). *Cdc11-119* cells continue to undergo the cell cycle, becoming multinucleated and growing from both ends after each mitosis (see Fig. 2 *c*). If an *orb*, *tea*, or *ban* mutant is defective in reestablishing polarity, then the *orb cdc11*, *tea cdc11*, or *ban cdc11* double mutants will change shape after mitosis at the restrictive temperature. Mutants in three genes, *orb10*, *orb11*, and *ban5*, take too long (more than 6 h) to show the mutant phenotype after shift to 36°C, and for this reason have been excluded from this analysis.

As predicted, the analysis of single and double mutant phenotypes has allowed us to categorize the *orb*, *tea*, and *ban* genes into the two classes: genes necessary for polarized growth throughout the cell cycle and genes required after mitosis, for the reestablishment of polarity. We also identified a novel class of genes, which is essential for NETO and the transition to bipolar growth.

***Genes Required for Polarized Growth throughout the Cell Cycle.*** The double mutants between *cdc2-33* and temperature-sensitive mutants of *orb1*, *sts5*, *orb6*, *cwg2*, and *orb12* lose growth polarity at the restrictive temperature. For example, *orb6* becomes round even though progression through mitosis is blocked (Fig. 2, *d* and *e*), and cytokinesis is prevented using *cdc11-119* (Fig. 2 *f*). Thus, *orb1*, *sts5*, *orb6*, *cwg2*, and *orb12* are continuously required during the cell cycle for the maintenance of polarized growth and a cylindrical cell shape. The double mutants between *cdc2-33* and *ban1*, *ban2*, *ban3*, and *ban4* mutants become bent and distorted (data not shown), suggesting that *ban1*, *ban2*, *ban3*, and *ban4* genes are also necessary at all times during cell growth and tip elongation, to maintain the correct direction of growth.

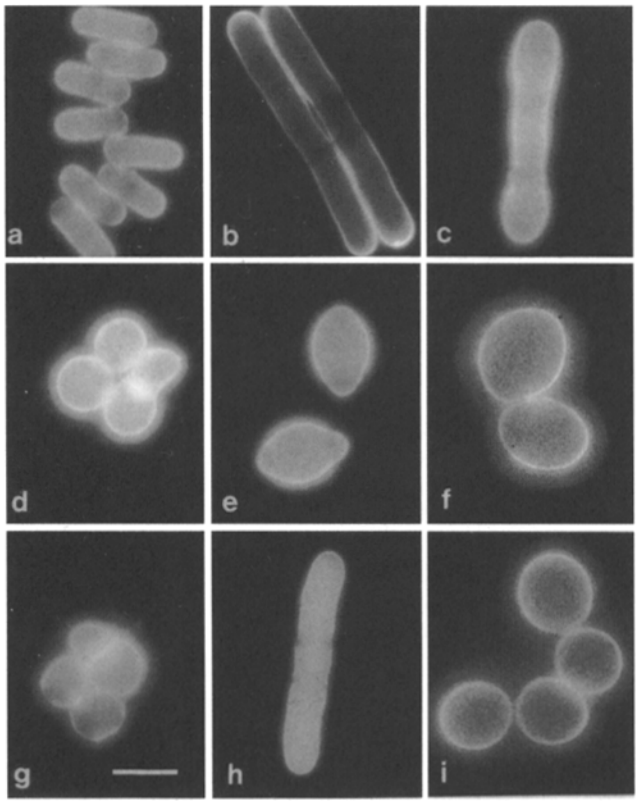


*Figure 2. Orb6-25* cells lose polarity of growth while arrested in G2, but *orb3-26* cells lose polarity only after mitosis. (*a*) Wild-type (972, h−). (*b*) *cdc2-33*. (*c*) *cdc11-119*. (*d*) *orb6-25*. (*e*) *orb6-25 cdc2-33*. (*f*) *orb6-25 cdc11-119*. (*g*): *orb3-167*, after 4 h incubation. (*h*) *orb3-167 cdc2-33*, after 4 h incubation. (*i*) *orb3-167 cdc11-119*, after 4 h incubation. Cells were incubated for 5 h, unless otherwise indicated, in YE medium at 36°C, and then stained with Calcofluor and photographed. Bar, 10 mm.

***Genes Required to Reestablish Polarized Growth after Mitosis.*** The double mutants between *cdc2-33* and mutants of the *orb3*, *orb8*, and *orb9* genes continue to grow in a polarized manner when arrested in G2, behaving like the *cdc2-33* single mutant (for an example see *orb3* in Fig. 2, *g* and *h*). However, if cells are allowed to undergo mitosis but not cytokinesis (double mutant with *cdc11-119*), polarity of growth is lost (see Fig. 2 *i*) and cells become large and round. Thus *orb3*, *orb8*, and *orb9* are required to establish polarity at the beginning of the cell cycle, but once polarity is established, they have no further role in maintaining polarized growth during the remainder of the cell cycle. Several of these gene products may interact, as pairwise combinations of *orb8*, *orb9*, and *orb11* mutants are synthetically lethal.

***Activation of Bipolar Growth.*** One-third of a cell cycle after cell division, actin localizes to the new end and bipolar growth begins. Transition from monopolar to bipolar growth is called NETO. Three genes identified in our morphogenetic screen were found to be also required for the initiation of bipolar growth: *orb2*, *tea1*, and *ban2* mutants fail to activate a second growing tip, as detected by Calcofluor staining (Table III) and thus grow from only one end. Growing tips are brightly stained while nongrowing tips, that have not undergone NETO, are not stained. *Orb2* mutant cells grow with only one end both at 25 and 36°C, with bipolar growth being detected in only 0.25% of cells (Table III). Two examples of *orb2* mutant cells at 25°C are shown in Fig. 3 *a*. At 36°C *orb2* mutant cells are shorter and fatter, but do not completely lose polarity (Fig. 3 *b*). The defect in the activation of a second growing end is permanent and is not overcome by delaying cells in G2 or by allowing them to undergo multiple cell cycles, because in *orb2-34 cdc2-33* and *orb2-34 cdc11-119* double mutants, cells still grow at one end only (Fig. 3, *c* and *d*).

*Tea1* and *ban2* mutants also fail to activate the new end (Figs. 4, *a* and *b*, and 1 *c* and Table III). *Ban2* cells increase in length at 36°C (Fig. 1 *d*). In *tea1* mutant cells, the NETO defect is evident already at the permissive temperature (Fig. 4 *a*). In *tea1* mutants, the failure to activate the new end can be partly overcome by delaying cells in G2 (Fig. 4

*Table III. NETO in orb2-34, tea1-3, and ban2-92 Mutants*

| Strain | 25°C 1 end | 25°C NETO | 25°C sept. | 36°C 1 end | 36°C NETO | 36°C sept. |
|---|---|---|---|---|---|---|
| | | % | | | % | |
| wild type | 27 | 58 | 15 | 25 | 60 | 14 |
| *orb2-34* | 77* | 0.25 | 23 | ‡ | ‡ | ‡ |
| *tea1-3* | 70 | 13 | 17 | 78 | 6 | 16 |
| *ban2-92* | 45 | 38 | 16 | 78 | 13 | 8 |

*~17% of these cells (~13% of the total cell number) are delayed in growth activation after mitosis, and show no end staining by Calcofluor or actin immunofluorescence.
‡At 36°C *orb2* cells become rounder, and growth patterns difficult to visualize.

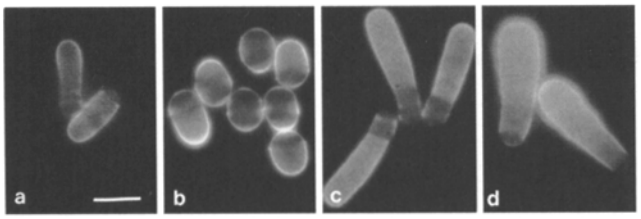


*Figure 3.* *Orb2-34* mutants do not activate bipolar growth. (*a*) *orb2-34* at 25°C. (*b*) *orb2-34* at 36°C. (*c*) *orb2-34 cdc2-33* at 36°C. (*d*) *orb2-34 cdc11-119* at 36°C. Cells were incubated for 5 h in YE medium at 25 or 36°C, then stained with Calcofluor and photographed. Bar, 10 mm.

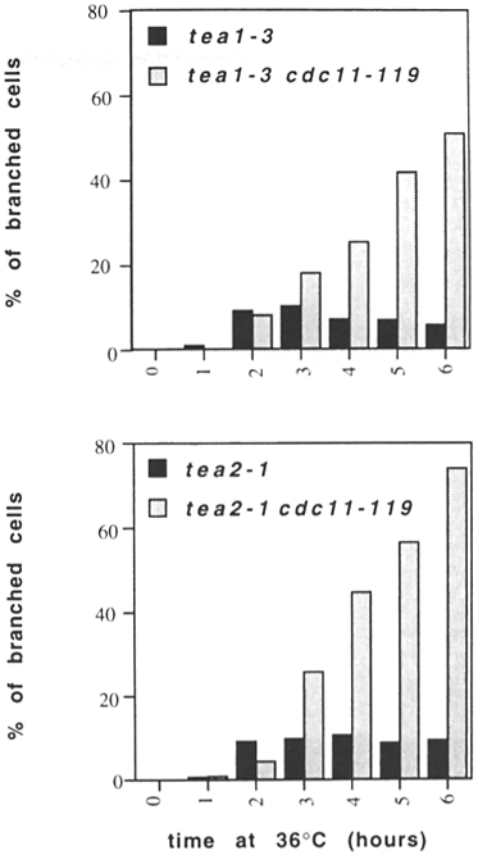


*Figure 5.* The percentage of branched cells in double mutants *tea1-3 cdc11-119* and *tea2-1 cdc11-119* cell populations increases with the number of nuclear cycles. Cells were grown in EMM medium at 25°C, and then transferred to 36°C. Samples were collected at 1-h intervals, fixed, and stained with Calcofluor. 500 cells were counted for each time point.

*c*: *tea1-3 cdc2-33* double mutant) or by allowing cells to undergo additional rounds of mitosis (example in Fig. 4 *d*: *tea1-3 cdc11-119* double mutant). This suggests that the failure to accomplish NETO might be due to different mechanisms in the *orb*2 and *tea1* mutant cells. The regulation of bipolar growth in fission yeast has not previously been studied by a genetic approach, and *orb*2, *tea1*, and *ban2* are the first genes to be identified that specifically affect this process.

***Genes Required after Mitosis for Correct Localization of the Growing Tips.*** At the restrictive temperature both *tea1* and *tea2* mutants show a branched phenotype, although this occurs only at a low frequency of ~10% (Fig. 5). The frequency of branched cells increases dramatically with time at 36°C in the double mutants *tea1-3 cdc11-119* or *tea2-1 cdc11-119* (Fig. 5). No increase in branching frequency with time is seen in double mutants of *tea1-3* or *tea2-1* with *cdc2-33* (data not shown) suggesting that progression through the nuclear cell cycle is required for expression of the branched phenotype.

Calcofluor staining shows that *tea1* mutant cells grow from one end (Fig. 4 *b*) while *tea2* mutant cells grow from either one or two ends (data not shown). This indicates that in both cases cell branching is caused by mislocalization of one of the two growing poles, and not by activation of an additional (i.e., a third) end. A proportion of the branched cells in the double mutants with *cdc11-119* activate supernumerary growing ends (~5% for *tea1-3 cdc11-119* and 30% for *tea2-1cdc11-119* after 4 h at 36°C), as determined by Calcofluor (Fig. 4 *d*) and actin staining. The number of these supernumerary growing ends never exceeds the number of intervening cell cycles. This result indicates that *tea1* and *tea2* mutants, with a certain frequency, fail to recognize the old ends after mitosis and consequently activate a new growing site at an incorrect position. We conclude that the *tea1* and *tea2* gene products may function as markers that are necessary to redirect the cytoskeleton and cell growth to the old ends after mitosis.

## *Cytological Analysis of the Mutants*

***The orb Mutants.*** During interphase, the actin cytoskeleton localizes to the growing tip(s) in a wild-type cell (Fig. 6 *e*) (Marks et al., 1986). Microtubules are in a basketlike pattern, running along the main cell axis and converging onto the tips (Fig. 6 *a*). At the restrictive temperature, loss of polarity in the *orb* mutants is correlated with random distribution of actin dots throughout the cortex (*orb6*, as an example, is shown in Fig. 6, *h* and *h'*). Microtubules become disorganized (see *orb6*, in Fig. 6 *d*). This phenotype

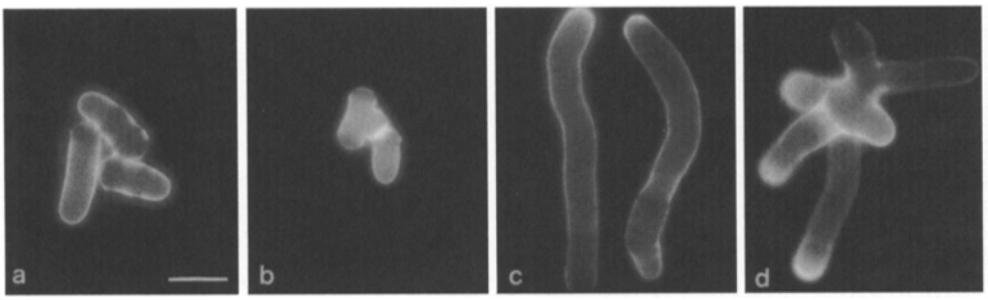


*Figure 4.* *Tea1-3* mutants do not activate bipolar growth and mislocalize their growing tip after mitosis. (*a*) *tea1-3* at 25°C. (*b*) *tea1-3* at 36°C. (*c*) *tea1-3 cdc2-33* at 36°C. (*d*) *tea1-3 cdc11-119* at 36°C. Cells were incubated for 6 h in YE medium at 25 or 36°C, then stained with Calcofluor and photographed. Bar, 10 mm.

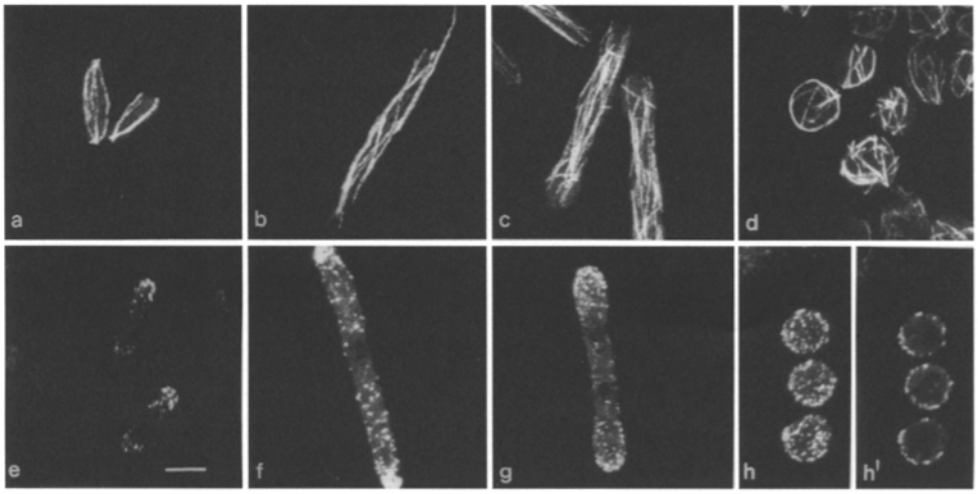


*Figure 6.* Microtubule and actin staining of wild-type 972 cells and of *cdc2-33*, *cdc11-119* and *orb6-25* mutant cells. Cells were incubated for 5 h in YE medium at 36°C, then fixed and stained for tubulin (*a–d*) and actin (*e–h*). (*a*) and (*e*) wt 972 h−. (*b* and *f*) *cdc2-33*. (*c*) and (*g*) *cdc11-119*. (*d* and *h*) *orb6-25*. (*h'*) One section of the *orb6-25* staining shown in *h*. Bar, 5 mm.

is observed in *orb1*, *orb3*, *sts5*, *orb6*, *cwg2*, *orb8*, *orb9*, *orb10*, *orb11*, and *orb12* mutants. In *orb3*, *orb8*, and *orb9* mutants, the actin and microtubule cytoskeleton are normal when cells are arrested in G2 using the *cdc2-33* mutation (compare *cdc2-33* single mutant in Fig. 6, *b* and *f* with *orb3-167 cdc2-33* in Fig. 7, *b* and *e*). After mitosis, in the double mutants with *cdc11-119*, microtubules become disorganized (Fig. 7 *c*) and actin redistributes throughout the cortex. Specifically in the *orb3-167 cdc11-119* mutant actin accumulates as a dense aggregation of actin patches (Fig. 7 *f*; compare with *cdc11-119* single mutant in Fig. 6, *c* and *g*). *Orb3-167 cdc11-119* cells lyse soon after 4 h incubation at restrictive temperature. The number of actin dots also increases in the *orb3* single mutant (Fig. 7 *d*). This suggests that *orb3*, which is required for reestablishing cell polarity after mitosis, might also be necessary for normal actin organization and dynamics.

***tea and ban Mutants.*** The *tea* and *ban* mutants are able to grow in a polarized fashion, but show defects in the choice of the site of growth or in the maintenance of the direction of growth. *Tea2* and all of the *ban* mutants clearly exhibit defects in the microtubule cytoskeleton. In *tea2* mutant cells microtubules are substantially shorter than the average wild-type length and never reach the cell tips, either at 25 or 36°C (Fig. 8, *c* and *d*). Microtubule average length is 5.2 ± 1 (SD) mm, substantially shorter than the wild-type value of 8.8 ± 2 (SD) mm (number of microtubules measured = 50). The microtubule cytoskeleton is also altered in all *ban* mutants (Fig. 9, *a–d*). Interphase microtubules bundle on one side of cells in *ban1* (data not shown), *ban2* (Fig. 9 *a*), *ban3* (Fig. 9 *b*) and *ban4* (Fig. 9 *c*). During mitosis spindles and telophase arrays appear abnormally short (Fig. 9, *a* and *c*). *Ban5-3* microtubules are also shorter during interphase (Fig. 9 *d*), with a microtubule average

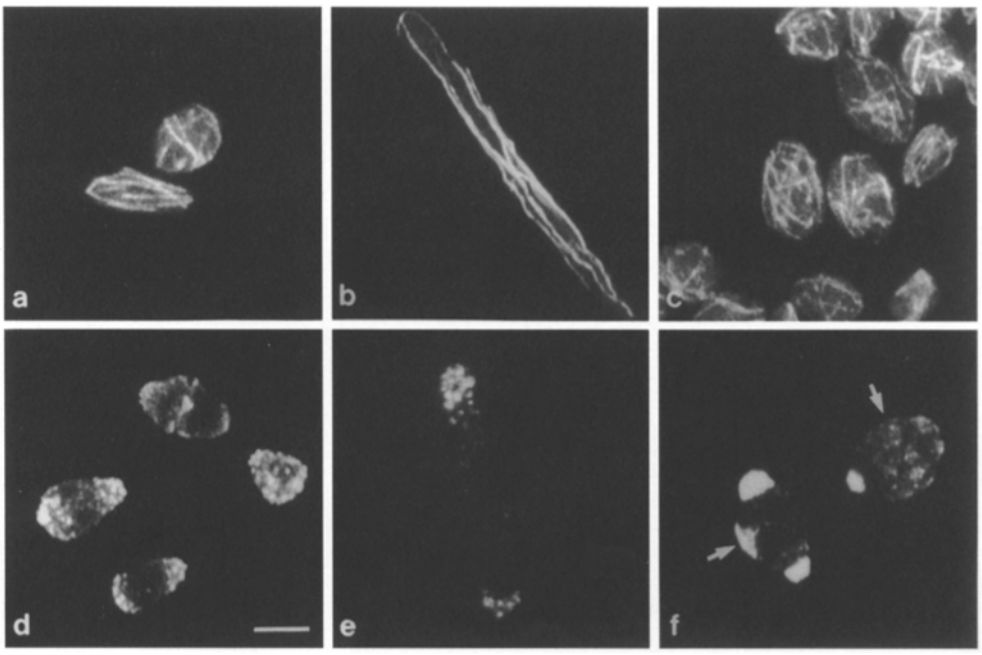


*Figure 7.* Microtubule and actin staining of the *orb3* mutant. Cells were incubated for 4 h in YE medium at 36°C, then fixed and stained for tubulin (*a–c*) and actin (*d–f*). (*a* and *d*) *orb3-167* at 36°C. (*b* and *e*) *orb3-167 cdc2-33* at 36°C. (*c* and *f*) *orb3-167 cdc11-119* at 36°C. In *orb3-167* single mutant cells the number of actin dots increases at the restrictive temperature (for direct comparison, *orb3* cells are shown after 4 h incubation, when they are still pear shaped; *orb3* cells become completely round after 5 h at 36°C). *Orb3-167 cdc2-33* cells, arrested in G2, grow normally throughout the 6-h incubation period (shown after 4 h at 36°C). *Orb3-167cdc11-119* cells lose polarity at 36°C and the number of actin dots increases at each cycle, creating large and mislocalized aggregates within the cells (*left arrow*). Actin also redistributes throughout the cell cortex (*right arrow*). *Orb3-167 cdc11-119* cells lyse shortly after 4 h incubation at 36°C. Bar, 5 mm.

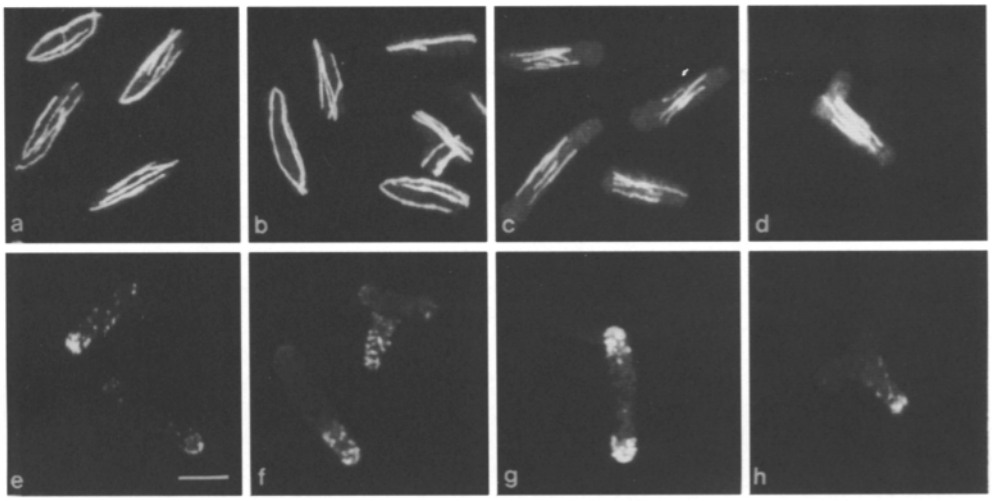


*Figure 8.* Microtubule and actin staining of the *tea1* and *tea2* mutants. Cells were incubated for 4 h in EMM medium at 25 or 36°C, and then fixed and stained for tubulin (*a–d*) and actin (*e–h*). (*a* and *e*) *tea1-3* at 25°C. (*b* and *f*) *tea1-3* at 36°C. (*c* and *g*) *tea2-1* at 25°C. (*d* and *h*) *tea2-1* at 36°C. Bar, 5 mm.

length of 4.3 ± 1 (SD) mm; cells often bend sharply and nuclei appear mislocalized (data not shown). In *tea1* mutants the microtubule cytoskeleton appears normal (Fig. 8, *a* and *b*).

No dramatic alteration is observed in the actin cytoskeleton that appears normally localized in all *tea* and *ban* mutants (see *tea1* in Fig. 8, *e* and *f*; *tea2* in Fig. 8, *g* and *h*; *ban2*, *ban3*, and *ban5* in Fig. 9, *e*, *f*, and *h*, respectively), although actin dots are significantly larger in *ban4* (Fig. 9 *g*).

These cytological observations suggest that mutations altering cell form and polarity also have significant effects on cytoskeletal organization, causing the misplacement of microtubule and actin structures. In some cases, cytoskeleton dynamics appears specifically altered, affecting actin reorganization after mitosis and microtubule average length.

## Discussion

We have isolated 64 fission yeast mutant strains displaying various morphological defects that define 19 different genes. By analyzing the mutants in various *cdc* (cell division cycle) backgrounds, we have established that these genes have distinct morphogenetic roles during the cell cycle. These roles are summarized in Fig. 10 and will now be considered in more detail.

### *Spatial Signals Mark the Cell Old Ends for the Correct Relocation of Growing Tips after Mitosis*

After cytokinesis, the two daughter cells restart growth from the old ends. In *tea1* and *tea2* mutants, a certain fraction of cells fail to start growth from the old ends and grow from illegitimate positions throughout the cell cortex. This fraction is much increased in the double mutants *tea1-3 cdc11-119* and *tea2-1 cdc11-119* blocking cytokinesis, and there is an increasing frequency in the activation of supernumerary growing tips as cells enlarge and accumulate more nuclei. The *tea1* and *tea2* gene products are required to establish correct spatial organization in the polarized cell and might function as end markers. BUD3 and BUD4 have also been proposed as positional markers in the budding yeast cell (Chant, 1994). The correlation between increased nuclear content and supernumerary growing tips in the *tea1-3 cdc11-119* and *tea2-1 cdc11-119* double mutants indicates that multiple nuclear cycles are necessary to activate additional growing tips. This suggests that passage through a stage of the nuclear cycle might be necessary to activate an extra tip, a mechanism that may be similar to the cell-cycle control of budding found in *Saccharomyces cerevisiae* (for review see Lew and Reed, 1995).

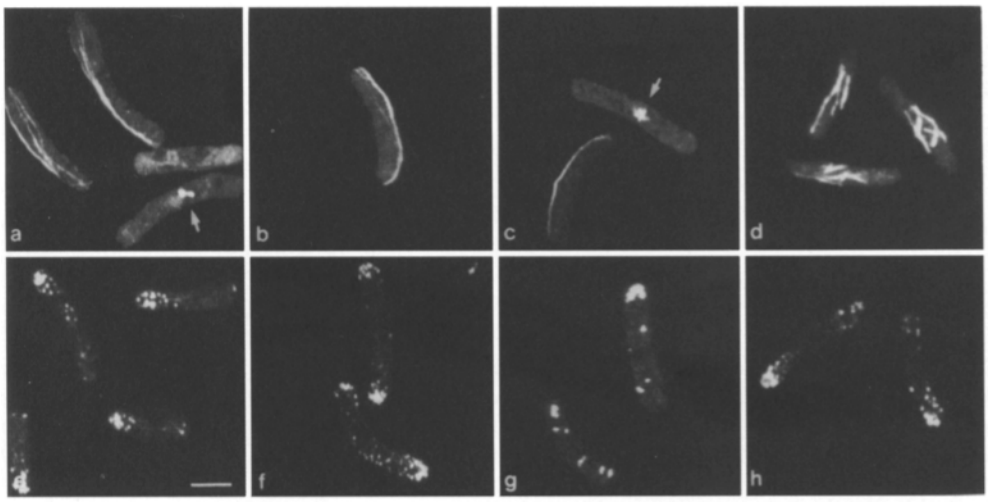


*Figure 9.* Microtubule and actin staining of the *ban* mutants. Cells were incubated for 7 h in YE medium at 36°C, and then fixed and stained for tubulin (*a–d*) and actin (*e–h*). (*a* and *e*) *ban2-92*. (*b* and *f*) *ban3-2*. (*c* and *g*) *ban4-81*. (*d* and *h*) *ban5-3*. Bar, 5 mm.

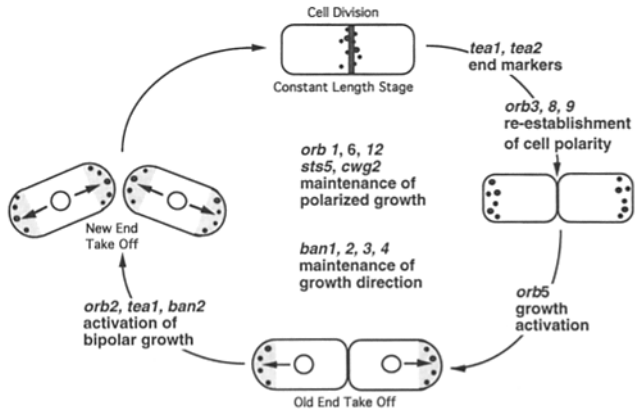


*Figure 10.* Control of fission yeast cell morphogenesis during the cell cycle: the role of *orb*, *tea*, and *ban* genes. ⁂, actin dots; ▩, areas of cell growth.

In *tea2* mutants, the interphase microtubule cytoskeleton is altered, and microtubules are shorter, being ~60% of the length seen in wild-type cells. Because *tea2* mutants do not display spindle abnormalities during mitosis, this alteration of microtubule dynamics must be specific to the interphase array. One possibility is that the *tea2* gene product functions as a tip marker as well as stabilizing microtubules locally at the cell tips. In fission yeast microtubules regrow from the area around the nucleus after cold-shock depolymerization (Snell, V., J. Cope, and J. Hyams, unpublished results), suggesting that microtubule plus-ends extend towards the cell tips. The *tea2* gene product might have a role in cytoskeletal organization by capturing or stabilizing microtubule plus ends.

### *Reestablishment of Cell Polarity after Mitosis and Reorganization of the Actin Cytoskeleton*

At the conclusion of mitosis, actin dots reappear at one end of the daughter cells (the old end), where polarized growth restarts. *Orb3*, *orb8*, and *orb9* gene functions are required for the correct reorganization of the actin cytoskeleton to the old ends. These gene products may act by recognizing positional markers at the old ends or could be necessary to reorganize cytoskeletal structures specifically after mitosis. *Orb3* cells show a progressive accumulation of actin dots, suggesting a role for the *orb3* gene product in actin dynamics and possibly destabilization of actin filaments.

*Orb5*, encoding the *S. pombe* homologue of casein kinase II, has been shown previously not to be involved directly in cell polarity. Rather, it is required for reinitiation of cell growth after actin reorganization has taken place (Fig. 10) (Snell and Nurse, 1994).

### *Activation of Bipolar Growth*

In early G2, one-third of a cell cycle after daughter cell separation, *S. pombe* cells switch to bipolar growth. The cell signals involved in the activation of a second growing tip or NETO require growth to a critical cell length and cell-cycle progression (Mitchison and Nurse, 1985). The nature of the signals that regulate this process is unknown. We identified three genes, *orb2*, *tea1*, and *ban2*, that are required for bipolar growth. The fact that *orb2*, *tea1*, and *ban2* mutants show also an alteration of cell shape suggests that elements required for cell polarity may also be necessary for detecting or generating the NETO signals. In *tea1* mutant cells, the NETO defect can be partly overcome by arresting cells in G2 for 5 h (*tea1-3 cdc2-33* double mutant); NETO then occurs at an increased cell size. Maybe in *tea1* mutant cells the mechanisms that transduce the measure of size to NETO are defective.

### *Maintenance of Polar Growth*

We have identified five genes necessary for the maintenance of cell polarity throughout the cell cycle: *sts5*, *orb12*, *cwg2*, *orb1*, and *orb6*. These gene functions might be required to create specific domains within the cell, defining and restricting the areas where cell growth can occur. A defect in these five gene functions might expand these domains leading to delocalized growth and, as a consequence, to a spherical cell.

*Cwg2*, allelic to *orb7*, encodes a homologue of budding yeast CDC43, the β subunit of the geranyl–geranyl transferase type I involved in the modification of CDC42 (Díaz et al., 1993). In budding yeast CDC42 is necessary for polarized growth (Adams et al., 1990) and this modification may facilitate its correct membrane attachment and localization (Johnson and Pringle, 1990; Finegold et al., 1991). Our allele of *cwg2* is perfectly spherical, but is still viable. Because the *cdc42sp*-encoded GTPase is essential for growth in *S. pombe* (Miller and Johnson, 1994), cdc42sp function might be mislocalized in our mutant, but still be able to support cell growth.

Two genes, *sts5* and *orb12*, are suppressed by multicopy expression of *pck1*$^+$ (a *S. pombe* protein kinase C homologue) (Toda et al., 1993) and *pyp1*$^+$ (a *S. pombe* tyrosine phosphatase) (Millar et al., 1992; Ottilie et al., 1992). This is consistent with the observation that protein kinase C *pck1* and *pck2* null mutants show shape abnormalities and defects in actin organization (Kobori et al., 1994). *Orb12* is also suppressed by expression of *ras1*$^+$, whose product interacts with cdc42sp and ral3/scd2 (BEM1 homologue) via ral1/scd1 (CDC24 homologue) (Chang et al., 1994). This interaction is reminiscent of mammalian systems where a GTPase cascade is involved in regulation of cell shape (for recent reviews see Ridley, 1995 and Chant and Stower, 1995; see Ridley et al., 1992; Nobes and Hall, 1995).

### *Maintenance of Directional Growth and the Microtubule Cytoskeleton*

The *ban* mutants are unable to maintain the correct direction of growth. In these mutants microtubules are very short or mislocalized to one side of the cell. This suggests a role for microtubules in the control of cell polarity, which is also supported by the observation that cold-sensitive *nda3* mutants (β-tubulin) and thiabendazole-treated cells also bend and branch (Umesono et al., 1983*a*,*b*; Hiraoka et al., 1984). Although microtubules are not essential for polarized growth in fission yeast (Ayscough et al., 1993), the microtubule cytoskeleton might provide structural support to the growing tip and be involved in isotropic deposition of the cell wall. Microtubules could also contribute to cell polarity by providing vectorial vesicle transport or by lo-

calizing polarity determinants. The crucial role of the microtubule cytoskeleton in intracellular trafficking is very well-established in a number of systems (Cole and Lippincott-Schwartz, 1995). One possibility is that the *ban* genes encode proteins directly involved in the control of the microtubule cytoskeleton, like microtubule-stabilizing MAPs or microtubule motors, that might alter the organization and dynamics of microtubules.

In *ban4* mutants, a smaller number of cortical patches are found at the cortex, which appear larger in size, suggesting a defect in the actin cytoskeleton. This phenotype has been previously described in *S. cerevisiae* for null alleles of the *SLA1* gene (Holtzman et al., 1993), which is thought to be involved in actin nucleation (Li et al., 1995). Thus the *ban4* gene product might have a role in the control of both the actin and the microtubule cytoskeleton.

## *Control of Cell Morphogenesis in Fission Yeast*

In conclusion, we have described 19 genes involved in fission yeast cell morphogenesis, 10 of which have not been previously identified, and we have ordered these genes according to their function during the cell cycle (Fig. 10). The *tea1* and *tea2* genes are required to correctly establish polarized growth at the preexisting cell tips after cytokinesis, and may act as positional markers defining the ends of the cell. The *orb3*, *orb8*, and *orb9* genes are necessary to reestablish cell polarity after mitosis and could act by recognizing positional cues and reorganizing the actin cytoskeleton to the cell ends. In contrast, *orb1*, *orb6*, *orb12*, *sts5*, and *cwg2* are required continuously throughout the cycle to maintain polarized growth, and might be important to delimit the growing domains to the cell ends. The *ban1*, *ban2*, *ban3*, and *ban4* genes are needed to maintain the correct direction of growth and are also defective in microtubule organization. Finally, the *orb2*, *tea1*, and *ban2* genes have a second role in activating bipolar growth midway through the cell cycle. Our results have allowed us to provide a framework for subsequent molecular analysis of these genes, which will contribute to the understanding of the biochemical mechanisms controlling cell architecture and form in *S. pombe*, and which we expect will be of relevance to other organisms.

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