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Article

A new F-actin structure in fungi: actin ring formation around the cell nucleus of *Cryptococcus neoformans*

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Abstract The F-actin cytoskeleton of *Cryptococcus neoformans* is known to comprise actin cables, cortical patches and cytokinetic ring. Here, we describe a new F-actin structure in fungi, a perinuclear F-actin collar ring around the cell nucleus, by fluorescent microscopic imaging of rhodamine phalloidin-stained F-actin. Perinuclear F-actin rings form in *Cryptococcus neoformans* treated with the microtubule inhibitor Nocodazole or with the drug solvent dimethyl sulfoxide (DMSO) or grown in yeast extract peptone dextrose (YEPD) medium, but they are absent in cells treated with Latrunculin A. Perinuclear F-actin rings may function as 'funicular cabin' for the cell nucleus, and actin cables as intracellular 'funicular' suspending nucleus in the central position in the cell and moving nucleus along the polarity axis along actin cables.

Keywords perinuclear F-actin ring, nucleus, *Cryptococcus neoformans*

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Introduction

The actin cytoskeleton of *Cryptococcus neoformans* consists of cables, patches and cytokinetic ring. The cables project from the mother cell to the daughter. The patches are located at the mother's cortex, at the apex of the growing stalk, at the growing bud and at the daughter's cortex. Before mitosis, cytoplasmic microtubules disappear, and the nucleus migrates from the mother through a neck to the daughter for unknown reasons by an unknown mechanism. The mitotic spindle originates eccentrically positioned in the nucleus that has migrated to the daughter cell for mitosis. During mitosis, one nucleus is delivered to the mother and the other remains in the daughter. Mitosis is followed by cytokinesis that proceeds with the actin cytokinetic ring and with septum formation in the bud neck [1].

Recently, we started investigation of actin and microtubule inhibitors in *Cryptococcus neoformans*. We are investigating their ability to block yeast proliferation and induce cell death and whether resistant cells appear among inhibited cells and how to prevent the birth of resistant cells. In the related yeast *Fellomyces fuzhouensis* [2] treated with these cytoskeleton inhibitors [3,4], we discovered perinuclear F-actin rings [5]. During this line of experimentation, we noticed perinuclear F-actin rings also in *Cryptococcus neoformans* inhibited with Nocodazole or with DMSO or cells grown in YEPD medium.

This paper describes the first demonstration of an F-actin perinuclear ring collars in the human yeast pathogen *Cryptococcus neoformans* by fluorescent microscopy.

Materials and methods

Yeast strains

The human pathogen strains *Cryptococcus neoformans* IFM 41464 (serotype A, haploid) and 40042

(serotype AD, diploid) were from Medical Mycology Research Center, Chiba University, Japan [1,6].

Media, cell cultivation and application of cytoskeleton inhibitors and DMSO

The strains were maintained on 2.0% (w/v) agar containing YEPD medium [1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) glucose] [7] at laboratory temperature. For experiments, cells were inoculated to 1% YEPD medium and cultivated on a shaker at 23°C, usually overnight. The same dilution of 10^6 cells ml^{-1} was used [5]. The inhibitors, Nocodazole (Fluka) and Latrunculin A (Molecular Probes, Eugene, Oregon, USA), were dissolved in DMSO (1% final concentration). Stock solutions were prepared as 10 mM in DMSO, stored at –20°C and added to a 100 μM concentration to 1% YEPD medium. Control samples without DMSO and with DMSO were used with 1 and 5% concentrations. All other procedures were done as we reported [5].

Fixation for fluorescent microscopy, nuclear staining, phase-contrast and fluorescence microscopy was done as reported [5].

Cell proliferation was determined by measuring the absorbance of a suspension of cells at 590 nm.

Freeze-substitution electron microscopy

The cells were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C at Medical Faculty in Brno, Czech Republic and sent by airmail for freeze-substitution electron microscopy to Chiba University, Japan. The cells were collected by centrifugation (12 000 rpm, 3 s), sandwiched between two copper disks and snap-frozen with melting propane kept in liquid nitrogen. After freeze-substitution in acetone containing 2% osmium tetroxide at –80°C, they were embedded in epoxy resin, ultrathin sectioned and observed in a JEM-1400 electron microscope (JEOL, Tokyo, Japan) [8,9].

Results

Fluorescent microscopy

4,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining was used with fluorescence microscopy to visualize cell nuclei. Microtubules were detected using an anti-α tubulin monoclonal antibody, and rhodamine-phalloidin staining was used to detect the F-actin cytoskeleton consisting of actin cables, actin patches and cytokinetic ring [1].

However, in cells from the exponential culture, we discovered F-actin rings around the nuclei. Because the rings were stained with rhodamine phalloidin, they must contain F-actin; therefore, we used the term 'perinuclear F-actin ring' for this new F-actin cytoskeleton structure.

We observed F-actin rings around the cell nuclei of haploid and diploid strains *Cryptococcus neoformans* in three different conditions.

First finding: The F-actin ring (Fig. 1a) around the cell nucleus (Fig. 1b) was observed in cells that were in exponential phase growth in YEPD medium after 20 h. While many cells had F-actin cables, patches and cytokinetic rings, only very few cells had a visible F-actin ring around the nucleus.

Second finding: F-actin rings around the cell nuclei were visible in control cells cultivated in YEPD medium containing 5% DMSO after 24 h. Cells had actin cytoskeleton structures and a visible F-actin perinuclear ring (Fig. 1c, e and g) around the nucleus (Fig. 1d, f and h).

The F-actin rings are likely localized near the external surface of the nucleus, as double labeling of the nuclei with DAPI staining and F-actin with rhodamine-phalloidin suggested that the F-actin rings localize to the periphery of the nucleus. Each cell nucleus contained only a single F-actin ring. The rings were in different planes, but we were able to detect only those rings that were positioned in the plane parallel to the glass.

When we investigated the occurrence of F-actin rings during growth with 5% DMSO, we detected the actin rings after 24, 48, 96 and 144 h. However, after 11 days, cells were already dead, and no actin rings occurred in empty cell walls remaining from dead cells; in contrast, perinuclear actin rings (PARs) occurred in alive control cells in YEPG medium after 11 days.

We were interested to know whether 5% DMSO influences cell proliferation. Therefore, we measured the optical density (OD) of cell samples during 6 days of cultivation. At the beginning of the experiment, all cell samples had OD at $T_0 = 0.005$. After 1 day of cultivation, cells with 5% DMSO had an OD of 0.55, whereas the control cells had a

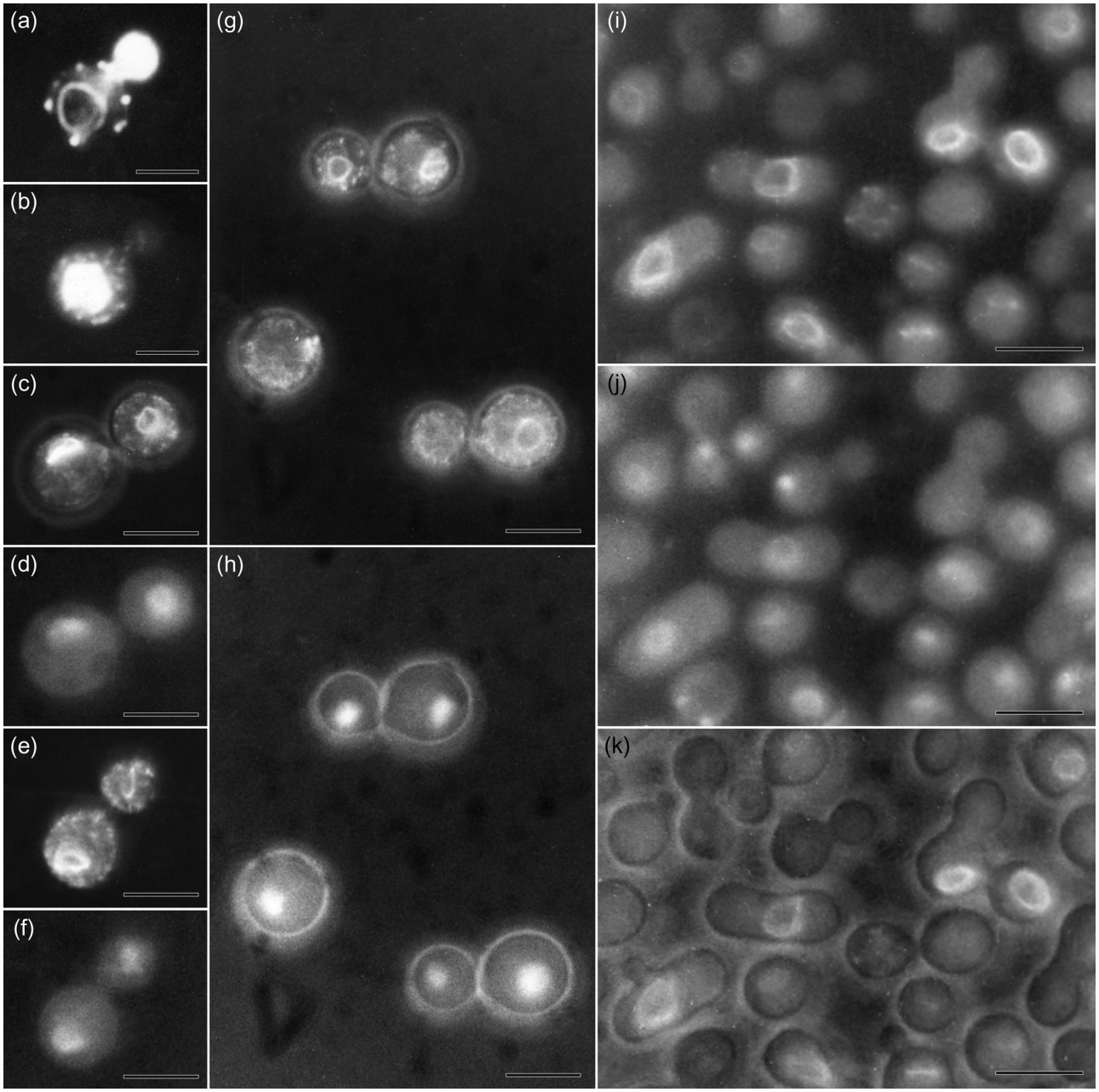

Fig. 1 Fluorescent microscopy of *Cryptococcus neoformans*. (a and b) *C. neoformans* IFM 41464 cells were cultured in 1% YEPD for 20 h. The actin cytoskeleton detected by rhodamine-phalloidin staining revealed PARs (a) and the nuclei were detected by DAPI (b). Scale bar = 10 µm. (c–h) *C. neoformans* IFM 40042 cells were cultured in 1% YEPD with 5% DMSO for 24 h. Nuclei were detected by DAPI (d, f, h), and the actin cytoskeleton detected by rhodamine-phalloidin staining also revealed PARs (c, e, g). G and H – Images acquired by both phase contrast and actin and DAPI fluorescence microscopy. Scale bar = 10 µm. (i–k) Cells were cultured in YEPD medium with 100 µM Nocodazole containing 1% DMSO for 6 days. The actin cytoskeleton was detected by rhodamine-phalloidin staining, and PARs are shown (i and k). Nuclei were detected by DAPI (j). Scale bar = 10 µm.

higher OD of 0.75. After 2 days, cells with 5% DMSO had an OD of 1.15, whereas the control cells had a higher OD of 1.25. After 4 days, cells with 5% DMSO had an OD of 1.25, whereas the control cells had a higher OD of 1.35. After 6 days, proliferation of cells with 5% DMSO and control cells reached a maximum OD of 1.5 (Fig. 2). These results show that proliferation of cells in 5% DMSO is slower than in control cells without DMSO.

Third finding: The F-actin rings (Fig. 1i and k) around the cell nucleus (Fig. 1j) occurred also in exponentially growing cells cultured in YEPD

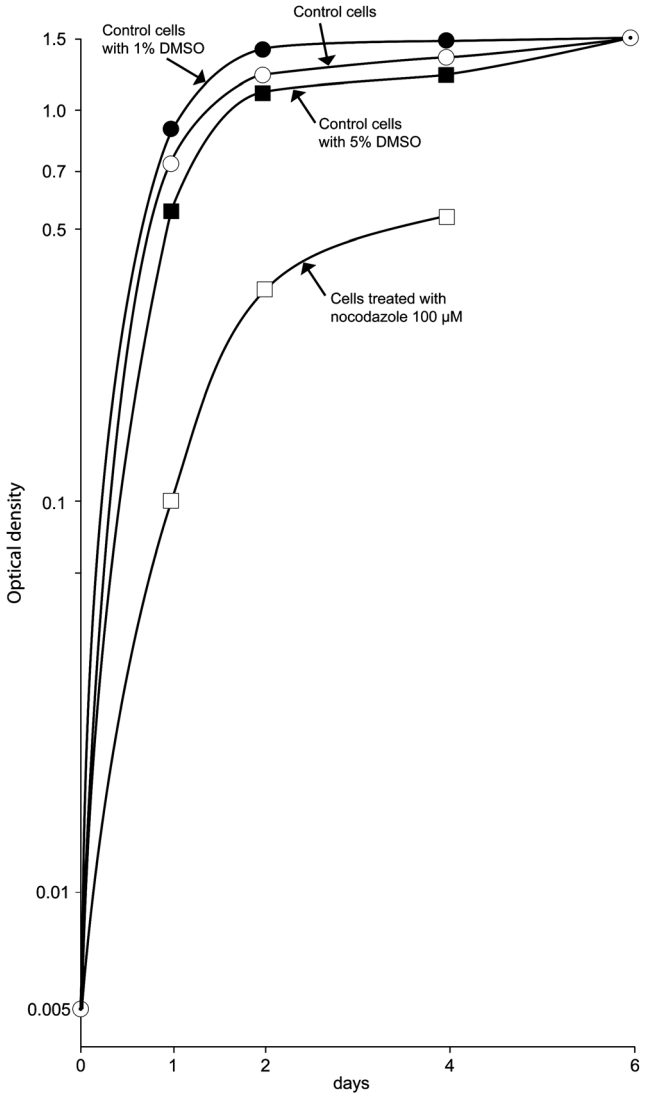

Fig. 2 OD of *Cryptococcus neoformans* cultures measured as absorbance.

medium and treated with the microtubule inhibitor Nocodazole at a 100 µM concentration that also delayed cell proliferation (Fig. 2). Actin rings were identical to those observed in cells treated with 5% DMSO alone (Fig. 1c, e and g). Also, the dynamics of occurrence was the same. They were visible in about 50% of cells (Fig. 1i and k). In contrast, control cells with a final DMSO concentration of 1% without Nocodazole did not reveal actin rings, and cells corresponded to control cells in YEPD (first finding).

Fourth finding: After treatment with the actin inhibitor, 100 µM Latrunculin A, all actin structures disappeared. From this observation, we deduce that Latrunculin A also disrupts perinuclear rings containing F-actin.

Freeze-substitution electron microscopy

We used transmission electron microscopy of freeze-substituted cells to investigate perinuclear F-actin rings at ultrastructural level. *Cryptococcus neoformans* cells had in electron micrographs typical fungal ultrastructure: many mitochondria with cristae, endoplasmic reticulum, vacuoles, plasma membranes, ribosomes, 'wall' vesicles, cell walls and spindle pole bodies, as we described previously [1,10]. Figure 3 shows ultrathin sections of cells treated with 5% DMSO for 5 days in YEPD medium, in which perinuclear F-actin rings around nucleus were clearly observed by fluorescent microscopy. Although many organelles, such as nucleus, nucleolus, nuclear membrane, endoplasmic reticula and mitochondria, were observed in the micrographs, no filamentous structures that may correspond to F-actin filaments of PARs were observed around the cell nuclei. It is possible that electron microscopy may not be able to detect 7-nm F-actin filament embedded in other cell components in ultrathin section (e.g., in ribosomes or nuclear membrane), whereas fluorescent microscopy is capable of detecting a few molecules of F-actin filament.

Discussion

This paper demonstrates for the first time a new F-actin cytoskeleton structure in the human pathogenic yeast *Cryptococcus neoformans* – an F-actin ring associated with the nucleus – by fluorescence microscopy of F-actin after rhodamine-phalloidin staining. Double labeling of F-actin and the cell nucleus for fluorescent microscopy demonstrated the localization of the actin ring to the periphery of the nucleus. The exact molecular mechanism that governs the localization of PARs is unknown.

Which *Cryptococcus neoformans* cells have PARs around the nucleus?

We think that all *Cryptococcus neoformans* cells may have PARs, but PARs are hardly visible in control cells rich in actin cytoskeleton patches and cables because of the random positioning of actin rings in different focal planes within cells. Only those PARs oriented in a position parallel to the glass can be detected by fluorescent microscopy.

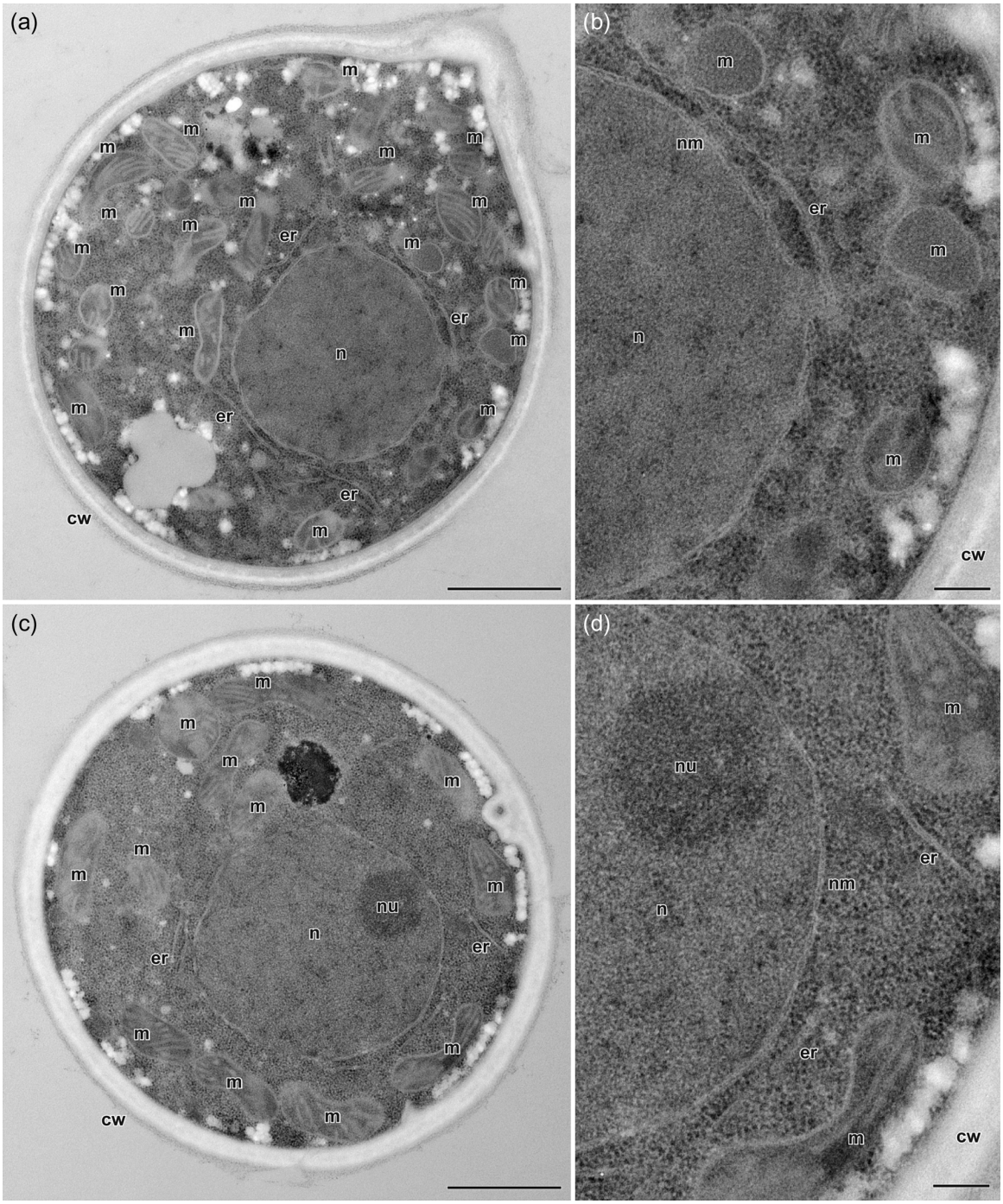

Fig. 3 Ultrathin sections of freeze-substituted *Cryptococcus neoformans*. Cells of IFM 41464 (a and b) and IFM 40042 (c and d) were cultivated in YEPD medium with 5% DMSO for 5 days at 23°C. cw, cell wall; er, endoplasmic reticulum; m, mitochondria; n, nucleus; nm, nuclear membrane.

Detection of PARs by electron microscopy is even more difficult because of their random positioning in the cells and random cutting of ultrathin sections through a cell that can contain many cross-sectioned actin filaments, hardly detectable, while tangential sections through PARs are very scarce. It is a problem in analogy to looking for actin cables [1,11]. It is possible that electron microscopy may not be able to detect 7-nm F-actin filament embedded in other cell components in ultrathin section, whereas fluorescent microscopy is capable of detecting a few molecules of F-actin filament.

Are PARs around the cell nuclei present in other organisms and what are their functions?

PARs occur in the yeasts: *Fellomyces fuzhouensis* [5] and in *Schizosaccharomyces pombe* mutant *ben4* (cold-treated arrested at mitotic prophase), where PARs may in fact be actin cytokinetic rings [12]. PARs also occur in the filamentous fungi: *Saprolegnia ferax* treated with cytochalasin E, in the mite-infecting zygomycete *Neozygites sp.* [13] and in the plant pathogen *Uromyces phaseoli* [14]. In algae, they are related to nuclear migration and nuclear rotation [15]. In human breast adenocarcinoma cells treated with taxol and taxothere, PARS can be related to apoptosis [16].

What hypothetical functions can PARs have in *Cryptococcus neoformans*?

(i) We observed PARs in *Cryptococcus neoformans* joined to cytoplasmic actin cables. This indicates that actin cytoskeleton cables may function as intracellular 'funicular' or suspensor apparatus for cell nucleus in the central position in the cell and for nuclear movement by means of PARs functioning as 'funicular cabin'or a 'horse collar' to draw the nucleus along the polarity axis along actin cables from the mother to the daughter, when cytoplasmic microtubules disappear; unfortunately, experimental data for such a function are still lacking. However, Wasteneys and Williamson [15] suggested in algal cells a similar role for the F-actin ring around the nucleus in nuclear migration and nuclear rotation. We identified nuclear abnormalities in the actin mutant of budding yeast *act1-1* [17]: eccentrically placed nuclei and affected nuclear migration to the bud neck, which caused deficient actin cables, which in turn affected the actin-dependent pathway in the cell cycle [11]. This led us to relate actin cables and PARs to nuclear positioning and movement also in *Cryptococcus neoformans* (this paper).

(ii) In the fungal kingdom, the nuclear envelope persists during nuclear division partially or completely, when the segregation of chromosome pairs to the opposite ends of the elongating nucleus is accomplished by the mitotic spindle. PARs may either constrict the elongating anaphase nucleus to divide the nuclear membrane of one nucleus into two nuclear membranes of two originating nuclei or be involved in the reconstitution of nuclear membrane after mitosis.

(iii) PARs may be a temporary storage deposit of F-actin in slowly proliferating, resting or stressed cells. Hundred micromolar Nocodazole dissolved in 1% final DMSO delayed cell proliferation by disruption of microtubules [18], and 5% DMSO delayed cell proliferation by an unknown effect (Fig. 2). A constitutive protein actin transcribed continuously can accumulate in PARs that may become better visible. Cells treated with 5% DMSO and Nocodazole 100 μM with 1% DMSO had visible PARS in about 50% of cells, but 50% of PARs may be on the opposite half of the nucleus, and therefore invisible. This means that in fact 100% cells may have PARs.

We discovered a new F-actin cytoskeleton structure – perinuclear F-actin rings in the human pathogenic yeast *Cryptococcus neoformans* (this paper) and in the basidiomycetous yeast *Fellomyces fuzhouensis* [5], which were earlier observed in the ascomycetous yeast [12], in several filamentous fungi [13,14], in algae [15] and in human adenocarcinoma cells [16].

Conclusion

Cryptococcus neoformans has visible F-actin rings around the cell nucleus in cells treated with Nocodazole or DMSO or in cells grown in YEPG, which disappear after Latrunculin A treatment. The PARs may be involved in the central positioning of yeast nucleus suspended by means of actin cables and in nuclear movement and in nuclear division, and as F-actin deposit in slowly proliferating, resting or stressed cells.

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References

1 Kopecká M, Gabriel M, Takeo K, Yamaguchi M, Svoboda A, Ohkusu M, Hata K, and Yoshida S (2001) Microtubules and actin cytoskeleton in *Cryptococcus neoformans* compared with ascomycetous budding and fission yeasts. *Eur. J. Cell Biol.* **80**: 303–311.

2 Gabriel M, Kopecká M, Yamaguchi M, Svoboda A, Takeo K, Yoshida S, Ohkusu M, Sugita T, Nakase T, *et al.* (2006) The cytoskeleton in the unique cell reproduction by conidiogenesis of the longneck yeast *Fellomyces (Sterigmatomyces) fuzhouensis*. *Protoplasma* **229**: 33–44.

3 Kopecká M and Gabriel M (2009) Microtubules and actin cytoskeleton of potentially pathogenic basidiomycetous yeast as targets for antifungals. *Chemotherapy* **55**: 278–286 (doi: 10.1159/000224655).

4 Kopecká M, Ilkovics L, Ramíková V, and Yamaguchi M (2010) Effect of cytoskeleton inhibitors on conidiogenesis and capsule in the long neck yeast *Fellomyces* examined by scanning electron microscopy. *Chemotherapy* **56**: 197–202. (doi:10.1159/000316330).

5 Kopecká M, Yoshida S, and Yamaguchi M (2012) Actin ring formation around the cell nucleus of long-neck yeast. *J. Electron Microsc.* **61**: 249–255.

6 Nishimura K, Miyaji M, Takeo K, Mikami Y, Kamei K, Yokoyama K, and Tanaka R (1998) *IFM List of Pathogenic Fungi and Actinomycetes with Photomicrographs*, 2nd edn (Culture Collection of Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Chiba, Seibunsha).

7 Novick P and Botstein D (1985) Phenotypic analysis of temperature-sensitive yeast actin mutants. *Cell* **40**: 405–406.

8 Yamaguchi M, Ohkusu M, Sameshima M, and Kawamoto S (2005) Safe specimen preparation for electron microscopy of pathogenic fungi by freeze-substitution after glutaraldehyde fixation. *Jpn. J. Med. Mycol.* **46**: 187–192.

9 Yamaguchi M, Namiki Y, Okada H, Uematsu K, Tame A, Maruyama T, and Kozuka Y (2011) Improved preservation of fine structure of deep-sea microorganisms by freeze-substitution after glutaraldehyde fixation. *J. Electron Microsc.* **60**: 283–287.

10 Yamaguchi M, Biswas S K, Kuwabara Y, Ohkusu M, Shimizu M, and Takeo K (2010) The spindle pole body of the pathogenic yeast *Cryptococcus neoformans*: variation in morphology and positional relationship to the nucleolus and the bud in interphase cells. *J. Electron Microsc.* **59**: 165–172.

11 Kopecká M and Yamaguchi M (2011) Ultrastructural disorder of actin mutant suggests uncoupling of actin- dependent pathway from microtubule-dependent pathway in budding yeast. *J. Electron Microsc.* **60**: 79–391.

12 Marks J, Hagan I M, and Hyams J S (1986) Growth polarity and cytokinesis in fission yeast: the role of the cytoskeleton. *J. Cell Sci.* **15** (Suppl. 5): 229–241.

13 Heath I B (1990) The roles of actin in tip growth of fungi. *Int. Rev. Cytol.* **123**: 95–127.

14 Hoch H C and Staples R C (1983) Visualization of actin in situ by rhodamine-conjugated phalloidin in the fungus *Uromyces phaseoli*. *Eur. J. Cell Biol.* **32**: 52–58.

15 Wasteneys G O and Williamson R E (1991) Endoplasmic microtubules and nucleus-associated actin rings in *Nitella* internodal cells. *Protoplasma* **162**: 86–98.

16 Rosenblum M D and Shivers R R (2000) Rings of F-actin from around the nucleus in cultures human MCF7 adenocarcinoma cells upon exposure to taxol and taxothere. *Comp. Biochem. Physiol. Part C* **125**: 121–131.

17 Kopecká M and Gabriel M (1998) The aberrant positioning of nuclei and the microtubule cytoskeleton in *Saccharomyces cerevisiae* due to improper actin function. *Microbiology (UK)* **144**: 1783–1797.

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