Effects of the F-actin inhibitor latrunculin A on the budding yeast Saccharomyces cerevisiae

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Our basic cell biology research was aimed at investigating the effect on eukaryotic cells of the sudden loss of the F-actin cytoskeleton. Cells treated with latrunculin A (LA) in yeast extract peptone dextrose (YEPD) medium were examined using phase-contrast and fluorescent microscopy, freeze-substitution, transmission and scanning electron microscopy, counted using a Bürker chamber and their absorbance measured. The cells responded to the presence of LA, an F-actin inhibitor, with the disappearance of actin patches, actin cables and actin rings. This resulted in the formation of larger spherical cells with irregular morphology in the cell walls and ultrastructural disorder of the cell organelles and secretory vesicles. Instead of buds, LA-inhibited cells formed only ‘table-mountain-like’ wide flattened swellings without apical growth with a thinner glucan cell-wall layer containing β-1,3-glucan microfibrils. The LA-inhibited cells lysed. Actin cables and patches were required for bud formation and bud growth. In addition, actin patches were required for the formation of β-1,3-glucan microfibrils in the bud cell wall. LA has fungistatic, fungicidal and fungilytic effects on the budding yeast Saccharomyces cerevisiae.

INTRODUCTION

The organization of F-actin in budding yeast includes submembraneous actin patches (AP) at the growing poles and the site of septum formation, and longitudinally running actin cables (AC) and actin rings (AR) in the cytokinesis region (Adams & Pringle, 1984; Kilmartin & Adams, 1984). A single actin gene act1 enabled the isolation of the first two ts actin mutants, act1-1 and act1-2 (Shortle et al., 1984). A shift to 37 °C caused disruption of the AC, delocalization of the AP, defects in bud formation, in the localization of chitin, in secretion of invertase and in the directed movement of the secretory vesicles (SV), suggesting that the main function of the actin cytoskeleton was to direct growth towards the bud (Novick & Botstein, 1985). Other roles for actin include bud site selection (Drubin, 1991), nuclear positioning and migration (Palmer et al., 1992; Kopecká & Gabriel, 1998), organelle movement (Pruyne & Bretscher, 2000; Irazoqui & Lew, 2004; Moseley & Goode, 2006), cytokinesis (Gabriel et al., 1998), cell wall regeneration (Gabriel et al., 1992; Kopecká & Gabriel, 1995), endocytosis (Kübler & Riezman, 1993) and the response to extracellular cues (Schmidt & Hall, 1998). Our reinvestigation of the phenotypes of act1-1 and act1-2 mutant cells at 37 °C proved that AC are necessary for the correct spatial positioning and orientation of secretory pathways to the bud and septum, and for vectorial movement of the SV along the AC (Yamaguchi & Kopecká, 2010); act1-1 cells at 23 °C have ‘faint actin cables’ that delay bud formation, bud growth and nuclear migration to the bud neck, in contrast to nuclear division (Kopecká & Gabriel, 1998; Kopecká & Yamaguchi, 2011). This indicated the existence of two cytoskeleton-dependent pathways: (i) the ‘actin pathway’ for bud formation, bud growth and nuclear migration, and (ii) the microtubule pathway for nuclear division (Kopecká & Yamaguchi, 2011) that may correspond to the two cell-cycle pathways identified by CDC mutants (Hartwell et al., 1974).

Disruption of the actin cytoskeleton in the act1-1 mutant of budding yeast resulted in the formation of an aberrant cell wall containing β-1,3-glucan microfibrils in colocalization with the evenly distributed AP (Gabriel & Kopecká, 1995).

The yeast cell wall consists of an inner glucan layer containing β-1,3-glucan microfibrils masked with amorphous β-1,3- and β-1,6-glucans (Kopecká et al., 1974, 1995) and an equally thick outer cell wall layer containing mannanproteins (Klis, 1994; Cid et al., 1995; Orlean, 2012; Teparić &
Mřsa, 2013; Klis et al., 2014) that under examination with electron microscopy have an amorphous appearance (Kopecká et al., 1974).

β-1,3-glucans are synthesized by β-1,3-glucan synthases (GSs) consisting of catalytic and regulatory subunits (Cabib et al., 2001) that co-localize with movable actin cortical patches at the cell wall remodelling sites found on the plasma membrane (Yamochi et al., 1994; Inoue et al., 1996; Qadota et al., 1996; Utsugi et al., 2002). Actin patch motility is required for the directed movement of Fks1p, a multidomain enzyme, and is required for cell wall construction (Utsugi et al., 2002; Ohyaa et al., 2005; Okada et al., 2010). The movement of yeast β-1,3-GSs is essential for uniform cell wall synthesis (Utsugi et al., 2002; Ohyaa et al., 2005).

Mulholland et al. (1994) identified the AP as finger-like invaginations (FLI) of the plasma membrane and suggested their function in endocytosis and cell wall assembly. We believe that the interior of the actin cortical patches (i.e. the FLI) is an ideal cylindrical space for the synthesis and assembly of β-1,3-glucan chains into triple-helices (elementary nanofibrils) and into β-1,3-glucan microfibrils that grow on the cell wall (Kopecká & Kreger, 1986; Kopecká & Gabriel, 1992, 1995).

In contrast, the synthesis of amorphous cell wall glycoproteins and their post-translational modification proceeds in the secretory pathways that start at the rough endoplasmic reticulum (ER), continue to the Golgi apparatus (GA) and end with their transport from the secretory vesicles (SV) to the cell wall (Novick et al., 1981; Novick & Schekman, 1983).

Herein, we investigate the actin-monomer binding drug, latrunculin A (LA) (Spector et al., 1983), which disrupts AC, AP and AR in Saccharomyces cerevisiae (Ayscough et al., 1997; Kopecká & Yamaguchi, 2011) and follow its effects on the cells. Our main question was ‘What will happen to bud cell wall formation after disruption of AP, which are normally strongly accumulated at the growing buds?’ (Adams & Pringle, 1984; Kilmartin & Adams, 1984; Novick & Botstein, 1985). We demonstrate that the F-actin cytoskeleton is necessary for bud development and cell wall formation in the bud, and that actin patches are required for the formation of β-1,3-glucan microfibrils on the yeast cell wall.

**METHODS**

**Yeast strain.** The S. cerevisiae WT DBY 1690 strain with standard actin allele (ACT1+/ACT1−) was kindly provided by D. Botstein and P. Grisafi (MIT).

**Media and cell cultivation.** The strain was maintained on 2.0 % (w/v) agar-containing YED medium [1 % (w/v) yeast extract, 2 % (w/v) peptone and 2 % (w/v) glucose] at laboratory temperature. To obtain an exponential culture, cells were cultivated in YED medium [1 % (w/v) yeast extract, 1 % (w/v) peptone and 1 % (w/v) glucose] on a shaker overnight (about 18 h) at 23 °C, then diluted to 5 × 10^5 to 1 × 10^6 cells ml^-1 using 1 % YEPD medium and used for the application of the inhibitor.

**Latrunculin A treatment.** A 10 mM stock solution was prepared by dissolving 100 μg of LA (Molecular Probes or Sigma) in 25 μl of DMSO, which was kept at −20 °C (Kopecká & Gabriel, 2009; Kopecká et al., 2010). The 10 mM stock solution was added to cells in 1 % YEPD to final concentrations of 100 μM. Five-hundred-micro-litre volumes of the cultures in test tubes were shaken in the dark in a water bath for 20 h; samples for fluorescent, transmission and scanning electron microscopy were taken at T0, and after 4.5 h and 20 h of inhibition, and fixed. Living cells were continuously observed using phase-contrast microscopy.

**Fixation for fluorescent microscopy.** Fixation was carried out in accordance with a previously published procedure (Gabriel & Kopecká, 1995).

**Cell proliferation.** Cell proliferation was determined by cell counting in a Bürker chamber, and measuring the absorbance of a suspension of cells at 590 nm.

**Freeze-substitution and transmission electron microscopy.** Freeze-substitution and transmission electron microscopy were conducted as previously described (Yamaguchi et al., 2009, 2011).

**Scanning electron microscopy.** Scanning electron microscopy was conducted as previously described (Kopecká et al., 2010; Kopecká & Yamaguchi, 2011).

**Image processing software.** Adobe Photoshop CS5 and Adobe InDesign CS5 for Windows were used.

**RESULTS**

Phase-contrast microscopy and fluorescent microscopy of the LA-treated cells

**Phase-contrast microscopy.** The control budding cells were ovoid at T0 (Fig. 1a, b) and after 20 h of cultivation (Fig. 1e). The LA effect after 4.5 h caused the mother cells and buds to gradually form a spherical shape (Fig. 1c, d) that predominated after 20 h (Fig. 1f–l). Many of the inhibited cells lysed and their cytoplasm was released into the culture medium (Fig. 1h–l). Lysis occurred in regions of cell wall growth in the bud and septum (Fig. 1k, l) and in the cells without buds in the areas of future bud formation (Fig. 1h–j). This indicated that the F-actin cytoskeleton was required for budding, bud wall formation, bud growth and septum formation.

**Fluorescent microscopy: F-actin staining.** The AP in the control cells were strongly accumulated in areas of cell wall growth in the bud and septum. AC proceeded along the longitudinal cell axis (Fig. 1m) and AR occurred at cytokinesis. The LA-inhibited cells had no AP, AC and AR; however, diffuse fluorescence occurred (Fig. 1n).

**Fluorescent microscopy: calcofluor staining.** The control cells stained with calcofluor (CA) revealed intensive fluorescence of bud scars and septa, while the lateral cell walls revealed only weak fluorescence (Fig. 1o).
The LA-inhibited cells stained with CA revealed intensive fluorescence of the mother cell walls (Fig. 1p) indicating delocalized chitin (Fig. 1p).

The effect of latrunculin A on cell proliferation

**Control cells.** The cell number at $T_0$ ($7.5 \times 10^5$ cells ml$^{-1}$) increased to $1.5 \times 10^8$ cells ml$^{-1}$ after 20 h (Table 1). Measurement of the OD$_{590}$ at $T_0$ (0.02) increased after 20 h to 1.05 (Table 1). These results indicated that 100 $\mu$M LA inhibited cell proliferation.

**Freeze-substitution and transmission electron microscopy of the LA-treated cells**

**Control cells.** The control budding cells were ovoid and had a standard ultrastructure with few organelles in the secretory pathway (ER, GA and SV) (Fig. 2a–e). The mother cells and buds had a uniform cell wall and outer electron-transparent cell wall layer thickness. In addition, the inner electron-dense cell wall layer of the mother cells and buds had a uniform thickness of approximately 100 nm. Their reverse stainability was attributed to combined glutaraldehyde fixation prior to freeze-substitution. Upon higher magnification, the peripheral darker fibres of mannoproteins (Klis, 1994) on the external cell wall surface (Fig. 2f, g) were observed, while the inner electron-dense glucan cell-wall layer revealed a fine microfibrillar texture in the mother cells (Fig. 2f) and buds (Fig. 2g) of $\beta$-1,3-glucan microfibrils (Kopecká et al., 1974). A few cell wall ridges and plasma membrane invaginations (Fig. 2a–e) were distorted, which was attributed to their sudden chemical fixation (Kopecká et al., 1973).

**The effect of LA.** The cells inhibited with LA for 4.5 h revealed ultrastructural disorder (Fig. 3a–e) similar to the actin mutant cells, act1-1/act1-1. The SV were randomly dispersed in the cytoplasm and did not accumulate in the buds (Fig. 3a–e) in contrast to that found with the control cells (Yamaguchi & Kopecká, 2010) (Fig. 1c, d). Vacuoles were in eccentric positions, and the ER traversed the cytoplasm and bud or bud base (Fig. 3a, d, e). Instead of buds, the LA-inhibited cells contained ‘table-mountain-like’ wide flattened swellings (Fig. 3a–e) that were not seen in the control cells (Fig. 2) or actin mutant cells (Gabriel & Kopecká, 1995; Yamaguchi & Kopecká, 2010; Kopecká & Yamaguchi, 2011). The LA-inhibited cells had thinner cell walls and thinner inner electron-dense glucan layers (Fig. 3a–e) in ‘table-mountain-like’ wide flattened swellings (Fig. 3a–e). Only a few LA-inhibited cells were found to have small buds with abnormally thick cell walls, or small buds separated by an aberrant leaky septum.

**Table 1. Number and OD$_{590}$ of cells inhibited by LA for 4.5 h and 20 h**

<table>
<thead>
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<th></th>
<th>0 h</th>
<th>4.5 h</th>
<th>20 h</th>
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<tbody>
<tr>
<td>Number of cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control cells</td>
<td>$7.5 \times 10^5$ ml$^{-1}$</td>
<td>$2.8 \times 10^6$ ml$^{-1}$</td>
<td>$1.5 \times 10^8$ ml$^{-1}$</td>
</tr>
<tr>
<td>Cells treated with LA</td>
<td>$7.5 \times 10^5$ ml$^{-1}$</td>
<td>$1.0 \times 10^6$ ml$^{-1}$</td>
<td>$1.0 \times 10^8$ ml$^{-1}$</td>
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<tr>
<td>OD$_{590}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control cells</td>
<td>0.02</td>
<td>0.08</td>
<td>1.05</td>
</tr>
<tr>
<td>Cells treated with LA</td>
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<td>0.06</td>
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The LA-inhibited cells stained with CA revealed intensive fluorescence of the mother cell walls (Fig. 1p) indicating delocalized chitin (Fig. 1p).

The effect of latrunculin A on cell proliferation

**Control cells.** The cell number at $T_0$ ($7.5 \times 10^5$ cells ml$^{-1}$) increased to $1.5 \times 10^8$ cells ml$^{-1}$ after 20 h (Table 1). Measurement of the OD$_{590}$ at $T_0$ (0.02) increased after 20 h to 1.05 (Table 1). These results indicated that 100 $\mu$M LA inhibited cell proliferation.
The cells inhibited with LA for 20 h revealed many lysed cells and collapsed empty cell walls persisting among the cells (Fig. 4a), that had ultrastructural disorder of the delocalized SV, one or two nuclei which were randomly positioned, vacuoles and parallel cisternae of the ER and GA (Fig. 4b–f). The shapes of the cells inhibited with LA for 20 h were irregular with a decreased cell size (Fig. 4c). The decrease in cell size due to dead cells was attributed to the loss of turgor pressure. The ultrastructural disorder after inhibition with LA for 20 h (Fig. 4a–f) was analogous to that found after 4.5 h of inhibition, as if cells inhibited for 4.5 h were paralysed in their inhibited state without growth, without budding and without cell division until 20 h of inhibition. 'Table-mountain-like' aberrant swellings (Fig. 4b–d, f) were similar to those after 4.5 h of inhibition, indicating that almost no apical growth occurred during 20 h of inhibition. In addition, their cell walls were significantly thinner, especially the electron-dense inner glucan cell-wall layer (Fig. 4b–f). Our measurements for the inner electron-dense glucan cell-wall layer of the bud showed a reduced thickness (approximately 86 nm), while the control cell walls have a glucan cell-wall layer thickness of approximately 100 nm. This indicated that the 'bud' cell walls in the inhibited cells did not grow and became thinner with a reduced electron-dense glucan cell-wall layer. In addition, some cells with larger buds have thin cell walls (Fig. 4e, f). At the base of some buds or swellings, local thickening of the cell wall occurred (Fig. 4d–f), which indicate the random chaotic processes occurring in the walls when septum formation is LA-inhibited.

Many buds or mother cells lysed and their cytoplasm was released into the external environment (Figs. 1h–l, 5a, b). In contrast to the thinner 'bud' cell walls, the mother cell walls (Fig. 4b, c, 5a) were approximately similar to that found for the control cells (Fig. 2a–g) with the exception of a few areas of localized aberrant cell wall thickening (Figs. 4c–f, 5a). This indicated that the mother cell...
walls were not influenced by LA disruption of the actin cytoskeleton in terms of their thickness or ultrastructure, with the exception of wall thickening (Figs. 4c–f, 5a) and lysed perforated cell walls (Fig. 5a, b).

**Scanning electron microscopy of LA treated cells**

The control cells were ovoid and had smooth cell wall surfaces with bud scars and birth scars (Fig. 5c). The LA-inhibited cells were larger spherical cells (Fig. 5d–f). The unique finding was a microfibrillar skeleton in the cell wall of the mother cells, which was visible by scanning electron microscopy probably due to the release of the external amorphous wall components upon the addition of 1 % DMSO to the culture medium. However, no microfibrils were seen in the small buds (Fig. 5f). Scanning electron microscopy also showed lysed cells with their released cytoplasm (Fig. 5e, f) and the collapsed cell walls of the lysed cells (Fig. 5d–f), which differed from the WT cell walls isolated by cell disruption using ballotini. The WT cells retained their ovoid shape (Kopecká et al., 1974). This indicated that the rigidity of the cell walls in the LA-inhibited cells had been affected. The cell wall integrity pathway (Levin, 2011) was unable to ensure the cell wall integrity after actin disruption.

**DISCUSSION**

**The actin cytoskeleton and cell wall formation**

WT control cells (ACT1+/ACT1+) that have AC in the mother cells and AP in the buds and septa show a correlation between the growing regions of the yeast cell surface and the
regions with a concentration of AP, which enables cell budding and formation with a uniform bud cell wall thickness containing two cell-wall layers as found in the mother cells. The inner electron-dense glucan cell wall layer thickness of approximately 100 nm contained β-1,3-glucan microfibrils and the outer electron-transparent layer contained mannoproteins (Kopecka et al., 1974).

The ts actin mutant cells (act1-1/act1-1) at 23 °C have ‘faint actin cables’ with a random arrangement (Kopecka & Yamaguchi, 2011) and AP on the buds, but also on the mother cells with a longer generation time (Novick & Botstein, 1985). ‘Faint actin cables’ delay bud formation, bud growth and bud wall construction due to the slow transport of delocalized SV; exocytosis proceeded, as the mother cells have thicker cell walls than the buds (Kopecka & Yamaguchi, 2011).

The ts actin mutant cells (act1-1/act1-1), after an increase in temperature to 37 °C, had absolutely no AC. Budding did not occur and budding cells lysed (Novick & Botstein, 1985). Only cells without buds persisted and grew spherical to form an aberrant cell wall in co-localization with AP (Gabriel & Kopecka, 1995).

LA-inhibited WT cells did not proliferate because AC, AP and AR were removed by LA. Instead of buds, only aberrant ‘table-mountain-like’ flattened wide swellings were formed. When the ring of AP which co-localized with the formation of the chitin ring in the bud neck was disrupted by LA, the bud neck did not develop because the chitin ring did not stabilize the bud neck. Apical growth of the swellings did not occur because LA removed AC, the secretory pathway was not directed to the buds and the buds did not grow. In addition, the glucan layer of the ‘bud’ cell wall did not grow and became thin because the new Fks1p were not delivered to the plasma membrane of the buds from the SV. These three examples document that budding without AC was impossible.

**Disruption of AC does not block the formation of β-1,3-glucan microfibrils in the cell wall**

The actin mutant cells (act1-1/act1-1) at 37 °C have disrupted AC and formed new β-1,3-glucan microfibrils on the cell wall (Gabriel & Kopecka, 1995) similar to protoplasts in the actin mutant cells (act1-1/act1-1) and protoplasts in the WT yeast (ACT1+/ACT1+), which both show AC disruption by ‘protoplasting’. However, this AC disruption did not block the formation of β-1,3-glucan microfibrils on the cell walls (Gabriel et al., 1992; Kopecka & Gabriel, 1995). These three examples demonstrate that the removal of AC did not block the formation of β-1,3-glucan microfibrils on the cell walls.

**The formation of β-1,3-glucan microfibrils on the cell wall requires AP**

WT protoplasts having no AC regenerated cell walls containing β-1,3-glucan microfibrils in co-localization with AP (Gabriel et al., 1992 and unpublished data). Protoplasts in the ts actin mutant, (act1-1/act1-1), also have no AC but form β-1,3-glucan microfibrils in co-localization with AP (Kopecka & Gabriel, 1995). The ts actin mutant cells (act1-1/act1-1) at 37 °C have no AC and form an aberrant cell wall containing β-1,3-glucan microfibrils in co-localization with AP (Gabriel & Kopecka, 1995). These three examples document the co-localization of AP and formation of β-1,3-glucan microfibrils on the cell walls.

It has been previously shown that movable AP co-localize with GS subunits Fks1 and probably also with Rho1, and that their movement was required for the formation of a uniform cell wall (Utsugi et al., 2002; Ohya et al., 2005). This explains why β-1,3-glucan microfibrils were formed in the actin mutant cells, WT protoplasts and actin mutant protoplasts in co-localization with AP (Gabriel et al., 1992 and unpublished data; Gabriel & Kopecka 1995; Kopecka & Gabriel, 1995).

We propose that the formation of the β-1,3-glucan microfibrils requires the synthesis of many glucan chains at the same time and place, ideally inside of the cylindrical AP/FLI on the plasma membrane where the synthesized β-1,3-glucan chains can immediately assemble into triple-helices, and triple-helices then into microfibrils via an ‘enzyme-directed assembly’ mechanism (Kopecka & Kreger, 1986; Kopecka & Gabriel, 1992, 1995). However, the inner cell wall glucan layer of the bud containing β-1,3-glucan microfibrils cannot be formed in LA treated cells. After the disruption of AP by LA, the GS molecules can escape from the buds and can diffuse randomly along the plasma membrane. Single GS Fks1p released from AP cannot synthesize glucan microfibrils because each GS molecule can only synthesize one glucan chain, while one β-1,3-glucan microfibril of 10 nm width contains about 30 hexagonally arranged triple helices of 1.53 nm width consisting of about 90 individual glucan chains (Kopecka & Kreger, 1986; Kopecka 1994). Theoretically, about 90 GS molecules Fkslp should be accumulated in one AP/FLI to produce one β-1,3-glucan microfibril of 10 nm in width. A single GS can synthesize (in cooperation with other enzymes) (Klis 1994; Orlean 2012; Teparić & Mrša, 2013; Klis et al., 2014) only a single branched β-1,3-glucan molecule of an amorphous glucan matrix along the cell surface. However, we have no evidence to confirm this, because the cell wall layer of the mother cells remained unchanged after LA treatment; the cells only became spherical and larger. Delocalized glucan synthesis may occur during the restructuring of the cell wall during the change in cell shape from an ovoid cell shape to spherical cell shape. We know that amorphous β-1,3-glucan masks the microfibrils in the inner glucan layer of the yeast cell walls, as proven by electron microscopy after treatment of the yeast cell walls with purified bacterial endo-β-1,3-glucanase. Bacterial endo-β-1,3-glucanase digests only amorphous β-1,3-glucan and releases it from the cell walls. However, β-1,3-glucan microfibrils on the cell walls
were resistant to this bacterial enzyme and persisted in the yeast cell walls (Kopecká et al., 1974, 1995).

Disruption of AP by LA in the bud neck can lead to delocalized chitin in the walls. After disruption of AP by LA, chitin synthases in the bud neck can escape and diffuse randomly along the cell surface and synthesize chitin at random locations.

**Cell lysis induced by the actin inhibitor, LA**

Cell lysis induced by Papulacandin B (Kopecká, 1984a), a specific inhibitor of synthesis of yeast β-1,3-glucan and β-1,3-glucan microfibrils (Kopecká, 1984a, b) indicates that the formation of β-1,3-glucan microfibrils requires not only GS Fks1p to synthesize the β-1,3-glucan chains, but also AP/FLI of the plasma membrane, which seem ideal cylindrical structures for the synthesis of many β-1,3-glucan chains and their regular assembly into triple helices and microfibrils by an ‘enzyme-directed assembly’ mechanism.

**CONCLUSIONS**

LA disrupted AP, AC and AR; inhibited the ‘actin pathway’ for bud formation, bud growth and the formation of the inner glucan cell wall layer containing β-1,3-glucan microfibrils; and LA-inhibited cells lysed. AP which accumulated in the buds were required for the ‘enzyme-directed assembly’ of β-1,3-glucan microfibrils in the yeast cell walls. AP in the bud neck was required for the formation of the chitin ring to stabilize the bud neck. In addition, both AC and AP are required for bud formation and bud growth.

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Latrunculin A, F-actin and cell wall formation in yeast

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