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Isolation and characterization of fission yeast mutants defective in the assembly and placement of the contractile actin ring

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SUMMARY

Fission yeast cells divide by medial cleavage using an actin-based contractile ring. We have conducted a genetic screen for temperature-sensitive mutants defective in the assembly and placement of this actin ring. Six genes necessary for actin ring formation and one gene necessary for placement of the actin ring have now been identified. The genes can be further organized into different phenotypic groups, suggesting that the gene products may have different functions in actin ring formation. Mutants of *cdc3* and *cdc8,* which encode profilin and tropomyosin respectively, display disorganized actin patches in all cells. *cdc12* and *cdc15* mutants display disorganized actin patches during mitosis, but normal interphase actin patterns. *cdc4* and *rng2* mutants display disorganized actin cables during mitosis, but normal interphase actin patterns. In *mid1* mutants, the actin ring and septum are positioned at random locations and angles on the cell surface, although the nucleus is positioned normally, indicating that the *mid1* gene product is required to couple the division site to the position of the nucleus. *mid1* mutant cells may reveal a new cell cycle checkpoint in telophase that coordinates cell division and the proper distribution of nuclei. The actin ring forms medially in a β-tubulin mutant, showing that actin ring formation and placement are not dependent on the mitotic spindle.

Key words: *Schizosaccharomyces pombe*, Contractile ring, Actin, Cell division mutant

INTRODUCTION

An important problem in cell biology is how cells divide into two functional daughters. Cell division in many eukaryotic cells occurs through the action of an actin-based contractile ring. However, little is understood about how it is assembled at the cell membrane and what cell cycle signals trigger its assembly and contraction (for reviews, see Fishkind and Wang, 1995; Satterwhite and Pollard, 1992). Perhaps even more mysterious is how the ring and the subsequent cleavage furrow are positioned in the cell. Early studies have suggested that, in animal cells, the mitotic spindle poles may dictate the position of the cleavage furrow between them (see Rappaport, 1986), but the molecular nature of these effects remains unknown.

The fission yeast *Schizosaccharomyces pombe* is particularly well suited for the study of cytokinesis on a molecular genetic level (Fankhauser and Simanis, 1994). In a manner similar to that for animal cells, yeast divides by medial cleavage via an actin-based contractile ring. The actin ring is assembled at the plasma membrane around the circumference in the middle of the rod-shaped cell (Marks et al., 1986; Marks and Hyams, 1985). It initially appears in early mitosis before anaphase as a thin ring, and thickens as the cell progresses through mitosis. Following anaphase, septation begins with the deposition of septum cell wall materials outside the membrane at the site marked by the actin ring. As the septum grows in a centripetal manner into the cell, the actin ring contracts at the leading edge, presumably closing the membrane (Jochová et al., 1991; F.C., unpublished observations). The actin ring can be seen in electronmicrographs as filaments arranged between the arms of the developing septum (Kanbe et al., 1989). In addition, many actin dots, which may represent vesicles carrying materials for building the septum, cluster around the septum site (Marks and Hyams, 1985; Kanbe et al., 1989). Following completion of the septum, it is digested away, leading to separation of the two daughter cells.

During interphase, filamentous actin is present primarily in multiple cortical patches at the growing end or ends of cells. These actin patches are involved in polarized cell growth and may represent small actin-rich invaginations in the plasma membrane, as has been proposed in budding yeast (Mulholland et al., 1994), or actin-rich vesicles (Kanbe et al., 1989). During mitosis, the actin patches disappear, and growth ceases.

How the actin ring is regulated in the cell cycle and positioned in the cell are not known. In contrast to the contractile ring in animal cells, which forms at the end of mitosis, the fission yeast ring forms early in mitosis,. The mitotic protein kinase $p34^{cdc2}$/cyclin B is necessary for actin ring formation, in that *cdc2* mutant cells arrested in the G_2 phase of the cell cycle maintain interphase actin staining at the ends and do not form an actin ring (Snell and Nurse, 1994). How the nucleus and ring are positioned in the middle of the cell is not known, but the position of the premitotic nucleus and ring are always coupled (see Discussion).

Numerous genes required for cell division in fission yeast have been identified in a screen for cell cycle (*cdc*) mutants (Nurse et al., 1976). These mutants were isolated as temperature-sensitive lethal mutants that became multinucleate and elongated at restrictive temperature. The mutants were shown to continue nuclear division, DNA replication, and growth cycles in the absence of cell division (Nurse et al., 1976). These cell division mutants were further organized into two groups based on septum morphology: the early cell plate mutants (*cdc7*, *cdc11*, *cdc14* and *cdc15*) and the late cell plate mutants (*cdc3*, *cdc4*, *cdc8* and *cdc12*). It was proposed that the early cell plate mutants, which formed no septal material, have a defect in an early step in cell plate formation, and that the late cell plate mutants, which form abnormal accumulations of septal material, have a defect in a later step in cell plate formation. However, subsequent analyses (Marks et al., 1987; this work) suggest that the late cell plate mutants in fact are defective in an earlier step in cell division, namely in actin ring formation. *cdc3,4,8,12* mutants exhibit abnormal actin distribution (Balasubramanian et al., 1992, 1994; Marks et al., 1987; this work). Early cell plate mutants may be involved in later steps in cell division such as septation and actin ring contraction (Fankhauser and Simanis, 1994); an early cell plate mutant *cdc11* exhibits normal actin rings, but subsequently does not form a septum (Marks et al., 1987). The identification of *cdc8* as a homologue of tropomyosin (Balasubramanian et al., 1992) and *cdc3* as a homologue of profilin (Balasubramanian et al., 1994) has further supported the proposal that the *cdc3,4,8,12* class of cell division genes is involved in the organization of the contractile actin ring.

Very few alleles of the cell division genes were identified in the original screen (Nurse et al., 1976). In this paper, we have performed a genetic screen to identify much larger numbers of actin ring mutants, in order to identify new genes in actin ring assembly and to isolate mutants with more severe, or different phenotypes for the previously identified *cdc* genes. The identification and characterization of these mutants provides a genetic framework for the different roles of proteins involved in ring assembly. In addition, this screen also has identified a novel gene necessary for ring placement, which should give insights into the spatial problem of how the ring is positioned in the middle of the cell.

MATERIALS AND METHODS

Genetic and physiological techniques

Standard *S. pombe* media and genetic manipulations were employed (Moreno et al., 1991). All strains were isogenic to wild-type $972h^-$ (Leupold, 1970). *mam2 leu1-32* h^{90} (Broek et al., 1991) was the parent strain used for mutagenesis in the genetic screen. Mutants were outcrossed to wild-type strains (h^+ *wt,* h^- *ura4-D18 leu1-32* or h^+ *ura4-D18 leu1-32*) three times: twice using random spore analysis and finally using tetrad analysis. Outcrossed strains used as representative strains in this paper were *cdc3-313* h^- (FC114), *cdc8-382* h^- (FC131), *cdc12-299* h^- (FC127), *cdc15-287 ura4-D18* h^- (FC55), *cdc4-377* h^- (FC123), *rng2-346* h^- (FC166), and *mid1-366* h^- (FC164).

For recessive-dominance testing, diploids were constructed by mating h^- *leu1-132* or *ura4* mutant strains to *mat2-102 ura1-171 his3-237 ade6-M210* h^{90} (Kohli et al., 1977). For temperature shift experiments, exponential phase cultures were grown in YE5S at the permissive temperature of 25°C and were shifted to the restrictive temperature of 35.5°C at various times. Cells counts were determined on fixed cells (Moreno et al., 1991) using a Sysmex F-800 microcell counter.

Mutant screening

mam2 leu1-32 h^{90} cells (Broek et al., 1991) were mutagenized with nitrosoguanidine (Moreno et al., 1991) to 30% survival and enriched 20- to 50-fold for diploidizing mutants as previously described (Broek et al., 1991; Hayles et al., 1994). From 1×10^5 diploidizing mutants, 1200 mutants showing temperature-sensitive lethality or reduced growth were identified: 30% of these were cell divison mutants. This screen enriches for cell division mutants because these mutants become multinucleate, and the nuclei in a homothallic background are able to undergo karyogamy, resulting in diploid formation without mating (Broek et al., 1991). This screen was also used to identify *cut* mutants (A. Woollard, unpublished observations) and rereplicating mutants (Hayles et al., 1994).

Cytology

Rhodamine/phalloidin staining was adapted from Marks and Hyams (1985), with some minor changes. Cells grown in 5 ml YE5S were fixed by the quick addition of 5 ml formaldehyde-PM (40% of EM-grade methanol-free formaldehyde 16% (Polyscience) and 60% PM (35 mM KPO_4, pH 6.8, 0.5 mM $MgSO_4$)) for 15-40 minutes at the growth temperature. Cells were washed 3× with PM, permeabilized with PM+1% Triton X-100 for 2 minutes, and washed 3× with PM. Cell pellets were incubated in rhodamine/phalloidin (stored in MeOH, reconstituted in PM at 200 units/ml; Molecular Probes) for 30 minutes at room temperature and stored at 4°C. Cells were dried on a coverslip and mounted in PM with 50 µg/ml DAPI (4′,6′-diamidino-2-phenylindole dihydrochloride) and 0.3 mg/ml *p*-phenlenediamine. Cells fixed in the same way were also stained with 50 µg/ml Calcofluor. Anti-tubulin staining was performed as described by Hagan and Hyams (1988), using methanol fixation, TAT1 anti-tubulin antibody, which was kindly provided by K. Gull, and a Cy3-conjugated secondary antibody (Sigma). Tubulin-stained cells were stained with the nuclear stain TOTO-1 (Molecular Probes). Cells were observed and photographed with a Zeiss Axioscope microscope using TMAX400 film (Kodak). For photographs of microtubules (see Fig. 8), cells were photographed with a Bio-Rad 600 confocal microscope and analyzed with Bio-Rad software. Septation patterns in Fig. 7 were analyzed from drawings of septa taken from microscopic examination of Calcofluor-stained cells in several focal planes. The analysis was facilitated by the fact that the cells are a constant cell length.

RESULTS

Genetic screen for contractile ring mutants

We identified *S. pombe* temperature-sensitive contractile actin ring mutants using a 3-step screen. In the first step, mutagenized cells were enriched for the ability to diploidize DNA content without mating (Broek et al., 1991; see Materials and Methods). Cells were then screened for reduced growth at the restrictive temperature of 36°C. These steps effectively enrich for mutant cells with either cell or nuclear division defects, or for rereplicating mutants (Hayles et al., 1994). In the second step, mutant cells were fixed, stained with the nuclear stain DAPI, and screened visually for mutants that become multinucleate and elongated after a shift to restrictive temperature, indicating a defect in cell division. In the third step, representative mutants were examined for actin distribution and septum formation. In this step, cell division mutants were screened and characterized for those with defects specifically in actin ring assembly, since some cell division mutants make normal actin

Table 1. Results of a genetic screen for contractile actin ring mutants

Gene	No. of alleles	Allele numbers
cdc3	10	313*, 327, 339, 340, 342, 360, 367, 373, 358*, 316
cdc4	19	377*, 347, 383, 319, 307, 312*, 341, 311, 309, 315, 306, 355, 357, 375, 317, 320, 324, 338, 363
cdc8	8	382*, 326, 372, 390, 343, 327, 353, 374
cdc12	3	299*, 354, 369
cdc15 (rng1)	3	287*, 332, 318
rng2	2	301*, 346
mid1	6	366*, 391*, 371*, 386*, 368*, 333

Allele numbers are arranged in approximate severity of the mutant phenotype, based on actin distribution.
*Representative alleles that have been outcrossed. The underlined alleles were used for phenotypic analysis in this paper.

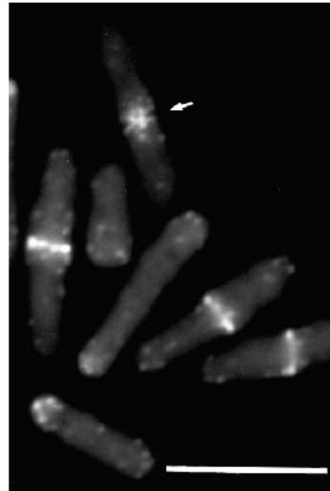

Fig. 1. Actin distribution in wild-type *S. pombe* cells. Wild-type *ura4-D18 h*$^-$ (FC43) cells were grown at 25°C, fixed and stained for actin with rhodamine/phalloidin. Note the numerous actin rings before contraction, and a contracting actin ring during septation (arrow). Bar, 10 μm.

rings but exhibit other defects in cell division (Marks et al., 1986). In this manner, 53 mutants defective in actin ring formation and cell division were isolated.

Linkage analysis showed that these mutants define 7 linkage groups (Table 1). Five of the genes are allelic to previously identified cell division genes: *cdc3*, *cdc4*, *cdc8*, *cdc12* and *cdc15*; two are new genes we named *rng 2*(ring) and *mid1*. *rng1* was initially thought be a novel locus, but was subsequently found to be allelic with *cdc15*. The number of alleles for the genes was highly variable, ranging from 19 alleles for *cdc4* to two alleles for *rng2*. The relatively small number of linkage groups suggests that this screen may be near saturation for the particular phenotype pursued in the screen. Since the mutants were collected from a small number of mutagenized pools, it is possible that the mutants were not all derived independently. However, minimal amplification procedures and the variety of different mutant phenotypes suggest that most of the mutants represent different mutational events. Characterization of these mutants, as discussed below, shows that *cdc3*, *cdc4*, *cdc8*, *cdc12*, *cdc15* and *rng2* are involved in three different aspects in the assembly of the ring, and *mid1* is involved in placement of the ring.

Characterization of actin ring mutants

In wild-type *S. pombe* cells, actin is organized primarily as cortical actin patches at the cell tips during interphase and the medial contractile ring during mitosis (Marks et al., 1986; Marks and Hyams, 1985). Fig. 1 shows actin distribution in wild-type cells visualized under our staining conditions using rhodamine/phalloidin.

The actin ring mutants described in the following sections have temperature-sensitive defects in actin ring formation. We examined actin distribution 1, 2 and 4 hours after a shift from the permissive temperature of 25°C to the restrictive temperature of 35.5°C. All the mutant cells grow and form normal actin rings at the permissive temperature, and develop abnormal actin distribution and cease cell division within the first cell cycle after shift to restrictive temperature. Nuclear division cycles continue, although at an approximately twofold slower rate than in wild-type cells (data not shown). In this analysis, we present pictures of actin distribution after 4 hours at restrictive temperature when cells have 2 to 4 nuclei, but similar phenotypes were seen after 1 hour at restrictive temperature (*wt* generation time at 35.5°C is 2 hours), demonstrating that these actin phenotypes are not caused by having too many nuclei or by impending death.

The different alleles for each gene have a range of phenotypes, from mild to severe effects on actin organization. For instance, in certain alleles of *cdc4* and *cdc8*, the actin rings are only slightly abnormal, and the cells form a septum but are unable to separate (data not shown). Although the strength of the defects varied for different alleles of a given gene, the general character of the defects was consistent in each allelic series. Representative alleles of each gene exhibiting the most severe actin defects of each allelic series were outcrossed and further analyzed. Tetrad analysis and recessive-dominance tests revealed that these mutants contain a single recessive gene trait. These new alleles, described below for *cdc3*, *cdc8* and *cdc4*, have a more severe actin distribution phenotype than the temperature-sensitive alleles described previously (Balasubramanian et al., 1994; McCollum et al., 1995; Marks et al., 1986; Nurse et al., 1976).

The genes required for actin ring assembly were organized into three groups based on two phenotypic criteria. First, we asked if these genes are required specifically for the organization of the actin ring, or for other actin structures as well. Mutations in factors involved specifically in actin ring assembly during mitosis would affect only the actin ring, and not interphase actin patches. Mutations in general actin-organizing factors would affect both the organization of the interphase actin patches and the actin ring. Second, we ascertained what kind of aberrant actin structures are made instead of the actin ring during mitosis.

Genes involved in general actin organization

cdc3 (profilin homologue) and *cdc8* (tropomyosin homologue) mutants display severely disrupted actin distributions, affecting both the actin ring and interphase actin patches. This phenotype suggests that these genes have a role in actin organization during all phases of the cell cycle.

The *cdc3-313* mutant exhibits abnormal actin organization in all cells (Fig. 2). Actin staining shows abnormal cortical patches localized in an uneven distribution throughout the

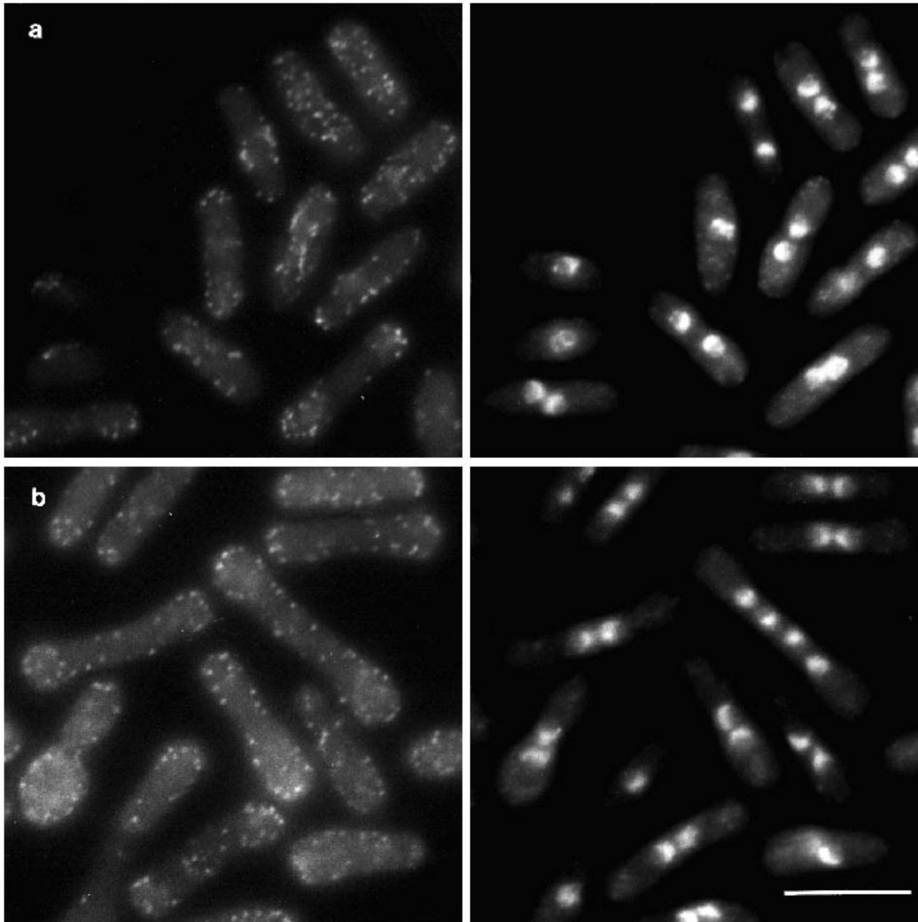

Fig. 2. Actin distribution in *cdc3* and *cdc8* mutant cells. (a) *cdc3-313 h*$^-$ (FC114); and (b) *cdc8-382 h*$^-$ (FC131). Cells were grown for 4 hours at the restrictive temperature of 35.5°C, fixed and stained for actin with rhodamine/phalloidin (left), and for DNA with DAPI (right). Bar, 10 μm.

cortex. The patches are not concentrated at the ends, as in normal interphase cells, and have abnormal and variable morphologies and sizes, often appearing filamentous and stringy. Some *cdc3-313* mutant cells exhibit larger actin patches at the middle of cell, suggesting an abnormal attempt at actin ring formation. Calcofluor staining shows large patches of septal material on the cell surface in the middle of the cell (see Fig. 5a).

The *cdc8-382* mutant displays actin patches uniformly distributed on the cell cortex in all cells (Fig. 2b). In contrast to the uneven patches in *cdc3* mutant cells, the patches in *cdc8* mutant cells are more uniform in morphology, distribution, and size. There is little or no concentration of actin patches at the ends or in the middle of the cells. Calcofluor staining showed that most *cdc8-382* cells have no septum staining (see Fig. 5b), although a small fraction of cells have a single or multiple small patches of septum material.

Genes involved in an early step in ring formation

cdc12 and *cdc15* mutants have normal interphase actin patterns, but minimal actin structures during mitosis. These phenotypes suggest that these gene products have a specific role in actin ring formation during mitosis.

The *cdc12-299* mutant displays normal actin patches concentrated at the ends of the cells during interphase (Fig. 3). The interphase patches are uniform and normal in morphology. During mitosis, actin redistributes into cortical patches that are delocalized or clustered around the middle of the cell (Fig. 3). The patches in mitosis are generally larger and variable in morphology, similar to patches seen in the *cdc3* mutant. Some *cdc12-299* mutant cells also exhibit very light loose whisps of actin filaments in the areas where the actin ring would form. Calcofluor staining shows small, irregular patches in the middle of the cell (see Fig. 5c). Cells are long, suggesting that growth is not impaired.

The *cdc15-287* mutant has a very similar phenotype to *cdc12* mutants (Fig. 3b). Actin distribution appears normal during interphase. In mitosis, actin redistributes into cortical patches with irregular distribution and morphology. Unlike those in *cdc12* mutants, the patches are not clustered in the middle of the cell. Up to 20% of the mitotic cells exhibit a very faint actin ring (data not shown), which may be due to incomplete penetrance of the mutant allele. Since cells with delocalized actin patches can be found in all stages of mitosis, it is unlikely, for instance, that rings form transiently at specific period in mitosis in all cells. Calcofluor staining shows that the cells have no septum staining (see Fig. 5d).

Genes involved in a late step in ring formation

cdc4 and *rng2* mutants both exhibit disorganized actin cables in place of the actin ring, suggesting that these genes may be involved in organizing cables of actin around the circumference of the cell into a ring.

Abnormal actin cables seen in *cdc4-377*and *rng2-346*

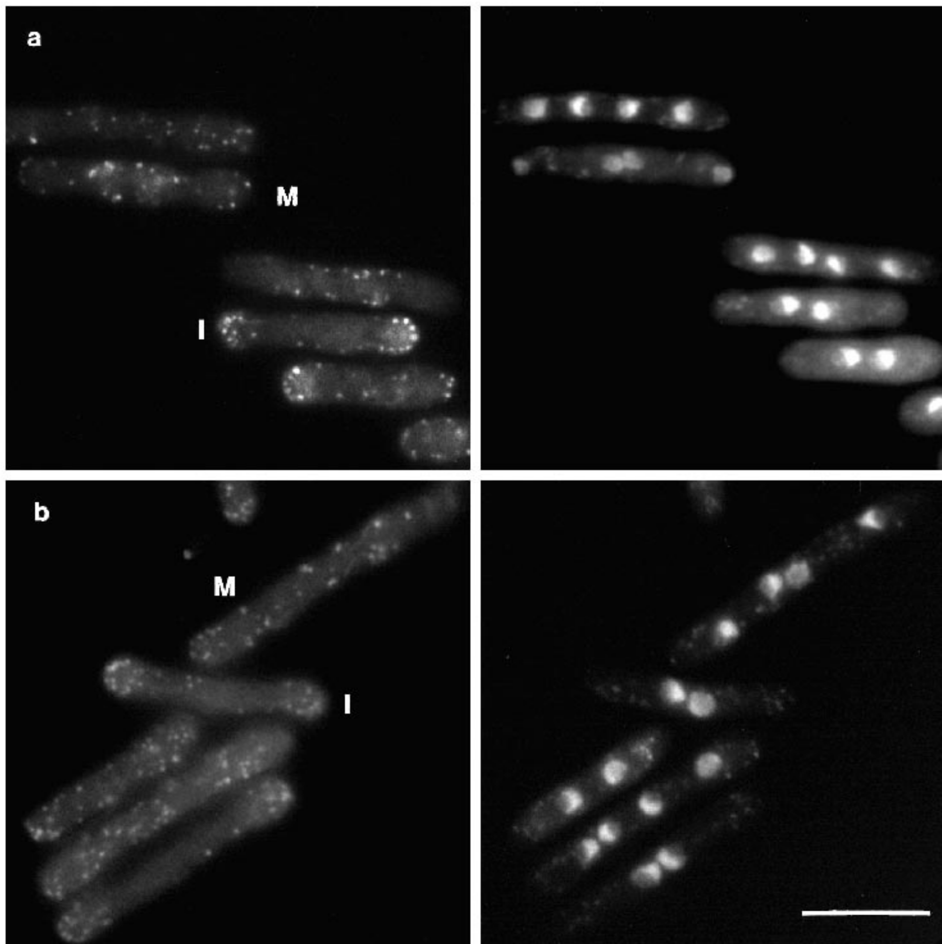

Fig. 3. Actin distribution in *cdc12* and *cdc15* mutant cells. (a) *cdc12-299 h*$^-$ (FC127); and (b) *cdc15-287 ura4-D18 h*$^-$ (FC55). Cells were grown for 4 hours at the restrictive temperature of 35.5°C, fixed and stained for actin with rhodamine/phalloidin (left panels), and for DNA with DAPI (right panels). M denotes mitotic cells, as determined by characteristic nuclear position and morphology. I denotes cells in interphase with normal distributions of actin patches at the ends of the cell. Bar, 10 μm.

mutants are either disorganized (Fig. 4c) or originate from a single point, forming an 'actin aster' (Fig. 4a). Both the asters and the disorganized filaments occur in mitotic cells at the time and place where the actin ring would normally assemble, suggesting that these abnormal actin structures arise as a consequence of defective actin ring assembly. In addition, the asters and filaments are present in post-mitotic cells and thus may persist after mitosis. The actin asters are commonly located near the middle of a cell body between two nuclei, where the actin ring would have formed, although they exist in other locations as well. The centre of the aster is most often close to the cell surface, especially in mitotic cells, but also can be found in the cell interior in post-mitotic cells. These different features vary in the different alleles of *cdc4* and *rng2*. *cdc4-377* mutants have prominent actin asters, which are present in 15% of the cells (Fig. 4a). The *rng2-346* allele and some alleles of *cdc4* display disorganized cables more often than the asters (Fig. 4b).

A less common feature is striking broad actin cables that run through the cell or wrap around the ends, as shown in Fig. 4b. These are generally present in interphase cells and are sometimes associated with an abnormal septum. *cdc4* and *rng2* mutants exhibit normal interphase actin patches at the ends of some cells (Fig. 4) and grow into long cells, indicating that these genes are not necessary for organization of the interphase actin patches at the cell tips.

In *cdc4* and *rng2* mutants, an abnormal septum forms on the surface from one side of the cell and then extends in a disorganized manner into the interior (Fig. 5e,f). This pattern is consistent with the abnormal actin structures that often also originate from a point or on one side on the cell surface.

Thus, *cdc4* and *rng2* mutants initiate formation of actin cables from the cell surface, but cannot properly organize the actin into an intact ring. We propose that these gene products may act to organize the actin cables into a ring, possibly by bundling the cables together or by anchoring the cables to the plasma membrane.

A gene involved in placement of the actin ring

Six mutants with a novel phenotype of actin rings and septa in random locations at the cell surface defined one gene that we have named *mid1* (division in the middle). No alleles of the other actin ring mutants possess a similar phenotype.

The *mid1-366* mutant forms an actin ring at highly variable locations and angles on the cell surface (Fig. 6a). The actin ring is thinner when the ring is more angled and larger, but is normal in morphology when the ring is orthogonal to the cell surface. No actin rings were found that were positioned longitudinally from one end of the cell to the other, but in some cells a single broad actin cable runs the length of the cell (data not shown).

The septa form in similar but broader distributions, suggesting that the misplaced actin rings mark the site for septum formation (Fig. 6). Many cells with an asymmetric septum

Fig. 4. Actin distribution in *cdc4* and *rng2* mutant cells. (a and b) *cdc4-377 h*$^{-}$ (FC123); and (c) *rng2-346 h*$^{-}$ (FC166). Cells were grown for 4 hours at the restrictive temperature of 35.5°C, fixed, and stained for actin with rhodamine/phalloidin (left panels) and for DNA with DAPI (right panels). M denotes cells in mitosis, as determined by nuclear position and morphology. I denotes cells in interphase with normal distributions of actin patches at the ends of the cell. (a and b) Images from two different fields and different focal planes (a is focused on the interior of the cell) illustrating the variety of actin structures in *cdc4-377* cells. Bar, 10 µm.

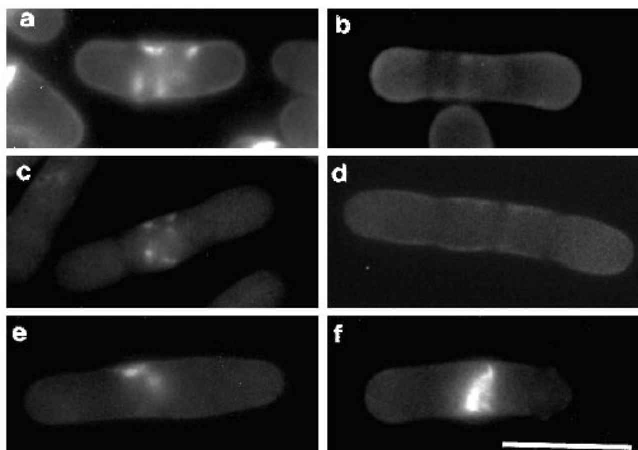

Fig. 5. Calcofluor staining in actin ring mutants. (a) *cdc3-313 h*$^{-}$ (FC114); (b) *cdc8-382 h*$^{-}$ (FC131); (c) *cdc12-299 h*$^{-}$ (FC127); (d) *cdc15-287 ura4-D18 h*$^{-}$ (FC55); (e) *cdc4-377 h*$^{-}$ (FC123); (f) *rng2-346 h*$^{-}$ (FC166). Cells were grown for 4 hours at the restrictive temperature of 35.5°C, fixed, and stained for septum cell wall material with Calcofluor. For comparision, note a wild-type pattern of staining in the upper left cell in Fig. 6b. Bar, 10 µm.

divide asymmetrically into a large and a small cell (Fig. 6b, bottom right cell). In other cells, a septum appears to start at one point on the cell surface and then extend longitudinally in broad sheets in the cell interior. Electron microscopy shows that the septal formations are outside the plasma membrane and do not represent intracellular organelles (N. Hajibagheri and F. Chang, unpublished observations). These ‘longitudinal’ septa may arise from severely displaced actin cables, or grow off from actin filaments unrelated to the actin ring. Occasionally (<1%), cells with two actin rings or two septa (Fig. 6) can be

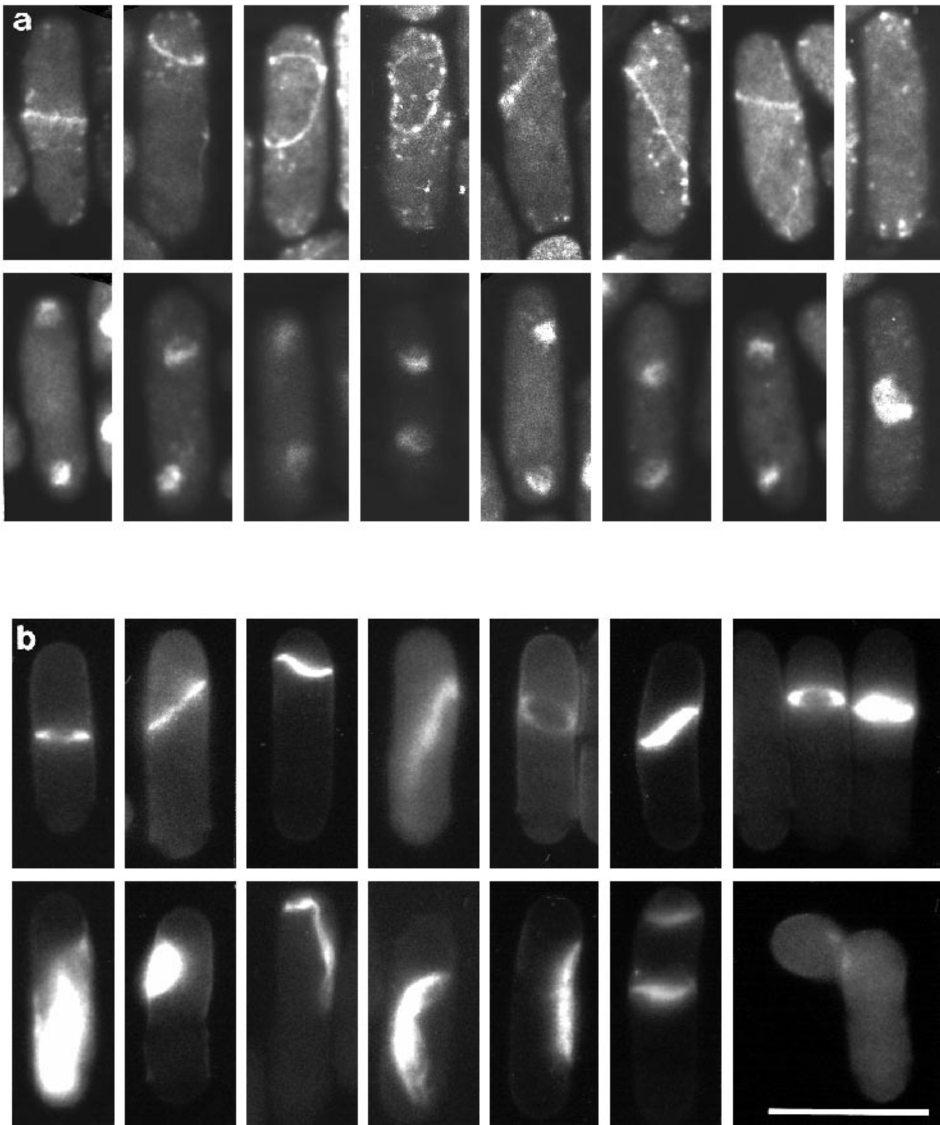

Fig. 6. Misplacement of actin rings and septa in *mid1-366* mutant cells. (a) Actin and nuclear localization. *mid1-366 h⁻* (FC164) were shifted to 35.5°C for 2 hours, fixed and stained for actin with rhodamine/phalloidin (upper panel) and for DNA with DAPI (lower panel). The cell on the right is an interphase cell, showing normal localization of the single nucleus in the middle of the cell. (b) Septum localization. *mid1-366* (FC 164) shifted to 35.5°C for 3 hours, fixed and stained for septum with Calcofluor. Bar, 10 μm.

seen. At later time points, many cells fill up with septal material (Fig. 6b, bottom left cell).

Fig. 7 shows quantification of different aspects of the *mid1* mutant. The septa have a random distribution in placement and angle along the long axis of the cell (Fig. 7A,B). In contrast, the nucleus during interphase is normally positioned in the middle of the cell (Fig. 7C; Fig. 6a, right cell). Thus, *mid1* mutants are defective in coupling the position of the division site with the position of the nucleus.

The microtubule cytoskeleton appears largely unaffected in the *mid1* mutant. Mitotic spindle assembly, nuclear division and the interphase microtubule networks are normal (Fig. 8). However, one interesting observation is that the post-anaphase microtubule organizing centres, which nucleate microtubules after mitosis, form at the asymmetric division site (Fig. 8b). In wild-type cells, these structures are positioned in the middle of the cell (Hagan and Hyams, 1988). This observation suggests that the same signals that position the actin ring also position the post-anaphase microtubule organizing centre.

Many *mid1* cells exhibit cell cycle arrest or delay. Rates of cell and nuclear division are reduced (doubling time=5 hours) in the *mid1* cells compared to wild-type cells (Fig. 7E). After 3 hours at the restrictive temperature, cells accumulate as binucleate cells with an abnormal or misplaced septum and an interphase array of microtubules (Figs 7D, 8c). The cells do not elongate, and actin distribution is not polarized (data not shown). The septation process continues, leading to septal material growing from a single septum and filling up the cell (Fig. 6B, bottom left cell). Thus, these cells appear to be arrested in a post-mitotic state, telophase. *mid1* cells may arrest at the same point in the cell cycle as *cdc16* mutant cells, which arrest in telophase and produce multiple septa without cell separation and or anucleate cells (Minet et al., 1979). Some aspects of the arrest in *mid1* cells are relieved approximately 5 hours after temperature shift, when cells begin to grow, branch around abnormal septa, and divide.

The telophase arrest may only occur in a portion of the cells: namely, those with an abnormal septum, or those with a misplaced septum forming an anucleate compartment. No anucleate cells are produced (Fig. 7D), indicating that cells with a misplaced septum forming an anucleate compartment does not divide. In contrast, cells with a misplaced septum with a nucleus on either side proceed to divide to form a large

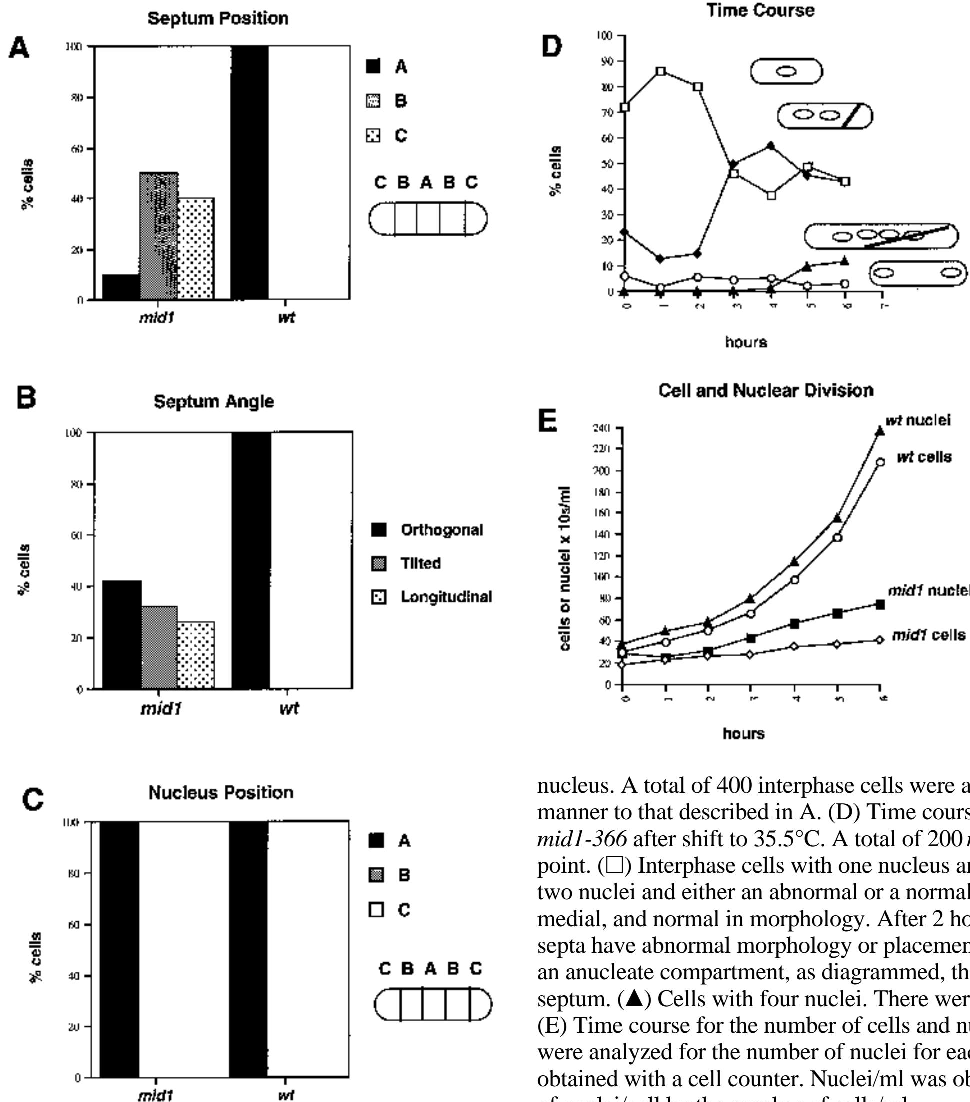

Fig. 7. Properties of septa and division in *mid1-366* mutant cells. *mid1-366 h*$^-$ (FC164) or wild-type *h*$^-$ (972) cells were shifted to 35.5°C for 3 hours (A,B,C) or at various times for a time course (D,E), fixed, stained with Calcofluor or DAPI and examined by flourescence microscopy. (A) Septum position. Septation patterns were analyzed on 600 *mid1* cells and 200 *wt* cells stained with Calcofluor. The position of the most distal part of the septum was quantified relative to the length of the cell divided into five equal parts, as diagrammed. This analysis was facilitated by the fact that the septated cells were a constant length. (B) Septum angle. The same cells were also analyzed for the angle of the septa relative to the long axis of the cell. Septa 78°-90° were designated as 'orthogonal' (the normal angle). Septa roughly parallel to the long axis that did not communicate with the cell surface at one end (see examples in Fig. 6, bottom row) were designated as 'longitudinal'. The remainder of the septa were designated as 'tilted'. (C) Position of the premitotic single nucleus. A total of 400 interphase cells were analyzed for nuclear position in a similar manner to that described in A. (D) Time course for the development of phenotypes in *mid1-366* after shift to 35.5°C. A total of 200 *mid1* cells were examined for each time point. (□) Interphase cells with one nucleus and no septum. (◆) Post-mitotic cells with two nuclei and either an abnormal or a normal septum. At time 0, > 90% of septa are medial, and normal in morphology. After 2 hours at restrictive temperature, >90% of septa have abnormal morphology or placement. The abnormal septa did not all produce an anucleate compartment, as diagrammed, though many did. (○) Mitotic cells without a septum. (▲) Cells with four nuclei. There were no anucleate cells seen (<0.1%). (E) Time course for the number of cells and nuclei per ml of culture. A total of 200 cells were analyzed for the number of nuclei for each time point (see D), and cell number was obtained with a cell counter. Nuclei/ml was obtained by multiplying the average number of nuclei/cell by the number of cells/ml.

cell and a small cell (see Fig. 6b, bottom right cell). These observations suggest that the cell may check if there is a nucleus (or some other associated organelle) on either side of the septum or if the septum is normal before commencing with cell separation.

The mitotic spindle is not involved in actin ring formation or placement

In animal cells, the mitotic spindle is thought to induce and position the cleavage furrow. We tested the role of the mitotic spindle in fission yeast by examining a mitotic spindle mutant. The *nda3-311* mutant is a cold-sensitive β-tubulin mutant that arrests in mitosis, with no mitotic spindle, an unduplicated spindle pole body, displaced, condensed chromosomes, and high mitotic p34^{cdc2} protein kinase activity (Hiraoka et al., 1984; Kanbe et al., 1990; Moreno et al., 1989; Toda et al., 1983). *nda3-311* mutant cells were grown at the permissive temperature of 30°C and then shifted to the restrictive temperature of 20°C for 10 hours. Rhodamine/phalloidin staining of *nda3*-arrested cells showed actin ring staining in the great majority of the cells (Fig. 9). The actin ring is filamentous and often slightly broader than normal. A fraction of the cells are also septated. Tubulin staining showed that a majority of the cells have no microtubule structures at all, and most of the remainder have only a small dot of tubulin staining, presumably at the spindle pole body (data not shown). Greater than 90% of cells have condensed chromosomes. Since most cells were in interphase and did not have rings before the shift to restrictive temperature, we infer that rings formed after the shift to restrictive temperature in the absence of mitotic spindle. These results demonstrate two key aspects in the spatial and cell cycle regulation of the actin ring: first, the mitotic spindle is not necessary for actin ring formation or placement; second, the actin ring can form in the presence of high p34^{cdc2} protein kinase activity.

A small fraction (12%) of *nda3*-arrested cells have an actin ring displaced from the middle of the cell (Fig. 9, see arrow). These rings are orthogonal to the long axis of the cell, unlike the angled rings in *mid1* cells. Most of the displaced rings lie above a displaced nucleus. Since most cells have displaced nuclei, it is not clear why only a subset of cells exhibit displaced rings. One possibility is that the nucleus specifies the

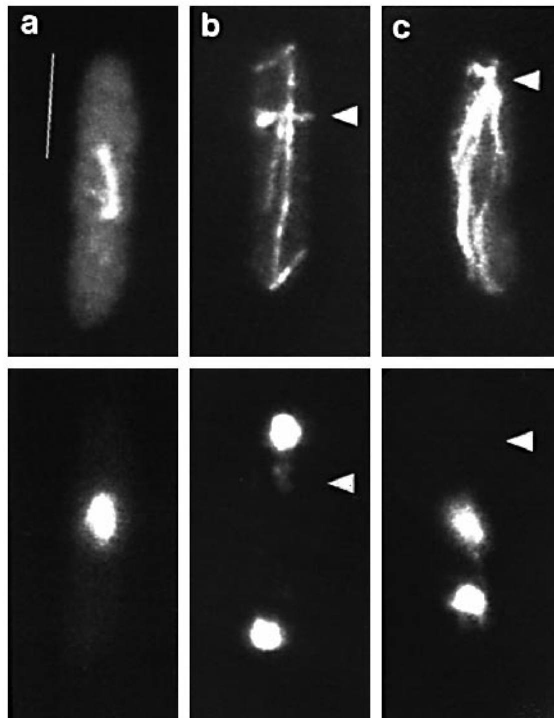

Fig. 8. Mitosis with a misplaced septum in *mid1-366* mutant cells. *mid1-366 h*$^{-}$ (FC164) were shifted to 35.5°C for 2 hours, fixed and stained for tubulin (upper panels) and DNA (lower panels). These panels show three representative cells in: (a) early mitosis; (b) late anaphase; (c) post-mitosis (telophase). The arrows indicate the asymmetric septum site. In (b) upper panel, the tubulin structures marked by the arrow are the post-anaphase MTOCs that form at the septum site. These MTOCs normally form in the middle of wild-type cells. In (c), the septum has produced a small anucleate compartment (above the arrow) and a large binucleate compartment (below the arrow). These cells typically do not divide. Bar (top left), 5 µm.

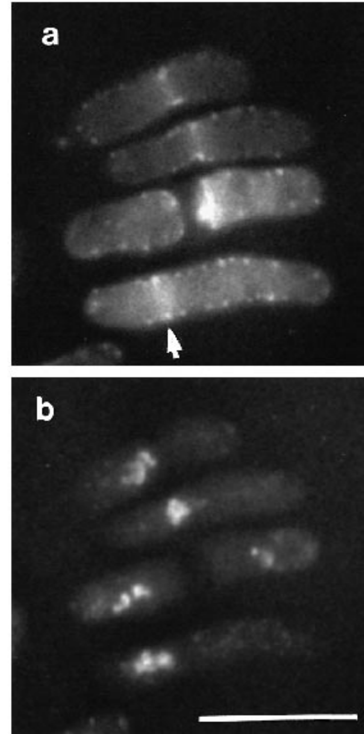

Fig. 9. Actin rings in a β-tubulin mutant, *nda3-311. nda3-311* (PN780) was shifted to the restrictive temperature of 20°C for 10 hours, fixed and stained with (a) rhodamine/phalloidin and (b) DAPI. Most cells exhibit a medially placed actin ring (top two cells). Some cells septate (third cell down) or exhibit an asymmetrically placed actin ring (bottom cell, arrow). DAPI staining shows condensed, misplaced chromosomes, indicative of mitotic arrest. Cells were also stained with anti-tubulin antibody, which showed a lack of microtubule structures in the majority of the cells (data not shown). Bar, 10 µm.

position of the ring before mitosis (see Discussion). In a small fraction of *nda3* cells, the nucleus may be displaced early in the cell cycle, and thus the actin ring may be positioned asymmetrically. In most *nda3* cells, the nuclei may be displaced only after entry into mitosis, after the site of the ring has been determined. Another explanation for a displaced ring is that the cell might grow asymmetrically after the point in the cell cycle when the division site has been determined.

Table 2. *S. pombe* contractile actin ring genes

Gene	Product	Mutant phenotype: actin distribution		Predicted function
		Mitosis	Interphase	
cdc3	Profilin*			General actin organization
cdc8	Tropomyosin†			
cdc12	*dia-BNI1*-like‡			Early actin ring formation
cdc15				
cdc4	EF hand§			Late actin ring formation
rng2				
mid1				Actin ring placement
Wild type				

See text for description.
*Balasubramanian et al. (1992).
†Balasubramanian et al. (1994).
‡F. Chang and P. Nurse, unpublished observation.
§McCollum et al. (1995).

DISCUSSION

S. pombe contractile actin ring genes

We have carried out a large genetic screen for fission yeast mutants defective in the assembly or placement of the contractile actin ring. These mutants define six genes necessary for contractile ring assembly, and one gene necessary for the proper localization of the ring. In addition to the identification of two novel genes, we have also isolated many new alleles of the previously identified cell division genes with more severe phenotypes. These have given new insights into their function. The genes necessary for actin ring assembly can be divided into three groups based on the phenotypes of the mutants (Table 2). *cdc3* and *cdc8* may be general actin organizing factors, because the mutants exhibit abnormal actin distribution in all phases of the cell cycle. *cdc12* and *cdc15* may have a role in an early step in actin ring formation, possibly in the nucleation of the actin ring, because these mutants show minimal actin structures during mitosis. *cdc4* and *rng2* may play a role in a late step in actin ring assembly, because these mutants make disorganized actin cables in place of a ring. The latter two classes of genes may have roles specifically in actin ring organization, since actin patches during interphase are not affected in these

mutants. Finally, *mid1* has a role in positioning the actin ring in the cell, because *mid1* mutants exhibit actin rings and septa in random locations and angles on the cell surface.

The mutant phenotypes of the actin ring genes are consistent with their molecular characterization. *cdc3* encodes fission yeast profilin (Balasubramanian et al., 1994), which is a small actin-binding protein proposed to have a role in regulating actin filament assembly, perhaps by sequestering monomer actin and preventing actin polymerization in inappropriate locations (see Theriot and Mitchison, 1993). The *cdc3* mutant develops a cortex filled with actin patches with a stringy, abnormal morphology, suggesting inappropriate actin filament assembly and organization. cdc3p is localized to broad bands at the tips of interphase cells and at the middle of mitotic cells (Balasubramanian et al., 1994), and thus may control the organization of multiple actin structures. Similar disruption of actin structures and cytokinesis have been seen in profilin mutants of *Saccharomyces cerevisiae*, *Drosophila*, and *Dictyostelium* (Haarer et al., 1990, 1993; Cooley et al., 1992; Haugwitz et al., 1994).

cdc8 encodes a homologue of tropomyosin (Balasubramanian et al., 1992), a small, conserved coiled-coil protein that binds along the actin filament. Like a budding yeast tropomyosin mutant (*tpm1*; Liu and Bretscher, 1989), fission yeast tropomyosin mutants have a defect in actin cable structures such as the actin ring and display large, well defined actin patches uniformly distributed on the cell surface (this work; Balasubramanian et al., 1992). cdc8p may colocalize with all filamentous actin structures (Balasubramanian et al., 1992) and thus, like profilin, appears to be necessary for the organization of multiple actin structures.

cdc12, one of the genes required for an early step in actin ring formation, encodes a large proline-rich protein (F.C., unpublished obseravtions) with similarity to a new family of genes including *diaphanous*, a *Drosophila* gene required for cytokinesis (Castrillon and Wasserman, 1994), and *BNI1*, a *S. cereivisiae* gene involved in bud site placement (H. Fares and J. Pringle, personal communication). In contrast to the general actin organizing proteins described above, cdc12p is localized at the contractile ring and not at the interphase actin patches (F. C., unpublished observations). This localization and the specificity of the *cdc12* mutant phenotype suggest that cdc12p may have a specific role in actin ring formation.

cdc4, one of the genes involved in a late step in actin ring formation, encodes a small EF-hand protein with properties of a myosin light chain (McCollum et al., 1995). Like cdc12p, cdc4p is also localized specifically to the actin ring. Our phenotypic analysis predicts that *cdc4* may be needed to bundle actin cables, or help anchor the cables to the membrane during ring formation.

There are certainly other gene products involved in actin ring assembly that we have not identified in this screen. Although near saturation, our screen was able to identify only those genes that could be mutated to give a temperature-sensitive elongated, multinucleate phenotype. Examples of proteins implicated in cytokinesis in other organisms, but not identified in our fission yeast screen, include actin (Mertins and Gallwitz, 1987), myosin II heavy chain, the septins (Sanders and Field, 1994), and a small GTPase such as rho (Kishi et al., 1993). It may be that mutations in these genes give a different phenotype, or that these genes may be functionally redundant.

Regulation of actin ring assembly in time

The assembly and subsequent contraction of the actin ring is under tight control in the cell cycle. The ring forms in early mitosis sometime around metaphase, although the exact timing is not well defined. The $p34^{cdc2}$/cyclin B mitotic kinase (MPF) may be involved in triggering ring formation. A *cdc2-33* mutant arrests in G_2 of the cell cycle with interphase actin distribution and no actin ring (Snell and Nurse, 1994). We show here that the actin ring is assembled in a β-tubulin mutant arrested in mitosis with high MPF activity (Moreno et al., 1989). This observation suggests that ring assembly may be triggered by MPF activity, not by the destruction of MPF.

plo1, a member of the polo protein kinase family, has been recently implicated as a link between MPF and actin ring formation in the cell cycle control of cell division (Ohkura et al., 1995). In fission yeast, loss of plo1p activity causes defects in both mitotic spindle formation and actin ring assembly. The actin and septation phenotypes are very similar to those found in *cdc15* mutants, suggesting that *plo1*and *cdc15* may interact. Remarkably, overexpression of *plo1* drives septation even in interphase cells, in the absence of MPF activity. Thus, the plo1 protein kinase may act downstream of MPF in activating actin ring formation.

Regulation of actin ring assembly in space

How is the contractile ring localized in the cell? In animal cells, the mitotic spindle is thought to position the cleavage furrow (see Rappaport, 1986; Strome, 1993). In fission yeast, we have shown by using a β-tubulin mutant that the actin ring forms normally even in the absence of the mitotic spindle. This finding is consistent with observations that a central actin ring may form in the presence of an interphase microtubule network and no mitotic spindle in a *cdc13-117* (cyclin B) mutant as it 'leaks' from G_2 into mitosis (Hagan, 1988) and in interphase cells driven to septate by the overexpression of *plo1* (Ohkura et al., 1995). Thus, in contrast to the situation in animal cells, the mitotic spindle is not required in actin ring positioning or formation in fission yeast.

An alternative model is that the position of the actin ring may be dictated by the position of the premitotic nucleus. The positions of the division site and the premitotic nucleus always correlate. In wild-type cells, both are positioned in the middle of the cell. In the septation mutant *cdc 11*, during the mitosis when two nuclei divide into four, two actin rings form at the positions of the two premitotic nuclei (Marks et al., 1986). In mutants with distorted patterns of growth, such as branched, round or monopolar mutants (Snell and Nurse, 1994; F. Chang, unpublished observations), the positions of the nucleus and the division site are still coupled. The medial positioning of the division site in some of these growth mutants makes it unlikely that the pattern of growth or a fixed point such as a previous division scar dictate the division site, as has been shown to be the case for bud site positioning in *S. cerevisiae* (Chant, 1994). Further, it has been shown in the hyphal fungus *Trametes versicolor* that displacement of the nucleus by micromanipulation displaces the site of the actin ring (Girbardt, 1979). Thus, one model is that a signal from the nucleus may position the ring.

The identification of *mid1* represents the first step in elucidating a molecular mechanism for positioning the division site in fission yeast. We have identified temperature-sensitive alleles of *mid1* that allow us to examine the initial effects of

disturbing *mid1*+ function. In the *mid1* mutant, the positions of the nucleus and actin ring are uncoupled, so while the nucleus is positioned normally in the middle, actin rings and septa form in random locations and angles on the cell surface. One possibility is that the *mid1* gene product may be involved in the signal between the nucleus and the ring. The *mid* phenotype is analogous to those of *bud* mutants in *S. cerevisiae* that exhibit random placement of the bud site (its subsequent division site) (Bender and Pringle, 1989; Chant and Herskowitz, 1991).

mid1 has been recently found to be allelic to the gene *dmf1* (data not shown; V. Simanis, personal communication), a gene isolated as an overexpression suppresser of the septation gene *cdc7* (V. Simanis, personal communication). A deletion allele of *mid1/dmf1* is viable and has similar defects in placement of division sites. *mid1/dmf1* has been cloned, and the sequence shows no similarity with any previously isolated gene (V. Simanis, personal communication).

Links between cell division with microtubule organizing centres and nuclear division

The post-anaphase microtubule organizing centres (MTOCs), which nucleate the interphase microtubule network following mitosis, normally form in the middle of cell just before septation (Hagan and Hyams, 1988). In the *mid1* mutant, both the division site and these MTOCs are misplaced together. This observation suggests that division site and microtubule organizing centres may be linked. However, septum formation is not necessary for MTOC formation, since they still form in septation mutants *cdc11* and *cdc14* (Hagan and Hyams, 1988). An intact actin ring also may not be necessary, since MTOCs form in some actin ring mutants (F. C., unpublished observations). Also, MTOCs are probably not required for actin ring formation, because the actin ring assembles before the MTOCs and also assembles in a β-tubulin mutant. Thus actin ring and the MTOCs may be positioned independently by a common signal.

The *mid1* phenotype also may reveal a cell cycle checkpoint control that coordinates nuclear and cell division. Many *mid1* cells with a displaced or abnormal septum do not complete cell division and exhibit cell cycle arrest or delay in telophase. No anucleate cells are produced. This cell cycle arrest suggests that there may be a checkpoint mechanism ensuring that mitosis has placed a nucleus on either side of the division site before cell separation commences. A similar checkpoint has also been demonstrated in *S. cerevisiae* dynein mutants, in which a defect in spindle orientation that causes the dividing nucleus not to enter into the daughter bud results in cell cycle arrest (Li et al., 1993; McMillan and Tatchell, 1994).

Link between the actin ring and formation of the septum

The two processes of actin ring formation and septum formation are coupled in that the actin ring marks the site of subsequent septum formation, and faulty assembly or placement of the actin ring leads to defects in septum formation or placement. Thus, actin or another contractile ring protein may nucleate the extracellular machinery required for septum cell wall biosynthesis. It is unlikely that actin itself recruits the septum machinery, for two reasons. First, although all the actin ring mutants exhibit actin structures on the cell surface, they exhibit different patterns of septum deposition, ranging from chunks in *cdc3* to no staining in *cdc15* and *cdc8* mutants. Second, in mutants such as *cdc3* the abnormal septum depositions are restricted to the middle region of the cell, even though actin is delocalized throughout the cortex. Thus, another contractile ring protein close to the membrane may be involved in recruiting the extracellular septum machinery. A possible candidate is *cdc15*, since no *cdc15* mutant exhibits any Calcofluor staining. The distinction between cell division mutants based on septum formations may not signify 'early versus late' or 'actin versus not actin' properties as previously suggested (Nurse et al., 1976; Balasubramanian et al., 1994; Fankhauser and Simanis, 1994), but may reflect the presence or absence of a distinct factor involved in recruiting the septum machinery.

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