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Actin ring formation around the cell nucleus of long-neck yeast

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Abstract The unique long-neck yeast *Fellomyces fuzhouensis* has F-actin cables and cortical patches. Here, we describe a new F-actin structure present in fungi, a perinuclear F-actin collar ring around the cell nucleus. This F-actin structure can be visualized by fluorescent microscopic imaging of rhodamine–phalloidin-stained F-actin in cells treated with the mitotic drug isopropyl *N*-(3-chlorophenyl) carbamate or the microtubule inhibitor thiabendazol or when cells were grown in cut dried radish medium or yeast extract pepton dextrose (YEPD) medium. In contrast, these structures were absent in cells treated with Latrunculin A. The hypothetical functions of the F-actin ring are discussed.

Keywords F-actin, yeast, nucleus, perinuclear actin ring

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Introduction

Fellomyces fuzhouensis is a unique 'long-neck yeast' that reproduces by conidiogenesis, a form of asexual reproduction with unusual features that distinguish it from known asexual forms of reproduction in budding and fission yeasts. A mother cell first develops a unique hyphal stalk, at the end of which a daughter yeast cell develops with the stalk forming a long neck between the mother and the daughter. The mature daughter cell gets separated from the mother by breaking this stalk and as a result, a long empty neck remains on the mother cell [1–3].

The actin cytoskeleton of *F. fuzhouensis* consists of cables and patches. The cables project from the mother cell to the hyphal stalk and to the daughter. The actin patches are located at the mother's cortex, at the apex of the growing stalk and at the daughter's cortex, but an actin cytokinetic ring was not found [1]. Before mitosis, the nucleus migrates from the mother, through a long narrow neck, to the daughter for unknown reasons by an unknown mechanism. Before mitosis, cytoplasmic microtubules disappear, and the mitotic spindle originates eccentrically positioned in the nucleus that migrated to the daughter cell for mitosis. During mitosis, one nucleus is delivered to the mother, and the other remains in the daughter [1]. The eccentrically triggered mitosis is followed by an eccentrically located cytokinesis that proceeds without an actin cytokinetic ring and without septum formation [1,2], which stands in contrast to the process that occurs in budding and fission yeast [4–7]. Small actin rings observed at the base of the conidia before conidial separation from hyphae in *Aureobasidium pullulans* [8] were not found in *F. fuzhouensis* [1].

Recently, we investigated actin and microtubule inhibitors in *F. fuzhouensis* for their ability to block yeast proliferation, to prevent the birth of

resistant cells and to induce cell death. This yeast species, due to its unusual cell morphology, has the potential to yield significant insights into these processes because the microtubule and actin inhibitors lead to different phenotypes [9,3]. During this line of experimentation, we noticed unusual perinuclear F-actin rings in cells inhibited with thiabendazol (TBZ) and isopropyl *N*-(3-chlorophenyl) carbamate (CIPC). We had previously observed this pattern only in cells grown in cut dried radish (CDR) medium (Kopecká M. and Yoshida S., unpublished data) and in a few cells grown in yeast extract pepton dextrose (YEPD) medium. This paper describes the first demonstration of an F-actin perinuclear ring collar in the long-neck yeast *F. fuzhouensis*.

Materials and methods

Yeast strain

Fellomyces fuzhouensis CBS 8243 (Yeast Culture Collection, The Netherlands) was kindly provided by Prof. Teun Boekhout, CBS Utrecht, The Netherlands.

Media and cell cultivation

Fellomyces fuzhouensis was maintained on plates at 25°C for 2–7 days on YEPD medium consisting of 1% glucose, 2% yeast extract and 2% polypeptone supplemented with 1.5% agar [9].

CDR medium (S. Yoshida, unpublished data): 10 g of dry radish (produced in Japan) was placed in 100 ml of redistilled water, homogenized, and extracted and refrigerated for approximately 24 h at 5–10°C. The extract obtained was filtered through a filter paper and sterilized by boiling in a water bath for 20 min and was then placed in a refrigerator. The obtained amount of medium, approximately 40 ml, was used to cultivate *F. fuzhouensis cells*.

Application of cytoskeleton inhibitors

For experiments, the cells were inoculated in liquid 1% YEPD (containing 1% each of yeast extract, peptone and glucose) and cultivated on a shaker at 27°C, usually overnight. Two-milliliter quantities of 1% YEPD medium were pipetted into 50 ml Erlenmeyer flasks. Yeast cultures in log-phase growth were added, usually in 100-µl volumes, to generate cultures that contained approximately 10^6 cells ml^{-1}. The inhibitors, CIPC and TBZ, dissolved in dimethyl sulfoxide (DMSO), were added to cells in order to produce final concentrations of 50 and 100 µM, respectively. Latrunculin A was used at a concentration of 100 µM. Control samples without DMSO and with DMSO were used. After applying the inhibitor, the culture was shaken in the dark in a water bath.

Fixation for fluorescent microscopy

Actin and microtubules were visualized as previously described [1,9].

Nuclear staining

Nuclei were stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) diluted to a final concentration of 1 µg ml^{-1} in Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA, USA).

Phase-contrast and fluorescence microscopy

Olympus BH2-RFCA and Leica Laborlux S Leitz fluorescent microscopes were used with standard filter blocks for violet (355–425 nm), blue (450–490 nm) and green (515–560 nm) excitation wavelengths. A Plan Phaco 3 100/1.25 objective and an equipment for phase-contrast microscopy were employed. The primary magnifications used in this study were 412.5× with the Olympus microscope and 375× with the Laborlux microscope. The preparations were photographed using Kodak-Tri-X-pan 400, Provia Fujichrome 400, Ilford 400 and Kodak Ektachrome 400 films.

Cell proliferation

Cell proliferation was determined by measuring the absorbance of suspension of cells at 550 nm.

Results

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 and actin patches [1].

However, in some single daughter and mother cells from the exponential culture or in the reproducing mother cells, we observed 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 *F. fuzhouensis* in five different conditions.

First finding

Many F-actin rings around the cell nuclei were observed in *F. fuzhouensis* cells cultivated in CDR medium. Some cells had normal actin patches and cables, but many cells had an F-actin ring (Fig. 1b, d and e) around the nucleus (Fig. 1a, c and f).

The F-actin rings are likely localized to the external surface of the nucleus, as double labeling of the nuclei with DAPI staining and F-actin with rhodamine–phalloidin (Fig. 1c–f) 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 the rings positioned in the plane parallel to the glass.

The rings were observed in individual interphase cells (Fig. 2a and b) and in M-phase cells when mother cell and daughter cell were present after mitosis but before cytokinesis (Fig. 2c–f).

When we investigated the dynamics of the occurrence of F-actin rings during growth in CDR medium, we often detected actin rings after approximately 3 or 4 days, when many young cells appeared in the culture possessing actin cables and patches. Later, the cells aggregated to form large clumps containing tens of cells that lacked actin cables and patches but contained actin rings around the cell nucleus in different focal planes (Figs. 1b and 2a and b). These cells became increasingly transparent and gradually dead.

To determine why actin rings were more frequently visible in CDR medium than in YEPG medium (see later), we measured the optical density of cells cultured in YEPD and CDR medium. At the beginning of cultivation, the optical density of both samples was 0.05. However, cells grown in CDR

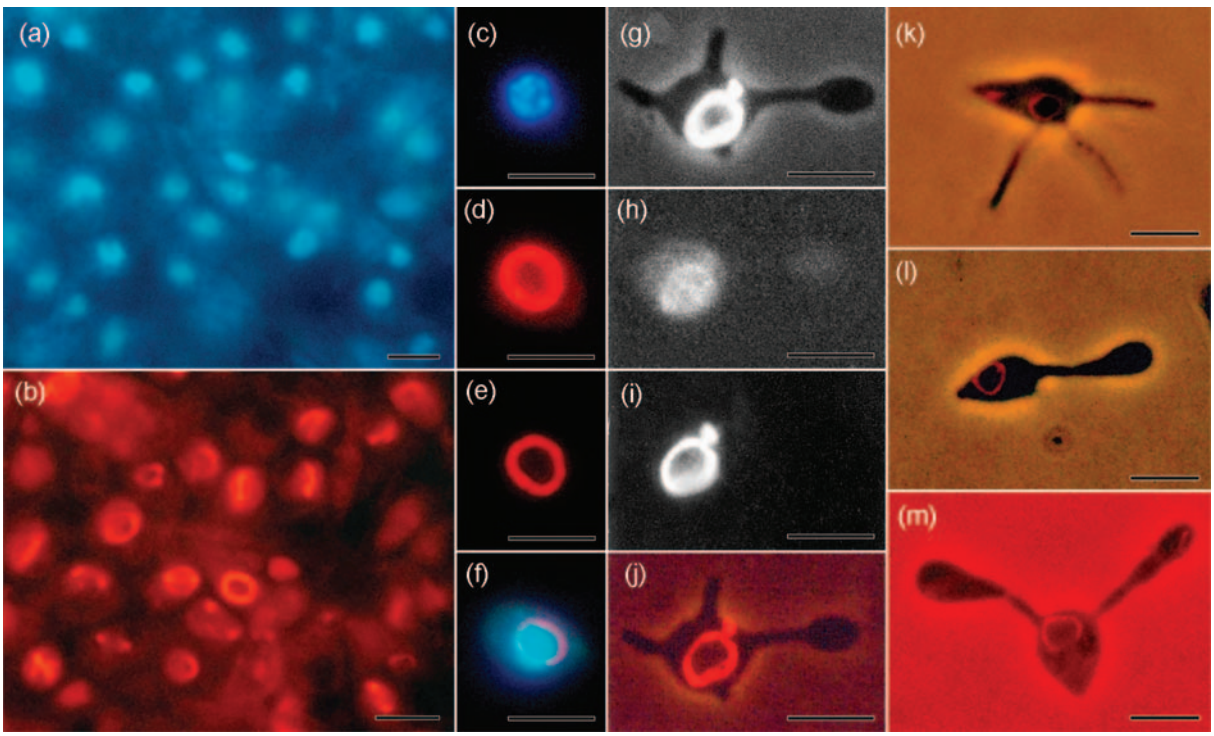

Fig. 1. Fluorescent and phase-contrast microscopy of 'long-neck' yeast *F. fuzhouensis* cells. (a–f) Cells were cultured in CDR medium for 4 days. Many cells were in the stationary phase, whereas others reproduced by conidiogenesis. Nuclei were detected by DAPI staining (a, c, f), and the actin cytoskeleton was detected by rhodamine–phalloidin staining, which revealed perinuclear actin rings (b, d, e, f). (g–j) Cells were cultured in YEPD medium and inhibited with 50 µM CIPC for 6 h. The actin cytoskeleton was detected by rhodamine–phalloidin, and one ring is shown in a mother cell (g, i, j). Nuclei were detected by DAPI staining (h). (g, j) Images acquired by both phase-contrast and actin fluorescence microscopy. (k–m) Cells were cultured in YEPD medium and inhibited with 100 µM CIPC for 6 h. The actin cytoskeleton was detected by rhodamine–phalloidin, and an actin ring is shown in each cell. (k–m) Images were acquired by both phase-contrast and actin fluorescence microscopy. Scale bar, 5 µm.

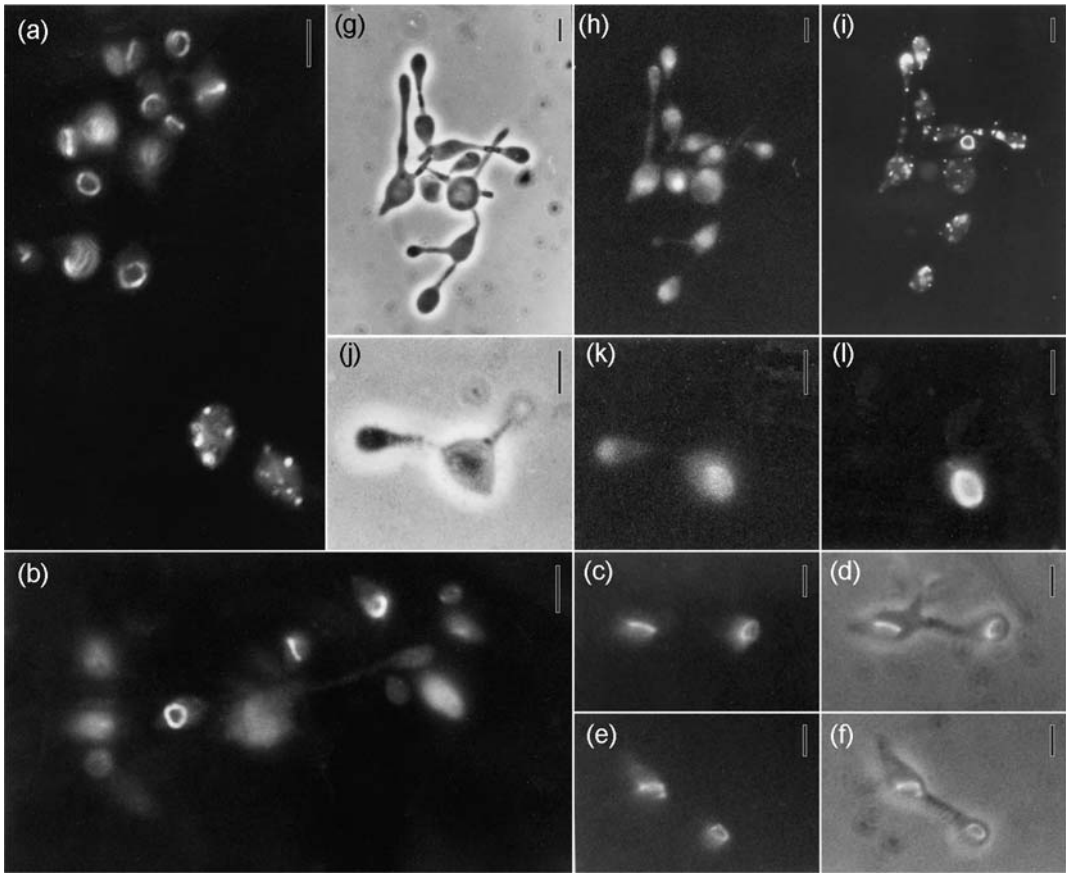

Fig. 2. Fluorescent and phase-contrast microscopy of long-neck yeast *F. fuzhouensis* cells. (a–f) Cells were cultured in CDR medium for 3 days. While many cells were in the stationary phase (a, b), a few cells were still reproducing by conidiogenesis and were in the mitosis stage prior to cytokinesis (c–f). The actin cytoskeleton was detected by rhodamine–phalloidin, and perinuclear actin rings are shown (a–f). In (a), actin patches are in two cells (bottom right). (d, f) Images acquired by both phase-contrast and actin fluorescence microscopy. (g–i) Cells were cultured in YEPD medium for 20 h. (g) Cells acquired using phase-contrast microscopy. (h) Nuclei of the same cells stained with DAPI. (i) F-actin using rhodamine–phalloidin. Almost all of the cells contain actin patches, whereas one actin ring is in a cell near the middle (upper right). (j–l) Cells were cultured in YEPD medium for 20 h and TBZ was added to cells at 100 µM for 6 h. (j) Cells in phase-contrast. (k) Nuclear staining by DAPI. (l) One actin ring around the mother's nucleus. Scale bar, 5 µm.

medium proliferated very slowly, reaching an optical density of 0.4 after 4 days, whereas cells grown in YEPD medium reached an optical density of 1.1 after 4 days.

Second finding

The F-actin ring around the nucleus occurred also in exponentially growing cells cultured in YEPD medium and treated with the mitotic spindle poison CIPC, which is known to disrupt microtubules through an interaction of CIPC with the microtubule-organizing center [10]. In 50 µM CIPC, we observed disrupted actin cables and patches, but after approximately 6 h, the cells had F-actin rings (Fig. 1g, i and j) that co-localized with the nuclei (Fig. 1g-1h, 1i–1h, 1j–1h). Actin rings were also observed in cells treated with 100 µM CIPC for 6 h (Fig. 1k–m). These inhibited cells did not proliferate (as determined by optical density measurements) and had small vacuoles in the cytoplasm that could be visualized using phase-contrast microscopy. The cells gradually became increasingly transparent and dead. The perinuclear actin ring in these inhibited cells might therefore be related to cell death.

Third finding

As mentioned above, the F-actin ring around the cell nucleus was also observed in cells that are in exponential phase growth in YEPD medium. Although many cells had F-actin cables and

patches, only a few cells had an F-actin ring around the nucleus (Fig. 2g–i).

Fourth finding

The F-actin ring was also observed after treatment with the microtubule inhibitor TBZ (Fig. 2j–l), and it seemed identical to what was observed in cells treated with CIPC.

Fifth finding

After treatment with the actin inhibitor, 100 µM Latrunculin A, all actin structures disappeared [9] and after approximately 17 h, the cells gradually became spherical in shape. From this observation, we deduce that Latrunculin A also disrupts perinuclear rings containing F-actin.

Discussion

This paper demonstrates for the first time a new F-actin cytoskeleton structure in yeast – an F-actin ring that is associated with the nucleus – by fluorescence microscopy of F-actin after rhodamine–phalloidin staining. Double labeling of F-actin and the cell nucleus demonstrated the localization of the actin ring to the periphery of the nucleus. The actin ring is most likely localized to the external surface of the nucleus and is most likely joined to the outer nuclear membrane; however, the exact molecular mechanism that governs its localization is unknown. The appearance of this structure does not indicate its function; therefore, we will investigate its possible functions by determining which cells have this unusual actin structure, which conditions it appears during and which other organisms have actin rings.

Which long-neck yeast cells have an F-actin ring around the cell nucleus?

We think that actin rings may be present in all interphase and in M-phase cells cultivated in YEPD or CDR media and that they form as a result of rich actin patches and cables. However, because of the positioning of actin rings in different focal planes within cells, they are not always easily observable under a microscope. Only a few cells grown in YEPD do not have rich actin cytoskeletons (these are most likely cells at the beginning of the cell cycle in the G1 stage), and in these cells, an actin ring can be seen. In CDR medium, cells grow very slowly; therefore, they stay in the G1 stage of the cell cycle for a long time, a stage of the cell cycles that is the most prolonged stage in unfavorable culture conditions. This phenomenon may explain why actin rings are present more often in CDR medium than in YEPD medium. Both CIPC and TBZ gradually affect the cytoskeleton, which permits for a visualization of actin rings in treated cells. This result suggests that perinuclear actin rings can be an integral structure of the yeast actin cytoskeleton in the unusual long-neck yeast *F. fuzhouensis*, which has an actin cytoskeleton that is comprised of actin patches, actin cables and a perinuclear actin ring; however, the actin cytokinetic ring has not yet been identified.

What hypothetical function can the F-actin ring around the cell nucleus of long-neck yeast have?

(i) Our results from cultivating cells in CDR medium suggest that the perinuclear actin ring may be a temporary storage deposit of F-actin in slowly proliferating or resting cells. When the cells begin to proliferate again, F-actin can be released from F-actin ring deposits by signal from the nucleus and re-organized to form actin patches, cables and actin involved in cytokinesis. From the inhibitory effect of CIPC and TBZ, we suggest that microtubule inhibitors indirectly influence the actin cytoskeleton that assembles to form perinuclear actin rings in these stressed cells. This phenomenon is in contrast to that of mutant cells with deficient actin cables, which primarily affects actin-dependent pathways in the cell cycle [11], disrupting the secretory pathway [12] and cell wall formation [13] and secondarily affecting the microtubule pathway of nuclear division [11]. It appears that the actin and microtubule cytoskeletons in fungal cells are closely interrelated and finely tuned with cell-cycle progression, such that stressing one cytoskeleton subsystem influences the other and vice versa.

(ii) The second hypothetical function is that the perinuclear F-actin ring may be involved in nuclear migration through the long neck from mother to daughter before mitosis. We noticed that before nuclear migration to the long narrow neck, the actin ring-like structure appeared around the nucleus like a ‘horse collar’ to draw the nucleus

through the long neck during nuclear migration. Actin cables proceeded around the nucleus in a parallel way along the long neck [1]. At the beginning of nuclear migration, we also noticed a very small actin ring in front of the migrating nucleus that was reminiscent of 'a small ring in the bull's nose to draw the bull' [1]. From these interesting findings, we can hypothesize a potential function of actin rings in nuclear migration through the long neck prior to mitosis, although experimental data for such a function are still lacking. Wasteneys and Williamson [14] suggested a similar role for the F-actin ring around the nucleus in nuclear migration and nuclear rotation in algal cells.

(iii) It has been known for a long time that, during nuclear division, the segregation of chromosome pairs to opposite ends of the elongating nucleus is accomplished through the actions of the mitotic spindle. However, in the fungal kingdom, where the nuclear envelope persists during mitosis, the F-actin ring can hypothetically constrict the elongating nucleus in the middle to divide the nuclear membrane of one nucleus into two nuclear membranes for two nuclei in anaphase.

(iv) Recently, we discovered that actin cytoskeleton cables are involved in nuclear positioning and inheritance in budding yeast [11]; however, we do not know whether the perinuclear actin rings found in *F. fuzhouensis* are also involved in nuclear positioning and whether perinuclear actin rings also occur in budding yeast.

Are F-actin rings around the nuclei present in other organisms?

Perinuclear actin rings have been found in cold-treated *ben4* mutant yeast *Schizosaccharomyces pombe* cells arrested at mitotic prophase, and these perinuclear rings may in fact be actin cytokinetic rings [4]. In *Cryptococcus neoformans* [15], the hyphal fungus *Saprolegnia ferax* treated with cytochalasin E, in the mite-infecting fungus zygomycete *Neozygites* sp. [16], and in the plant pathogen *Uromyces phaseoli*, an incomplete ring-like structure has been observed [17]. The actin rings around the nuclei of algae are related to nuclear migration or rotation [14], and in human breast adenocarcinoma cells treated with taxol and taxothere, the actin rings around the nuclei are related to apoptosis [18].

Conclusion

We have identified a new F-actin cytoskeleton structure in the yeast *F. fuzhouensis* – F-actin rings around the cell nucleus – which form during growth in CDR medium and YEPD medium and in cells treated with CIPC and TBZ, whereas they disappear following Latrunculin A treatment. The effects of these inhibitors suggest a close relationship between microtubules and the actin cytoskeleton. Microtubule inhibitors indirectly influence the actin cytoskeleton, which accumulates into a perinuclear actin ring as a result. The F-actin ring around the cell nucleus may be an F-actin deposit in slowly proliferating cells, in resting cells and in stressed cells. In dividing cells, the perinuclear actin ring could hypothetically be involved in nuclear migration through the long neck and in nuclear division to divide the nuclear envelope of one nucleus into two.

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