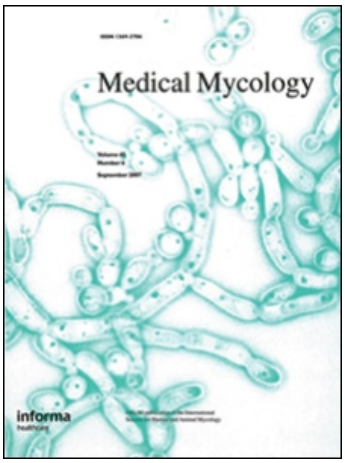

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Pseudohypha budding patterns of Candida albicans

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Pseudohypha budding patterns of *Candida albicans*

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Pseudohyphal growth of *Candida albicans* has been recognized as a morphological growth form that exhibits characteristics that are distinct from those of the budding yeast phase and true hyphal form of this pathogenic fungus. In *Saccharomyces cerevisiae*, pseudohypha growth involves synchronous unipolar cell divisions that are a modification of the bipolar budding pattern of diploid cells. While pseudohyphae of *C. albicans* also exhibit unipolar cell divisions, live cell imaging demonstrated departures from the normal unipolar pattern. Buds occasionally followed a bipolar or axial budding event in which buds could be formed from the proximal or distal ends of a parent pseudohypha. This extends the known morphological repertoire of cell division patterns in *C. albicans* pseudohyphal cells.

Keywords budding, *Candida albicans*, cell cycle, pseudohyphae

Introduction

Candida albicans is a pleiomorphic fungus, with three different vegetative growth forms: yeast, pseudohyphae and true hyphae [1,2]. In contrast, bakers' yeast *Saccharomyces cerevisiae* exhibits either unicellular budding or pseudomycelial growth, but does not form parallel-sided unconstricted true hyphae [3]. The original formal definition of a pseudohypha is that it is comprised of a series of conjoined elongated yeast cells that have obvious constrictions at septal sites. A colony of pseudohyphal cells is sometimes termed a pseudomycelium. The extent of cell elongation of pseudohyphal cells can vary considerably depending on the growth conditions [4,5]. Morphology indices have been formulated that describe the extent of elongation relative to the maximum and minimum diameters of the cell [4]. However, all pseudohyphal cells, irrespective of their morphology indices, share properties, such as synchronous cell divisions and septation at the mother-bud neck, that distinguishes them from true hyphae [1,2]. Pseudohyphae are formed by a wide variety of yeast species including most pathogenic *Candida* species and many pleiomorphic fungi that exhibit transitions between filamentous and unicellular forms of growth [6,7]. Amongst the *Candida* species, true hyphae are normally formed only by *C. albicans* and *C. dubliniensis* [8,9], many isolates of *C. tropicalis* and a few exotic and rare *Candida* species [10].

Recent years have seen mounting evidence to support the view that pseudohyphae should be regarded as distinct from true hyphae (e.g., [11,12] and reviewed in [1,13,14]). Nuclear division in pseudohyphae occurs at the point of maximum constriction between the mother and daughter cells and septum formation occurs at the position of minimum cell diameter at the neck. In contrast, in true hyphae of *C. albicans* the site of mitosis, septin ring formation and subsequent septum formation is normally located some distance within the germ tube of a true hypha. In addition, cell divisions are characteristically near synchronous in pseudohyphae whilst in true hyphae sub-apical cells are often arrested in G1 for several cell cycles. The latter phenomenon may be related to the abnormal partitioning of the vacuole during cytokinesis [13,15] and may account for the linear kinetics of hyphal extension [8,13,16]. Several mutations result in cells being unable to form hyphae whilst retaining the ability to form pseudohyphae [2,17]. There are also significant departures from normal cell cycle control of the evagination event in *C. albicans* true hyphae [12,18] and in the pattern of gene expression in pseudohyphal and true hyphal cells [19]. Therefore,

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pseudohyphal growth can be regarded as a defined developmental state of *C. albicans* and presumably fungi in general.

The study of the budding pattern of yeasts has been inspired by the genetic analysis of this process in *S. cerevisiae* [20–25]. Yeast cells of *S. cerevisiae* and *C. albicans* grow by budding, with an initial period of apical growth followed by a period of isotropic development [1,26]. In *S. cerevisiae*, the budding pattern is ploidy-dependent. Haploid cells exhibit an axial budding pattern where haploid cells form consecutive buds adjacent to previous bud sites in an axial row. In diploids, budding is bipolar in which buds are formed at both poles with the first daughter cell normally formed on the opposite pole and subsequent buds forming at either pole [22–24,27,28]. In *C. albicans*, yeast cells are diploid and budding follows the *S. cerevisiae* pattern although the pattern is affected by temperature so that higher temperatures see more randomness in the bud site selection process [29]. For yeast cells of *C. albicans*, 58% of buds were reported to emerge with an axial pattern and 42% bipolar – this ratio being altered in the *int1*Δ mutant background [30]. Site selection for germ tube evagination of *C. albicans* has also been reported to be more random [29,31].

Pseudohyphal growth in *S. cerevisiae* has been demonstrated to exhibit unipolar budding, where daughter cells are produced at the opposite side of the cell to the birth pole, and then subsequently at the proximal end of the compartments within the pseudohypha [20,21,32]. However, the polarized growth period of the cell cycle is extended leading to an elongated cell morphology. The mother and daughter cells are approximately the same size immediately after cytokinesis and consequently they re-enter the cell cycle semi-synchronously. In *C. albicans*, considerably more attention has been given to the characterization of true hyphal growth than to pseudohyphal growth. Here, we describe the use of live cell imaging and digital time-lapse microscopy to describe the budding patterns of *C. albicans* pseudohyphae. We report several new developmental modes for pseudohyphal budding, including bipolar, axial, and distal/proximal budding patterns, thus extending the morphological repertoire of budding patterns observed for *C. albicans* pseudohyphae.

Materials and methods

Strains and culture conditions

C. albicans CAF2 strain was used exclusively in this study [33]. Pseudohyphal development was induced following published protocols with minor modifications [34]. Cells were grown overnight in modified glucose-phosphate-proline (GPP) medium containing as final concentrations; 12.5 mM KH_2PO_4 (pH 6.5), 51 mM l-proline, 13 mM N-acetyl-glucosamine, 15 mM $MgSO_47H_2O$, and 2% glucose. Cells were then harvested, and inoculated in fresh GPP medium supplemented with 300 mM potassium phosphate.

Live-cell imaging

Cavity glass slides (Agar Scientific, UK) containing 125 µl of molten GPP medium supplemented with 300 mM phosphate and 2% agarose were used for the time-lapse videos of live cells. Pseudohyphal cells from an overnight culture were inoculated on the agarose surface, a cover slip was applied and the cavity was sealed using a mixture of lanoline, Vaseline and paraffin wax (1:1:1). Cells were then incubated in the environmental chamber of a Delta-VisionRT microscope (Applied Precision Instruments, USA) at 35°C.

Software

Cells were imaged over a period of 12 h, and images were recorded at fixed intervals of 5 min, using the Softworx version 3.5.0 software (Applied Precision Instruments). The 'point re-visiting' function of the Softworx software was used to track the growth of several cells in the same sample. Still images were exported as tiff files and processed with Adobe Photoshop CS3. Time-lapse movies were assembled using the Softworx software and saved as Quicktime files.

Results

Candida albicans *pseudohyphal cells exhibit different morphologies*

Pseudohyphal growth was induced in the presence of high concentrations of phosphate (300 mM). Liquid cultures of filament forming cells tend to result in the washing off of buds from pseudohyphal mother cells thus preventing analyses of the sequential order of budding events. This problem can be circumvented using live-cell imaging of cells growing on solid media. We therefore used time-lapse live cell imaging to characterize the pattern of growth and budding in *C. albicans* pseudohyphal cells. Cells were plated sparsely to prevent crowding and encroachment of neighbouring pseudohyphal colonies. Control experiments involving the imaging of cells after 12 h growth in the absence of time lapse showed that observation of cells

using the Delta-Vision point-revisiting automation did not affect the growth rate or budding pattern of cells growing in the slide-culture chamber. These conditions generated a different range of cell morphologies, from ovoid elongated yeast cells, to highly elongated pseudohyphae with constrictions at septal sites (Fig. 1). In *S. cerevisiae*, filamentous pseudohyphal cells have been demonstrated to grow with synchronous kinetics in a unipolar budding pattern [20,21]. We observed individual pseudohyphae with visible constrictions in which synchronous development of the cells within the pseudohypha did not occur (Fig. 1C). In addition, a range of budding patterns was observed in different cells, some of which were departures from the normal unipolar mode of growth typical of *S. cerevisiae* pseudohyphae (below).

Combined axial, bipolar and unipolar budding

Pseudohyphae, and true hyphae, often produce multiple buds at septal junctions leading to the formation of clusters of yeast cells of elongated pseudohyphal buds at these positions. Pseudohyphal filaments grew predominantly in a unipolar manner, with new buds emerging from the proximal poles of the leading pseudohyphal cells and the sub-apical compartments (Fig. 2; Supplementary material [on-line version only], Movie 1; Fig. 5A). Subsequent buds emerged from sub-apical cells, adjacent to the previous budding place in an axial pattern (Fig. 2; Fig. 5B). New buds continued to grow some remained attached to the filament trunk and some displaced previous buds and their conjoined daughter cells. Lateral buds generated from the trunk of the pseudohyphae normally exhibited bipolar and

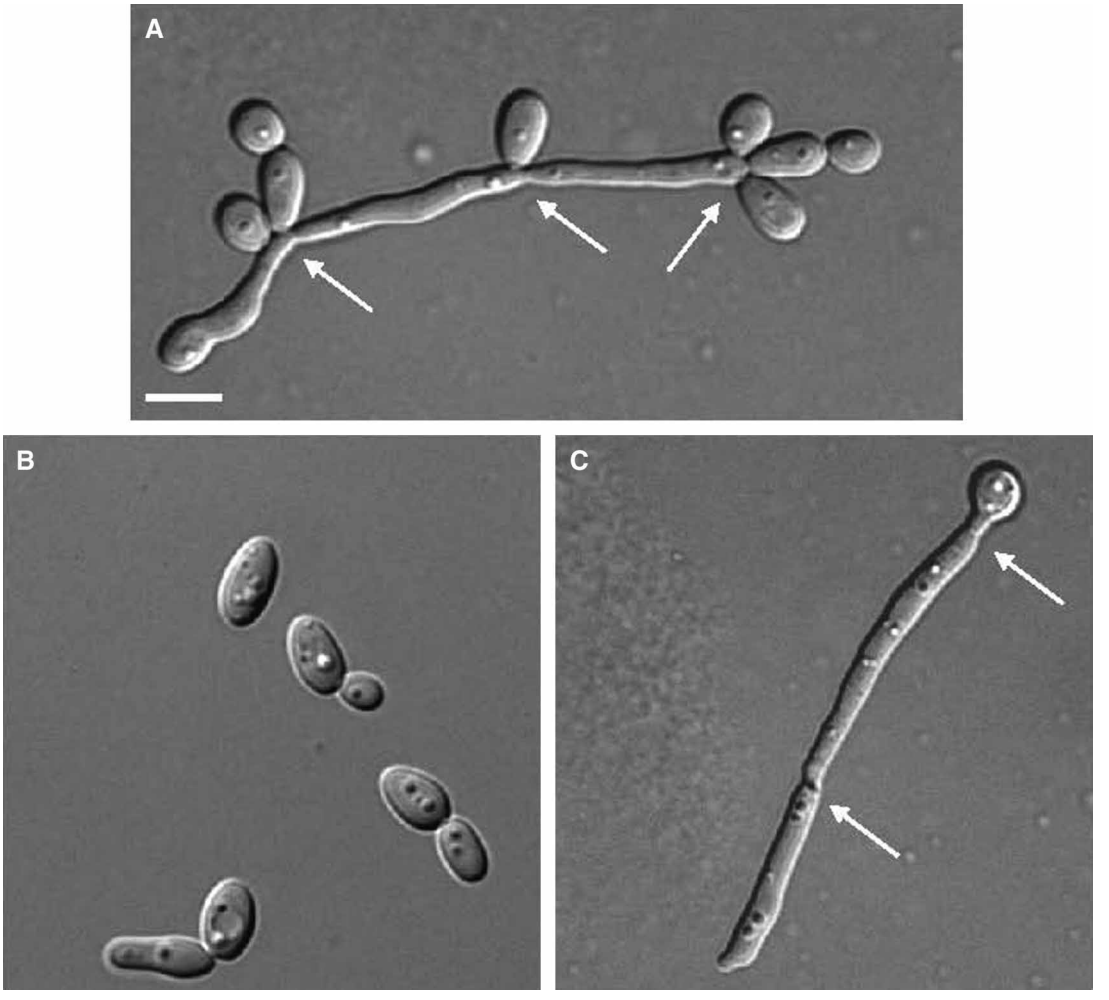

Fig. 1 A range of morphologies of *Candida albicans* cells under pseudohyphal-inducing conditions on solid medium including; (A) branched pseudohyphal structure with lateral buds, (B) yeast cells, and (C) highly elongated pseudohyphal filament. Arrows point to constrictions at septal sites. The scale bar is 10 μm.

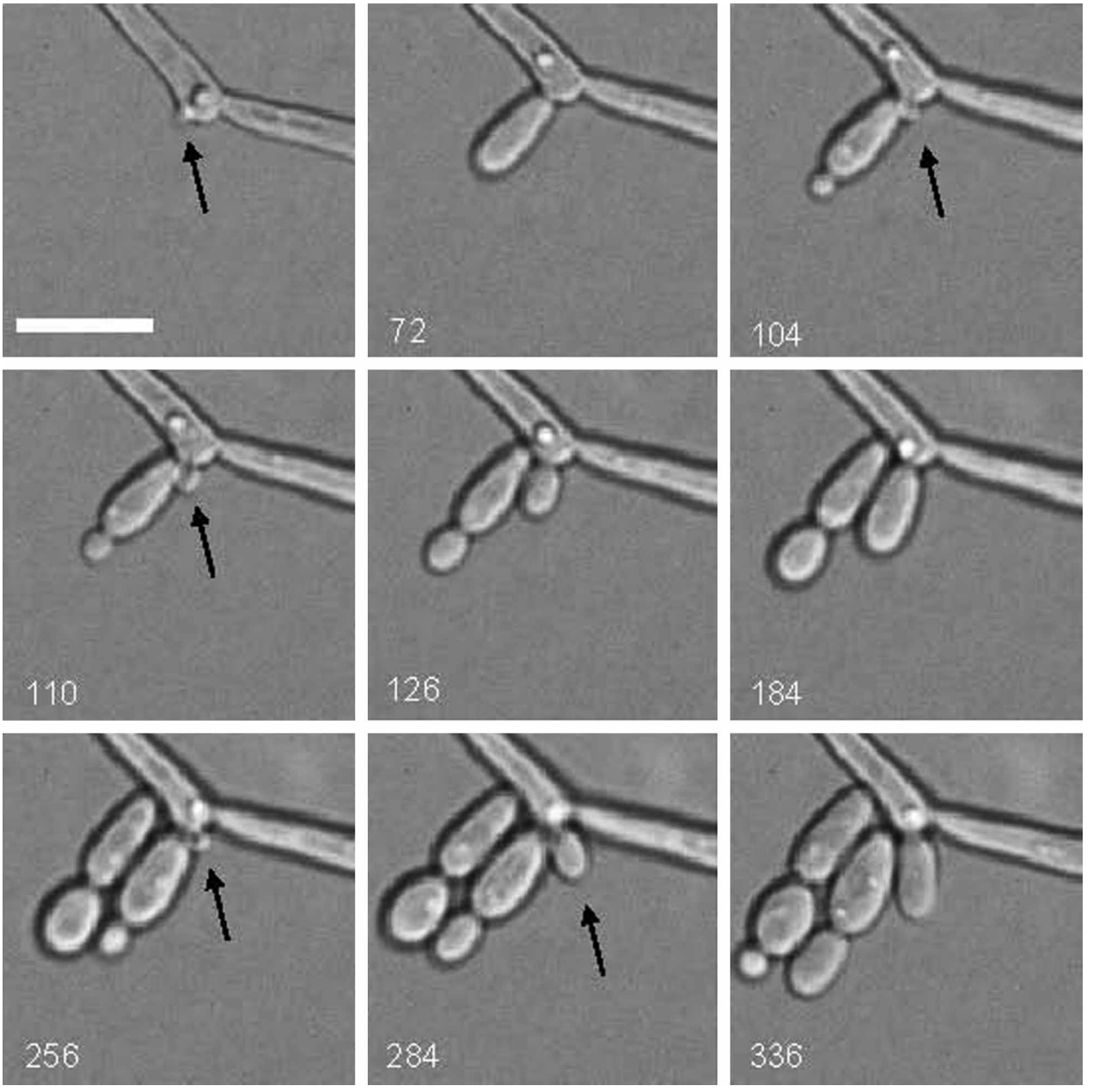

Fig. 2 *Candida albicans* pseudohyphal cells can exhibit both unipolar, bipolar and axial budding patterns. The arrows indicate axial budding events, while the buds that emerge from the main pseudohyphal stem continue to bud in a unipolar fashion. Elapsed time (min) from the recording start is shown at the bottom left of each frame. The scale bar is 10 μm. This sequence is also available as Supplementary movie 1 (on-line version only).

subsequently unipolar growth to develop pseudohyphal branches (Fig. 2; Fig. 5B).

Unipolar and bipolar budding patterns

For most pseudohyphae, unipolar budding sustained the forward progress of the pseudomycelium into fresh growth medium, however in some instances individual pseudohyphal compartments formed a bud that was distal to the direction of pseudomycelium expansion (Fig. 3 dotted arrow; Supplementary material [on-line version only], Movie 2; Fig. 5C). Such filaments generated buds both from the proximal and distal ends of the pseudohypha. This resulted in individual compartments generating buds exhibiting near synchronous cell cycles growing at both poles of the compartment (Fig. 3; Fig. 5C).

Distal-proximal pattern

In some pseudohyphal cells, buds were seen to arise synchronously from adjacent compartments (Fig. 4; Supplementary material [on-line version only], Movie 3). In the example shown (Fig. 4), these buds emerged at the distal pole of the proximal cell and the proximal pole of the distal cell. These buds then budded in a bipolar fashion at the opposite pole and then subsequently by unipolar budding to generate two parallel pseudohyphal branches. Therefore, proximal and distal budding can occur in adjacent pseudohyphal compartments (Fig. 5D).

A total of 26 time lapse sequences representing 180 h of growth were analysed and more than 200 budding

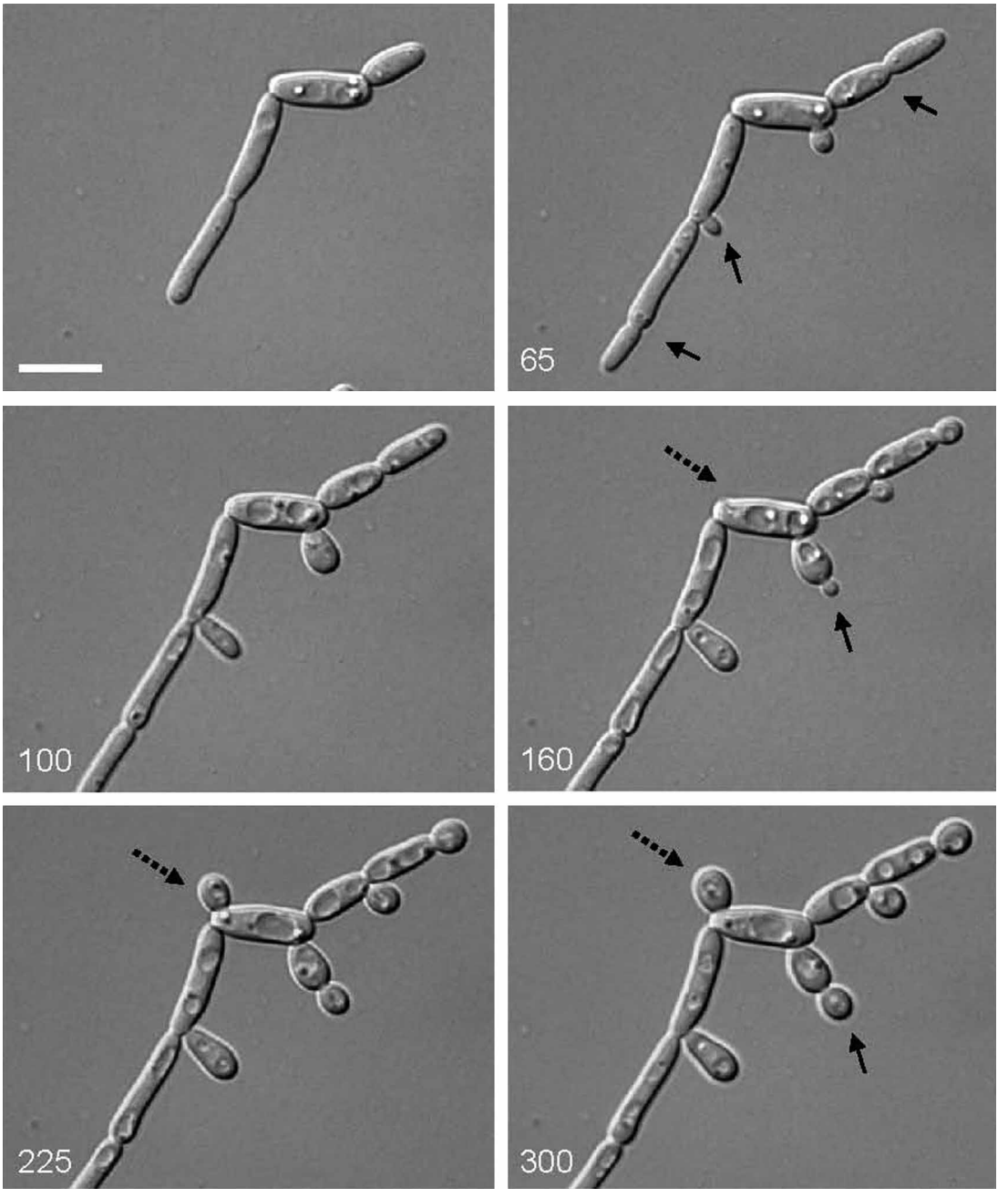

Fig. 3 Unipolar and bipolar budding pattern in *Candida albicans* pseudohyphal cells. Arrows point to unipolar budding sites and dashed arrow points to a bipolar budding event at the opposite pole of the mother cell. The scale bar is 10 μm and the elapsed time in minutes is shown in the bottom left of each panel. This sequence is also available as Supplementary movie 2 (on-line version only).

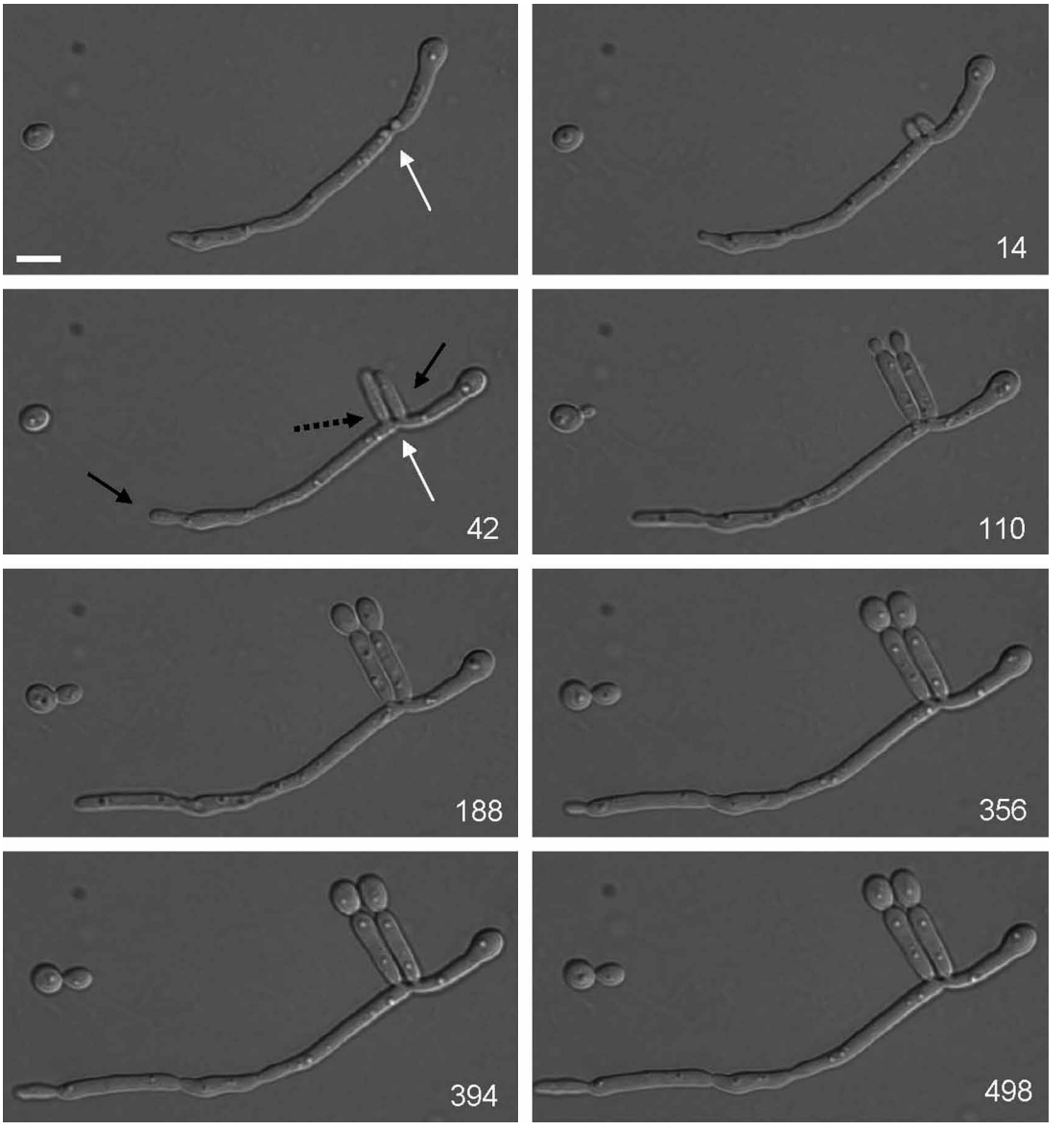

Fig. 4 Distal and proximal budding in *Candida albicans* pseudohyphal cells. Different budding patterns are seen within a single pseudohypha. Unipolar budding is seen at the apical mother cell (arrow), while a distal (dashed arrow) and a proximal bud (solid arrow) is seen emerging from either side of a septum (white arrow) between two adjacent pseudohyphal compartments. These buds continue to develop in a unipolar fashion. The scale bar is 10 μm and the elapsed time in minutes is shown at the bottom right. This sequence is also available as Supplementary movie 3 (on-line version only).

events were recorded and scored. From this, 62% of new buds were unipolar, 24% axial, 12% bipolar and 2% distal-proximal. These various patterns, with the exception of the distal-proximal pattern were observed both of buds emerging from a pseudomycelium and from a single yeast cell in the same culture. There was no obvious correlation between the extent of cellular elongation of the pseudohyphal cells, quantifiable by morphology index, and the type of budding pattern observed. Therefore, although unipolar budding predominates in pseudomycelial growth, other budding patterns account for almost a third of all new buds emerging under these conditions.

Discussion

As a major human fungal pathogen, *C. albicans* has been the focus of extensive research in recent years but the basic cell cycle biology of each of the major vegetative growth forms have not yet been fully

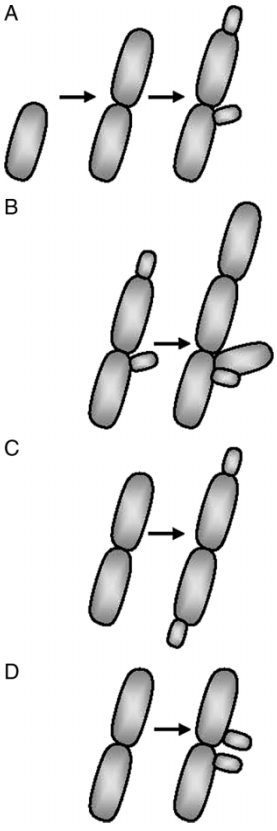

Fig. 5 Summary of pseudohyphal budding patterns in *Candida albicans*. In all cases, the oldest mother cell is at the bottom left of each panel. The arrows indicate the progression of budding events over the period of a single cell cycle. Different budding patterns observed in *C. albicans* pseudohyphal cells are summarized here. (A) Unipolar, synchronous budding in which the first and subsequent daughter buds are formed at the opposite pole from the birth pole. (B) Unipolar-axial budding of cells to form clusters of buds behind pseudohyphal septal junctions. (C) Bipolar budding in which daughter cells are formed at both poles of a pseudohypha. (D) Budding in which daughter cells are formed from both the distal and proximal ends of adjacent cells within a pseudohypha.

described. Since the morphological plasticity of the cell is widely regarded as an important key virulence trait [35,36], it is important to understand the basic methods of cellular development that operate during mitotic cell divisions. For example, it is known that the transcription of genes encoding many of the cell wall glycoproteins that represent major adhesins in the cell wall are highly regulated [37] and so yeast, pseudohyphae and true hyphae have different physical, chemical, immunological and behavioural properties [13,38]. Here, growth media with high concentrations of phosphate were used to induce preferentially pseudohyphal growth. Although the underlying molecular mechanism that triggers the pseudohyphal developmental program under these conditions is not known, this method is effective in obtaining a population of predominantly pseudohyphal cells [34]. We observed a wide range of cell morphologies, including cells with significant differences in the extent of cell elongation in this medium. Live-cell imaging of *C. albicans* cells over long periods made it possible to observe the different budding patterns occurring during the pseudohyphal growth.

Several mutants with constitutive pseudohyphal phenotypes have been described, including *tup1*Δ, *nrg1*Δ, *fkh2*Δ, *tcc1*Δ, *rfg1*Δ and *grr1*Δ [17,37,39]. Many of these are transcriptional factor mutants in genes involved in cell cycle regulation and morphogenesis, and few of these have been characterized in terms of the pseudohyphal morphology and budding pattern. It is not known whether the proportion of cells exhibiting the various modes of budding described here would be affected in such mutants.

The regulation of bud site selection has been highly studied in *S. cerevisiae* and has been shown to be regulated by many genes including *BUD* genes and others that encode components of signalling GTPase modules, septins, cortical marker proteins that orient the actin-based cytoskeleton and the vectorial secretion of vesicles and proteins of the polarosome complex [28]. The axial pattern of budding is thought to favour the orientation of haploid sexual gametes for efficient mating while the bipolar and unipolar budding patterns facilitate the foraging of fronds of filamentous cells that can explore their environment and assimilate nutrients. The discovery that the pseudohyphal mode of growth is more plastic than previously realized may offer new opportunities for the screening of morphological mutants and the analysis of genes whose role is to regulate the selection of new growth axes during cellular development.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Legends to supplementary movies

Supplementary Movie 1. Quicktime Movie File (14.5 MB). Imaging of pseudohyphal development of *Candida albicans* CAF2 cells in phosphate-rich medium at 35°C. Frames were recorded every 2 min over a period of 12 h.

Supplementary Movie 2. Quicktime Movie File (2.78 MB). Imaging of pseudohyphal development of *Candida albicans* CAF2 cells in phosphate-rich medium at 35°C. Frames taken every 5 min over 6 h of growth are shown in the movie.

Supplementary Movie 3. Quicktime Movie File (3.49 MB). Imaging of pseudohyphal development of *Candida albicans* CAF2 cells in phosphate-rich medium at 35°C. Frames were recorded every 2 min for 12 h.

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