Sterol-rich plasma membrane domains in the fission yeast *Schizosaccharomyces pombe*

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Summary

Sterol-rich membrane domains exist in unicellular and multicellular eukaryotes. They are thought to provide a structural framework for interactions among a subset of proteins by selectively incorporating some proteins while excluding others. Although most studies have focused on the biophysical and biochemical properties of sterol-rich membrane domains and incorporated proteins, relatively little is known about their intracellular distribution. Using a cytological approach we show here that in the fission yeast *Schizosaccharomyces pombe*, sterols are enriched in the plasma membrane at the growing cell tips and at the site of cytokinesis. The distribution of sterols is regulated in a cell-cycle-dependent manner and requires a functional secretory pathway. By manipulating the integrity of sterol-rich membrane domains using sterol sequestering agents and genetic means, we show that these domains are important for multiple processes regulating cytokinesis. In these cells, defects in proper maintenance of the actomyosin ring and/or its attachment to the overlying plasma membrane were observed. Furthermore, the stability of a plasma membrane protein that colocalises with sterol-rich membrane domains was compromised. Taken together, our studies establish *S. pombe* as a genetically tractable model organism in which to study the role(s) of sterol-rich membrane domains in cell polarity and cytokinesis.

Key words: Sterol, Plasma membrane, Polarity, Cytokinesis, *Schizosaccharomyces pombe*

Introduction

Sterol-rich membrane domains that are insoluble in cold non-ionic detergents have been implicated in sorting, trafficking, actin cytoskeletal function and signalling events in mammalian cells (Simons and Ikonen, 1997; Brown and London, 1998; Oliferenko et al., 1999; Caroni, 2001; Ikonen, 2001; Martin, 2001). Studies in T cells and neutrophils have shown that detergent-resistant membrane domains accumulate in distinct areas of the plasma membrane during polarisation of these cells (Gomez-Mouton et al., 2001; Seveau et al., 2001). Membrane domains that are insoluble in Triton X-100 have also been isolated from the unicellular eukaryote *Saccharomyces cerevisiae* (Kubler et al., 1996). Sphingolipids and ergosterol, the most abundant sterol in yeast (Harmouch et al., 1995), were shown to be detergent-resistant lipid components (Bagnat et al., 2000).

S. pombe grows in a polarised manner at the cell tips and divides by medial fission by the constriction of an actomyosin ring and concomitant synthesis and deposition of septum material (Chang, 2001). These processes require the polarised localisation of proteins involved in cell wall and septum synthesis, as well as the vectorial addition of new membrane material to the existing plasma membrane. Therefore, we asked whether inherent differences in the lipid composition of the plasma membrane might be important for proper targeting of the growth and division machinery. To this end, we decided to examine the localisation of sterols in *S. pombe* using the fluorescent probe filipin, a polyene antibiotic that forms specific complexes with free 3-β-hydroxysterols. Excitation of filipin at 360 nm results in strong fluorescence with an emission maximum at 480 nm (Drabikowski et al., 1973).

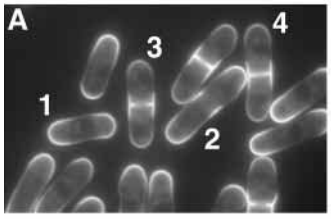
In this study, we report that sterols are localised to distinct regions of the plasma membrane in a cell-cycle-dependent manner. Of particular importance is our finding that membrane sterols are detected at the division site and appear to play multiple roles in cytokinesis.

Materials and Methods

Media for cell culture were as described previously (Moreno et al., 1991). Temperature-sensitive mutant strains were grown at the restrictive temperature of 36°C for 4 hours before visualisation or release to the permissive temperature. Filipin was purchased from Polysciences (Warrington, PA), dissolved in dimethyl sulfoxide (DMSO) and used at a final concentration of 5 μg/ml. For staining purposes filipin was added to the medium and cells were observed immediately. Filipin treatment for disruption of sterol-rich membrane domains was carried out for 1 hour. Thiabendazole (TBZ) and Brefeldin A (BFA) were purchased from Sigma. Latrunculin A (LatA) and anti-green fluorescent protein (GFP) antibodies were purchased from Molecular Probes (Eugene, OR). Quantitative data are based on at least two independent experiments and a counting of at least 800 cells per sample. Fluorescence microscopy was done with a Leica DMLB microscope and appropriate sets of filters. Images were captured using an Optronics DEI-750T cooled CCD camera and Leica QWIN software. Image processing was done on Adobe PhotoShop 5.5. NIH Image (National Institutes of Health, Bethesda, MD) was used for quantitation of protein levels after immunoblotting.

Results

When we used filipin to probe sterol-rich domains in the plasma membrane of live wild-type *S. pombe* cells grown to



B staining pattern	% cells
1	82.75±0.9
2	6.35±1.3
3	4.65±0.8
4	6.25±0.4

Fig. 1. Sterols localise to distinct sites of the plasma membrane. (A) Sterol localisation detected by filipin staining in wild-type cells. (B) Frequency of filipin staining patterns denoted in A in an exponentially growing wild-type culture.

logarithmic phase, we observed various staining patterns (Fig. 1A). The most prevalent pattern showed intensive staining at the tips of the cell (denoted as pattern 1 in Fig. 1A). More elongated cells had an additional medial band (pattern 2), bright staining of membrane invaginations at the site of cytokinesis (pattern 3) or a brightly stained line along the completed septum (pattern 4). In the two latter cases the fluorescence intensity at the tips of the cell was decreased. Interestingly, the relative distribution of cells with respect to the staining pattern resembled the distribution of cells in interphase or cell division, respectively, that is typical of exponentially growing wild-type cultures (Fig. 1B). Our observations support freeze-fracture electron microscopy studies that had shown that alterations in the membrane structure induced by high filipin concentrations are found at the cell poles and in the middle in dividing cells, but not in the proximal regions between the poles and the middle (Takeo, 1985). We conclude that sterols are enriched at the tips of interphase cells and at the medial division site in cells undergoing cytokinesis.

Sterols are enriched at the growing cell tips and at the site of cytokinesis

To determine whether the localisation of sterols to the cell tips is connected to cell growth, we used filipin to probe mutant strains or cells grown under conditions that cause characteristically altered growth patterns. Nitrogen starvation was induced by incubating for 24 hours a prototrophic wild-type strain in medium lacking any source of nitrogen, which led to the appearance of small, rounded, non-growing cells. These cells exhibited greatly reduced levels of fluorescence when stained with filipin (Fig. 2A). After release from nitrogen starvation, cells resumed growth and exhibited wild-type fluorescence intensity and pattern (Fig. 2B). Thus, the presence of sterols in the plasma membrane can be correlated to sites of cell growth.

Using temperature-sensitive mutants we tested whether an altered growth pattern could be reflected in the sterol distribution. Shift of *cdc10*-V50 cells to the restrictive temperature causes a cell-cycle block in G_1-phase before growth at the new end is initiated, leading to a monopolar growth pattern (Nurse et al., 1976). Filipin staining of arrested cells resulted in strong fluorescence at one end of the cell only (Fig. 2C). Using GFP fused to the actin-binding calponin homology domain of Rng2p (CHD-GFP; K. Eng and M.K.B., unpublished), we confirmed that actin patches concentrate at the same end as sterols (Fig. 2D), suggesting that this is the growing old end of the cell. The new end showed staining that was relatively weak in intensity and which covered a smaller membrane area compared with the old end or both growing ends in a wild-type strain. Hence, the distribution of sterols in the plasma membrane of cells growing at the old end only is biased towards the growing tip. We assume that the sterols detected at the new end stem from the previous cytokinesis that occurred before the cell-cycle arrest.

Glucose-starved cells differ from cells starved from nitrogen in the cell-cycle stage at which they arrest (the majority are in G_2 for glucose starvation and in G_1 for nitrogen starvation) and are therefore not at

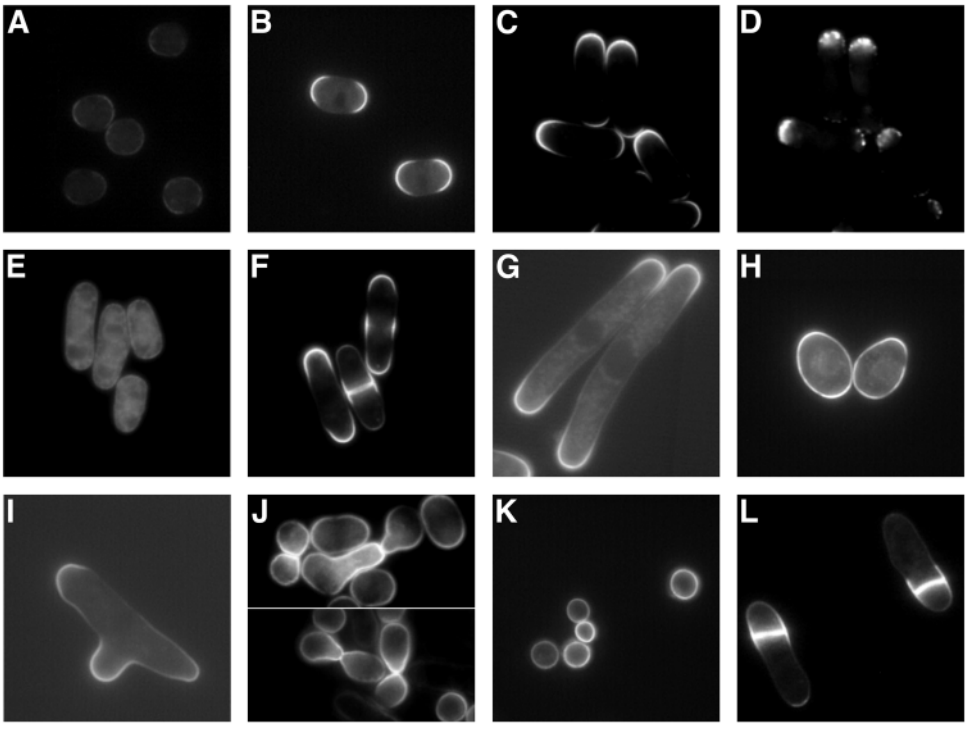


Fig. 2. Sterol localisation correlates with sites of active cell growth and cytokinesis. Sterol localisation detected by filipin staining in (A) nitrogen-starved cells, (B) cells released from nitrogen starvation for 80 minutes, (C) *cdc10*-V50 cells at 36°C, (E) glucose-starved cells, (F) cells grown in medium with glucose, (G) *cdc25*-22 cells at 36°C, (H) *orb1*-13 cells at 36°C, (I) a branched TBZ-treated *cdc10*-V50 cell, (J) mating cells, (K) ascospores and (L) *mid1*-18 cells at 36°C. (D) Localisation of CHD-GFP in *cdc10*-V50 cells.

the verge of initiating mating, which might require the reorganisation of the membrane. Glucose starvation was induced by incubating for 24 hours a prototrophic wild-type strain in medium lacking glucose. When stained with filipin, these cells exhibited greatly reduced levels of fluorescence (Fig. 2E) as compared with control cells grown in medium with glucose (Fig. 2F). Hence, the situation in glucose-starved cells resembles nitrogen-starved cells rather than the new end of arrested *cdc10*-V50 cells. However, it is possible that the uptake of sterols from the plasma membrane is a rather slow process and therefore, sterols are reduced, but still clearly detectable, at the new end of *cdc10*-V50 cells after the temperature shift, which is short lasting compared with the induction of starvation.

The shift of *cdc25*-22 cells to the restrictive temperature causes a cell-cycle block at the G_2/M-boundary – a stage of the cell cycle at which growth occurs at both tips (Russell and Nurse, 1986). Arrested cells showed filipin staining at both ends of the cell but never in a medial band (Fig. 2G). Thus, localisation of sterols in a medial band depends on entry into M-phase.

orb mutants that are shifted to the restrictive temperature have been shown to exhibit unpolarised growth, leading to large, rounded cells (Snell and Nurse, 1994). Filipin-stained *orb1*-13 cells showed fluorescence covering the entire circumference of the cell (Fig. 2H). We conclude that cells lacking growth polarity also lack polarity in the distribution of sterols within the plasma membrane.

We then assessed whether sterols were detected at ectopic new ends generated by treatment of *cdc10*-V50 cells with the microtubule-depolymerising drug TBZ (Sawin and Nurse, 1998). We probed branched cells with filipin and observed that the ectopically growing tip was brightly stained, in many cells with an even higher fluorescence intensity than at the endogenous tips (Fig. 2I). Our results with cells growing in monopolar, unpolarised or ectopical patterns indicate that the distribution of sterols in the plasma membrane is correlated to the growth pattern of the cell.

Mating projections constitute a specialised form of naturally occurring polarised growth in *S. pombe* in which morphogenesis directed towards pheromones overrides the usual antipodal growth observed in vegetative cells. To examine whether sterols can be detected in mating projections, we induced synchronous meiosis in a homothallic wild-type strain as described (Beach et al., 1985). Filipin staining showed that sterols in the plasma membrane were polarised towards the projection (Fig. 2J), indicating that enrichment of sterols in the plasma membrane also occurs during mating projection formation. Our observations support recent studies in *S. cerevisiae* that had shown that lipid rafts cluster at the mating projection in budding yeast (Bagnat and Simons, 2002).

Sexual development and meiosis in *S. pombe* results in the formation of four ascospores. As cell wall synthesis in ascospores requires enzymes that are different from those required for cell wall formation during vegetative growth (Liu et al., 2000; Martin et al., 2000), we tested to see whether sterol localisation was also evident in the plasma membrane underlying spore walls. Filipin uniformly stained the plasma membrane of spores generated from a cross between wild-type cells of opposite mating types (Fig. 2K). Interestingly, although the fluorescence intensity was approximately the same as that at the growing tips of cells in vegetative development, photobleaching of filipin occurred much more rapidly in spores than in vegetative cells. It would be interesting to perform further experiments to address the function of sterols in the plasma membrane of ascospores.

A shift of *mid1*-18 cells to the restrictive temperature leads to mispositioned actomyosin rings and septa (Chang et al., 1996; Sohrmann et al., 1996; Balasubramanian et al., 1998). We found that the plasma membrane invaginations associated with the site of cell division in *mid1*-18 cells are stained by filipin even when these sites are misplaced (Fig. 2L). The medial band of sterols next to the invaginated membrane was found to localise in a similar manner. Hence, we conclude that the sterol enrichment of the plasma membrane at the site of cytokinesis is coupled to the localisation of the division machinery.

The distribution of sterols is regulated in a cell-cycle-dependent manner

To characterise the emergence of sterols in the medial region of the cell, *cdc25*-22 cells expressing Hht2-GFP (Wang et al., 2002) and Rlc1-GFP (Naqvi et al., 2000) as nuclear and actomyosin ring markers were released from a cell-cycle block at 36°C to 18°C, which allowed a better temporal resolution of mitotic and cytokinetic events than release to 24°C. We found that the formation of the actomyosin ring and nuclear division occurred before the concentration of sterols in a medial band (Fig. 3; 0-45 minutes). However, constriction of the actomyosin ring was observed after sterol targeting to the middle of the cell (Fig. 3; 60-90 minutes). Filipin also stained the plasma membrane invaginations that follow the constricting ring (Fig. 3; 105 minutes). After constriction of the ring to a dot the plasma membrane separating the two daughter cells exhibited intense fluorescence (Fig. 3; 120 minutes). These experiments establish that sterols are concentrated at the division site following assembly of the actomyosin ring, and persist during ring constriction and septum assembly. A medial band of sterols was observed only in cells undergoing cell division. Hence, the localisation of sterols to the middle of the cell is regulated in a cell-cycle-dependent manner.

Sterol localisation requires a functional secretory pathway

Our time-course studies led to the possibility that the formation of the actomyosin ring might serve as a spatial and temporal landmark for sterol localisation to the middle of the cell. To test this idea, we investigated whether an intact F-actin cytoskeleton is required for sterol localisation to the plasma membrane overlying the actomyosin ring. On release of *cdc25*-22 cells from the cell-cycle arrest we added the actin-polymerisation inhibitor LatA or DMSO as solvent control. The effective disruption of the actin cytoskeleton in LatA-treated cells was confirmed by rhodamin-phalloidin staining (data not shown). Sixty minutes after release we found that similar fractions of LatA-treated cells and of control cells exhibited medial sterol localisation (Fig. 4A; arrows). This localisation was observed at the centre of the cell and symmetrically on both sides, suggesting that it forms a band circumventing the cell. We conclude that the F-actin

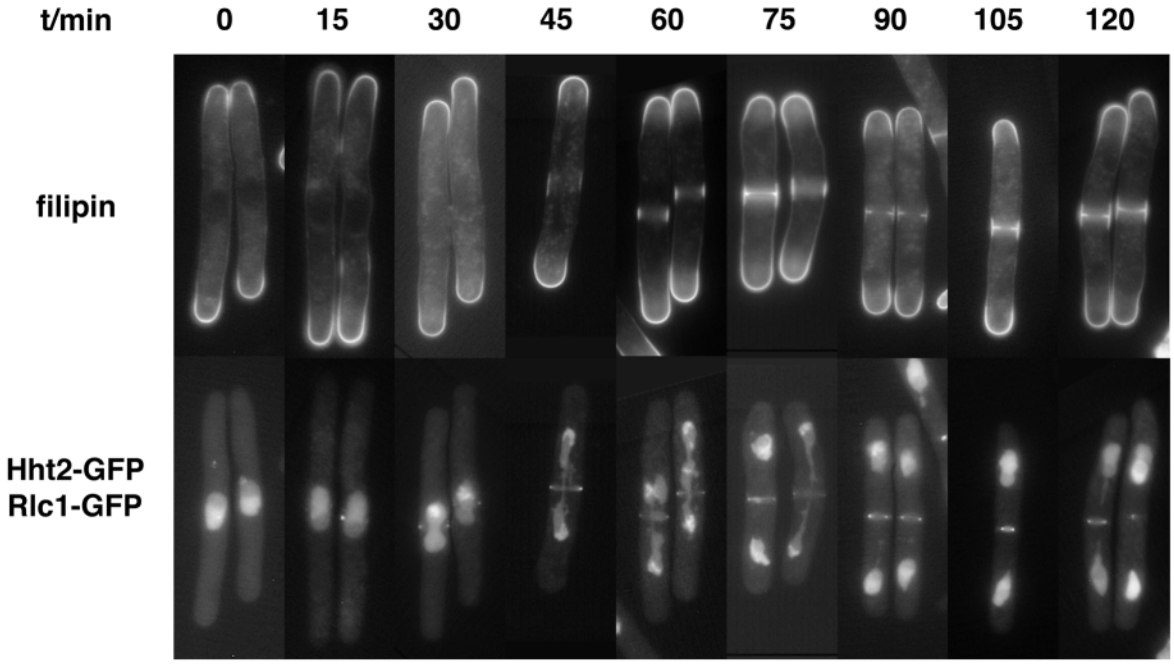


Fig. 3. Sterol localisation to the middle of the cell during mitosis and cytokinesis. *cdc25*-22 *hht2*GFP *rlc1*GFP cells were synchronised and released to the permissive temperature (18°C). Samples were taken at the indicated time points after release, stained with filipin and observed for GFP and filipin fluorescence.

cytoskeleton is not required for the localisation of sterols to the middle of the cell. In addition to the medial band, many LatA-treated cells showed patches of filipin staining at varying positions between the tips and the middle of the cell (Fig. 4A; arrowheads). In contrast to the medial band, these patches were frequently distributed in an asymmetric manner, indicating that they did not form bands around the long axis of the cell. The emergence of sterol patches at various sites of the plasma membrane may be due to continued secretion of sterol-rich membrane material with a simultaneous lack of endocytosis caused by disruption of the F-actin cytoskeleton. Such disturbance of the equilibrium between secretion and endocytosis of sterols may cause an excess of sterols at the plasma membrane, leading to the formation of additional sterol patches.

To address the role of the microtubule cytoskeleton in sterol localisation before cytokinesis, we used *nda3*-KM311, a cold-sensitive mutant that does not form microtubules when shifted to 18°C (Toda et al., 1983). We counted cells with medial sterol localisation over a course of 6 hours after downshift. The percentage of cells with a medial band increased with the time of incubation at low temperature (Fig. 4B). We conclude that microtubules are not necessary to localise sterols to the middle of the cell. Additionally, this result suggests that arrest of cells at metaphase by the spindle assembly checkpoint does not prevent the medial localisation. To test if this was the case, we overexpressed *mad2*$^{+}$ as an alternative means to activate the spindle assembly checkpoint, thereby leading to metaphase arrest (He et al., 1997). Overexpression of *mad2*$^{+}$ led to a high percentage of cells with a medial band of sterols compared with the control, confirming that cells arrested at metaphase localise sterols in a medial band (Fig. 4B). Although previous experiments using *cdc25*-22 synchrony indicated that sterols are detected at the division site at later stages of cytokinesis, the *mad2*$^{+}$ overexpression and *nda3*-KM311 experiments indicate that sterols are detected there in metaphase-arrested cells. A possible explanation for this inconsistency is that low levels of sterols may accumulate in cycling cells at metaphase. Although these low levels may be difficult to detect readily, this localisation may become more obvious in metaphase-arrested cells due to the continued accumulation of sterols.

We then investigated whether a functional secretory pathway was necessary for sterol localisation to the plasma membrane overlying the actomyosin ring. On the release of *cdc25*-22 cells from the cell-cycle arrest we added BFA or ethanol (EtOH) as solvent control. It has been shown that in yeast, BFA inhibits transport from the endoplasmic reticulum (ER) to the Golgi apparatus (Graham et al., 1993). Sixty minutes after release we scored these cells for medial sterol localisation. A high percentage of control cells exhibited bright medial filipin staining (Fig. 4C), but almost all BFA-treated cells showed none or only faint fluorescence in the middle of the cell (Fig. 4C; arrow). This indicates that a functional secretory pathway is required for efficient medial sterol localisation. The faint band in BFA-treated cells may be caused by an incomplete block of secretion. Alternatively, it is possible that a pool of sterols may be present in a post-Golgi compartment that can still be targeted to the plasma membrane when the ER-to-Golgi transport is blocked. Moreover, we observed that BFA-treated cells exhibited a low level of fluorescence throughout the plasma membrane whereby the tips no longer appeared brighter than the rest of the plasma membrane. One explanation for this finding is that endocytosis of sterol-rich membrane material may occur at the cell tips. Studies in mammalian cells have shown that a pool of intracellular cholesterol is present in the endocytic recycling compartment (Mukherjee et al., 1998;

Hao et al., 2002). In case of an intact secretory pathway, this membrane material may be recycled in a polarised manner to the plasma membrane at the cell tips, whereas a block of secretion may result in the accumulation of endocytic vesicles and subsequent fusion with the plasma membrane in a locally unrestricted manner.

Because the strongest filipin fluorescence is observed during septum deposition, we investigated whether the Septum Initiation Network (SIN) might be involved in regulating the localisation of sterols to the site of cytokinesis. To test this idea we used *cdc7*-24, a temperature-sensitive mutant that renders the SIN pathway inactive (Nurse et al., 1976). These cells expressed Cdc4-GFP as an actomyosin ring marker (V. M. D'souza and M.K.B., unpublished). After shift to 36°C for 4 hours, hardly any cells showed medial staining as observed during cytokinesis (Fig. 4D), regardless of the presence or absence of an actomyosin ring (data not shown). However, the majority of cells exhibited a faint filipin staining in the middle of the cell (Fig. 4D; arrows). This staining was often asymmetrical and extended over a varying length of the cell. Many cells formed bulges at the site displaying faint staining, this being reminiscent of cells beginning to form ectopic cell tips after TBZ treatment. We therefore assume that the faint staining that we observe in *cdc7*-24 is due to an attempted initiation of growth at a site where the new cell end would be, had the cell undergone cytokinesis. The lack of strong filipin staining in the middle of the cell in a *cdc7*-24 mutant seems to conflict with our findings for cells arrested at metaphase, i.e. a stage of the cell cycle that occurs before the SIN pathway is activated. A possible explanation is the bypass of the post-anaphase array stage of the cell cycle in SIN mutants. Hence, we propose that not an active SIN pathway but the cell-cycle stage (between metaphase spindle stage and post-anaphase array stage) is crucial for sterol localisation as a medial band. This would correlate the staining with the timing of the orientation of the secretion machinery towards the medial region.

Manipulating the integrity of sterol-rich membrane domains leads to defects in cytokinesis

The localisation pattern of sterol-rich membrane domains suggests multiple roles for sterols in polarity and/or growth on the one hand and in cytokinesis on the other hand. To gain insight into the possible functions of sterols, we attempted to alter the structure of sterol-rich membrane domains in the plasma membrane of *S. pombe* cells. We have chosen to do so in two different ways: by extended incubation in filipin and by overexpression of C-4 sterol methyl oxidase.

In addition to the use of filipin as a probe for sterols in fluorescence assays, incubation in high filipin concentrations and/or for an extended time-span has been shown to induce deformations in sterol-containing membranes that can be observed by freeze-fracture and electron microscopy

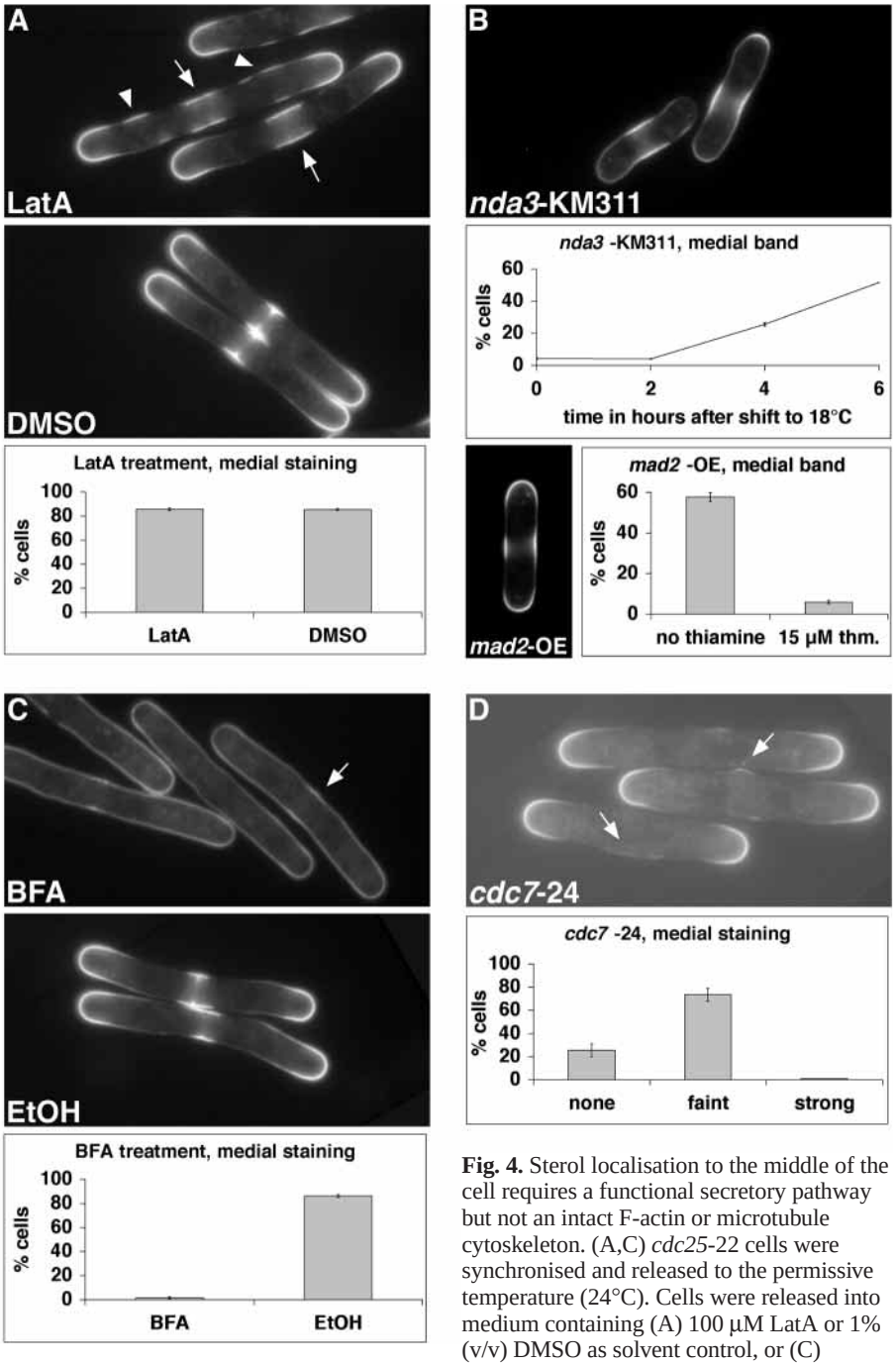


Fig. 4. Sterol localisation to the middle of the cell requires a functional secretory pathway but not an intact F-actin or microtubule cytoskeleton. (A,C) *cdc25*-22 cells were synchronised and released to the permissive temperature (24°C). Cells were released into medium containing (A) 100 µM LatA or 1% (v/v) DMSO as solvent control, or (C) 100 µM BFA or 1% (v/v) EtOH as solvent control. Samples were taken 60 minutes after release and stained with filipin. (B,D) Sterol localisation detected by filipin staining in (B) *nda3*-KM311 cells at 18°C and cells overexpressing *mad2*$^{+}$ under the *nmt1*-promotor, and (D) *cdc7*-24 cells at 36°C. Arrows indicate strong medial staining in A and faint medial staining in C and D. Arrowheads indicate additional asymmetric patches of sterol-rich membrane.

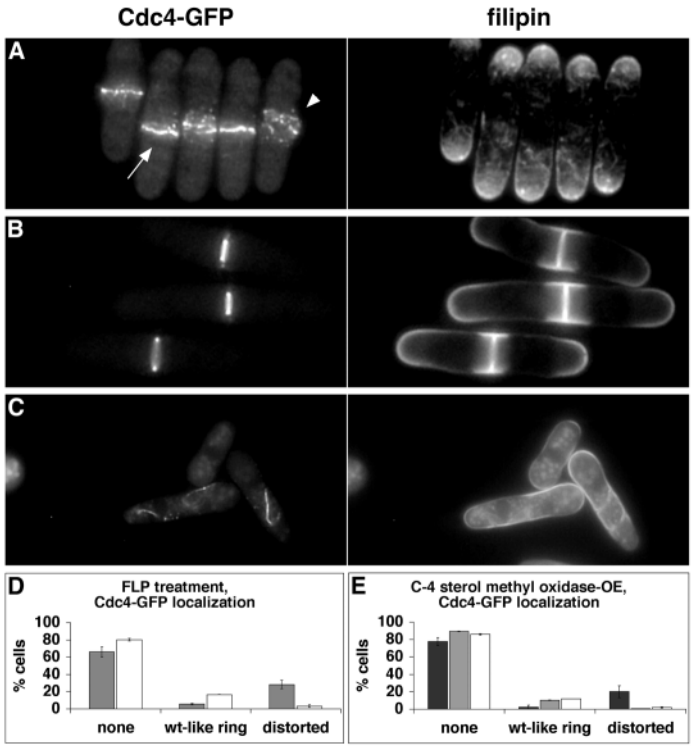


Fig. 5. Structural alterations of sterol-rich membrane domains affect cytokinesis in *S. pombe*. (A,B) *cdc4*GFP cells were grown for 1 hour in medium containing 5 μg/ml filipin or 0.1% (v/v) DMSO as solvent control. Arrow indicates mis-shapen Cdc4-GFP ring; arrowhead indicates Cdc4-GFP spots. (C) *cdc4*GFP cells overexpressing C-4 sterol methyl oxidase under the *nmt1*-promotor. (D,E) Quantitation of localisation patterns. Bars in D represent filipin-treated cells (grey) and DMSO-treated cells (white); bars in E represent cells overexpressing C-4 sterol methyl oxidase (dark grey), control cells with empty vector (light grey) and control cells in 15 μM thiamine (white).

techniques (Friend and Bearer, 1981; Severs and Robenek, 1983). Filipin also disrupts the structure and function of caveolae, non-coated cholesterol-rich invaginations in the plasma membrane of mammalian cells (Rothberg et al., 1990). Cells expressing Cdc4-GFP were grown to logarithmic phase. Filipin or DMSO as solvent control were added and Cdc4-GFP epifluorescence was visualised after 60 minutes. In a high proportion of filipin-treated cells the localisation of Cdc4-GFP was distorted (Fig. 5A), whereas control cells showed proper formation and positioning of uniform Cdc4-GFP rings (Fig. 5B). The observed phenotypes ranged from occurrence of mis-shapen ring-like structures in the medial region of the cell (Fig. 5A; arrow) to the accumulation of spots of various intensities (Fig. 5A; arrowhead). Occasionally, we observed rings that had drifted out of their original position. The higher percentage of cells with Cdc4-GFP staining after filipin treatment (33.9±5.7% compared with 19.7±1.6% of control cells) indicates that filipin-treated cells divide inefficiently. Filipin staining in the medial region of the cell was observed in some cells (Fig. 5A, Fig. 6A). However, we observed numerous cells without medial staining in which distorted Cdc4-GFP rings were approximately in their original position. The filipin signal at the cell tips was visible in all examined cells.

Using a screen for gene products which, on overexpression, lead to cell-division abnormalities, we have isolated C-4 sterol methyl oxidase encoded by an *S. pombe* homologue of *S. cerevisiae ERG25* (Bard et al., 1996; Li and Kaplan, 1996), an enzyme in the ergosterol biosynthetic pathway. Interestingly, when we overexpressed C-4 sterol methyl oxidase, we observed a similar range of Cdc4-GFP localisation phenotypes as with filipin treatment: Cdc4-GFP rings were mis-shapen and mispositioned, and more frequently formed cables that ran along the long axis of the cell (Fig. 5C). Control cells in which C-4 sterol methyl oxidase was repressed by thiamine or which were transformed with an empty vector showed wild-type-looking Cdc4-GFP rings and sterol distribution (data not shown). After extended overexpression of C-4 sterol methyl oxidase, some cells became elongated and branched (data not shown). The filipin staining was not restricted to distinct areas of the plasma membrane though the cell tips, and the middle of the cell showed the strongest fluorescence (Fig. 5C). The overall fluorescence intensity was higher than in control cells. Similar to *mid1*-18 cells, septum material was deposited at aberrant positions according to the misplaced actomyosin ring. Studies in *S. cerevisiae* had reported that overexpression of *ERG25* led to an overall pattern of lipids and sterols similar to wild type (Li and Kaplan, 1996). However, possible changes in the relative abundance of sterol intermediates would not have been detected by this analysis. Because Erg25p is involved in the C-4 demethylation of 4,4-dimethylzymosterol, it is possible that its overexpression may lead to an accumulation of downstream intermediates in the ergosterol biosynthetic pathway. We conclude that changes in the structure or composition of sterol-rich membrane domains cause various defects in actomyosin ring positioning and/or maintenance.

Manipulating the integrity of sterol-rich membrane domains destabilises a colocalising plasma membrane protein

To examine the effects of extended filipin treatment on the plasma membrane, we observed the intracellular distribution of the integral membrane protein Bgs4p, a 1,3-β-glucan synthase subunit that localises to the plasma membrane at the growing cell tips and at the site of cytokinesis (Liu and Balasubramanian, 2001). Cells expressing Bgs4-GFP were grown to logarithmic phase. Filipin or DMSO as solvent control was added and Bgs4-GFP epifluorescence was visualised after 60 minutes. In filipin-treated cells the localisation to distinct sites of the plasma membrane was completely abolished (Fig. 6A). Instead, a weak GFP signal was distributed throughout the cell. By contrast, control cells showed Bgs4-GFP localisation at the tips and the division site (Fig. 6B). The loss of Bgs4-GFP from the tips and the division site could be due to relocalisation from the plasma membrane or to a reduction in Bgs4-GFP levels. To discriminate between these possibilities we analysed equal

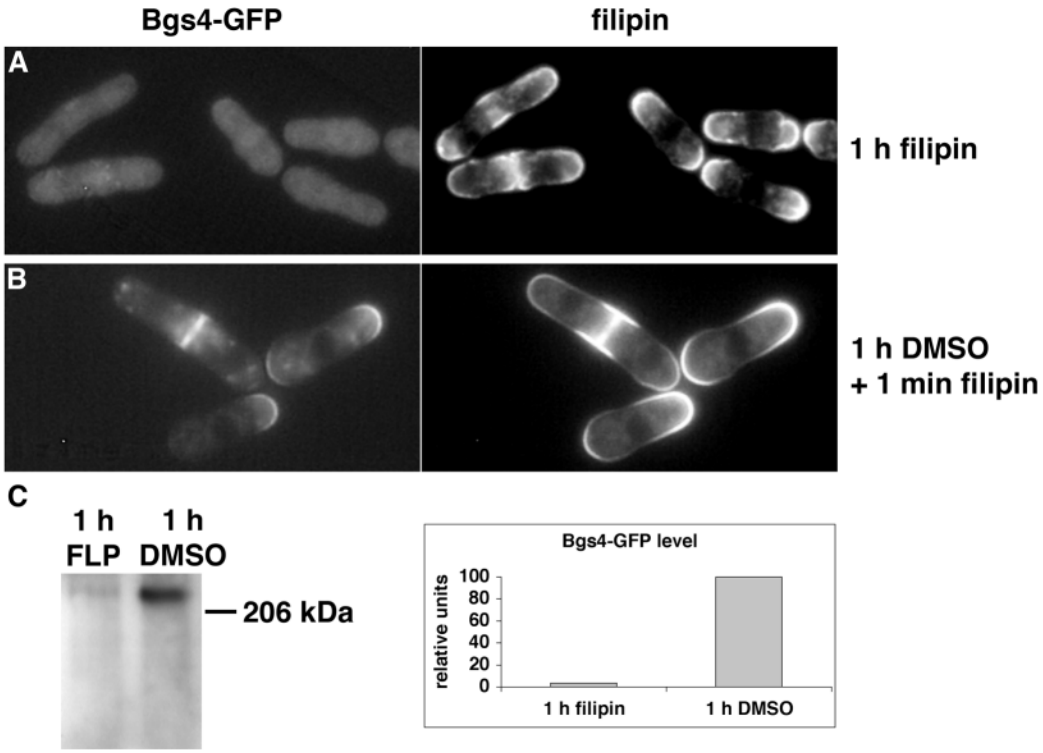


Fig. 6. Structural alterations of sterol-rich membrane domains compromise the stability of a colocalising plasma membrane protein. (A,B) *bgs4*GFP cells were grown for 1 hour in medium containing 5 μg/ml filipin or 0.1% (v/v) DMSO as solvent control. (C) Equal amounts (11.5 μg each) of total protein from filipin- or DMSO-treated cells were analysed by immunoblotting and probed with anti-GFP antibodies. The signal strength was quantified and plotted as relative units.

amounts of total protein from filipin-treated cells and from control cells treated with DMSO. On immunoblots, anti-GFP antibodies recognised a single protein, with the predicted molecular weight of 252 kDa. The band was easily detectable in the control, but only a very weak signal was observed in the filipin-treated sample. Quantitation showed that the signal strength in filipin-treated samples was reduced to below 4% of the strength in control samples (Fig. 6C). Our observations show that the stability of a membrane protein that colocalises with sterol-rich membrane domains is compromised when the structure of these domains is altered.

These results suggest that sterol-rich membrane domains play an important role in positioning and/or maintenance of the actomyosin ring. One possible explanation is that proteins involved in ring maintenance may localise to intact sterol-rich membrane domains. The disruption of these membrane structures may affect the stability, localisation and/or function of these proteins. Another possibility is that sterol-rich membrane domains may be involved in anchoring the ring to the plasma membrane. The identification of proteins linking the actomyosin ring and the plasma membrane will provide further insight into the relevance of sterols in the regulation of cytokinesis.

Discussion

In this study we report the cell-cycle-regulated and secretion-dependent localisation of sterol-rich membrane domains to distinct sites in the plasma membrane of *S. pombe*. The localisation correlates with sites of active cell growth and cytokinesis. We have shown that the integrity of these domains is crucial for positioning and/or maintenance of the actomyosin ring, as well as for the stability of an integral membrane protein. Sterols may play multiple roles in cell growth and division: on the basis of their selective incorporation of proteins, sterol-rich membrane domains may act as a structural framework within the plasma membrane (Simons and Ikonen, 1997). Such a framework may facilitate interactions among proteins involved in establishing cell polarity and in cell growth, including cell-wall synthesis and targeted membrane addition. Similarly, proteins involved in cytokinetic processes such as ring constriction and septum deposition may require concentration in distinct areas of the plasma membrane for their interaction and function.

It is crucial for the morphogenesis of *S. pombe* that it tightly regulates where and when cell growth and division occur (Verde, 1998). Therefore, another possible role of sterol-rich membrane domains may be the spatial limitation of the growth machinery and the division machinery to the growing cell tips and to the site of cytokinesis, respectively.

Finally, recent work on cellulose synthesis in plants has implicated sitosterol-β-glucosides in 1,4-β-glucan (cellulose) chain initiation (Peng et al., 2002). 1,3-β-glucan is an abundant cell-wall component in *S. pombe*, and in growing cells 1,3-β-glucan synthases localise to the cell tips and the division site (Liu and Balasubramanian, 2001; Liu et al., 2002). Hence, sterol-linked molecules may play a role in the synthesis of the cell wall and septum as a primer or substrate. Being amenable to molecular biology as well as to genetics and having a well-studied cell cycle, *S. pombe* will be a suitable model organism to test the models discussed above and to define the function of sterol-rich membrane domains in cell polarity and cytokinesis.

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