Endocytosis in fission yeast is spatially associated with the actin cytoskeleton during polarised cell growth and cytokinesis

Yannick Gachet* and Jeremy S. Hyams‡,§

Department of Biology, University College London, Gower Street, London, WC1E 6BT, UK

*Present address: LBCMCP-CNRS UMR5088, Institut d'Exploration Fonctionelle des Génomes (IFR109), Université Paul Sabatier, 118 route de Narbonne, 31062 Toulouse, France
‡Present address: Institute of Molecular BioSciences, Massey University, Private Bag 11-222, Palmerston North, New Zealand
§Author for correspondence (e-mail: j.hyams@massey.ac.nz)

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Summary

In the fission yeast, *Schizosaccharomyces pombe*, uptake of the fluorescent styryl dye FM4-64 via the endocytic pathway to the vacuole was localised to the poles of growing, interphase cells and to the cell equator during cell division, regions of cell wall deposition that are rich in actin. When the pattern of growth or the plane of cytokinesis was altered, the relationship between the actin cytoskeleton and the site of endocytosis was maintained. Transfer of the label to the vacuolar membrane was dependent upon the Rab GTPase Ypt7 and, hence, vesicle fusion. Endocytic vesicles transiently colocalised with actin patches and endocytosis was inhibited in mutants that affected actin patch integrity and by the actin inhibitor latrunculin A. Concentrations of latrunculin that removed actin cables but left patches unaffected had no effect on endocytosis at the poles, but abolished endocytosis at the cell equator. Equatorial, but not polar, endocytosis was also inhibited in cells lacking the formin For3 (which have selectively destabilised actin cables), in mutants of the exocyst complex and in cells treated with brefeldin A. Differential effects on endocytosis at the cell poles and equator were also observed in the actin mutant *cps8* and the Arp2/3 complex mutant *arp2*. The redirection of endocytosis from the cell poles to the cell equator in M phase coincided with the anaphase separation of sister chromatids and was abolished in the septation initiation network (SIN) mutants *cdc7*, *sid1* and *sid2*, demonstrating that the spatial reorganisation of the endocytic pathway in the *S. pombe* cell cycle requires a functional SIN pathway. We conclude that endocytosis in fission yeast has two distinct components, both of which are actin-based, but which are mechanistically distinct, as well as being spatially and temporally separated in the *S. pombe* cell cycle.

Key words: Endocytosis, FM4-64, Actin, Fission yeast

Introduction

Fission yeast cells exhibit a defined pattern of cell growth and division. Following cytokinesis, each daughter cell consists of an 'old' cell pole that existed in the previous cell cycle and a 'new' cell pole that was created following the separation of the two daughter cells. Growth in the following cell cycle is initially confined to the old end until cells attain a critical mass whereupon new end growth is initiated. This transition is known as NETO (for 'new-end take-off') (Mitchison and Nurse, 1985). Following NETO, cells grow in a bipolar manner, albeit faster at the old end. Upon entry to M phase, growth ceases and cell wall synthesis is redirected from the poles to the cell equator for the formation and cleavage of the cytokinetic septum (Cortés et al., 2002). The distribution of actin through the fission yeast cell cycle precisely parallels this pattern of growth and cytokinesis (Marks and Hyams, 1985). Fission yeast cells contain three F-actin-based structures: patches, cables and the cytokinetic actomyosin ring (CAR) (Marks and Hyams, 1985; Gachet et al., 2004a). Actin patches are confined to the old cell end prior to NETO but become associated with both poles following the switch to bipolar growth. This association of actin with the growing poles is quantitative as well as qualitative. In post-NETO cells the density of actin at the two poles is roughly 1.5:1 (old:new), close to the contribution of the respective poles to cell growth (Y.G. and J.S.H., unpublished observations) (Mitchison and Nurse, 1985). At cell division, actin patches disappear from the poles and the CAR forms at the cell equator from a combination of the reorganisation of the F-actin cables that extend along the axis of the interphase cell and de novo actin assembly (Arai and Mabuchi, 2002; Pelham and Chang, 2002). The CAR, which both predicts the site of septation and serves to orient the mitotic spindle (Gachet et al., 2004b), forms early in mitosis and contracts during late anaphase, directing the accurate assembly of the centripetally directed septum (Mulvihill and Hyams, 2003). As the septum matures, patches of actin appear at the cell mid-zone and the contracted CAR separates into two dots (Wong et al., 2000; Mulvihill and Hyams, 2003).

Although the role of actin in CAR assembly and contraction is becoming increasingly well understood, the role of actin patches at the cell poles and equator is less well established. As in budding yeast (Doyle and Botstein, 1996; Waddle et al., 1996), actin patches are dynamic (Pelham and Chang, 2001) but

whether they share the molecular complexity of their budding yeast counterparts (Pruyne and Bretscher, 2000) remains to be investigated. Cortical actin is required for the localisation of two classes of enzymes concerned with cell wall synthesis: α-glucan synthases (Katayama et al., 1999; Win et al., 2001) and β-glucan synthases (Cortés et al., 2002) but other functions almost certainly remain to be revealed. Studies in a range of eukaryotic cell types have linked the cortical actin cytoskeleton to endocytosis (Geli and Riezman, 1998; Jeng and Welch, 2001). However, until very recently, the structural relationship between actin and the endocytic pathway remained imprecisely defined (Qualmann et al., 2000). Actin was thought to localise endocytosis to particular regions of the cell cortex, or to play a role in membrane internalisation and/or to move vesicles to the cell interior. Recent reports have gone some way to resolve some of these issues (Kaksonen et al., 2003; Huckaba et al., 2004). In the budding yeast *Saccharomyces cerevisiae*, actin patches are assembled at sites of endocytosis and serve to aid the internalisation of endocytic vesicles. Actin polymerisation at the patch (Kaksonen et al., 2003) or the dynamic properties of actin cables (Huckaba et al., 2004) then drives the vesicle into the cell. The association between patch and vesicle is transient, accounting for the short life span of actin patches (Smith et al., 2001; Kaksonen et al., 2003).

Here we present the first attempt to describe the endocytic pathway in fission yeast. We show that endocytosis precisely mirrors the pattern of cell growth and cytokinesis in *S. pombe*. We show that the internalisation step of endocytosis at the cell poles is dependent upon actin patches and that, as in budding yeast, endocytic vesicles and actin patches transiently colocalise. However, endocytosis at the cell equator additionally requires actin cables. The cytological clarity of endocytosis in fission yeast and the ability to isolate endocytosis to particular regions of the cell at different cell cycle stages suggest that *S. pombe* will become a valuable model system in which to dissect the mechanisms and function of this fundamental cellular process.

Materials and Methods

Cells were grown in minimal medium (EMM2) or EMM2 lacking nitrogen as described (Moreno et al., 1991). The strains used in this study are listed in Table 1. Cells were visualised using a Zeiss Axiophot microscope with a Plan-Neofluar 63×/1.25 objective. Images were captured with a Hamamatsu C2400-08 digital camera with a C2400 controller using Openlab software (Improvision, Coventry, UK). Visualisation of endocytosis with FM4-64 was essentially as described (Vida and Emr, 1995). Cells were grown in minimum medium to an optical density (OD_{600}) of 0.5, harvested by centrifugation and resuspended at OD_{600} 3-5 and placed on ice. FM4-64 (Molecular Probes) was dissolved in DMSO at a concentration of 1.63 mM. Two μl FM4-64 stock solution was added to 400 μl cold cells (final concentration 8.15 μM). Cells were then transferred to an imaging chamber CoverWell (Grace Bio-Labs) filled with agarose to 1% concentration (SeaKem, Molecular Biology Grade) in EMM2. The chamber was warmed to 29°C under the microscope and cells visualised using a FITC filter set. For staining with CDCFDA [5-(and-6)-carboxy-2′, 7′-dichlorofluorescein diacetate] (Molecular Probes), cells were grown and harvested as above. CDCFDA was dissolved in DMSO at 100 mg/ml. One μl was added to 1 ml ice-cold cells and mounted in the ice-cold imaging chamber. Cells were visualised using a FITC filter set. For staining with Hoechst 33342 (Molecular Probes) 5 ml cells were harvested by centrifugation and resuspended at 3-5 OD_{600} and incubated at 29°C at a final concentration of 17 μM for 10 minutes. Cells were washed three times in ice-cold EMM2 and left on ice for a maximum of 10 minutes before being visualised. For co-labelling experiments, cells were prelabelled with CDCFDA and then pulsed for 1 minute with FM4-64 and then washed with FM4-64-free medium. For vacuole fusion experiments, cells were prelabelled with CDCFDA and transferred to water overnight prior to the addition of FM4-64 as above. For quantification of FM4-64 fluorescence, images were captured in a single focal plan and the light intensity recorded for 20 cells at each time point using Openlab 2.25 and Windows Excel. Brefeldin A (BFA) was purchased from Sigma, dissolved in ethanol (10 mg/ml) and kept at 4°C and was used at a final concentration of 100 μg/ml. Cell expressing GFP-Gma12 were used as a positive control for BFA activity in equatorial endocytosis experiments (Brazer et al., 2000).

Results

Characterisation of the fission yeast endocytic pathway

In order to examine the spatial organisation of the endocytic pathway in fission yeast we followed the internalisation of the amphiphilic dye FM4-64, which in fungal cells is taken up by

Table 1. Yeast strains used in this study

Strain	Genotype	Source
Wild type	*ura4-D18 leu1-32 ade6-M210*	P. Fantes (Edinburgh, UK)
nda2-KM52	*nda2-KM52 ura4-D18 leu1-32 ade6*	T. Toda (London, UK)
atb2-gfp	*atb2::lys1 leu1-32 lys1+*	Y. Hiraoka (Kobe, Japan)
arp2-1	*arp2-1 man::LEU2 ura4-D18 leu1-32 ade6-M210*	K. Gould (Nashville, TN)
cps8-188	*cps8-188 ura4-D18*	W. Kobayashi (Kobe, Japan)
for3Δ	*for3::kan ura4-D18 leu1.32 ade6-M210*	F. Chang (New York, NY)
cdc10-129	*cdc10-129 leu1.32 ura4-D18 ade6-M210*	V. Simanis (Epalinges, Switzerland)
sid2-250 myo2-gfp	*sid2-250 myo2-gfp::kan ura4-D18 leu1-32 ade6-M210*	D. McCollum (Worcester, MA)
sid1-125 myo2-gfp	*sid1-125 myo2-gfp::kan leu1-32 ura4-D18 ade6-M210*	D. McCollum
cdc7-24 myo2-gfp	*cdc7-24 myo2-gfp::kan ura4-D18*	This study
mid1Δ	*mid1::ura4 ura4-D18 leu1-32 his3-d1ade6-M210*	F. Chang
crn1-gfp	*crn1-gfp ura4-D18 leu1-32 ade6-M210*	F. Chang
syb1-gfp	*syb1-gfp::kan ura4-D18 leu1-32 ade6-M210*	Y. Toyoshima (Tokyo, Japan)
tea2Δ	*tea2::his3 ura4-D18 leu1-32 his3-d1 ade6-M210*	P. Nurse (New York, NY)
ypt7Δ	*ypt7::ura4 ura4-D18 leu1-32 ade-M210*	J. Armstrong (Brighton, UK)
sec8-1	*sec8-1 leu1.32 ura4-D18*	M. Balasubramanian (Singapore)
sec8-gfp	*sec8-gfp::ura4 leu1.32*	M. Balasubramanian

*If no reference, insert institute, town and country/US state for each source.

endocytosis and transported to the vacuolar membrane (Vida and Emr, 1995; Bone et al., 1998; Fischer-Parton et al., 2000). A time course of FM4-64 uptake in living wild-type fission yeast cells (Fig. 1A) shows that within 1 minute, bright fluorescence was associated with the cell membrane at the cell poles, presumably reflecting the localisation of endocytosis at these sites. Within 5 minutes, small (<0.2 μm) vesicles were observed in the cytoplasm adjacent to the poles. Larger, ovoid-to-spherical compartments up to ~0.5 μm in diameter were evident at later stages and vacuoles (1.0-2.5 μm in diameter) were detected by approximately 30 minutes. Not all vacuoles became fluorescent at the same time; those proximal to the cell poles were labelled before those farther away from the site of FM4-64 entry. Closer examination of the pattern of FM4-64 uptake also revealed differences depending on the position of the cell in the cell cycle. In small (length <10 μm), pre-NETO cells, fluorescence was initially confined to a single cell pole (Fig. 1A, left-hand panel). As cells continued to grow through the experiment, labelling of the opposite pole was often evident (after about 30 minutes in the cell shown in Fig. 1A, left-hand panel), indicating that the cell had passed NETO during the period of observation. In cells that were post-NETO at the time of FM4-64 addition, fluorescence was immediately associated with both poles, albeit asymmetrically (Fig. 1A, centre panel).

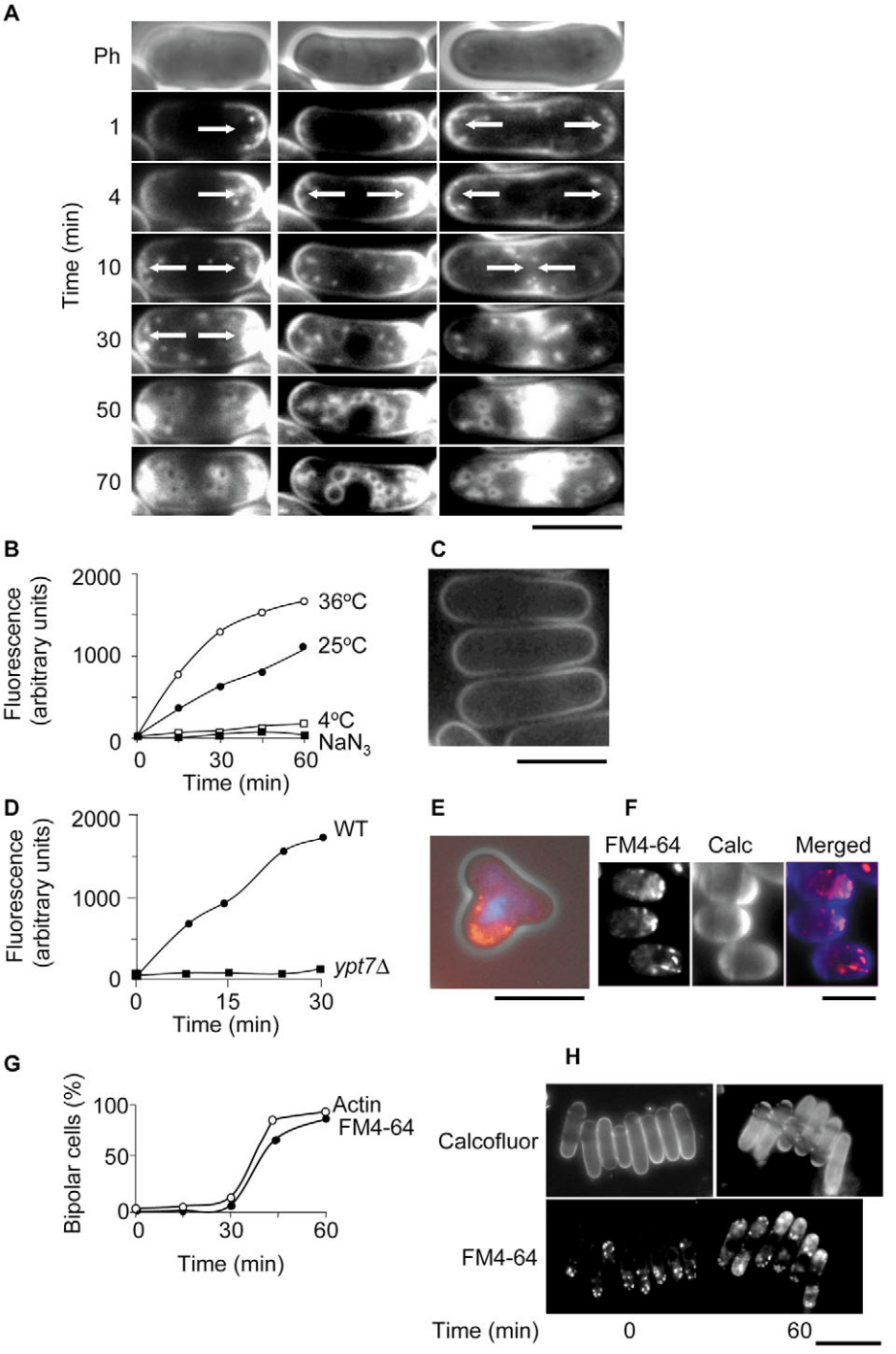

Fig. 1. Endocytosis in fission yeast is coincident with regions of cell growth and cytokinesis. (A) Time course of FM4-64 uptake in living cells at different stages of the cell cycle. Left-hand column, pre-NETO cell. Middle column, post-NETO cell. Right-hand column, dividing cell. Each cell is initially shown as a phase-contrast image (Ph). Cells are oriented with the old end to the right. In the pre-NETO cell fluorescence is initially associated with the old end only but begins to appear also at the new end as the cell passes NETO (50 minutes). In the post-NETO cell both poles are initially fluorescent but the old end is brighter. In dividing cells, fluorescence accumulates at the cell equator. Arrows indicate the changes in growth polarity as individual cells progress through the cell cycle. (B) FM4-64 uptake is by endocytosis. FM4-64 fluorescence was followed in live cells at different temperatures and in the presence of sodium azide. Inhibition at low temperature and energy dependence are characteristics of endocytosis. (C) Cells treated with sodium azide prior to addition of FM4-64. In these conditions the dye binds to the entire cell surface. (D) FM4-64 uptake is by endocytosis. FM4-64 uptake is inhibited in *ypt7Δ*. Ypt7 is a Rab GTPase that is essential for vesicle fusion. WT, wild type. (E) Localisation of endocytosis in *tea2Δ*. In cells lacking the kinesin-related protein Tea2, cell growth is directed to the tip of the lateral branch, which is also the site of endocytosis. The nucleus is stained with Hoechst. (F) Endocytosis in nitrogen-starved, G1-arrested wild-type cells co-stained with Calcofluor to reveal the cell wall. Bright staining with Calcofluor identifies the old end as the single site of endocytosis. (G,H) Endocytosis switches from monopolar to bipolar at NETO. In *cdc10-129* cells arrested in G1 by growth at 36°C, endocytosis is monopolar. Following shift to the permissive temperature (25°C), cells pass NETO and endocytosis becomes bipolar coincident with the appearance of actin at both cell poles. Bars, 10 μm (A,C,E,H); 4 μm (F).

Once again, because cell growth continued, it was possible to unambiguously identify the brightly staining pole as the fast growing (old) end. Quantification of fluorescence intensity at the two poles gave a ratio of ~2:1 old end: new end (data not shown). Dividing cells showed a third pattern, with little fluorescence at the poles but intense uptake at the cell equator, the future site of septum deposition (Fig. 1A right-hand panel). Uptake of FM4-64 was abolished at low temperatures and in the presence of sodium azide (Fig. 1B). In sodium azide-treated cells FM4-64 bound to the entire cell surface (Fig. 1C), indicating the intense polar and equatorial fluorescence seen in uninhibited cells was really due to endocytic uptake and not to selective binding of the dye in these regions. FM4-64 uptake was also dependent on the presence of Ypt7, the Rab GTPase required for vacuole fusion in fission yeast (Bone et al., 1998) (Fig. 1D).

To further investigate the correlation between the site of cell wall deposition/actin and endocytosis we investigated situations in which the pattern of cell growth was altered. Cells lacking the kinesin-like protein Tea2 form lateral branches with growth confined to the tip of the branch (Browning et al., 2000). Staining of *tea2Δ* cells with FM4-64 once again revealed a clear correlation between the site of cell growth/actin and FM4-64 uptake (Fig. 1E). Wild-type cells starved for nitrogen accumulate in G1, prior to NETO. Although endocytosis is reduced in such cells, FM4-64 uptake was uniquely associated with the old end (identified by its bright staining with the cell wall dye Calcofluor; Fig. 1F). Finally, we examined the situation in *cdc10-129* cells that were arrested in G1 by growth at the restrictive temperature and then allowed to pass NETO synchronously following return to the permissive temperature. Endocytosis changed from monopolar to bipolar in concert with the redistribution of actin as post-NETO cells switched to bipolar growth (Fig. 1G,H).

To distinguish between vacuoles and other vesicular compartments, we added FM4-64 to cells in which vacuoles were prelabelled with CDCFDA, a dye that enters vacuoles independently of the endocytic pathway. FM4-64 fluorescence was initially distinct from CDCFDA fluorescence and was associated with numerous small vesicular structures. These initially increased in number but then declined as fluorescence transferred to the vacuolar membrane (Fig. 2A,B). When CDCFDA-labelled vacuoles were enlarged by fusion prior to the addition of FM4-64, fluorescence remained associated with the small vesicular compartments (Fig. 2C,D). We tentatively identify these structures as prevacuolar compartments (PVC) although we cannot exclude possibility that they include vesicles that recycle back to the cell membrane or to other cellular compartments.

We next added FM4-64 to cells expressing GFP-Syb1, the fission yeast homologue of the SNARE protein synaptobrevin (Edamatsu and Toyoshima, 2003), a protein associated with

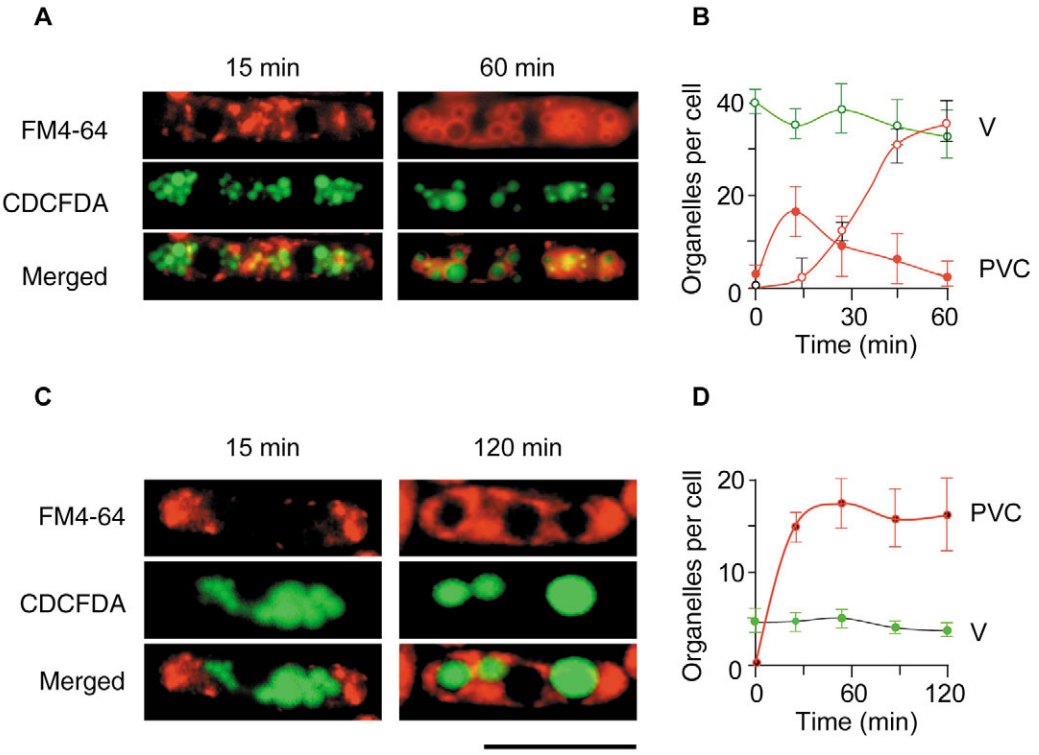

Fig. 2. Endocytosis in fission yeast passes from the cell membrane to the vacuole via intermediate prevacuolar compartments. (A) Vacuoles were prelabelled with CDCFDA and then exposed to FM4-64. After 15 minutes, FM4-64 labels prevacuolar compartments not stained with CDCFDA. (B) Quantification of the experiment in A. The number of CDCFDA-stained vacuoles (green line, V) remains constant throughout the experiment. The number of FM4-64-stained PVC initially rises but subsequently falls as fluorescence is transferred to the vacuolar membrane (red line, PVC). The number of FM4-64-labelled vacuoles (red line, V) increases with time. (C) The same experiment as in A but vacuoles were first fused by incubation in water for 60 minutes. (D) The number of CDCFDA-stained vacuoles (green line, V) remains constant through the experiment but the number of FM4-64-labelled PVC reaches a plateau as transfer to the vacuolar membrane is blocked (red line, PVC). Bar, 10 μm.

newly forming endocytic vesicles (Gurunathan et al., 2000). GFP-Syb1 vesicles are enriched at the ends and middle of *S. pombe* cells according to their position in the cell cycle (Edamatsu and Toyoshima, 2003). In pre-NETO cells, FM4-64 fluorescence appeared within seconds in the membranes of Syb1 vesicles at the growing cell pole (the old end) only (Fig. 3A,B). In post-NETO cells, fluorescence appeared in Syb1 vesicles at both cell poles (Fig. 3B), prior to transfer to the vacuolar membrane. Thus, FM4-64 unambiguously passes to the vacuole via a true endosomal compartment although it is likely that a subset of the Syb1-positive, FM4-64-positive vesicles are recycled back to the cell membrane (Gurunathan et al., 2000). Syb1 vesicles were stable during the transfer of fluorescence to the vacuole and hence the two compartments did not fuse.

Endocytosis in fission yeast is associated with actin

Given that FM4-64 is taken up at the poles and the equator of fission yeast cells, regions that are rich in actin, we further investigated this relationship, taking advantage of cells expressing GFP-tagged Crn1, the fission yeast coronin homologue, which is predominantly associated with actin patches (Pelham and Chang, 2001). FM4-64 labelling of Crn1-GFP cells showed that a subset of endocytic vesicles was spatially coincident with a subset of actin patches (Fig. 4A). An individual vesicle remained associated with an individual actin patch for <20 seconds, following which colocalisation was lost. When Crn1-GFP cells were treated with the actin inhibitor latrunculin B at a concentration that completely destroys the actin cytoskeleton prior to the addition of FM4-64, internalisation of the dye was largely abolished. Some coronin patches remained after this treatment (Pelham and Chang, 2001) and FM4-64 was internalised at these residual structures but fluorescence was not transferred to internal compartments (Fig. 4B). Bright fluorescence was also detected at the membrane of dividing cells in the region of the septum (Fig. 4B). Quantification of FM4-64 uptake confirmed the inhibitory effect of latrunculin B but not the microtubule inhibitor TBZ (Fig. 4C). Neither latrunculin nor TBZ inhibited the uptake of CDCFDA (Fig. 4D). To investigate whether, in addition to an early step in endocytosis, actin was also associated with later events (transfer to prevacuolar compartments and vacuole), we prelabelled vacuoles with CDCFDA then added FM4-64. After 3 minutes the FM4-64 was washed out and either latrunculin B or TBZ added. Cells were then observed to see whether fluorescence was transferred to the vacuole in the presence of either inhibitor. FM4-64

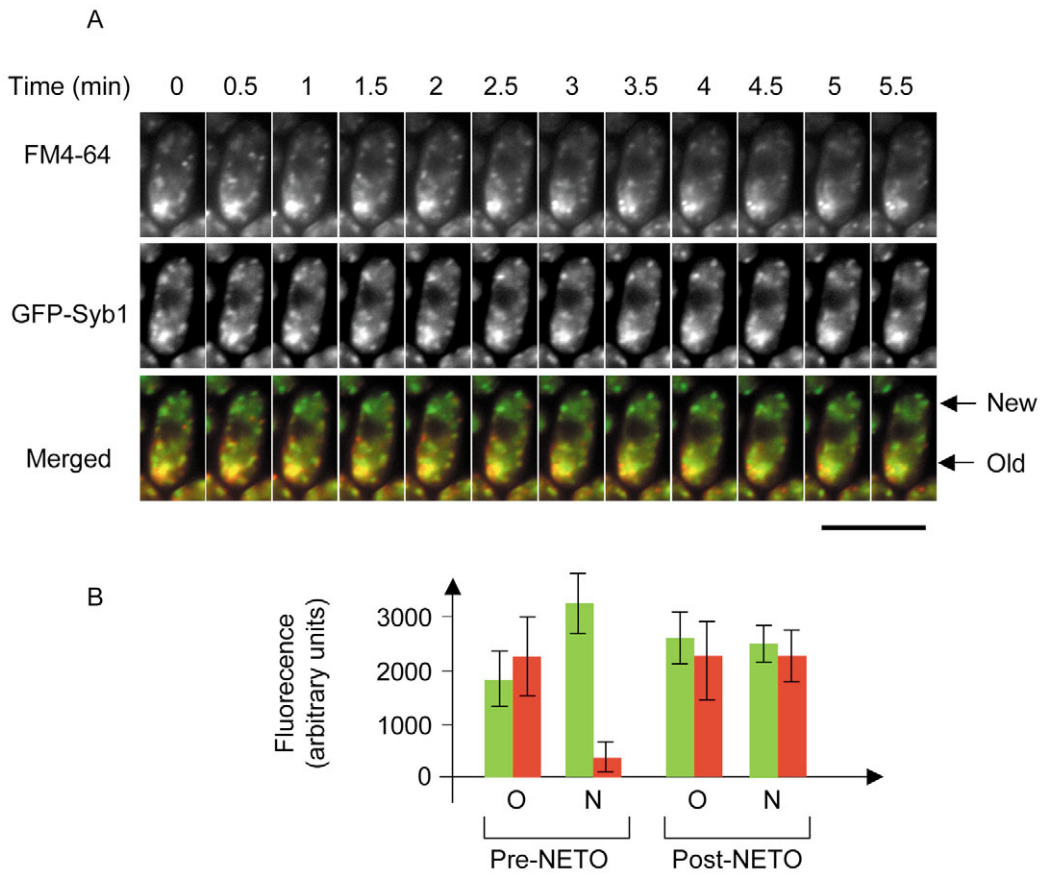

Fig. 3. Internalised FM4-64 is rapidly transferred to vesicles containing Syb1. (A) Endocytosed FM4-64 (red) is rapidly transferred to Syb1 vesicles (green) as judged by the overlap of the two signals (yellow) after less than 1 minute. This series shows a pre-NETO cell. Note that Syb1 vesicles at the non-growing new end remain green. (B) Ratio of green and red fluorescence at the old (O) and new (N) ends pre and post NETO. In pre-NETO cells, FM4-64 uptake (red bars) is confined to the old end despite there being a bias to Syb1 vesicles (green bars) at the opposite pole. In post-NETO cells the two poles are equivalent. Error bars indicate s.e.m. Bar, 10 μm.

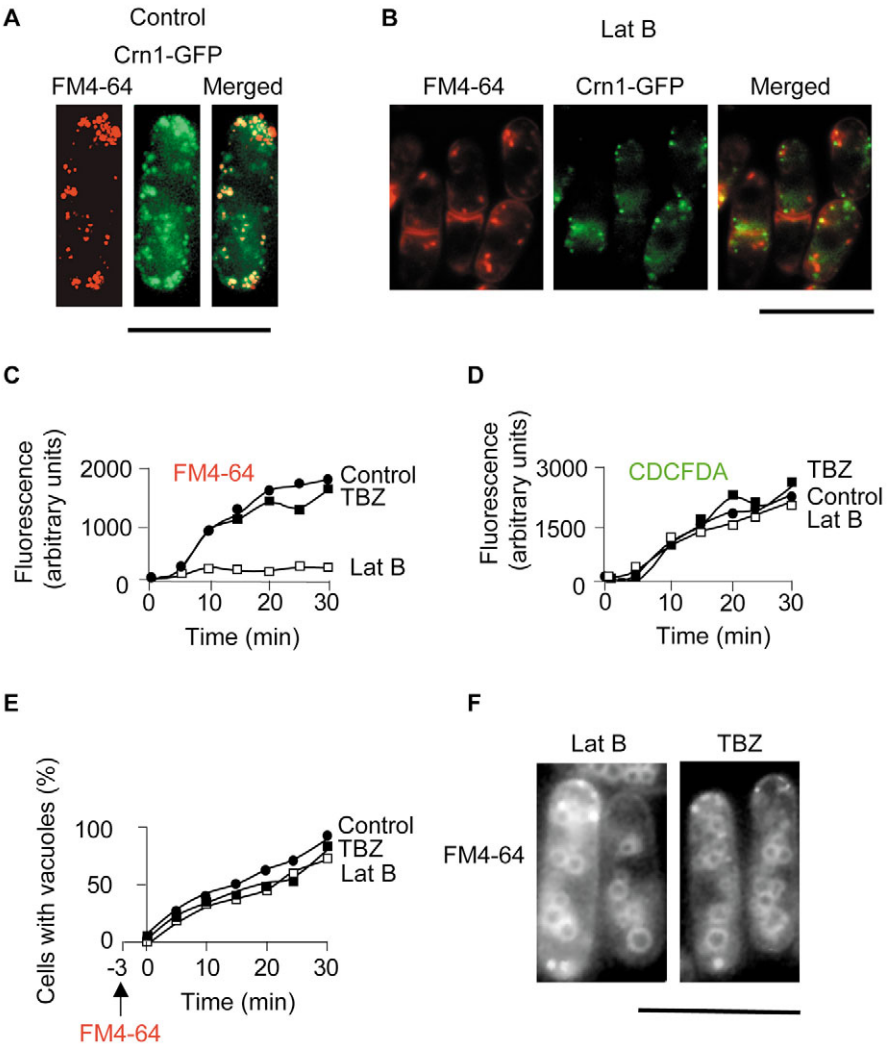

Fig. 4. Endocytosis in fission yeast is associated with actin. (A) Crn1-GFP cells were labelled with FM4-64 examined under the microscope within 1-2 minutes. A subset of endocytic vesicles (red) and Crn1-GFP spots (actin patches, green) are superimposable (yellow). (B) Endocytosis is inhibited Lat B. Crn1-GFP cells were treated with 10 μM Lat B for 10 minutes prior to the addition of FM4-64. Fluorescence remained associated with the cell membrane, particularly at the cell equator, and with some residual structures at the cell membrane. No transfer to internal compartments was observed. (C,D) Effect of drugs on FM4-64 and CDCFDA uptake. Whereas FM4-64 uptake was inhibited by Lat B, CDCFDA uptake was not. TBZ had no effect on the uptake of either probe. (E) In cells preloaded with FM4-64 3 minutes prior to the addition of Lat B or TBZ (arrow) the dye trafficked normally to the vacuole (F). Thus later events of the endocytosis pathway are independent of both actin and microtubules. Bars, 10 μm.

transferred effectively to the vacuole membrane (Fig. 4E,F), hence neither disruption of the actin cytoskeleton nor depolymerisation of the microtubule network affected the later events of endocytic pathway

To eliminate possible non-specific effects of latrunculin, we followed endocytosis in the temperature-sensitive actin mutant *cps8*. Even at the permissive temperature, FM4-64 uptake at the poles was markedly inhibited in this genetic background: bright patches of staining remained adjacent to the cell membrane but with little or no transfer to internal compartments (Fig. 5A,B). By contrast, bright fluorescence was associated with the membrane flanking the cytokinetic septum although transfer did not progress beyond this point. No inhibition was observed in the cold-sensitive α-tubulin mutant *nda2* at the restrictive temperature (Fig. 5A,C). FM4-64 uptake was also inhibited at the restrictive temperature in the mutant *arp2*, encoding a component of the Arp2/3 complex, in which actin patches are selectively destabilised (Morrell et al., 1999) (Fig. 5D) but not in *for3Δ*, which has normal patches and lacks actin cables (Feierbach and Chang, 2001) (Fig. 5E). As in *cps8*, patches of fluorescence were seen at the cell membrane in *arp2* and bright fluorescence was seen at the cell membrane at the poles and septa.

A possible explanation for these observations may lie in the latrunculin sensitivity of the Syb1-containing prevacuolar compartments. We have shown previously that actin patches persist at concentrations of latrunculin A that destroy actin cables (Tournier et al., 2004). When Syb1-GFP cells were exposed to a concentration series of latrunculin A, Syb1-GFP vesicles showed differential sensitivity depending on the position in the cell cycle (Fig. 5F,G). Whereas vesicles in dividing cells were sensitive to a latrunculin concentration that selectively removed actin cables (ID_{50}=1.25 μM), vesicles in interphase cells were sensitive only to higher concentrations (ID_{50}=5.0 μM), equivalent to that required to remove patches. Latrunculin-treated Syb1 cells showed a striking resemblance to equivalent FM4-64 images. Vesicles associated with the cell equator rapidly and completely dispersed Syb1 fluorescence, becoming largely associated with the cell membrane flanking the septum (Fig. 5G). Syb1-GFP vesicles also fused with the cell membrane at the poles, albeit over a longer timescale, leaving fluorescent foci adjacent to the cell membrane. These results confirm and extend the earlier findings of Edamatsu and Toyoshima (Edamatsu and Toyoshima, 2003) who concluded that the association of Syb1 vesicles at the medial region of the cell was dependent upon the actin cytoskeleton.

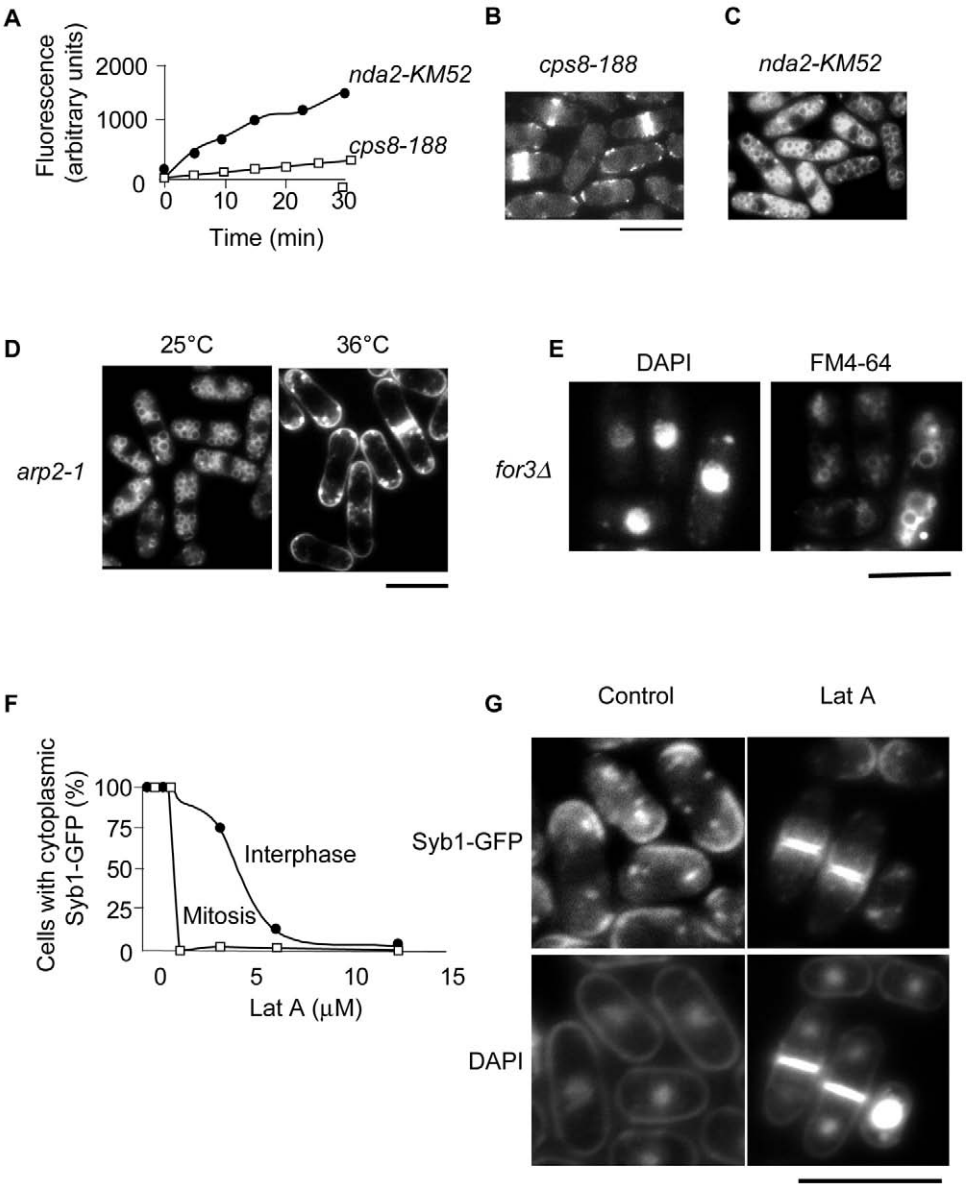

Fig. 5. Endocytosis in fission yeast is associated with actin. (A,B) FM4-64 uptake is inhibited in the actin mutant *cps8* even at the permissive temperature of 25°C (B). Note the bright fluorescence at the cell membrane, particularly at the equator of dividing cells. (A,C) FM4-64 uptake is not inhibited in the α-tubulin mutant *nda2* at the restrictive temperature (36°C). (D) FM4-64 internalisation is inhibited in the Arp2/3 complex mutant *arp2* at the restrictive temperature but not in the formin mutant *for3Δ* (E). (F,G) Syb1 vesicles at the poles and the equator show different sensitivity to latrunculin. Whereas vesicles in dividing cells collapse to the cell membrane in low latrunculin concentrations, higher concentrations are required to disrupt Syb1 vesicles at the poles of interphase cells. (G) Syb1-GFP collapses to the membrane in latrunculin-treated cells (Lat A). Bars, 10 μm.

Endocytosis at the cell equator is associated with actin patches and filaments

A feature of endocytosis in *cps8* and *arp2* cells was the persistence of staining at the cell membrane at the equatorial region of dividing cells (Fig. 5B,D). Because the actin dependence of equatorial FM4-64 uptake appeared to be somewhat different to that at the poles, we re-examined the colocalisation of FM4-64 uptake and actin patches in this region using the Crn1-GFP strain. As described previously for the cell poles, endocytic vesicles again showed a clear but transient colocalisation with actin patches (Fig. 6A).

The redirection of endocytosis from the poles to the cell equator was further examined in a strain in which the non-essential α-tubulin gene *atb2*$^{+}$ was fused to GFP (Ding et al., 1998). When living Atb2-GFP cells were followed through mitosis in the presence of FM4-64, endocytic vesicles were seen to appear in the region between the retreating anaphase chromosomes in a manner reminiscent of the accumulation of actin at the incipient division site (Fig. 6B). In light of this finding, we examined FM4-64 uptake in dividing *for3Δ* cells. Polar endocytosis appeared normal in this mutant but the relocation of endocytosis to the equator was delayed with FM4-64 uptake at the poles continuing until the completion of cytokinesis (Fig. 6C,D). A similar delay was seen in another formin mutant, *cdc12* (Fig. 6E). Finally, we examined the situation in cells lacking Mid1, a protein involved in the correct placement of the septum (Chang et al., 1996; Sohrmann et al., 1996). FM4-64 uptake in dividing *mid1Δ* cells was often observed displaced from the cell equator (Fig. 6F), consistent with the mis-positioning of the division plane in this strain. Thus, equatorial endocytosis is not a function of cell equator per se but is intimately associated with cytokinesis.

Endocytosis at the cell equator is associated with exocytosis and septation

To further examine the relationship between equatorial endocytosis and septum formation, we followed FM4-64

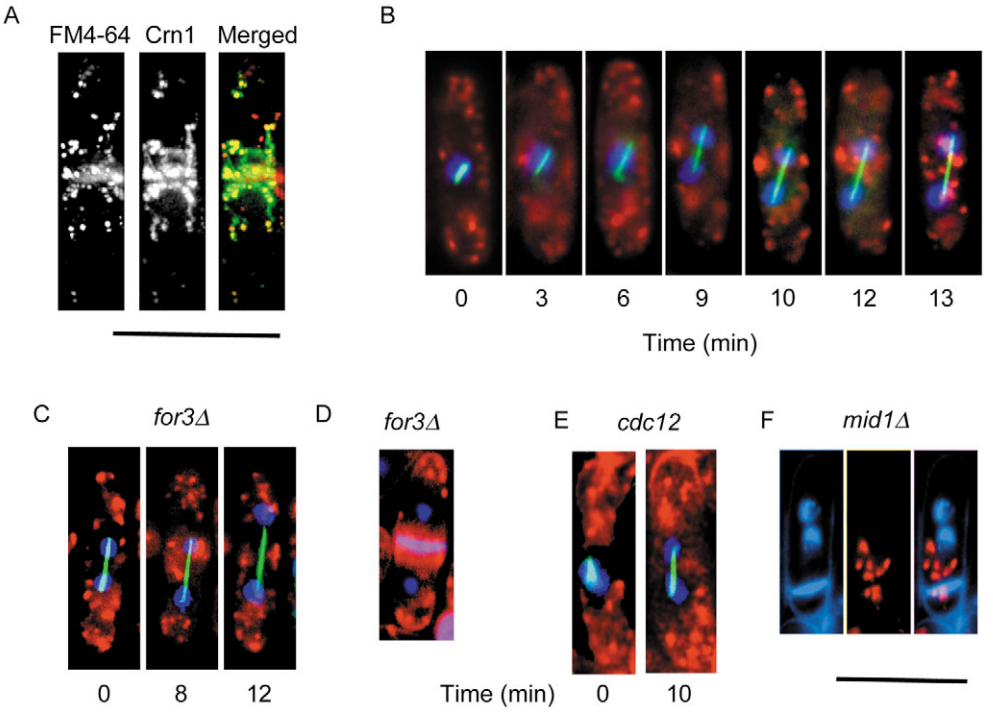

Fig. 6. Equatorial endocytosis in fission yeast is associated with actin. (A) A dividing Crn1-GFP cell treated with FM4-64. Transient colocalisation of endocytic vesicles and actin patches is observed at the equator as at the cell poles. (B) Mitosis in a *atb2-gfp* cell showing the accumulation of endocytic vesicles in the region between the separating sister chromatids and the disappearance of vesicles at the poles. As the sequence focuses on the elongation of the spindle, endocytic vesicles appear out of focus. (C) Endocytosis at cell division in *atb2-gfp for3Δ* showing the continuation of polar endocytosis in this mutant. (D) Polar endocytosis continues through cytokinesis in *for3Δ* mutants. (E) Polar endocytosis continues at cell division in *atb2-gfp cdc12*. (F) Equatorial endocytosis is misplaced in *mid1Δ* cells. Bars, 10 μm.

uptake in the SIN mutants *cdc7-24*, *sid1-125* and *sid2-250*. At the restrictive temperature, cytokinesis is inhibited in these strains, which nevertheless go through repeated nuclear divisions, becoming multinucleate. All three mutants were followed through two rounds of nuclear division in the presence of FM4-64 at the restrictive temperature. In no case was an accumulation of FM4-64 in the region of the dividing nuclei observed whereas endocytosis was still localised at the cell tips (Fig. 7A-C). Thus, the relocation of endocytosis requires the activation of the SIN pathway. We also investigated the relationship of equatorial endocytosis to the exocyst, a complex of proteins associated with exocytosis that has a specific role in cytokinesis in fission yeast (Wang et al., 2002). Exocyst components localise both to the cell poles and division plane in a F-actin-dependent manner in *S. pombe* but exocyst mutants are defective only for septum cleavage and cell separation. We therefore added FM4-64 to *sec8-1* labelled with DAPI and scored the percentage of cells showing FM4-64 fluorescence between the dividing nuclei at both the permissive and restrictive temperatures. Equatorial endocytosis was inhibited in this mutant (Fig. 8A), although endocytosis at the cell poles was apparently normal (data not shown). A similar result was observed in cells treated with BFA, an inhibitor of the ER-to-Golgi step of the secretory pathway (Fig. 8A). Thus, endocytosis at the division plane may serve to balance exocytosis specifically at this site and at this stage of the cell cycle. To confirm that the two processes are indeed independent, we examined FM4-64 uptake at the cell equator in cells expressing Sec8-GFP to label secretory vesicles. Endocytic vesicles and secretory vesicles were distinct, with

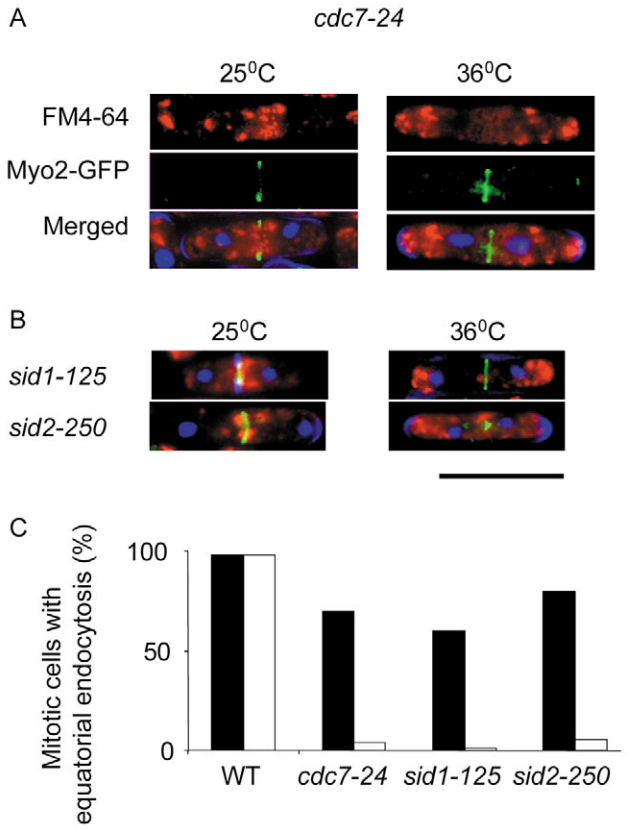

Fig. 7. Relocation of endocytosis from the poles to the equator requires a functional SIN. (A,B) In *cdc7*, *sid1* and *sid2* cells the site of endocytosis moves to the equator at the permissive (25°C), but not the restrictive (36°C) temperatures. Myo2 ring formation is independent of SIN function and hence, endocytosis. For *sid1* and *sid2* cells the images shown are merged Myo2-GFP, FM4-64 and DAPI. (C) Quantification of equatorial endocytosis in wild-type cells (WT) and SIN mutants at 25°C (closed bars) and 36°C (open bars). Bar, 10 μm.

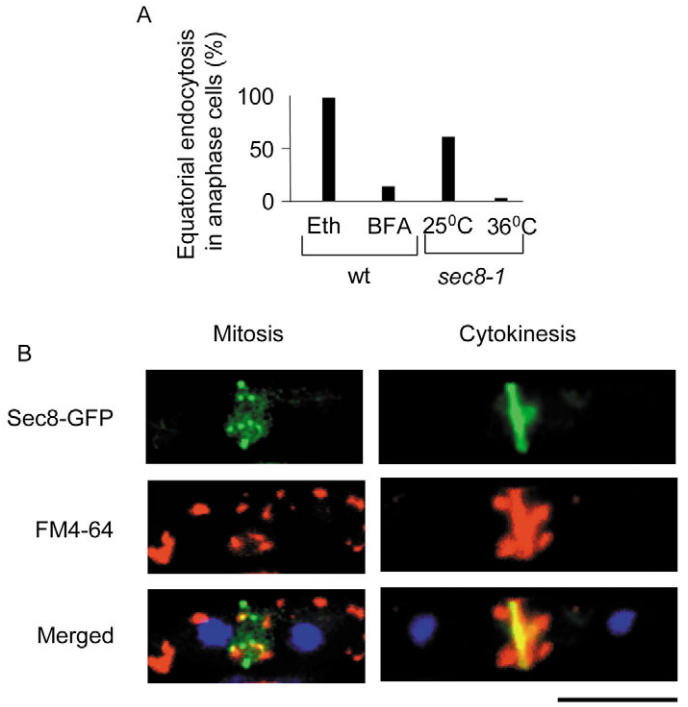

Fig. 8. Equatorial endocytosis is dependent upon exocyst function. (A) Equatorial endocytosis was scored in dividing cells in the presence of BFA using ethanol (Eth) as a control and in *sec8-1* cells at the permissive (25°C) and restrictive (36°C) temperatures. (B) Sec8-GFP vesicles and FM4-64-labelled vesicles are largely distinct although some exchange may occur between these compartments. Bar, 10 μm.

fluorescence only overlapping at the membrane flanking the septum (Fig. 8B).

Discussion

FM4-64 has been used extensively as a marker of the endocytic pathway in both unicellular and filamentous fungi (Vida and Emr, 1995; Bone et al., 1998; Fischer-Parton et al., 2000), although in budding yeast the utility of FM4-64 as a marker of endocytosis has been questioned as uptake of the dye occurs uniformly over the cell surface whereas actin is localised to the tip of the bud (Vida and Emr, 1995; Munn, 2001). FM4-64 has been widely used to stain the vacuolar membrane in fission yeast (Bone et al., 1998; Brazer et al., 2000; Edamatsu and Toyoshima, 2003; Feoktistova et al., 1999; Gaits and Russell, 1999; Iwakai et al., 2003; Iwakai et al., 2004; Morrell et al., 1999; Mulvihill et al., 2001; Murray and Johnson, 2000; Murray and Johnson, 2001; Routhier et al., 2003; Takegawa et al., 2003) but in no case has its transport to this compartment been systematically investigated. Here we show that uptake of FM4-64 in fission yeast is time-, temperature- and energy-dependent, characteristics of fluid phase endocytosis (Vida and Emr, 1995; Munn, 2000). It is also dependent upon vesicle fusion as judged by its inhibition in *ypt7Δ* (Bone et al., 1998; Iwaki et al., 2004). The first stage of FM4-64 uptake is intense labelling at the cell membrane, possibly reflecting the formation and internalisation of small (30-50 nm diameter) primary endocytic vesicles that are below the resolution of the light microscope (Prescianotto-Baschong and Riezman, 1998). Subsequently, fluorescence was associated with vesicular compartments containing the synaptobrevin homologue, Syb1. In budding yeast, delivery of FM4-64 to the vacuole is dependent upon the Syb1 homologue Suc1, which localises to newly forming endosomes (Gurunathan et al., 2000). In fission yeast, these compartments were previously shown to be associated with the cell poles during interphase and the septum at cytokinesis, consistent with a role in endocytosis at these sites (Edamatsu and Toyoshima, 2003). Fluorescence is subsequently transferred to larger compartments that then disappear with the arrival of fluorescence at the vacuolar membrane. When endocytosis is inhibited with sodium azide, FM4-64 fluorescence is associated with the entire cell surface. Thus, FM4-64 uptake at the cell poles and equator in uninhibited cells reflects the localisation of endocytosis to these sites and excludes other possibilities such as the selective permeability of the cell wall or increased affinity of the cell membrane for FM4-64 in these regions. In the latter regard, the polar and equatorial cell membranes of fission yeast cells are sterol-enriched (Wachtler et al., 2002). However, filipin-positive membrane arrives at the equator relatively late in cytokinesis whereas FM4-64 staining accumulates early and it is likely that the two are distinct.

Intermediate stages in the endocytic pathway appear to be easier to visualise in *S. pombe* than in budding yeast where they have proved difficult to capture cytologically (Munn, 2000). This may reflect differences in the pattern of cell growth or the fact that vacuolar organisation is very different in the two yeasts, *S. cerevisiae* possessing two or three large vacuoles (Wickner, 2002), fission yeast up to 80 small ones (Bone et al., 1998; Mulvihill et al., 2001; Takegawa et al., 2003). Both we (Mulvihill et al., 2001), and others have noted that FM4-64 enters fission yeast cells at the cell poles and at the division site but in no case was this investigated further (Brazer et al., 2000; Iwaki et al., 2004). Here we show that in both wild-type cells and in mutants in which growth is restricted to a single cell pole or in which the septum is misplaced, endocytosis is restricted to regions of cell growth and, hence, is associated with concentrations of actin patches (Marks and Hyams, 1985). Accumulating evidence from a variety of cell types links the internalisation step of endocytosis to the actin cytoskeleton (Engqvist-Goldstein and Drubin, 2003). Most persuasive are genetic studies in budding yeast reporting that actin mutants are defective in endocytosis (Munn, 2000; Munn, 2001; Shaw et al., 2001), and drug studies showing that endocytosis has an absolute requirement for a dynamic actin cytoskeleton (Ayscough, 2000). As in fission yeast, interphase budding yeast cells contain two actin structures, patches and cables. Whereas actin cables are thought to underlie the bud-directed transport of organelles (Bretscher, 2003), actin patches are associated with both cell wall deposition (Tang et al., 2000; Utsigi et al., 2002) and endocytosis. Actin patches contain at least 20 proteins (Pruyne and Bretscher, 2000) and mutations in many of these also show endocytosis defects (Munn, 2001). Although the precise role of actin remains unknown, an important recent study from Kaksonen et al. (Kaksonen et al., 2003) has shown that actin patches form at sites of endocytosis and are transiently associated with endocytic vesicles as they move away from the cell membrane. The latter may be coupled to the dynamic properties of actin cables (Huckaba et al.,

2004). Similar events are also thought to underlie endocytosis in mammalian cells (Merrifield, 2004).

Our results demonstrate that endocytosis in fission yeast is also actin dependent. The evidence for this is threefold: (1) the ratio of endocytosis at the two cells ends is roughly equivalent to the ratio of actin patches at these locations; (2) endocytic vesicles and actin patches transiently colocalise at the cell poles and equator; (3) endocytosis is abolished when treated with latrunculin and in the mutant *arp2*, in which actin patches are disrupted (Morrell et al., 1999; Feoktistova et al., 1999). As in budding yeast, later steps in the endocytic pathway in fission yeast are independent of actin (Kaksonen et al., 2003). However, the integrity of Syb1 vesicles is also actin dependent, despite the fact that Syb1 vesicles at the poles and equator show differential sensitivity to latrunculin (Edamatsu and Toyoshima, 2003). Based on the rapid appearance of FM4-64 fluorescence in these structures, Syb1 vesicles are an early component of the fission yeast endocytic pathway. However, their precise relationship to actin patches remains to be determined. Taken together, our findings indicate that the mechanism of endocytic uptake is fundamentally similar in the two yeasts. However, differences may exist. Actin patches persist for up to 3 minutes in fission yeast (Pelham and Chang, 2001), considerably longer than has been observed in *S. cerevisiae* (Smith et al., 2001; Kaksonen et al., 2003), and although two-thirds of patches move away from the cell poles in *S. pombe*, consistent with a role in directing endocytic vesicles away from the cell membrane, the remaining one-third move in the opposite direction (Pelham and Chang, 2001). In fission yeast, actin polymerisation is envisaged to drive patches along cables (Pelham and Chang, 2001), whereas in *S. cerevisiae* patches are thought to use the force generated by cable polymerisation (Huckaba et al., 2004). Our results show that endocytosis at the cell poles is insensitive to concentrations of latrunculin that depolymerise cables and, indeed, polar endocytosis is unaffected in cells lacking the formin, For3, in which these structures are absent. On the other hand, equatorial endocytosis does appear to require the presence of cables. The explanation for this difference probably lies in the type V myosin, Myo52 (Win et al., 2001; Motegi et al., 2001). Myo52 localises to the poles and equator of fission yeast cells and is associated with the redirection of the cell wall synthesising machinery between these two sites at cell division (D. P. Mulvihill and J.S.H., unpublished). The cell cycle redistribution of Syb1 vesicles is also dependent upon Myo52 (Edamatsu and Toyoshima, 2003) suggesting that Myo52 has a major role in the global redistribution of the cell growth and endocytosis machinery at G2-M. Mutant *for3Δ* cells lacking actin cables have aberrant Myo52 distribution (Feirbach and Chang, 2001) show a delay in the equatorial relocation of endocytosis during M phase. Intriguingly, these cells also have a NETO defect. One daughter cell from each division grows in a monopolar manner and, hence, exhibits no NETO whereas the other grows in a bipolar manner and thus exhibits premature NETO (Feierbach and Chang, 2001). Whether this phenotype is a consequence of the failure of endocytosis to properly relocalise to the cell equator at the previous cell division remains to be determined. Regardless, our findings emphasise the importance of membrane traffic to cytokinesis in fission yeast (Rajagopalan et al., 2003).

A number of protein kinases are involved in the control of NETO (Bähler and Pringle, 1998; Verde et al., 1998; Rupes et al., 1999), whereas a signal transduction pathway consisting of several protein kinases, a GTPase and a protein phosphatase (the SIN) governs septation (McCollum and Gould, 2001). Although both growth transitions involve the global reorganisation of the actin cytoskeleton and consequently, of both cell wall synthesis and endocytosis, the two events are in fact quite distinct. One involves the activation of growth at a pre-existing cell pole, the other the de novo creation of two cell poles following the synthesis and cleavage of the septum. It is therefore not surprising that the relationship of endocytosis to these two processes is distinct. The early steps of equatorial endocytosis persist in the actin mutants *cps8* and *arp2*, in which endocytosis at the poles was largely arrested. It is also selectively inhibited by BFA and in the exocyst mutant *sec8*, both of which block the secretory pathway (Liu et al., 2002). Thus, although the conventional explanation for endocytosis at sites of secretion, namely that the fusion of secretory vesicles with the cell membrane must be balanced by membrane internalisation by endocytosis (Gundelfinger et al., 2003), could well be true at the cell equator, it is unlikely to be the explanation for endocytosis at the cell poles. One possibility is that equatorial endocytosis restricts the lateral diffusion of the receptors of polar determinants within the cell membrane (Snaith and Sawin, 2003), resulting in their unequal distribution between the two daughter cells. The redirection of endocytosis to the division site requires a functional SIN. The latter conclusion was based on the inhibition of equatorial endocytosis in SIN mutants and the fact that endocytic vesicles become aligned across the equatorial region of the cell early in anaphase, the point at which SIN components become asymmetrically associated with the spindle poles (Cerutti and Simanis, 1999). A known target of the SIN includes Cps1/Bgs1, a β-glucan synthase involved in septum deposition (Liu et al., 2002), and our observations add to the view that the SIN is a global regulator of cytokinetic events. More than 100 genes or 2% of the budding yeast genome is estimated to be involved in endocytosis (D'Hondt et al., 2000; Wiederkehr et al., 2001), emphasising its importance to yeast biology. Although we are a long way from such a detailed analysis in *S. pombe*, our description of the endocytic pathway suggests that fission yeast can make a useful contribution to our understanding of the molecular mechanisms of endocytic membrane transport.

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