Mycoscience (2005) 46:85–89
DOI 10.1007/s10267-004-0216-6

FULL PAPER

Atsuhiro Suda · Kuniko Kusama-Eguchi · Yoshio Ogawa
Kazuko Watanabe

Novel actin ring structure in sporulation of *Zygosaccharomyces rouxii*

Received: August 23, 2004 / Accepted: November 24, 2004

Abstract The filamentous actin (F-actin) during sporulation of *Zygosaccharomyces rouxii* was visualized with rhodamine-phalloidin, and then the behavior was observed using confocal laser scanning microscopy. During spore formation, we found a novel actin ring structure that has not been reported in other yeasts and molds in sporulation. The ring surrounded each meiotic nucleus at the peripheral regions of spores. Three-dimensional observation suggested that the ring was not an artificial structure produced by spherical structure sectioning. The period and location of the ring's appearance suggest that the ring may have some relation to the spore membrane or wall development. In addition, this ring structure was more stable than other F-actin structures against latrunculin A, an F-actin disrupting agent.

Key words Actin ring · Filamentous actin · Latrunculin A · Sporulation · *Zygosaccharomyces rouxii*

Introduction

Filamentous actin (F-actin) is an essential cytoskeletal protein that plays an important role in the cell division of yeasts. Vigorous studies have been made to clarify the structural changes and functions of F-actin that correspond to the various stages of cell division (reviewed by Pruyne and Bretscher 2000; Schott et al. 2002). Regarding meiosis and subsequent spore formation, several F-actin structures have been reported. Smith et al. (1995) observed actin patches and cables that developed in the cortical region of *Saccharomyces cerevisiae* Meyen ex E.C. Hansen during the early stages of meiosis. Using mutants with the actin gene, they demonstrated that these F-actin patches and cables were closely related to the rearrangement of mitochondria. Doyle and Botstein (1996) showed that cortical actin patches remained in spores throughout spore formation. Petersen et al. (1998) and Toya et al. (2001) indicated that, in *Schizosaccharomyces pombe* Linder, F-actin appeared as concentrated dots at the projection tip during cell conjugation and karyogamy, around the nucleus during meiosis, and within the spores during spore maturation. In addition to these structures of F-actin, Thompson–Coffe and Zickler (1993, 1994) found a cage structure of F-actin in *Sordaria macrospora* Auersw and *Neurospora crassa* Shear & B.O. Dodge during their ascospore formation. They inferred that this cage structure was closely related to spore formation of these fungi because the cage enclosed the spore at the late stage of sporulation.

We investigated the behavior of F-actin during sporulation in an ascomycetous yeast *Zygosaccharomyces rouxii* (Boutroux) Yarrow to compare with that in other yeasts and molds which have already been reported. During that study, we discovered a novel ring structure that surrounded four nuclei resulting from the meiosis, but its function remains unclear. The present study describes the process of formation of this ring structure. Furthermore, the nature of this ring structure is discussed on the basis of the effect of latrunculin A, an F-actin-disrupting agent, on the ring.

Materials and methods

Two strains of *Z. rouxii* a (IFO 1876) and α (IFO 1877), which differ from one another in the mating types, were used for the present study. NaCl was supplied to all media because vegetative growth is enhanced by the presence of NaCl (Watanabe and Takakuwa 1984) and because sporulation only occurs in environments containing NaCl (Wickerham and Burton 1960; Mori and Onishi 1967). First, *Z. rouxii* a and α were cultured separately in growth medium (YPDN; 1% yeast extract, 2% peptone, 2% glucose, and 5% NaCl) at 25°C. When both cultures reached

A. Suda (✉) · K. Kusama-Eguchi · Y. Ogawa · K. Watanabe
College of Pharmacy, Nihon University, 7-7-1 Narashinodai, Funabashi, Chiba 274-8555, Japan
Tel. +81-47-465-3794; Fax +81-47-465-3794
e-mail: sudaatsu@pha.nihon-u.ac.jp

the early stationary phase, we mixed the cells of a and α in sporulation medium (MEN; 5% malt extract and 5% NaCl). The mixture was placed onto an MEN agar plate because *Z. rouxii* sporulation was not induced in the liquid medium. After about 48 h, we were able to observe spores in a zygotic ascus.

Filamentous actin was stained by the modified method described by Balasubramanian et al. (1997). The cells under sporulation were fixed in 3.5% paraformaldehyde for 2 h at 25°C. After fixation and washing with phosphate-buffered saline, the cells were permealized in 1% Triton X-100 solution for 1 min and were washed immediately. Permealized cells were then incubated in 0.075 μM rhodamine-phalloidin (Molecular Probes, Eugene, OR, USA) for 2 h at 25°C. The cells were washed and stained using 10 μM TO-PRO 3 (Molecular Probes), a fluorescent dye to stain nuclear DNA, in Fluoroguard antifade reagent (Bio-Rad Laboratories, Hercules, CA, USA). Fluorescence images were then taken with a confocal laser scanning microscope (LSM510; Carl Zeiss, Jena, Germany) equipped with a 100× oil immersion objective (Zeiss Plan-Neofluar; NA = 1.3). Rhodamine fluorescence was excited and detected using a 543-nm He/Ne laser and a 560- to 615-nm bandpass emission filter, respectively. TO-PRO3 fluorescence was excited and detected using a 633-nm He/Ne laser and a 650-nm long-pass emission filter, respectively. Images were taken as optical sections (0.2 to 0.3-μm intervals) through the entire cell and then projected onto a single image. Although TO-PRO3 exhibited deep red fluorescence ordinarily, the color was converted to light blue to distinguish it from rhodamine fluorescence. Differential interference contrast (DIC) images were taken simultaneously. To show the three-dimensional structure of F-actin in detail, the acquired optical section images were reconstructed to three-dimensional rotation images. Then, the reconstructed images were observed from various angles (30° intervals) by rotation. All images were converted into TIFF files and processed with Adobe Photoshop 7.0.

In the treatment of latrunculin A (Lat A), the cells under the sporulation on an MEN agar plate were suspended in MEN broth. Then, Lat A (Wako Pure Chemical, Tokyo, Japan) dissolved in dimethyl sulfoxide (DMSO) was added to the suspension so that the final concentration of Lat A became 200 μM. These cells were incubated for 5 and 15 min after Lat A addition at 25°C and stained by rhodamine-phalloidin and TO-PRO3. Images of the stained cells were then acquired by confocal laser scanning microscopy as already described. The parameters used for the creation of an image were kept uniform to compare rhodamine brightness among images of different incubation times.

Results and discussion

Figure 1 shows the process of sporulation of *Z. rouxii*. Because the cell cycle was not synchronized, cells of various stages were observed at the same time using confocal microscopy observation. The stages of cells were determined from nucleus behavior and DIC images. Actin dynamics throughout sporulation were followed by visualization with rhodamine-phalloidin. Shmoo, a peculiar structure for the conjugation, proliferated from a cell of each mating type to that of another mating type (Fig. 1.1). We easily recognized that dense actin patches are localized at the growing tip of the shmoo (Fig. 1.3). After conjugation, the two nuclei came close to each other through the isthmus of the zygote (Fig. 1.6); many actin patches occurred at the isthmus region (Fig. 1.7). Thereupon, these nuclei became a single nucleus through karyogamy, creating a diploid nucleus (Fig. 1.10). In this step, relatively small actin patches seemed to be scattered all over the zygote and the actin cables expanded along the longitudinal cell axis (Fig. 1.11). As the nucleus divided into four (Fig. 1.14), actin patches disappeared and faint F-actin distributed all over the zygote (Fig. 1.15). When the nucleus division finished (Fig. 1.18), bright actin dots appeared near each nucleus (Fig. 1.20). The bright dots of F-actin around each nucleus became more numerous (Fig. 1.24). When the spore shape became visible in the DIC image (Fig. 1.25), ring structures of F-actin appeared around each nucleus (Fig. 1.28). Observations of DIC and F-actin images suggest that the rings usually appear at the peripheral region of spores without changing their diameter dynamically. In addition to the ring structure, a few actin patches were located near the rings. With spore maturation (Fig. 1.29), the ring structures disappeared and actin patches increased (Fig. 1.31).

The ring structure is not a section of spherical structure of F-actin. It was shown to be a prominent ring structure because images were taken as optical sections (0.2 to 0.3-μm intervals) through the entire cell and then projected onto a single image using confocal microscopy. Furthermore, three-dimensional images also indicate this fact. Figure 2 shows three-dimensional images of the actin ring viewed from various angles. These images suggest that the ring structure is not an artifact caused by observation of the single section of the images (Fig. 2).

The role of the actin ring is obscure. The period and location of the appearance of the ring suggest that the ring may be related to spore membrane or wall development. Alternatively, the ring may have the function of arranging mitochondria around spore nuclei. Miyakawa et al. (1984) found that mitochondria distribute as a ring structure in the sporulation of *S. cerevisiae*. The position of the mitochondrial ring appears to be consistent with the observed actin ring in *Z. rouxii*.

The actin ring that occurred during sporulation seems to be characteristic of *Z. rouxii*. So far, an actin ring structure in yeasts has been shown in mitosis, but not in sporulation. In vegetatively growing cells, the actin ring appears at the division site in *S. cerevisiae* (Kilmartin and Adams 1984; Epp and Chant 1997; Bi et al. 1998; Lippincott and Li 1998) and *S. pombe* (Marks and Hyams 1985; Kitayama et al. 1997; Arai and Mabuchi 2002). These actin rings, which include myosin II protein, serve to separate these cells by constricting the ring (cytokinesis). The ring structure observed in the present study is distinct from them in both period of occurrence and function. In filamentous ascomyc-

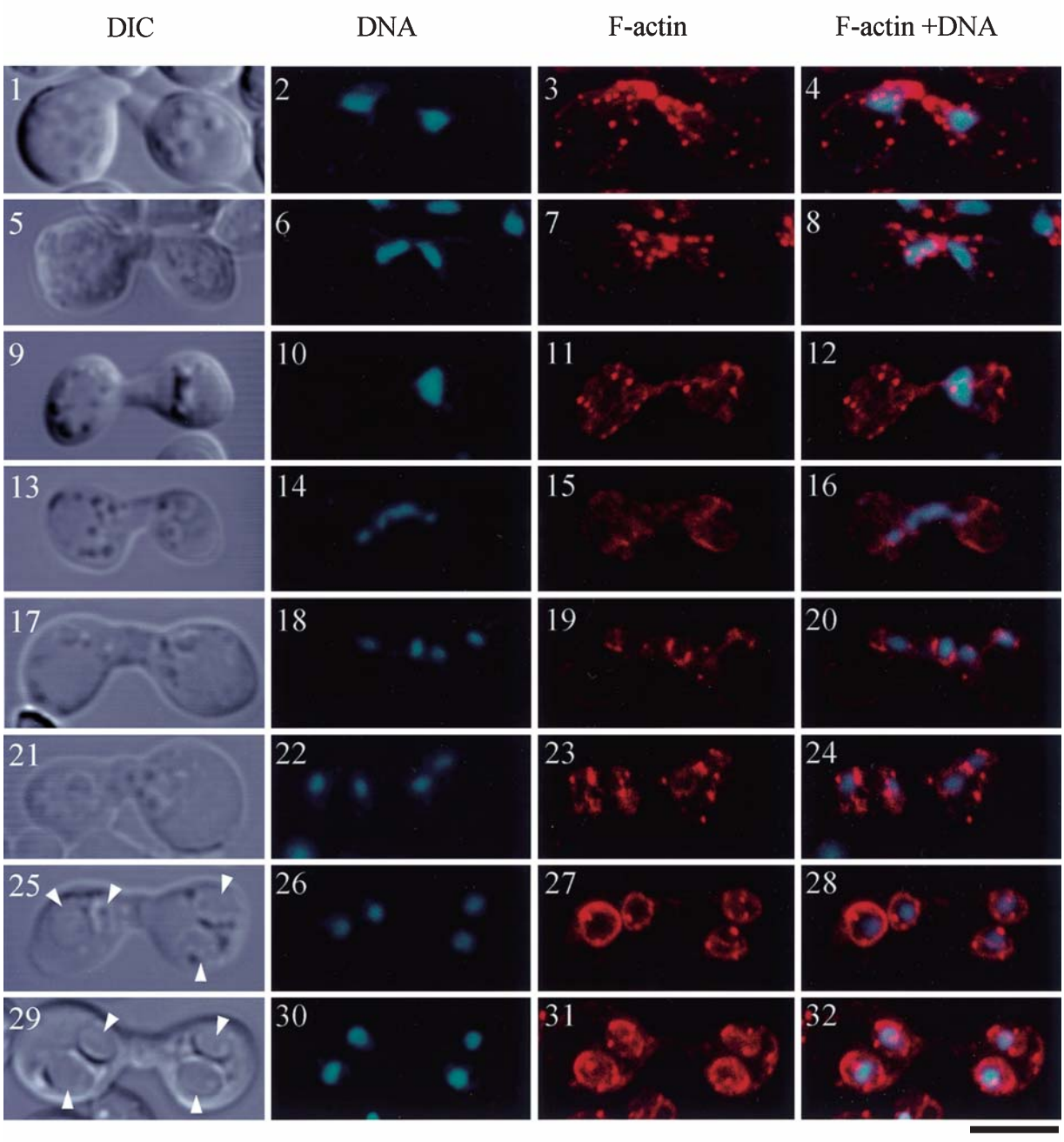

Fig. 1. F-actin localization in sporulation of *Zygosaccharomyces rouxii*: the *first column* shows differential interference contrast (DIC) images; the *second column* shows nuclear DNA stained by TO-PRO3 (*light blue*); the *third column* shows F-actin stained by rhodamine-phalloidin (*red*); and the *fourth column* shows the merged images of the second and third column. Although TO-PRO3 indicates deep red fluorescence ordinarily, the color is converted to light blue. *Z. rouxii* a and α were mixed and placed on an MEN agar plate; then mating and meiosis continued. The cells were then fixed and stained as described in Materials and methods. The images, taken as optical sections, were projected onto a single image. **1–4** indicate zygote formation, **5–8** and **9–12** indicate karyogamy, **13–16** and **17–20** indicate meiotic division, **17–20** and **21–24** indicate spore formation, and **25–28** and **29–32** indicate spore maturation. In DIC images, roughness that appears on the surface of zygotes does not indicate the nucleus position. *Arrowheads* in **25** and **29** indicate spores in the zygotic ascus. *Bar* 5 μm

etous *S. macrospora* and *N. crassa*, the actin cytoskeleton formed a cage structure that surrounds nascent spores with fine actin cables (Thompson-Coffe and Zickler 1993, 1994). The cage structure possesses a ringlike actin structure at the edge of the spore. This ring appears to separate each spore by narrowing it in a similar manner to the mitotic actin rings in these yeasts. The actin ring in *Z. rouxii* seems not to narrow the diameter. Therefore, it also differs from the rings observed in *S. macrospora* and *N. crassa*.

Distributions of actin patches in *Z. rouxii* appear to bear some similarities to those in *S. cerevisiae* (Hašek et al. 1987; Read et al. 1992; Smith et al. 2001; Doyle and Botstein 1996)

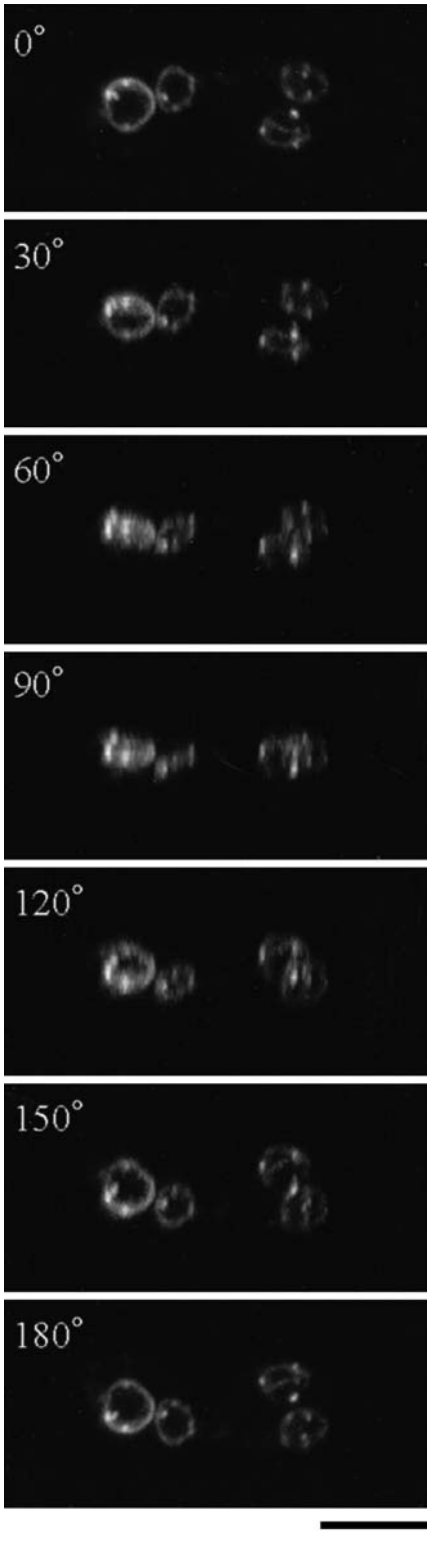

Fig. 2. A three-dimensional image of the actin ring structure. The optical sectioning images were projected onto three-dimensional images. The projected images were rotated at 30° intervals. This image was derived from the same cell image indicated on Fig. **1.25–28**. *Bar* 5 μm

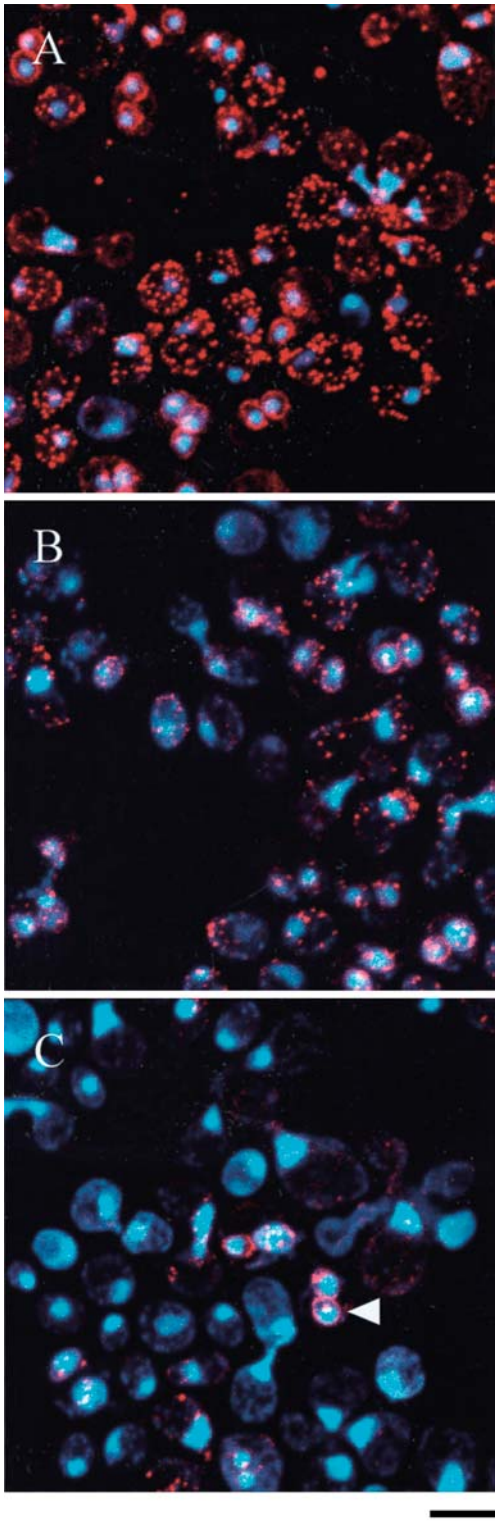

Fig. 3. The effect of latrunculin A (Lat A) on the actin cytoskeleton. Zygote cells under sporulation were suspended in MEN broth, fixed and stained with rhodamine-phalloidin (F-actin, *red*) and TO-PRO3 (nuclear DNA, *light blue*) before treatment (**A**), after 5 min (**B**), and after 15 min (**C**) of Lat A treatment. The *arrowhead* in **C** indicates actin ring structures. *Bar* 5 μm

and *S. pombe* (Petersen et al. 1998; Toya et al. 2001). For instance, actin patches were localized at projection tips, at the zygotic isthmus, and around divided nuclei.

We examined the effect of Lat A, an F-actin-disrupting agent, on the ring structure to elucidate the structure's characteristics. Figure 3 shows the effect of Lat A on the F-actin of *Z. rouxii* cells under sporulation. Because the cell stage was not synchronized, cells of various stages were observed in a single image. The F-actin, before Lat A treatment, was stained brightly using rhodamine-phalloidin (Fig. 3A). After 5 min of Lat A treatment, the brightness of F-actin patches and cables decreased remarkably (Fig. 3B). After 15 min, their brightness decreased further, but the brightness of actin rings around the nuclei remained (Fig. 3C). In the control treatment, the F-actin was stained as in the cells seen before Lat A treatment (data not shown).

Although most actin patches and cables were disrupted immediately by Lat A treatment, the actin ring showed more stability than other actin structures. A possible explanation for this fact is that the ring structure possesses a property by which the turnover rate of F-actin is lower than that of other actin structures. Lat A inhibits polymerization of actin monomer by binding and sequestering the monomer (Coué et al. 1987; Ayscough et al. 1997). Therefore, actin structures with a high turnover rate are disrupted by Lat A because new actin monomers are not supplied by the effect of Lat A. In contrast, actin structures with a low turnover rate are not disrupted readily by Lat A because the structure is maintained without the supply of new actin monomer. The low turnover rate may engender the stability of the ring against Lat A effect. Another explanation is that the spore membrane, and especially the wall, interfered with Lat A permeation into the spores. The spore wall in budding yeast has been shown to be a dense and multilayered structure different from the cell wall (Lynn and Magee 1970). Thus, it may be difficult for Lat A to reach and affect the actin ring through the spore wall.

This study analyzed the F-actin behavior of *Z. rouxii* forming the zygotic ascus. We found a characteristic actin ring structure in the process. In the zygotic ascus, four spores locate around the same plane. Consequently, they do not overlap each other. This feature may allow observation of detailed actin distribution and enable finding of a novel actin ring during sporulation. From this perspective, it is possible that *Z. rouxii* offers an advantage for analyzing the behavior of actin cytoskeleton during spore morphogenesis.

Acknowledgments We thank Ms. M. Ohtomo, Mr. N. Ohshima, Ms. H. Usagawa, and Mr. Y. Takahashi for their technical support. This work was supported in part by "Academic Frontier" Project for Private Universities: matching fund subsidy from MEXT (Ministry of Education, Culture, Sports, Science and Technology), 2002–2006.

References

Arai R, Mabuchi I (2002) F-actin ring formation and the role of F-actin cables in the fission yeast *Schizosaccharomyces pombe*. J Cell Sci 115:887–898

Ayscough KR, Stryker J, Pokala N, Sanders M, Crews P, Drubin DG (1997) High rates of actin filament turnover in budding yeast and roles for actin in establishment and maintenance of cell polarity revealed using the actin inhibitor latrunculin-A. J Cell Biol 137:399–416

Balasubramanian MK, McCollum D, Gould KL (1997) Cytokinesis in fission yeast *Schizosaccharomyces pombe*. Methods Enzymol 283:494–506

Bi E, Maddox P, Lew DJ, Salmon ED, McMillan JN, Yeh E, Pringle JR (1998) Involvement of an actomyosin contractile ring in *Saccharomyces cerevisiae* cytokinesis. J Cell Biol 142:1301–1312

Coué M, Brenner SL, Spector I, Korn ED (1987) Inhibition of actin polymerization by latrunculin A. FEBS Lett 213:316–318

Doyle T, Botstein D (1996) Movement of yeast cortical actin cytoskeleton visualized in vivo. Proc Natl Acad Sci USA 93:3886–3891

Epp JA, Chant J (1997) An IQGAP-related protein controls actin-ring formation and cytokinesis in yeast. Curr Biol 7:921–929

Hašek J, Rupeš I, Svobodová J, Streiblová E (1987) Tubulin and actin topology during zygote formation of *Saccharomyces cerevisiae*. J Gen Microbiol 133:3355–3363

Kilmartin JV, Adams AEM (1984) Structural rearrangements of tubulin and actin during the cell cycle of the yeast *Saccharomyces*. J Cell Biol 98:922–933

Kitayama C, Sugimoto A, Yamamoto M (1997) Type II myosin heavy chain encoded by the myo2 gene composes the contractile ring during cytokinesis in *Schizosaccharomyces pombe*. J Cell Biol 137:1309–1319

Lippincott J, Li R (1998) Sequential assembly of myosin II, an IQGAP-like protein, and filamentous actin to a ring structure involved in budding yeast cytokinesis. J Cell Biol 140:355–366

Lynn RR, Magee PT (1970) Development of the spore wall during ascospore formation in *Saccharomyces cerevisiae*. J Cell Biol 44:688–692

Marks J, Hyams JS (1985) Localization of F-actin through the cell-division cycle of *Schizosaccharomyces pombe*. Eur J Cell Biol 39:27–32

Miyakawa I, Aoi H, Sando N, Kuroiwa T (1984) Fluorescence microscopic studies of mitochondrial nucleoids during meiosis and sporulation in the yeast, *Saccharomyces cerevisiae*. J Cell Sci 66:21–38

Mori H, Onishi H (1967) Diploid hybridization in a heterothallic haploid yeast, *Saccharomyces rouxii*. Appl Microbiol 15:928–934

Petersen J, Nielsen O, Egel R, Hagan IM (1998) F-actin distribution and function during sexual differentiation in *Schizosaccharomyces pombe*. J Cell Sci 111:867–876

Pruyne D, Bretscher A (2000) Polarization of cell growth in yeast. II. The role of the cortical actin cytoskeleton. J Cell Sci 113:571–585

Read EB, Okamura HH, Drubin DG (1992) Actin- and tubulin-dependent functions during *Saccharomyces cerevisiae* mating projection formation. Mol Biol Cell 3:429–444

Schott D, Huffaker T, Bretscher A (2002) Microfilaments and microtubules: the news from yeast. Curr Opin Microbiol 5:564–574

Smith MG, Simon VR, O'Sullivan H, Pon LA (1995) Organelle-cytoskeletal interactions: actin mutations inhibit meiosis-dependent mitochondrial rearrangement in the budding yeast *Saccharomyces cerevisiae*. Mol Biol Cell 6:1381–1396

Smith MG, Swamy SR, Pon LA (2001) The life cycle of actin patches in mating yeast. J Cell Sci 114:1505–1513

Thompson-Coffe C, Zickler D (1993) Cytoskeleton interactions in the ascus development and sporulation of *Sordaria macrospora*. J Cell Sci 104:883–898

Thompson-Coffe C, Zickler D (1994) How the cytoskeleton recognizes and sorts nuclei of opposite mating type during the sexual cycle in filamentous ascomycetes. Dev Biol 165:257–271

Toya M, Motegi F, Nakano K, Mabuchi I, Yamamoto M (2001) Identification and functional analysis of the gene for type I myosin in fission yeast. Genes Cells 6:187–199

Watanabe Y, Takakuwa M (1984) Effect of sodium chloride on lipid composition of *Saccharomyces rouxii*. Agric Biol Chem 48:2415–2422

Wickerham LJ, Burton KA (1960) Heterothallism in *Saccharomyces rouxii*. J Bacteriol 80:492–495