Microtubules offset growth site from the cell centre in fission yeast

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Summary

The design principles that underlie cellular morphogenetic mechanisms are central to understanding the generation of cell form. We have investigated the constraints governing the formation and positioning of new growth zones in the fission yeast cell and have shown that establishment of a new axis of polarity is independent of microtubules and that in the absence of microtubules a new growth zone is activated near the nucleus in the middle of the cell. Activation of a new growth zone can occur at any stage of the cell cycle as long as the nucleus is a sufficient distance away from previously growing ends. The positioning of growth zones is regulated by the polarity marker Tea1 delivered by microtubules; cells with short microtubules locate the growth zone near the region where the microtubules terminate. We propose a model for the activation of new growth zones comprising a long-range laterally inhibitory component and a self-activating positive local component that is delivered to cell ends by Tea1 and the microtubules. The principle of this symmetry-breaking design may also apply to the morphogenesis of other cells.

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Introduction

Spatial organization is central to cell function, cell movement and cell division. Many factors involved in spatial organization have been identified in different organisms, but the overall mechanism that brings about cell shape has remained elusive. It is generally accepted that for microbial cells to break symmetry and acquire a non-spherical shape, growth has to be restricted to specific regions of the cell (Harold, 1990). In walled eukaryotic cells localized deposition of cell wall material ensures formation of a tip and consequent apical growth. In the budding yeast *Saccharomyces cerevisiae*, localized secretion and exocytosis account for bud emergence and growth. In spherical plant cells, polarized growth is achieved by targeting the growth machinery to local weakening in the cell boundary (Mathur, 2006). In the fission yeast *Schizosaccharomyces pombe*, the rod shape of the cell is maintained by restricting growth to both ends of the long cell axis (Chang, 2001; Hayles and Nurse, 2001). Because of this highly polarized organization, fission yeast has proven a good system to study cell morphogenesis. Newborn fission yeast cells, after division, start to grow in a monopolar fashion from the previously growing old end. Early in G2 activation of a new growth zone takes place at the opposite new cell end, maintaining a single straight axis. This transition from monopolar to bipolar growth is known as new end take off, or NETO (Mitchison and Nurse, 1985). The opposed positioning of growth zones relies on microtubules spanning the length of the cell (Brunner and Nurse, 2000; Tran et al., 2001) and delivering the polarity factor Tea1 (Feierbach et al., 2004; Mata and Nurse, 1997) and other polarity factors to cell ends. Tea1 is transported on the tips of growing microtubules (Behrens and Nurse, 2002) and released at cell ends where it associates with the cell cortex (Snaith et al., 2005; Snaith and Sawin, 2003) and recruits the growth machinery (Mata and Nurse, 1997; Feierback et al., 2004). In the presence of short microtubules, such as in cells treated with the microtubule inhibitor thiabendazole (TBZ) (Sawin and Nurse, 1998; Walker, 1982), or in a *tea2* kinesin mutant (Verde et al., 1995), Tea1 and the growth machinery become mislocalized, giving rise to branched cells (Sawin and Nurse, 1998; Sawin and Snaith, 2004) with a new axis of symmetry. Neither Tea1 nor microtubules appear to be required for polarized growth, although it has been suggested that both are necessary for the establishment of new sites of polarized growth (Sawin and Nurse, 1998; Sawin and Snaith, 2004).

The timing of the transition from monopolar to bipolar growth relies on completion of DNA replication and attainment of a minimal cell size (Mitchison and Nurse, 1985). In wild-type cells fulfilment of these two requirements results in NETO occurring early in G2. However, transient perturbation of the actin cytoskeleton to increase the number of actin monomers can induce the transition from monopolar to bipolar growth in the absence of DNA replication (Rupes et al., 1999). The transient increase in actin monomers activates a new site of growth at the new end, opposite the pre-existing growth zone. This observation suggests that the potential to activate growth

may be present throughout the cell cycle, but is restricted to early G2 by coupling to the DNA replication and cell size controls. In this study we have re-examined the timing and positioning of new growth activation in fission yeast and how it is influenced by S-phase completion and cell size.

Results

Activation of new sites of growth independently of DNA replication

In exponentially growing wild-type cells, only one new site of growth is activated per cell cycle, early in G2. It has long been understood that activation of a new growth zone requires completion of DNA replication and a minimal cell size (Mitchison and Nurse, 1985), but there is some evidence (Rupes et al., 1999) suggesting that the potential to activate growth is present throughout the cell cycle. To test this possibility further, we checked whether more than one growth zone could be activated per cell cycle. We used the temperature-sensitive cell cycle mutant, *cdc25-22*, which at the restrictive temperature (36.5°C) blocks in G2 giving rise to elongated cells with a bipolar actin distribution. After 3 hours at 36.5°C, cells were treated with 50 μg/ml methyl-2-benzimidazole-carbamate (MBC) to depolymerize microtubules (lower panel of Fig. 1A). After 4 hours in MBC, around 80% of these cells branched (Fig. 1A,B, upper panel). As shown in Fig. 1B, only small stubs of tubulin were detectable in these cells. Actin accumulation was detected in the cell middle when the branch started to form, and all three ends of the branching cells were found to have actin patches and therefore be actively growing (Fig. 1C). Growth at all ends was confirmed by calcofluor staining (Fig. 1C). Using time-lapse movies of MBC-treated *cdc25-22* cells at the restrictive temperature we estimated that the average cell length at branching was 31 μm, approximately twice the size of a bipolar fission yeast cell before mitosis. These results were confirmed using another cell cycle mutant, *cdc2-33*, most cells of which block early in G2. Under the same experimental conditions described for *cdc25-22*, 70% of *cdc2-33* cells branched after MBC treatment whereas only 4% did so in control dimethylsulfoxide (DMSO)-treated cells (data not shown). Hence, elongated bipolar cells can activate a third growth zone, indicating that establishment of new growth sites can occur in G2 cells in the absence of further rounds of DNA replication.

To confirm that DNA replication is not required for growth activation, we generated bipolar G1 cells using the double mutant *cdc10-129 cdc11-119*. At 36.5°C, *cdc10-129 cdc11-119* cells undergo a round of mitosis without septation and as a consequence block in G1 and stay bipolar. After MBC addition, 70% of cells became branched compared with only 7% in DMSO-treated cells (Fig. 2A,B). We conclude that activation of a new growth zone is independent of DNA replication and of cell cycle stage.

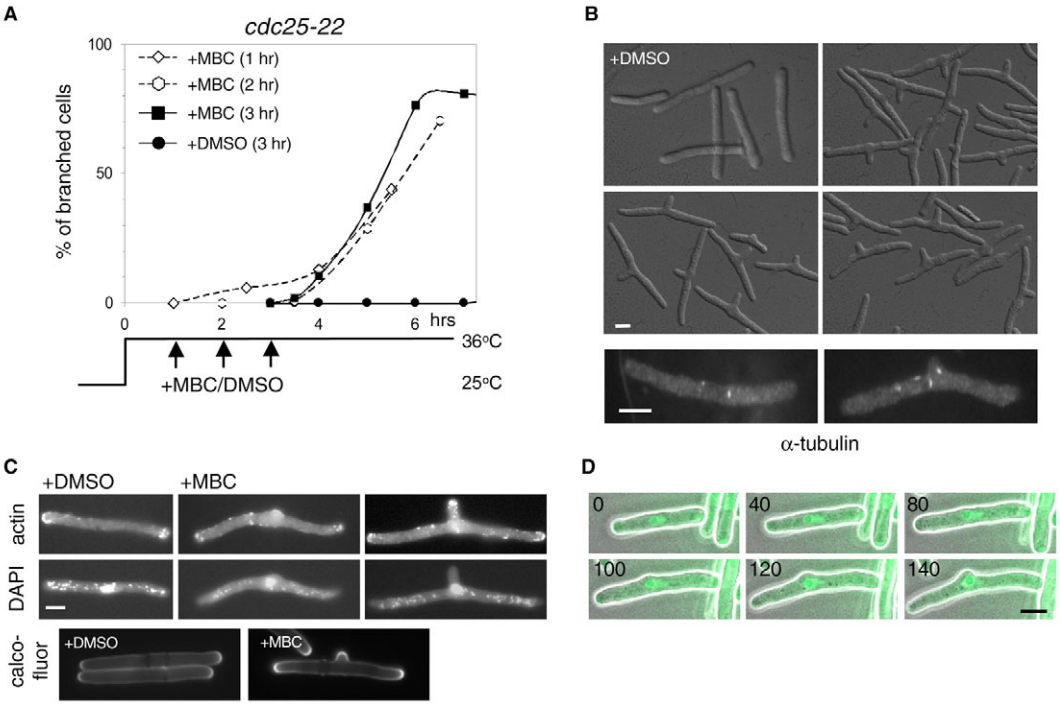

Fig. 1. Activation of new growth zones in bipolar G2 cells. (A) Branching cells during *cdc25-22* block. Cells were blocked at 36.5°C for 1, 2 and 3 hours, prior to MBC/DMSO addition (length of block prior to MBC/DMSO addition shown in parentheses in the graph). Black arrows indicate time of drug treatment. The percentage of branched cells is an average of three experiments (unless otherwise stated) with s.e.m. values smaller than 5%. 100-200 cells were counted at each time point. (B) Brightfield images of blocked (3 hours) cells treated for 3 hours with DMSO (top left) or with 50 μg/ml MBC (top right and lower panels). Staining with TAT-1 antibody of MBC-treated cells, treated as above (bottom). (C) *Cdc25-22* blocked (3 hours) cells treated with DMSO (left), MBC for 1 hour (middle) and for 3 hours (right), stained for actin (upper panel), with DAPI (middle panel) and with calcofluor (lower panel). (D) Frames from movie of *cdc25-22 cdc13-YFP* strains blocked at 36.5°C for 3 hours and then treated with MBC at *t*=0, at start of time-lapse. Frames were taken every 10 minutes, using an Axioplan Zeiss microscope. Bars, 5 μm.

Minimal cell size requirement for new growth zone activation

We next tested whether cells need to be a minimal length to activate an extra growth zone. Populations of cells of different average cell length were obtained by blocking the *cdc25-22* strain for different lengths of time (1, 2, 3, 4, 5 hours) before MBC addition. As shown in Fig. 1A, 50% of cells branched 5 to 6 hours after the cell cycle block had been imposed, irrespective of the time of MBC addition, which does not inhibit cell length extension (Sawin and Snaith, 2004), indicating that cell length is limiting for activation of an extra growth zone. We also tested branching efficiency in monopolar cells by adding MBC to blocked *cdc10-129* cells. As shown in Fig. 2B, only 13% of cells had a branched phenotype after 5 hours in MBC, which indicates that monopolar cells do not branch efficiently. To investigate this further we examined whether an increase in monopolar cell size would result in an increase in branching efficiency. We compared normal-sized cells to blocked *cdc25-22* cells that had been incubated at the restrictive temperature to double their size. The latter cells were then released to the permissive temperature of 25°C in the presence of the DNA synthesis inhibitor hydroxyurea (HU), which blocks cells in early S phase prior to NETO transition. Ninety minutes after release, cells were treated either with MBC or DMSO. After 3 hours of MBC treatment 25% of cells had a new growth zone in their middle. By contrast, only 4% of the normal-sized control cells activated a new growth zone in their middle (Fig. 2C). Taken together, these observations indicate that in the absence of microtubules a new growth zone can be activated in the middle of the cell but only once the cell has reached a critical minimal length.

If a minimal distance from a growing zone is necessary to activate growth, in monopolar cells the preferential site for growth activation should be at the new end, opposite the already growing zone. We therefore reasoned that in pre-elongated monopolar cells activation of growth at the new end could take place before S phase was completed. Hence, we repeated the experiment described above, leaving out the MBC treatment. The *cdc25-22* cells were blocked for 3 hours at 36.5°C and then shifted to permissive temperature (25°C) in the presence of 12 mM HU. After 1 hour in HU 80% of cells underwent septation, and after 3 hours, when cells were still

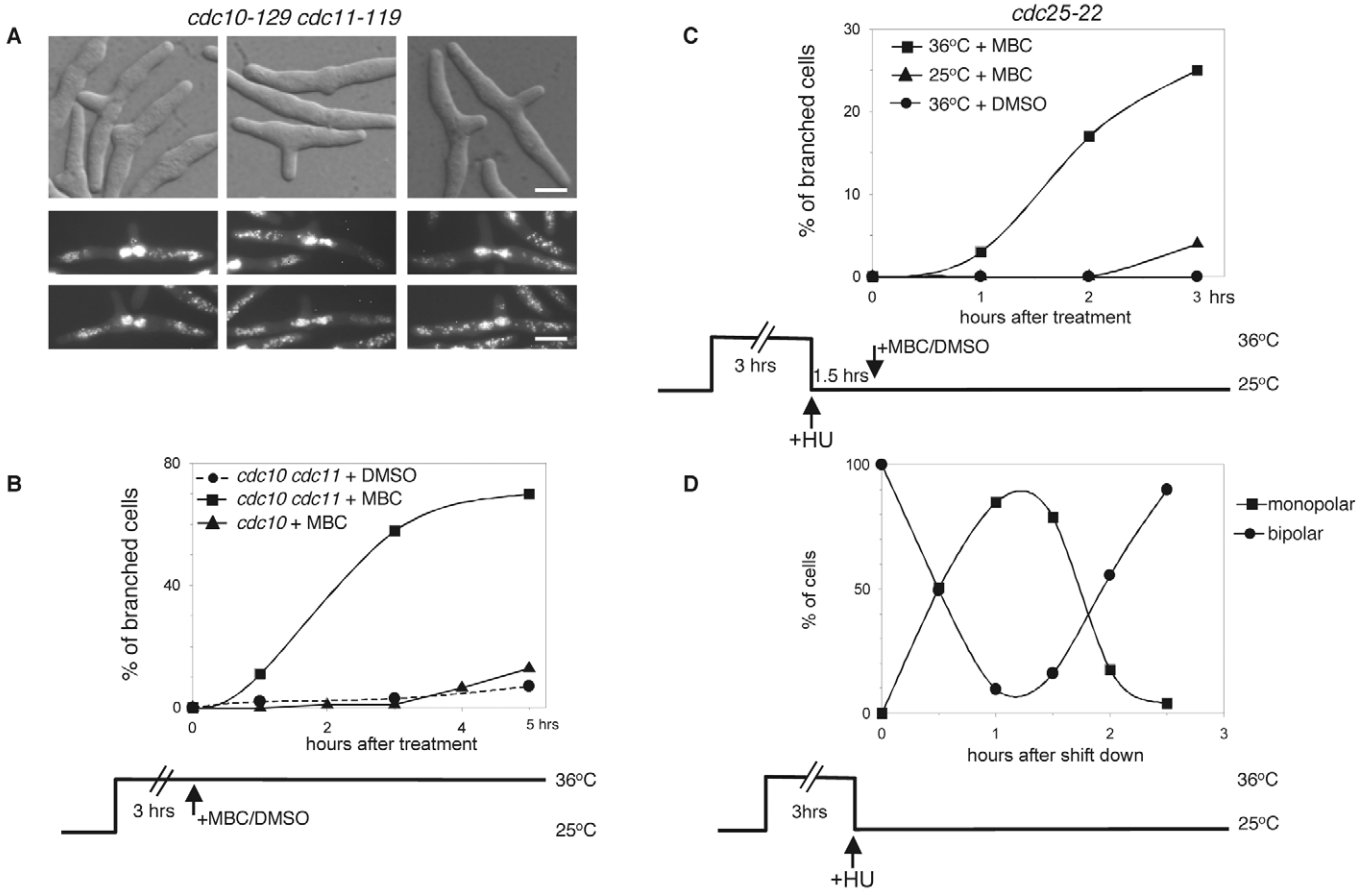

Fig. 2. Activation of new growth zones in G1- and S phase-arrested cells. (A) Images of *cdc10-129 cdc11-119* cells at 36.5°C, after treatment with 50 μg/ml MBC for 3 hours: Nomarski (top) and DAPI stained (bottom). (B) Scoring of branching *cdc10-129* and *cdc10-129 cdc11-119* cells. Both c*dc10-129* and *cdc10-129 cdc11-119* cells were blocked for 3 hours before MBC treatment. Black arrow indicates drug addition. (C) Scoring of branching *cdc25-22* cells. Cells were blocked for 3 hours at the restrictive temperature to block cells in G2 prior to mitosis. After 3 hours cells were released to the permissive temperature (25°C) to allow mitosis in the presence of 12 mM HU to prevent S phase to proceed. After 1.5 hours DMSO/MBC was added to the media. The effect of temperature and HU treatment was controlled using a wild-type strain. Both treatments had no effect on wild-type cells (data not shown). (D) Scoring of monopolar and bipolar cells in *cdc25-22* cells blocked at 36.5°C and then released to 25°C in 12 mM HU. Bars, 5 μm.

blocked in early S phase (FACS data not shown), 90% of the cells had actin patches at both ends (Fig. 2D), indicating that they had activated growth at the new end before the completion of S phase. To measure the cell size at NETO in the absence of DNA replication, we repeated the experiment above, varying the length of the block prior to shift down and HU treatment. Under these conditions we estimated that the average cell size at NETO in the absence of DNA replication was 17.2 μm, approximately 25% longer than a mitotic wild-type cell (see supplementary material Fig. S1).

We conclude that a new site of growth can be activated irrespective of S-phase completion and that the new site of growth is activated preferentially at the new end, which is furthest away from the actively growing old end.

The nucleus positions new sites of growth in the absence of microtubules

When microtubules are depolymerized in cells above the critical size, the new growth zone is positioned in the middle of the cell. DAPI staining in *cdc25-22* (Fig. 1C) and *cdc10-129 cdc11-119* (Fig. 2A) MBC-treated cells showed that the branch was always positioned in the vicinity of the nucleus. To confirm this correlation between nuclear position and location of the new growth zone, we performed time-lapse movies of *cdc25-22* cells carrying yellow fluorescent protein (YFP)-tagged Cdc13 to label the nucleus (Decottignies et al., 2001). Cells were blocked at the restrictive temperature and filmed after the addition of MBC. As shown in Fig. 1D, the branch formed in the area of the cell overlaying the nucleus.

To confirm that positioning of this new growth site is directed by the nucleus rather than by the geometric middle of the cell, we generated cells with misplaced nuclei by centrifugation. *Cdc25-22* cells were blocked for 3 hours at restrictive temperature and then centrifuged in the presence of 50 μg/ml MBC at 36°C, to move the nucleus away from the cell centre. Branch formation was followed for 4 hours. The position of the nucleus was assessed by DAPI staining and the distance between the middle of the nucleus and the centre of the branch measured in individual cells. The nucleus was displaced from the centre in more than 80% of cells (mean displacement of the nucleus was approximately 4.5 μm). As shown in Fig. 3A, in 77% of these cells the displaced nuclear position and branch position were less than 2 μm apart. These observations further support a role for the nucleus in positioning growth sites when microtubules are depolymerized. As MBC treatment leaves small tubulin stubs in the vicinity of the nucleus (Fig. 1B), we could not exclude a role for those stubs in establishment of polarized growth. To assess this possibility, we performed the same experiment in a *cdc25-22 mto1Δ* background. Mto1 promotes noncentrosomal

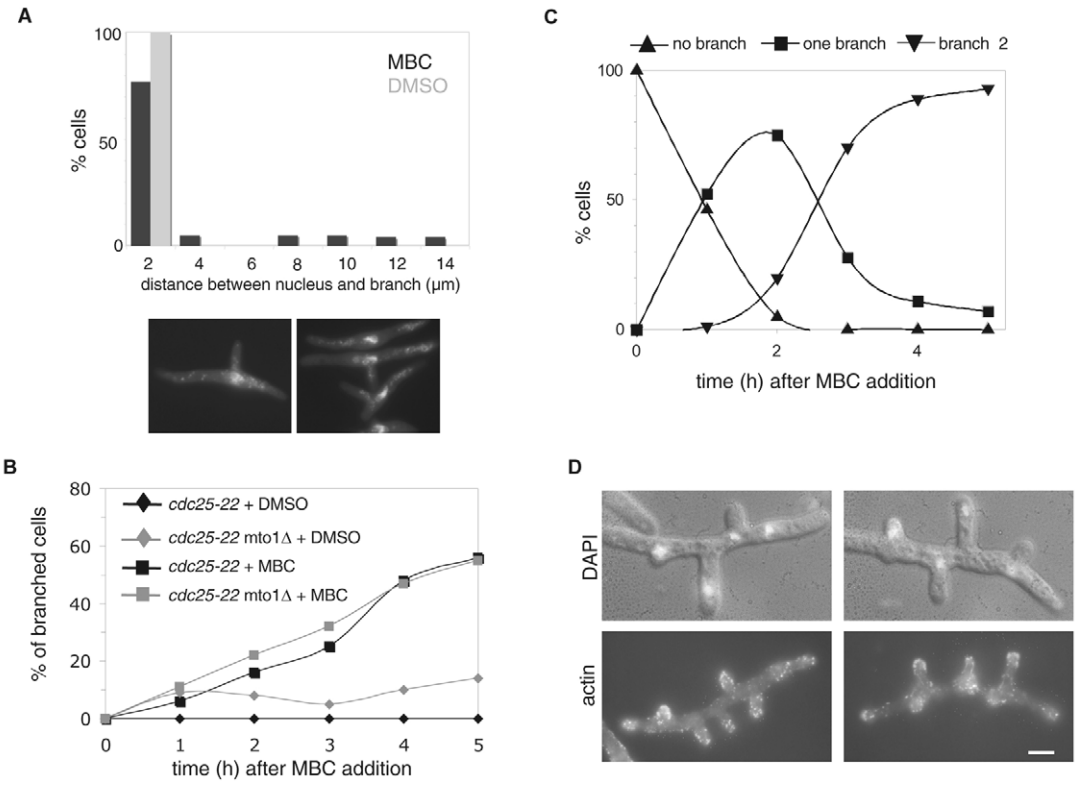

Fig. 3. Nuclei determine position of branches. (A) Quantification of distance between nucleus and branch in individual *cdc25-22*-blocked cells 4 hours after centrifugation in the presence of MBC (black) or without centrifugation (grey). 82 cells were measured. DAPI staining of branching *cdc25-22*-blocked cells in MBC after centrifugation (lower panel). (B) Scoring of branching in *cdc25-22*- and *cdc25-22 mto1Δ*-blocked cells in the presence of either DMSO or MBC. Prior to treatment cells were blocked for 3 hours at 36.5°C. (C) Branching *cdc11-119* cells treated with DMSO or MBC over a 5-hour time course. Cells were blocked at the restrictive temperature for 4 hours prior to drug treatment. (D) Images of DAPI- (top) and actin (bottom)-stained *cdc11-119* cells blocked at 36.5°C for 4 hours and then treated with MBC for 6 hours. Bar, 5 μm.

microtubule nucleation and in *mto1Δ* cells microtubule nucleation is highly impaired and no interphase microtubule nucleating centres (iMTOC) are present (Sawin et al., 2004). As shown in Fig. 3B, the branching efficiency of *cdc25-22 mto1Δ* cells in MBC was comparable to that of *cdc25-22* cells throughout the time course, suggesting that establishment of polarized growth is independent of microtubules. In *mto1Δ* cells the nucleus is off-centred (Sawin et al., 2004) and in *cdc25-22 mto1Δ* cells the branch was also off-centred together with the nucleus (data not shown).

We then tested whether the presence of multiple nuclei made cells competent to activate multiple growth sites. We used the *cdc11-119* mutant, which at the restrictive temperature undergoes mitosis without intervening cytokinesis giving rise to multinucleated cells. After 4 hours at the restrictive temperature, most *cdc11-119* cells have four nuclei. After MBC was added cells branched very efficiently; by 2 hours in MBC, 80% of cells had one branch and by 4 hours 85% of cells had at least two branches (Fig. 3C), with some cells having four to six branches. All of these branches were in the vicinity of nuclei (Fig. 3D), and actin staining showed that they were all actively growing (Fig. 3D). We further assayed branch formation in cells with different numbers of nuclei by adding MBC to *cdc11-119* cells blocked for 0, 2 and 4 hours, generating populations with an average of 1, 1.6 and 3.8 nuclei per cell, respectively. After 4 hours at the restrictive temperature in the presence of MBC, a strong correlation was observed between number of nuclei and number of branches (see supplementary material Fig. S2A). As the DNA content is also equally increased in *cdc11-119*-blocked cells, we checked branching in a cdc13 switch-off strain. Upon degradation of cdc13, cells re-replicate their DNA without intervening mitosis, giving rise to polyploid cells with one enlarged nucleus. Despite the increased DNA content, only one branch was observed upon MBC treatment (see supplementary material Fig. S2B).

We observed that the branches in the long multinucleated cells occurred in a sequential order, with the first branch appearing near the nucleus in the middle of the cell, and with subsequent branches occurring later near nuclei located more to the periphery of the cell. Centrifugation of blocked *cdc11-119* cells in the presence of MBC resulted in the appearance of a first branch in 60% of the cells, which was displaced away from the cell centre near where the nuclei were clustered (see supplementary material Fig. S2C,D).

We conclude that in the absence of microtubules the position of a new growth zone is determined by the location of the nucleus, and that the final number of branches is determined by the number of nuclei within the cell rather than by ploidy. However, nuclei are not equivalent, and growth is activated preferentially near nuclei that are furthest away from other existing growth zones.

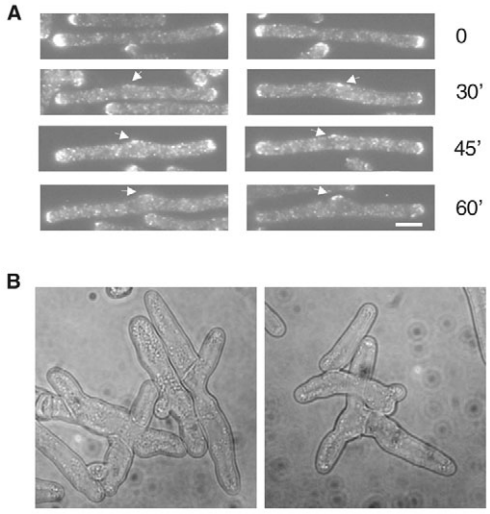

Fig. 4. Tea1 accumulates in the middle of the cell in the absence of microtubules. (A) Anti-Tea1 antibody staining of *cdc25-22* cells blocked for 3 hours at 36.5°C and treated with DMSO (top) or MBC for 30, 45 and 60 minutes (below). Cells were fixed in TCA to allow visualization of Tea1 in the cell middle. Tea1 staining in the cell centre is lost with methanol fixation. (B) Brightfield images of *cdc25-22 mid1Δ* cells blocked for 3 hours at 36.5°C and then for 1 hour in 50 μg/ml MBC. Bar, 5 μm.

Microtubules reposition new growth zones away from the nucleus

When microtubules are present the activation of a new growth zone does not occur in the vicinity of the nucleus, suggesting that a positive factor activating growth may be transported away from the nucleus by the microtubules. One candidate for such a factor is the polarity marker Tea1 (Behrens and Nurse, 2002; Mata and Nurse, 1997). Fig. 4A shows that in the absence of microtubules Tea1 accumulates in the middle of the cell, in the vicinity of the nucleus, prior to the appearance of a branch. To obtain more direct evidence for the role of Tea1, or a component of the Tea1 polarity complex (Feierbach and Chang, 2001; Martin et al., 2005), being required for establishment of a new growth zone away from cell tips we deleted different known polarity factors in a *cdc25-22* background and checked for branch formation in the presence of MBC at the restrictive temperature. As reported in Table 1, approximately 25% of *cdc25-22 tea1Δ* cells branch but no significant increase in branching was observed upon MBC treatment. At the end of the experiment *cdc25-22* cells were not significantly longer than *cdc25-22 tea1Δ* cells (31.5 μm and 30 μm, respectively), excluding that the lack of branching of *cdc25-22 tea1Δ* cells was because of slower growth and consequently reduced cell size. As reported in Table 1, impairment of Tea3 (Arellano et al., 2002), Tea4 (Martin et al., 2005), Orb2 (Verde et al., 1998), Bud6 (Glynn et al., 2001) and Pom1 (Bahler and Pringle, 1998) function also caused a reduction in branching efficiency upon MBC addition. Deleting *ssp1* did not cause any change in branching behaviour compared with the single mutant, whereas deletion of *mid1* resulted in a great increase in branching efficiency. All *cdc25-22 mid1Δ* cells branched within 1 hour of MBC treatment as compared with 10% in control *cdc25-22* cells (Table 1 and Fig. 4B).

If the factors required for polarized growth are transported by the microtubules to position a new growth zone, then varying microtubular length should alter the position of the branch. To test this possibility we performed a titration of MBC in *cdc25-22*-blocked cells and generated long bipolar cells that contained different lengths of microtubules. Fig. 5 shows that at higher MBC concentrations microtubules were shorter (for

Table 1. Branching of double mutants after 4 hours in MBC

Genotype	DMSO	MBC
cdc25-22	0	64%
cdc25-22 tea1Δ	23%	30%
cdc25-22 tea1Δkeltch	26%	28%
cdc25-22 tea4Δ	25%	23%
cdc25-22 bud6Δ	1%	8%
cdc25-22 sla2Δtalin	0	10%
cdc25-22 mid1Δ	0	100% (after 1 hour)
cdc2-33	0	55%
cdc2-33 ssp1Δ	0	53%
cdc2-33 tea3Δ	0	18%
cdc2-33 orb2-34	0	4%
cdc2-33 pom1Δ	9%	25%

All cells were blocked for 3 hours at 36°C prior to drug treatment.

representative cells, see supplementary material Fig. S3), although the nucleus remained in the cell centre (see supplementary material Fig. S3), and the branch formed further away from the cell ends, supporting the proposal that the microtubule-Tea1 system positions the growth zone. The branching efficiency varied along the long cell axis, reaching a maximum in the cell middle furthest away from already growing tips, but decreasing significantly when the concentration of MBC was reduced and microtubules extended closer to the cell ends (Fig. 5C). Collectively, these observations suggest that microtubules are not required for the establishment of polarized cell growth but are necessary to position a new site of growth away from the nucleus.

Discussion

Previous work (Mitchison and Nurse, 1985; Sawin and Nurse, 1998) had suggested that in fission yeast, activation of a new site of growth could only take place upon completion of DNA replication. Here we show that the potential to activate a new growth zone is present throughout the fission yeast cell cycle and is independent of S phase. However, it does require attainment of a minimal cell size. The requirement for a minimal cell size can explain the higher branching efficiency observed in our experiments compared with those previously described (Sawin and Nurse, 1998; Toda et al., 1983; Walker, 1982), where shorter cells were examined.

In contrast to previous reports (Sawin and Snaith, 2004), we also show that microtubules are not required for establishment of a new site of growth, and that polarized growth is established in the vicinity of the nucleus when microtubules are fully depolymerized (MBC treatment). One possible explanation for the difference between our observations and the published data could reside in the use of MBC versus TBZ to depolymerize microtubules. Cells treated with low doses of MBC that leave short microtubules branch five times less efficiently than TBZ-treated cells with similar-length microtubules (Sawin and Snaith, 2004). TBZ was shown to cause a transient actin depolarization (Sawin and Nurse, 1998; Sawin and Snaith, 2004). A brief treatment with the actin-depolymerizing drug latranculin A (latA) together with MBC mimics TBZ treatment, resulting in an increase in branching efficiency in *cdc10-129* cells (see supplementary material Fig. S4).

In a wild-type background, microtubules are not required either for maintenance of polarized growth (Sawin and Snaith, 2004) or, as we have now shown, for establishment of new sites of growth. Instead, microtubules are essential for offsetting new growth zones from the centre of the cell, and therefore for correct positioning within the cell. Microtubules transport and position Tea1 and the polarity machinery: full-length microtubules that reach cell ends give rise to normal rod-shaped wild-type fission yeast cells. We propose that in the absence of microtubules, Tea1 accumulates in the vicinity of the nucleus, marking it for growth and giving rise to a centrally branching cell, whereas short microtubules transport Tea1 a partial way down the cell, giving rise to off-centred branches. However, we observe that the closer the microtubule ends come to a growing tip, the lower is the likelihood of a branch forming, suggesting that the potential to activate growth is not identical along the long cell axis. Fig. 6 depicts one possible model explaining these observations: we propose that lateral inhibition from growing cell ends ensures that a new growth zone is activated only at a minimal distance from a growing end. In a monopolar cell, the lateral inhibition would be relieved first at the new end, the site furthest away from the old

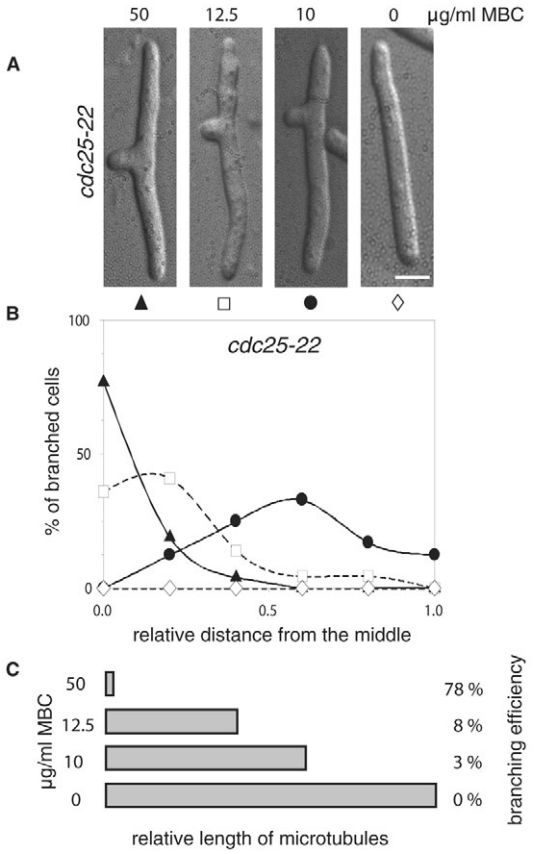

Fig. 5. Microtubules determine growth site position, but are not required for establishment of polarized growth. (A) Brightfield images of *cdc25-22*-blocked (3 hours prior to treatment) cells treated with MBC or DMSO. (B) Branch position at different MBC concentrations, measured as relative distance from the middle of the cell. Between 50 and 100 cells were scored for each MBC concentration. (C) Average microtubule length (bars) and branching efficiency (percentages) at different MBC concentrations. 25-35 cells were scored for each MBC concentration. Bar, 5 µm.

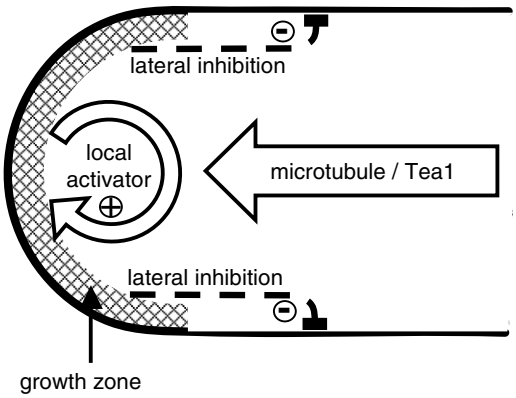

Fig. 6. Model for a morphogenetic mechanism in fission yeast. Microtubules transport Tea1 to cell ends where it recruits a spontaneous cell-polarization module. This module comprises a short-range activator, promoting growth zone formation, and a long-range lateral inhibitory component that prevents growth zone formation.

growing end. Consistently, we observed that monopolar cells tend to become bipolar rather than branch. In the absence of microtubules, Tea1 accumulation around the nucleus marks the middle of the cell as a potential site for growth, but this site is kept silent by the inhibition imposed by the growing ends. However, the lateral inhibition is weakened by distance, and as the cell grows a branch forms when the nucleus exceeds a minimal distance from growing ends. Lateral inhibition could also explain experiments in which a new growth zone is formed in monopolar cells after transient actin depolymerization by latA treatment (Rupes et al., 1999; Sawin and Snaith, 2004). Actin depolymerization disrupts the growth zone and thus the source of lateral inhibition. When actin is allowed to repolymerize, two growth zones can be formed. These are at opposite cell ends if microtubules are present (Rupes et al., 1999), or in the middle of the cell, as a branch, if microtubules are disrupted (Sawin and Snaith, 2004).

If such long-range lateral inhibition is combined with a self-activating positive local signal then spatial pattern can be generated in an initially symmetric or uniform system (Gierer and Meinhardt, 1972; Meinhardt and Gierer, 2000), a phenomenon known as spontaneous symmetry breaking (Sohrmann and Peter, 2003; Wedlich-Soldner et al., 2003). We suggest that these two components (local self-activation and lateral inhibition) might be responsible for activation of polarized growth in fission yeast cell (Fig. 6). In our model a locally acting positive component is delivered to the cell ends by the microtubules and Tea1, whilst a lateral inhibitory component prevents an adjacent growth zone from being activated if the polarity marker Tea1 becomes partially displaced from the cell end. In a wild-type fission yeast cell the doubling of the genome content following DNA replication might reduce the threshold for growth activation, thereby promoting NETO after S-phase completion.

Given the latA experiments one possible candidate for the inhibitory component is actin. A threshold of G-actin could be required to initiate actin polymerization and pre-existing growth zones could efficiently recruit G-actin into patches, reducing local G-actin levels and thus inhibiting activation of a new growth zone. Based on our observations, an alternative candidate could be Mid1. In the absence of microtubules, *cdc25-22 mid1Δ* cells branch very quickly and more efficiently than *mid1+* cells. Recent work (Celton-Morizur et al., 2006; Padte et al., 2006) suggests that, during interphase, cortical Mid1 is excluded from the tips of the cell through the inhibitory action of Pom1 and probably other polarity factors present at the tips. This inhibitory effect of the tip components could cause the formation of a Mid1 gradient peaking in the cell middle around the nucleus. In *mid1Δ* cells, where growth inhibition is absent, local accumulation of the positive component would be the only requirement for establishment of a new site of growth. Therefore, a new branch could form very quickly in the absence of microtubules once Tea1 and the growth machinery had concentrated around the nucleus. This would occur independently of the minimal cell size, as we have observed.

Similar mechanisms, involving a local activator and a long-range inhibitor, have been proposed to operate in other cell types. Local positive signals have been postulated in budding yeast where the generation of a new bud is brought about by local reinforcement of the signal involving Cdc42, Cdc24, Bem1 and actin filaments (Irazoqui et al., 2003; Wedlich-Soldner et al., 2003). In wild-type haploid cells the new bud forms adjacent to the previous bud scar, resulting in axial budding, whereas in diploid cells budding alternates between opposite poles, resulting in bipolar budding. The signalling pathway involved in bud site selection has been identified and mutations that disrupt the pathway do not affect budding per se, but randomize its location (Chant and Herskowitz, 1991). Limiting Cdc42 or actin have been proposed as possible long-range inhibitors. In fission yeast the Tea1-microtubule positioning mechanism continuously monitors the overall cell space of the cell (Mata and Nurse, 1997), acting as a dynamic landmark. In the absence of microtubules, polarization still takes place but positioning is altered. In the metazoan *Xenopus leavis*, rotation of the cortex relative to the cytoplasm within a newly fertilized egg results in establishment of the dorsal-ventral axis (Gerhart et al., 1989; Vincent and Gerhart, 1986). This polarization occurs in the presence of both a polarized or a uniform stimulus (Gerhart et al., 1989). Microtubules transport dorsalizing proteins, such as Dishevelled (Miller et al., 1999), and they could act by further focusing of microtubules or else attachment at the site of deposition (Wedlich-Soldner et al., 2003). In neurons, lateral growth cone formation induced by microtubular disruption (Bray et al., 1978) also shows similarities to the lateral inhibition by growth zones described here. Thus, the design principle described in this study, combining local self-activation and long-range lateral inhibition, may apply generally to symmetry breaking in eukaryotic cells.

Materials and Methods

Strains and media

All *S. pombe* strains used in this study are listed in Table 2. Standard methods were used for growth and genetic manipulation (Moreno et al., 1991). All experiments, unless otherwise stated, were performed in YE4S (yeast extract with added 250 mg/l histidine, adenine, leucine and uridine).

Treatment with MBC

Cells were grown at 25°C to 1-2×10^6 cell/ml density before shifting to the restrictive temperature (36.5°C). After 3 hours (unless otherwise stated), cultures

Table 2. Strains used in this study

Strain	Genotype	Source
PN7	*h– cdc25-22*	Lab collection
PN4092	*cdc25-22 nmt1-cdc13-YFP:sup3-5 leu1-32*	Lab collection
PN8	*h– cdc2-33*	Lab collection
PN17	*h– cdc10-129*	Lab collection
PN111	*h+ cdc10-129*	Lab collection
PN15	*h– cdc11-119*	Lab collection
PN1667	*h– cdc25-22 leu1-32 ade6-M210*	Lab collection
PN4671	*h+ cdc10-129 cdc11-119*	This study
PN4377	*h+ tea1-GFP:kanMX leu1-32*	Lab collection
SC150	*h+ cdc25-22 tea1-GFP:kanMX ade6M210*	This study
PN1414	*h– cdc13::ura4 ura4D-18, ade6-704, leu1-32 pREP45cdc13+*	Lab collection
SC157	*cdc25-22 tea1::ura4 ura4-D18*	This study
SC160	*cdc25-22 tea1Δkeltch*	This study
YSM144	*h+ tea4::kanMX ade6-M216 leu1-32 ura4-D18*	Martin et al., 2005
SC293	*cdc25-22 tea4::kanMX*	This study
KS1158	*h– mto1::kanMX6 ade6, leu1-32 ura4-D18*	Sawin et al., 2004
SC298	*cdc25-22 mto1::kanMX*	This study
FC846	*h– bud6::kanMX*	Glynn et al., 2001
SC130	*cdc25-22 bud6::kanMX*	This study
PN4726	*cdc2-33 ssp1::sup3-5 ade6-704*	This study
PN4723	*cdc2-33 tea3::kanMX*	This study
SC302	*cdc25-22 orb2-34*	This study
SC128	*cdc25-22 sla2Δtalin::kanMX*	This study
SC195	*cdc2-33 pom1::ura4 ura4-D18*	Lab collection
JB43	*h– cdc25-22 mid1Δ::ura4 ade6-2 leu1-32 ura4-D18*	Bahler et al., 1998

were split into halves and treated with either 50 μg/ml MBC (freshly made in DMSO) or DMSO at 36.5°C, unless otherwise stated. To count branching cells, 1.5 ml of culture was spun down and cells were counted immediately, using an Axioplan Zeiss microscope for pictures. For block and release experiments cells were blocked at 36.5°C for 3 hours and then quickly shifted to 25°C in the presence of 12 mM HU. After 90 minutes cells were filtered, washed twice with YE4S and resuspended in preconditioned medium at the same concentration as prior the wash. For time-lapse microscopy, cells were fixed to the coverslip of a MaTek dish precoated with lectin (100 μg/ml). Excess cells were removed and 3 ml of fresh medium was added to the dish. After 3 hours at 36.5°C, 50 μg/ml of freshly made MBC (in DMSO) was added to the medium and filming was started. Images were taken every 10 minutes using an Axioplan Zeiss microscope.

The percentage of branched cells is an average of two to three experiments with s.e.m. values smaller than 5%.

Nuclear displacement by centrifugation

Centrifugation experiments were performed as previously described (Carazo-Salas and Nurse, 2006; Daga et al., 2006), with a few modifications to take into account the temperature shift and the MBC treatment. Briefly, cells were blocked for 3 hours at 36.5°C. Then they were transferred to pre-warmed 1.5 ml Eppendorf tubes and centrifuged at 36°C for 8 minutes at 16,000 ***g*** using a table-top Eppendorf 5415R centrifuge. After centrifugation cells were resuspended in their own medium, pooled together and transferred back to 36.5°C. MBC was added immediately prior to centrifugation. After centrifugation cells were followed for 5 hours and fixed for DAPI staining. Position of branch and nucleus is measured as distance between the centre of either and cell tips.

Immunofluorescence, actin, DAPI and calcofluor staining

For immunofluorescence cells were collected by filtration and then fixed. Fixation methods varied depending on the staining performed. For microtubules cells were fixed in –80°C methanol for 1 hour, for standard Tea1 staining cells were fixed in –80°C methanol for 8 minutes, whereas to detect Tea1 in cell middles, cells were fixed in ice-cold 10% trichloroacetic acid (TCA) overnight. Cells were processed as previously described (Alfa et al., 1993). For Tea1 staining, we used an affinity-purified polyclonal α-Tea1 antibody at 1:50 dilution and Alexa Fluor-488-linked anti-rabbit (Molecular Probes) at 1:1000 dilution as secondary antibody. For microtubule detection, TAT1 antibody (α-tubulin antibody; a kind gift of K. Gull, University of Oxford, UK) was used at 1:200 dilution and Alexa Fluor-546-linked anti-mouse (Molecular Probes) at 1:1000 dilution as secondary antibody. Photographs were taken using a Deltavision microscope. In the titration experiment, single microtubules were measured in individual cells after 30 minutes of MBC treatment and the longest considered as the microtubule length in that cell.

For actin staining, cells were fixed by adding formaldehyde (final concentration 3.7%) to the medium for 25 minutes, washed twice with PEM (100 mM Pipes, 1 mM EGTA, 1 mM $MgSO_4$, pH 6.9), permeabilized with 1% Triton X-100, washed twice with PEM and stained with rhodamine phalloidin (Molecular Probes). Photographs were taken using an Axioplan Zeiss microscope.

For DAPI, cells were heat-fixed (70°C) for 10 seconds on a slide and then stained with 2 μl of 50% glycerol, 0.1 M Tris, pH 8, containing 1 μg/ml DAPI. Photographs were taken using an Axioplan Zeiss microscope.

For calcofluor, 5 μg/ml calcofluor (in DMSO) was added to live cells for a few seconds, excess was washed out and photographs were taken with an Axioplan Zeiss microscope.

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