Live cell imaging of the assembly, disassembly, and actin cable–dependent movement of endosomes and actin patches in the budding yeast, *Saccharomyces cerevisiae*

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U sing FM4-64 to label endosomes and Abp1p-GFP or Sac6p-GFP to label actin patches, we find that (1) endosomes colocalize with actin patches as they assemble at the bud cortex; (2) endosomes colocalize with actin patches as they undergo linear, retrograde movement from buds toward mother cells; and (3) actin patches interact with and disassemble at FM4-64–labeled internal compartments. We also show that retrograde flow of actin cables mediates retrograde actin patch movement. An Arp2/3 complex mutation decreases the frequency of cortical, nonlinear actin patch movements, but has no effect on the velocity of linear, retrograde actin patch movement. Rather, linear actin patch movement occurs at the same velocity and direction as the movement of actin cables. Moreover, actin patches require actin cables for retrograde movements and colocalize with actin cables as they undergo retrograde movement. Our studies support a mechanism whereby actin cables serve as "conveyor belts" for retrograde movement and delivery of actin patches/endosomes to FM4-64–labeled internal compartments.

Introduction

Actin patches and actin cables are the two F-actin–containing structures that persist throughout the cell cycle in budding yeast. Actin patches are punctate, cortical structures that predominantly localize to the bud and mother-bud neck. Actin cables are bundles of F-actin that align along the mother-bud axis and are implicated as tracks for the movement of secretory vesicles, mRNA, spindle alignment elements, mitochondria, Golgi, and vacuoles from the mother cell to the growing bud (Lazzarino et al., 1994; Hill et al., 1996; Simon et al., 1997; Takizawa et al., 1997; Pruyne et al., 1998; Rossanese et al., 2001; Schott, et al., 2002). Live cell imaging analyses revealed that both of these cytoskeletal structures are motile and highly dynamic (Doyle and Botstein, 1996; Waddle et al., 1996; Smith et al., 2001; Carlsson et al., 2002; Yang and Pon, 2002; Kaksonen et al., 2003). Actin cables assemble at the bud cortex and mother-bud neck. During this assembly process, new material is incorporated into the end of an existing actin cable at the assembly site. As a result, the distal ends of elongating actin cables undergo retrograde movement from the bud toward the mother cell at a rate of $\sim$0.3 μm/s.

Actin patches also assemble in the bud cortex and mother-bud neck, but display a more complex pattern of movement (Kaksonen et al., 2003). In vegetative yeast, actin patches assemble at the bud cortex, and undergo random cortical movement that is associated with movement away from the plasma membrane. Thereafter, some actin patches undergo linear, retrograde movement toward the mother cell. A similar pattern of actin patch movement occurs in yeast during mating projection formation (Smith et al., 2001). Here, we provide evidence that actin patches and actin cables play direct roles in endocytosis in budding yeast.

Several lines of evidence support a link between actin patches and endocytosis. First, mutations in proteins required for endocytosis map to actin, actin patch proteins, or proteins that affect actin organization (Kubler and Riezman, 1993). Second, many of the proteins required for endocytosis, including the Arp2/3 complex, Arp2/3 complex activators, and the actin-regulating protein kinases (Ark1p and Prk1p), localize to actin patches (Li, 1997; Moreau et al., 1997; Pruyne and Bretscher, 2000; Goode and Rodal, 2001; Sekiya-Kawasaki et al., 2003). Third, destabilization of actin using the drug jasplakinolide or mutation of actin patch proteins (e.g., cofilin, Sla2p, or Arp2p) produces defects in endocytosis (Lappalainen and Drubin, 1997; Moreau et al., 1997; Tang et al., 1997; Ayscough, 2000; Kaksonen et al., 2003). Fourth, biochemical studies indicate

that pheromone receptors, which undergo endocytosis during mating, can bind to Arp2/3 complex activators that localize to actin patches (Winter et al., 1999; Duncan et al., 2001; Goode et al., 2001; Howard et al., 2002).

Here, we provide the first direct evidence that yeast actin patches assemble at sites of endocytosis, move with endosomes, and disassemble when endosomes interact with FM4-64–labeled internal compartments. Moreover, we present evidence for a novel mechanism of movement of actin patches and endosomes from their site of formation in the developing bud to FM4-64–labeled internal compartments in the mother cell. We find that actin patches associate with actin cables and undergo linear, Arp2/3-independent retrograde movement using the forces that drive elongation and retrograde flow of actin cables.

Results

Membrane internalization at sites of actin patch formation

We used FM4-64 and Abp1p-GFP to explore the relationship between endosomes and actin patches in living yeast cells. FM4-64 is a lipophilic styryl dye that binds to the plasma membrane, is brought into the cell via endocytosis, and subsequently travels through endosomal sorting compartments to the vacuole (Vida and Emr, 1995; Wendland et al., 1996). Abp1p is an actin-binding protein that can activate the Arp2/3 complex and is found exclusively in actin patches in vegetative yeast (Drubin et al., 1988; Goode et al., 2001). Expression of Abp1p-GFP in living yeast cells produces a robust signal that localizes exclusively to actin patches and has no obvious effect on cell growth, mating efficiency, or organization of the actin cytoskeleton (Doyle and Botstein, 1996; Smith et al., 2001; Carlsson et al., 2002). Recently, Kaksonen et al. (2003) showed that Abp1p-GFP accumulates in assembling actin patches ~20 s after other resident actin patch proteins and shortly before actin patches move away from the plasma membrane.

We found that FM4-64 and Abp1p-GFP assemble into the same punctate structures in living yeast. In the example shown, Abp1p-GFP appears as a weakly fluorescent spot at the mother-bud neck of a yeast cell (Fig. 1). FM4-64 appears as a weakly fluorescent spot that colocalizes with the Abp1p-GFP–containing particle 0.6 s later. The fluorescence intensity of the FM4-64 and Abp1p-GFP in this particle increased over the next 3 s and then remained constant for 1.2 s. FM4-64 appears and accumulates at sites in the cell cortex that labeled with Abp1p-GFP in >97% instances observed ($n = 112$). In each of these cases, accumulation of FM4-64–labeled particles occurred <1 s after the appearance of Abp1p-GFP. Two-color, 4D-imaging (3D reconstruction combined with time-lapse imaging) revealed that the appearance and increase in fluorescent intensity of FM4-64 and Abp1p-GFP were not due to movement of the particle into the focal plane (Fig. 2). Rather, these observations indicate that endocytic compartments assemble at sites of actin patch assembly. Because Abp1p incorporates into actin patches ~20 s after other resident actin patch proteins (Kaksonen et al., 2003), the assembly of endocytic compartments must also occur during late stages in the assembly of actin patches.
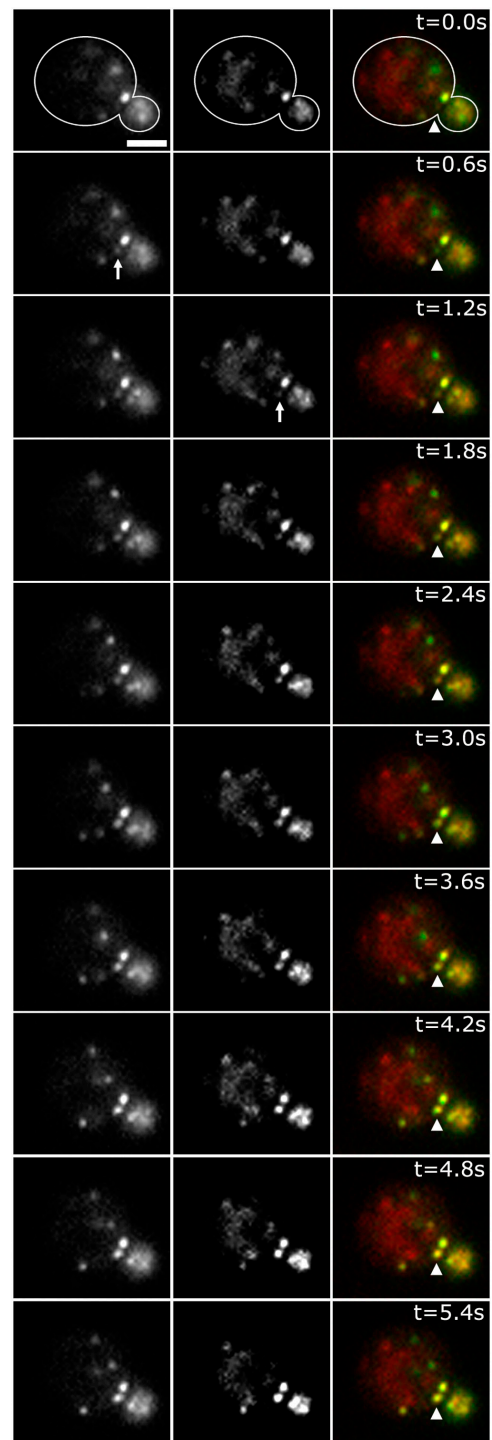
Figure 1. **FM4-64 and Abp1p-GFP assemble at the same punctate structures in living yeast.** Mid-log phase wild-type haploid cells expressing Abp1p-GFP from the chromosomal locus were incubated with FM4-64 for 30 s at RT. Cells were washed with lactate medium to remove excess FM4-64 and analyzed by time-lapse fluorescence imaging within 2 min after initial incubation with FM4-64. Under these staining and imaging conditions, FM4-64 localizes to sites of endocytosis and endosomes. Cells were imaged in a single, cortical focal plane at RT using an optical beam splitter that allows for simultaneous imaging of Abp1p-GFP and FM4-64 (see Materials and methods). Images are still frames from a time-lapse series showing Abp1p-GFP in the left column, FM4-64 in the middle column, and a merged image showing Abp1p-GFP (green) and FM4-64 (red) in the right column. Outline of the cell is shown at t = 0 s. Arrowheads in merged images mark the site of FM4-64 and Abp1p-GFP accumulation. Arrows indicate the first time-points in which a signal is detectable for Abp1p-GFP (left) and FM4-64 (middle). Bar, 2 μm.
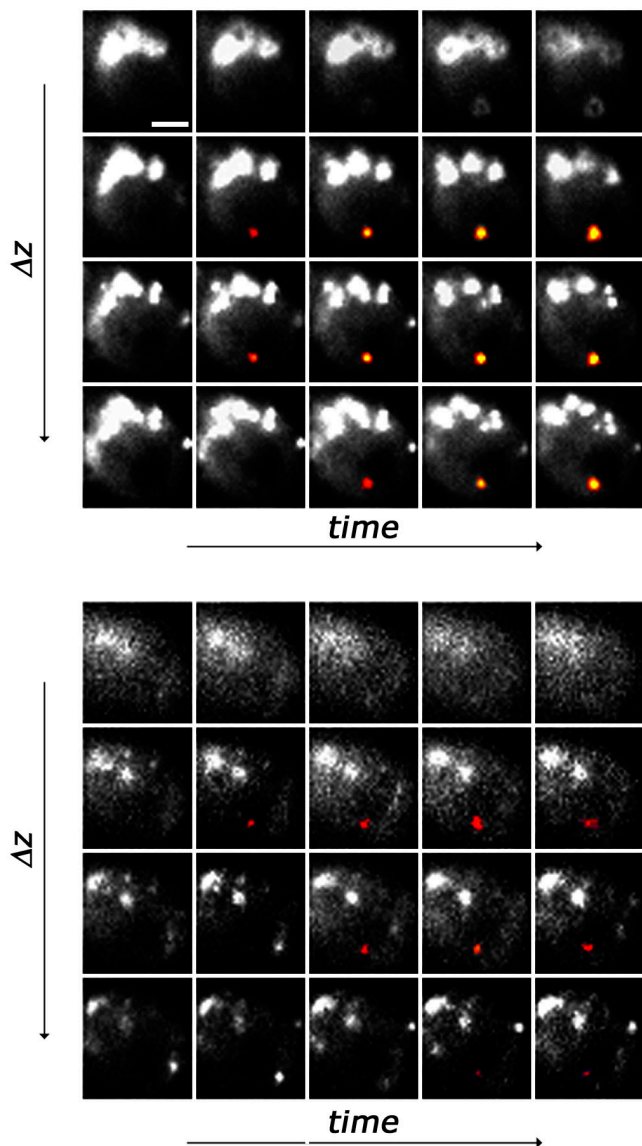
Figure 2. **Visualization of the assembly of FM4-64 and Abp1p-GFP by 3D reconstruction combined with time-lapse imaging.** Mid-log phase wild-type haploid cells expressing Abp1p-GFP from their chromosomal locus were incubated with FM4-64 as for Fig. 1. Cells were analyzed by simultaneous two-color imaging and 3D reconstruction combined with time-lapse imaging. Simultaneous two-color imaging was performed as for Fig. 1. Z-sections were obtained at 0.4-μm increments. The time interval between each successive set of z-sections is 1.6 s. The still frames shown are z-sections at focal planes that show sites of incorporation of Abp1p-GFP (top) and FM4-64 (bottom) at different time-points during the assembly process. The cell shown is unbudded and has polarized toward the presumptive bud site in the top of the cell. The structure of interest is pseudocolored according to the strength of the fluorescent signal, where yellow corresponds to greatest intensity, orange corresponds to medium intensity, and red corresponds to low intensity. Abp1p and FM4-64 accumulate in an intermediate focal plane in the second column and are not present either above or below the plane of appearance in the preceding column. Thus, the structures are indeed assembling, rather than moving into the focal plane. Bar, 2 μm.

Motile endosomes colocalize with Abp1p-labeled actin patches

Using simultaneous two-color time-lapse imaging in living yeast cells, we found that FM4-64 colocalized with motile Abp1p-GFP–labeled actin patches. In the representative exam-

ple shown, a particle labeled with FM4-64 and Abp1p-GFP undergoes linear, retrograde movement for a distance of ~2 μm over a 3.6-s period (Fig. 3). Abp1p-GFP–labeled actin patches colocalized with punctate FM4-64–labeled structures during linear, retrograde movement in >95% of instances observed ($n = 118$). This observation provides further support for the model that actin patches are intimately associated with endosomes.

Actin patches disassemble at internal, FM4-64–labeled structures

Previous work in mating yeast revealed that actin patches disassemble after undergoing retrograde movement (Smith et al., 2001). To determine whether actin patch disassembly is linked to endosome trafficking, we monitored the interaction between Abp1p-GFP–labeled actin patches and FM4-64–labeled internal compartments. Previous findings indicate that 10 min after its uptake, FM4-64 labels an endosomal sorting compartment that undergoes Pep12p-dependent fusion with the vacuole (Vida and Emr, 1995; Holthuis et al., 1998). Here, we incubated yeast with FM4-64 under conditions that label internal compartments and studied the interaction of Abp1p-GFP–labeled actin patches with internal FM4-64–labeled compartments by simultaneous two-color time-lapse imaging. In the example shown, linear retrograde movement of an Abp1p-GFP–labeled actin patch brings it in close proximity with an FM4-64–labeled internal compartment (Fig. 4). The two particles remain in close proximity for 1.5 s. Thereafter, the Abp1p-GFP particle is no longer detectable. 4D imaging revealed that the loss of Abp1p-GFP signal is not due to movement of the particle out of the focal plane (Fig. 5). Rather, we found that Abp1p-GFP–labeled actin patches disassembled after interacting with FM4-64–labeled internal compartments in 90% of the instances observed ($n = 58$). This finding provides additional support for the model that actin patches are intimately associated with endosomes. Moreover, they suggest that retrograde movement of actin patches/endosomes results in transport within the endomembrane system.

Yeast fimbrin Sac6p colocalizes with endosomes during their assembly, movement, and disassembly

Previous work in this laboratory and others has shown that Sac6p-GFP exhibits behavior that is similar to Abp1p-GFP; both proteins assemble into cortical, punctate structures, and can undergo linear, retrograde movement (Doyle and Botstein, 1996; Smith et al., 2001). Using simultaneous two-color imaging, we found that Sac6p-HcRed is recruited late in the life cycle of an actin patch, and colocalizes with Abp1p-GFP (Fig. 6). In addition, we found that Sac6p-HcRed colocalizes with Abp1p-GFP during linear, retrograde movement (unpublished data). Finally, we found that Sac6p-GFP, like Abp1p-GFP, colocalizes with FM4-64 during assembly and retrograde movement (Fig. 7 A), and that Sac6p-GFP–containing particles disassemble at FM4-64–labeled internal compartments (Fig. 7 B).

These results reinforce the model that actin patches are intimately associated with endosomes during their formation, retrograde movement, and disassembly at FM4-64–labeled
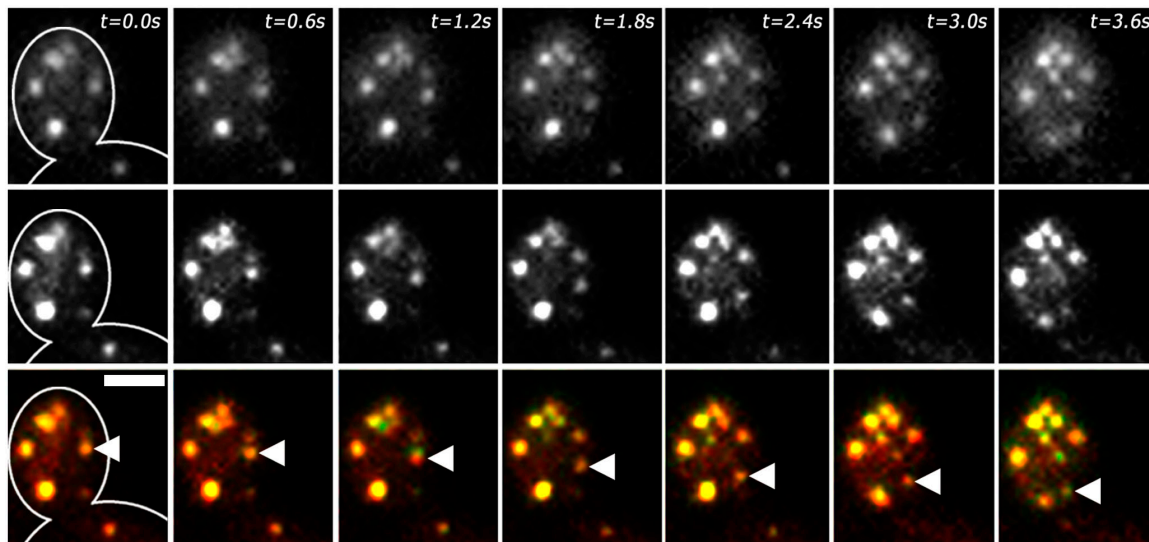
Figure 3. **Particles labeled with FM4-64 and Abp1p-GFP exhibit linear, retrograde movement.** Mid-log phase wild-type haploid cells expressing Abp1p-GFP from the chromosomal locus were incubated with FM4-64 for 1 min at RT. Cells were washed with lactate medium to remove excess FM4-64, and imaged within 3 min after initial incubation with FM4-64. Two-color time-lapse imaging was performed as described for Fig. 1. Images shown are still frames from a time-lapse series showing Abp1p-GFP in the top row, FM4-64 in the middle row, and a merged image showing Abp1p-GFP in green and FM4-64 in red in the bottom row. The outline of the cell is shown in panels at t = 0 s. The bud, mother-bud neck, and part of the mother cell are shown. Arrowheads in the merged images mark an actin patch/endosome undergoing linear movement. Bar, 2 µm.

internal compartments. Moreover, we find that 72.2% of the Sac6p-GFP–labeled actin patches analyzed display random movement in the plane of the membrane, and 23.1% of Sac6p-GFP–labeled actin patches analyzed display linear movement ($n = 377$). Finally, 86.2% of linear actin patch movements observed in the mother cell were directed in the retrograde direction, i.e., away from the bud ($n = 87$). Thus, there is a bias in linear movement in the retrograde direction.

Our studies indicate that actin patch disassembly can occur after linear retrograde actin patch movement. However, only 23% of all actin patches display this type of movement. Moreover, actin patches undergoing nonlinear cortical movement can interact with and disassemble at FM4-64–labeled internal compartments (unpublished data). Thus, linear, retrograde movements are not required for interaction of actin patches/endosomes with other endosomal compartments. However, because most actin patches form in the bud, we suspect that the linear retrograde movements are important for interaction of actin patches with endosomal compartments in the mother cell.

Motile actin patches interact with actin cables and require actin cables for linear, retrograde movement

Previously, we showed that Abp140p-GFP, a GFP fusion protein containing the resident actin cable protein Abp140p, labels actin cables but has no obvious effect on cell growth, actin organization, or actin function. Moreover, we showed that the intensity of fluorescence from Abp140p-GFP was not uniform along actin cables, and that the amount of Abp140p-GFP in actin cables was proportional to the amount of F-actin in actin cables. Finally, we demonstrated that bright spots of Abp140p-GFP could be used as fiduciary marks to analyze ac-

tin cable dynamics in living yeast cells (Yang and Pon, 2002). Here, we monitored movement of Abp140p-GFP fiduciary marks on actin cables and Abp1p-HcRed–labeled actin patches to determine the velocity of actin cable and patch movement. First, we found that all detectable actin cables exhibited retrograde flow ($n = 100$). Second, the velocity of retrograde actin cable flow was similar to the velocity of retrograde actin patch movement (Fig. 8 A).

Consistent with this, we find that destabilization of actin cables results in the loss of linear, retrograde actin patch movement. Previous reports indicate that a formin mutant bearing a conditional mutation in the *BNI1* gene and a deletion in the *BNR1* gene *(bni1-11 bnr1Δ)* displays a loss of actin cables after a shift to restrictive temperature (35°C) for 2 min, and the restoration of actin cables within a minute after return to permissive temperature (Evangelista et al., 2002; Sagot et al., 2002). We confirmed this result, and found that the pattern and velocity of movement of Abp1p-HcRed–labeled actin patches in the *bni1-11 bnr1Δ* strain at permissive temperature resembled that of actin patches in wild-type cells. However, short-term shift of the *bni1-11 bnr1Δ* to restrictive temperatures—conditions that destabilize all detectable actin cables—results in the loss of linear, retrograde movement of Abp1p-HcRed–labeled actin patches (Fig. 8 B). Thus, actin cables are required for linear, retrograde actin patch movement.

Finally, we found that Abp1p-HcRed–labeled actin patches undergoing linear, retrograde movement localize to Abp140p-GFP–labeled actin cables (Fig. 9; Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200404173/DC1). Because Abp140p-GFP fiduciary marks are rare and difficult to resolve, the frequency of detecting an actin patch undergoing a retrograde movement along an actin cable that also contained a resolved Abp140p-GFP fiduciary mark was low. However, we
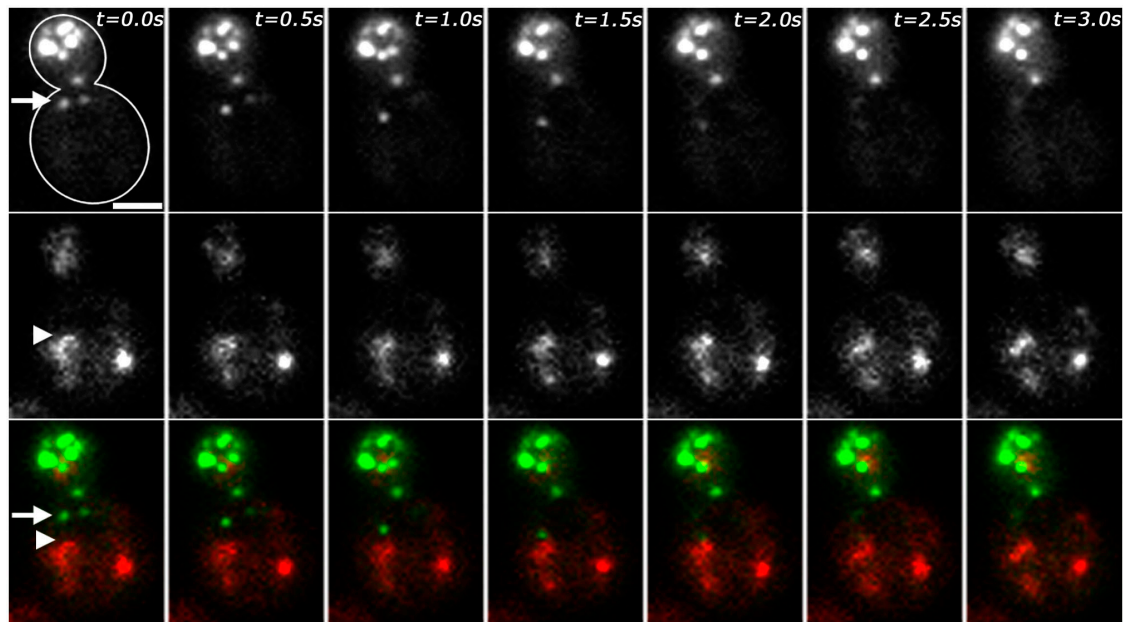
Figure 4. **Abp1p-GFP disassembles after actin patches interact with FM4-64–labeled internal compartments.** Mid-log phase wild-type haploid cells expressing Abp1p-GFP from the chromosomal locus were stained with FM4-64 for 2 min at RT. Cells were washed with lactate medium to remove excess FM4-64 and imaged 7 min after initial incubation of cells with FM4-64. Under these conditions, FM4-64 stains the endosomal sorting compartment (Holthuis et al., 1998). Two-color time-lapse imaging was performed as described for Fig. 1. Images shown are still frames from a time-lapse series showing Abp1p-GFP–labeled actin patches in the top row, FM4-64–labeled internal compartments in the middle row, and a merged image showing Abp1p-GFP in green and FM4-64 in red in the bottom row. The outline of the cell is shown in the top panel at t = 0 s. Arrows mark an Abp1p-GFP–labeled actin patch that undergoes retrograde movement and interacts with an FM4-64–labeled internal compartment, indicated by arrowheads. Bar, 2 μm.

observed retrograde actin patch movement in conjunction with retrograde actin cable movement in 90% of the instances observed ($n = 21$). Moreover, we found that 96.8% of the actin patches undergoing retrograde movement colocalized with actin cables ($n = 95$). This observation supports a role for actin cables in linear, retrograde actin patch movement.

Role of actin cables in retrograde actin patch movement

The findings that actin patches require and colocalize with actin cables during retrograde movement and that actin patches and cables undergo retrograde flow at the same velocity support a model in which actin patches remain fixed on actin cables and move as a result of retrograde actin cable flow. To test this model, we studied a possible role of actin patch–associated force generators on actin patch movement. In addition, we studied the motility of actin patches relative to actin cables.

There are two actin-dependent force generators that localize to actin patches: myosin I proteins (Myo3p and Myo5p) and the Arp2/3 complex. Previous reports indicate that deletion of *MYO3* and *MYO5* does not block actin patch movement in vegetative yeast, and has no effect on the velocity of linear, retrograde actin patch movement in mating yeast (Waddle et al., 1996; and Smith et al., 2001). Thus, type I myosins do not appear to be the motors for retrograde actin patch movement. Indeed, because all known myosin-driven movements along actin cables are anterograde and directed toward the bud (Schott et al., 2002), it is unlikely that any of the myosins of yeast drive retrograde actin patch movement.

In light of this, we tested whether the Arp2/3 complex contributes to this movement. We used Abp1p-GFP to monitor actin patch movement in a yeast strain (*arp2-1*) bearing a temperature-sensitive mutation in the Arp2p subunit of this complex. Previous work showed that the *arp2-1* strain did not display defects in actin cable orientation or trafficking of internalized endosomes to the vacuole. However, this mutation produces temperature-sensitive defects in actin patch polarization and endosome internalization (Moreau et al., 1997). At permissive temperature (23°C), the *arp2-1* strain displayed normal actin patch motility. However, incubation at restrictive temperature (37°C) for 30 min resulted in a change in actin patch movement. First, we observed a nearly 10-fold decrease in cortical, nonlinear actin patch movement (Table I). Second, we observed a threefold reduction in the frequency of linear, retrograde actin patch movements. Because the Arp2/3 complex is required for endosome internalization, the internalization defect in the *arp2-1* mutant would reduce the number of actin patches that can move away from the cell cortex. Nonetheless, the velocities of linear, retrograde actin patch movement in the mutant were similar to those observed in wild-type cells (Table I). These findings indicate that the Arp2/3 complex is not required for linear, retrograde actin patch movements, which occur during transport of endosomes to FM4-64–labeled internal compartments. Instead, our findings support a role for the Arp2/3 complex in cortical, nonlinear endosome movement that is required for or occurs during endosome internalization. Finally, we found that deletion of Abp1p, the Arp2/3 complex activator that assembles onto ac-
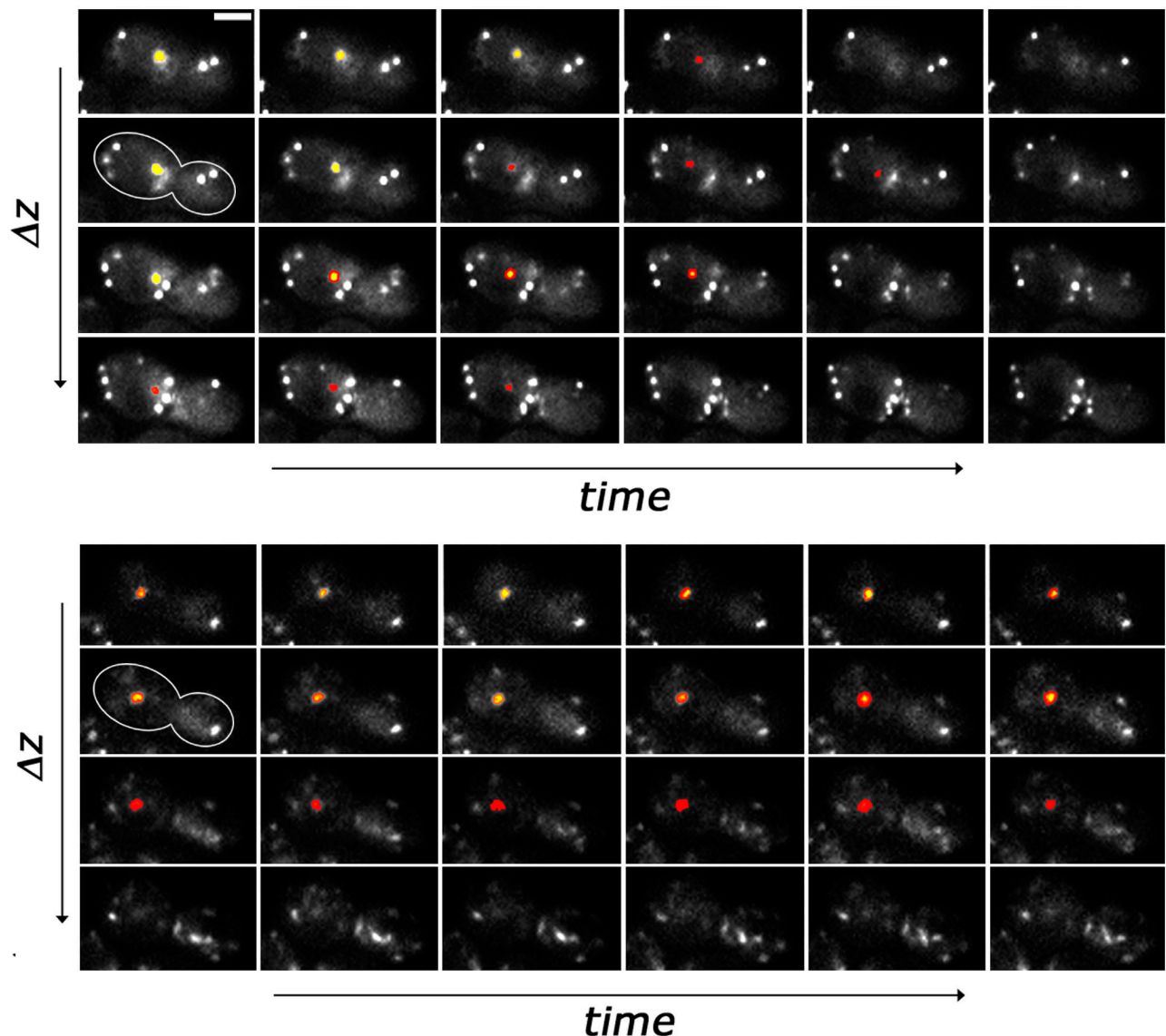
Figure 5. **Visualization of the disassembly of Abp1p-GFP at FM4-64–labeled internal compartments by 3D reconstruction combined with time-lapse imaging.** Mid-log phase wild-type haploid cells expressing Abp1p-GFP were stained with FM4-64 as for Fig. 4. Cells were analyzed by simultaneous two-color imaging and 3D reconstruction combined with time-lapse imaging. Simultaneous two-color imaging was performed as for Fig. 1. Z-sections were obtained at 0.4-μm increments. The time interval between each successive set of z-sections is 1.6 s. The still frames shown are z-sections at focal planes that show sites of disassembly of Abp1p-GFP (top) as the particle interacts with an FM4-64–labeled internal compartment (bottom) at different time-points during the disassembly process. The cell shown is a large-budded cell. The structures of interest are in the mother cell and are pseudocolored as in Fig. 2. Optical sections taken above and below the plane of Abp1p-GFP–labeled particle disassembly indicate that the loss of Abp1p-GFP signal is due to disassembly, and not to movement of the particle out of the plane of imaging. Bar, 2 μm.

tin patches immediately before internalization, had no significant effect on either the frequency or the velocity of linear actin patch movement (Table I).

The finding that the Arp2/3 complex and other force generators are not required for retrograde actin patch movement supports the model that retrograde actin cable flow drives this actin patch movement. In support of this, we found that actin patches make no net movement along the length of a motile actin cable. These studies were conducted using bright spots of Abp140p-GFP on actin cables or the tip of an elongating actin cable as fiduciary marks to study actin cable dynamics and Abp1p-HcRed to monitor actin patch dynamics. In the example shown, an actin patch remained associated with the tip of an elongating actin ca-

ble (Fig. 10). In addition, we observed instances in which there was no net change in the distance between an Abp1p-HcRed–labeled actin patch and an Abp140p-GFP fiduciary mark on an actin cable as both structures underwent linear, retrograde movement. These findings indicate that the retrograde movement of actin cable–associated actin patches depends solely on the elongation and retrograde movement of the actin cable.

Discussion

The first link between the actin cytoskeleton and endocytosis came from a yeast genetic screen, which showed that mutations that inhibit receptor-mediated endocytosis map to actin,
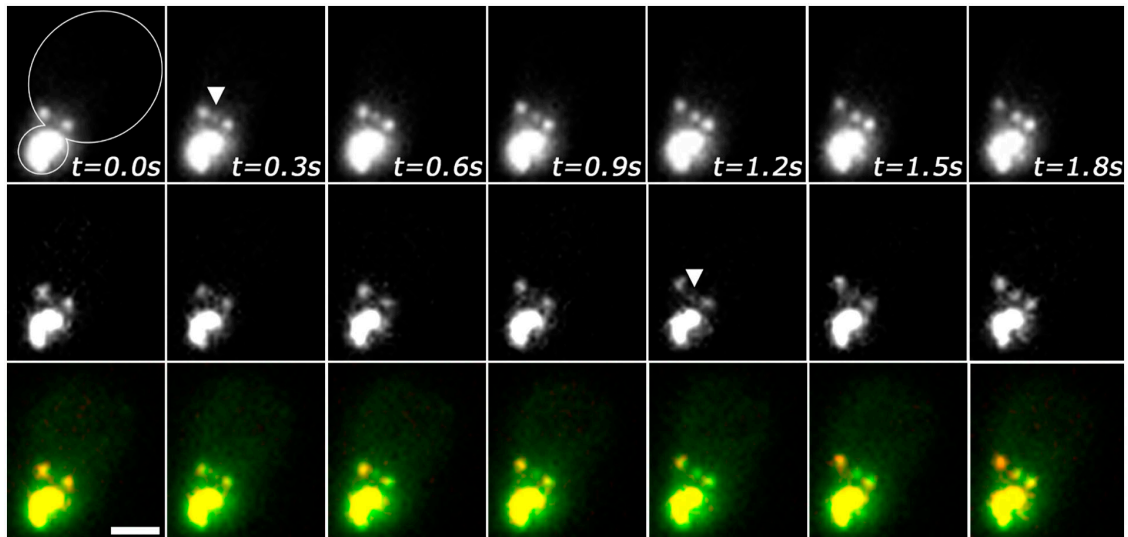
Figure 6. **Abp1p-GFP and Sac6p-HcRed assemble at the same punctate structures in living yeast.** Wild-type haploid cells expressing Abp1p-GFP and Sac6p-HcRed from the chromosomal loci were grown to mid-log phase in lactate medium and imaged in a single cortical focal plane using simultaneous two-color imaging as for Fig. 1. Images shown are still frames from a time-lapse series showing Abp1p-GFP–labeled actin patches in the top row, Sac6p-HcRed–labeled actin patches in the middle row, and a merged image showing Abp1p-GFP in green and Sac6p-HcRed in red in the bottom row. Arrowheads indicate the point of emergence of fluorescent signal in the top and middle rows. The outline of the cell is shown in the top panel at t = 0 s. Bar, 2 μm.

actin-binding proteins, and proteins that regulate actin organization (Kubler and Riezman, 1993). Because many of the cytoskeletal proteins that are required for endocytosis in yeast are conserved in other eukaryotes (for review see Enqvist-Goldstein and Drubin, 2003), it raised the possibility that the actin cytoskeleton plays a vital role in endocytosis in higher organisms. In support of this, the Arp2/3 complex, Arp2/3 complex activators, and/or actin polymerization have been implicated in membrane internalization and/or movement of endosomes in mast cells, NIH 3T3 cells, and *Xenopus* oocytes; of phagosomes in macrophages and *Dictyostelium*; and macropinosomes in PTK1 cells (Merrifield et al., 1999; Kaksonen et al., 2000; Schafer et al., 2000; Taunton et al., 2000; Insall et al., 2001; Zhang et al., 2002; Southwick et al., 2003). Moreover, dynamin, a molecule implicated in membrane fission,

can bind to actin-binding proteins and Arp2/3 complex activators in PTK1 and NIH 3T3 cells (Schafer et al., 2002; Krueger et al., 2003). Finally, elegant light microscopy analyses revealed that dynamin and actin assemble at sites of endocytosis in Swiss 3T3 cells and sites of SV40 uptake in caveolae in CV-1 cells (Merrifield et al., 2002; Pelkmans et al., 2002). Here, we provide evidence that actin cables and actin patches, the major F-actin containing structures in budding yeast, have direct roles in endocytosis.

Actin patches are intimately associated with endosomes

In budding yeast, buds form, grow, and separate from mother cells as a result of secretion that is directed to the bud and mother-bud neck. We provide the first direct evi-
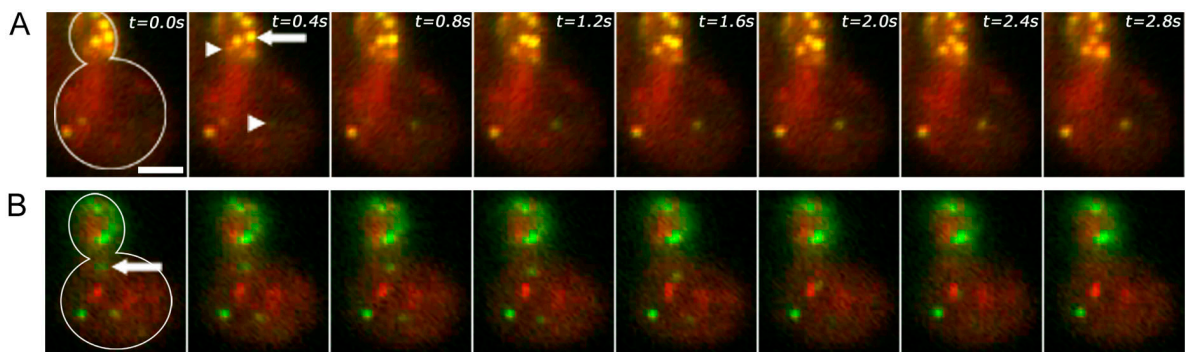

Figure 7. **Assembly, movement, and disassembly of Sac6p-GFP.** Mid-log phase wild-type haploid cells expressing Sac6p-GFP from the chromosomal locus. FM4-64 staining to detect endosomes in A was performed as described in Fig. 1. FM4-64 treatment to stain internal compartments in B was performed as described in Fig. 4. Two-color time-lapse imaging was performed as for Fig. 1. Images shown are still frames from time-lapse series showing Sac6p-GFP–labeled actin patches in green, FM4-64 in red, and sites of colocalization in yellow. The outline of each cell is shown in both panels at t = 0 s. In A, arrowheads indicate the coassembly of a Sac6p-containing actin patch and an FM4-64 labeled endosome. Arrow in A points to the beginning of a linear, retrograde movement of a Sac6p- and FM4-64-containing structure. In B, an arrow points to a Sac6p-labeled actin patch that undergoes linear, retrograde movement and subsequent disassembly at the FM4-64–labeled internal compartment. Bar, 2 μm.

Figure 8. Retrograde movement of actin patches occurs with the same velocity as retrograde actin cable movement and requires actin cables. (A) The velocity of actin cable and patch movement. Wild-type cells expressing either Abp140p-GFP or Abp1p-GFP from the chromosomal loci were grown to mid-log phase in lactate medium. Cells were imaged by time-lapse fluorescence imaging and the velocities of linear, retrograde movements of actin cables ($n = 41$) and actin patches ($n = 42$) was determined as described in Materials and methods. (B) Destabilization of actin cables results in loss of linear, retrograde actin patch movement. Abp1p was tagged at its chromosomal locus with HcRed in wild-type cells and yeast bearing a deletion of the *BNR1* gene and a temperature-sensitive mutation in the *BNI1* gene (*bni1-11 bnr1Δ*). Cells were grown to mid-log phase in lactate medium. At t = 0, aliquots of the liquid culture were removed and either maintained at permissive temperature (RT) or incubated at restrictive temperature (35°C) for 2 min. Time-lapse imaging of Abp1p-HcRed–labeled actin patches was performed at 23 and 35°C. The frequency of linear retrograde actin patch movement was defined by the number of linear retrograde actin patch movements per mother cell in the 20-s imaging period ($n = 67$–108 cells). Linear retrograde movement was defined as a movement away from the bud neck in the mother cell over three consecutive time-points.
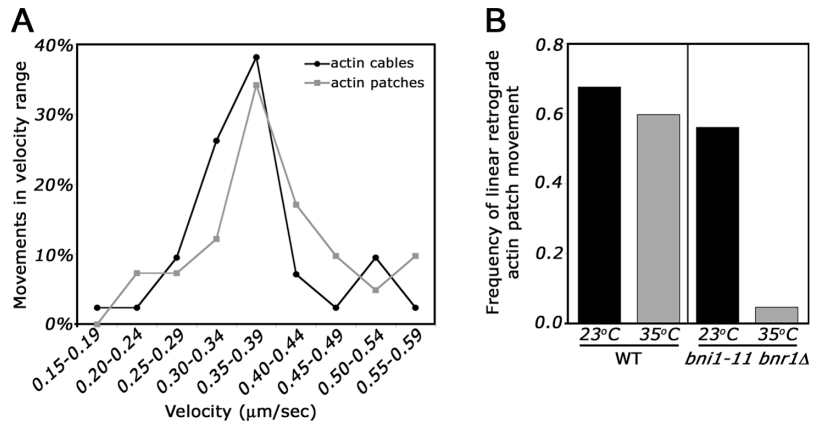
dence that actin patches, structures that localize to sites of polarized secretion in budding yeast, are intimately associated with endosomes. That is, we find that FM4-64 incorporates into structures that are labeled with Abp1p-GFP or Sac6p-GFP at late stages of actin patch assembly. In addition, we find that actin patches colocalize with FM4-64–labeled endosomes as they undergo linear retrograde movement. This is the first documentation of linear, retrograde movement by an FM4-64–labeled structure. Finally, we find that these structures use actin cables for movement from the bud to FM4-64–labeled internal compartments, and disassemble upon interaction with FM4-64–labeled internal compartments.

Our observations support the model that actin patches in budding yeast are endosomes. That is, actin and actin patch proteins are present on the surface of endosomes during endosome formation, fission, and movement. This interpretation is supported by previous findings that (1) actin and actin patches are linked to endocytosis; (2) actin assembles on plasma membrane invaginations in budding yeast; and (3) F-actin assembles at the plasma membrane at sites of endocytosis, phagocytosis, and macropinocytosis and forms comet tails on endosomes in animal cells (Mulholland et al., 1994; Merrifield et al., 1999; Taunton et al., 2000; Zhang et al., 2002; Enqvist-Goldstein and Drubin, 2003; Southwick et al., 2003). In light of the finding that vertebrate cells contain cortical punctate structures con-
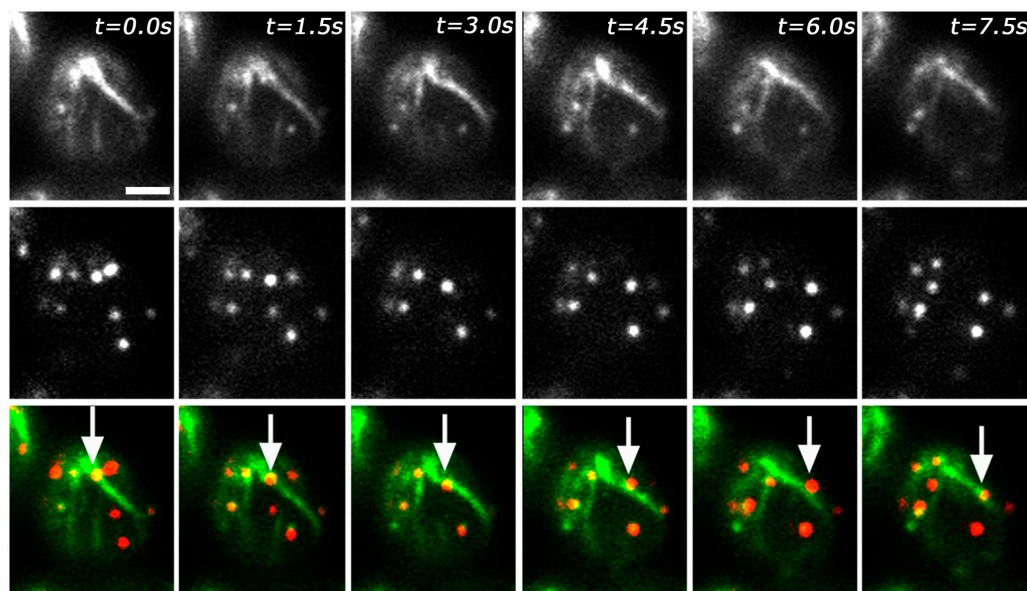
Figure 9. Colocalization of actin patches and actin cables during retrograde movement. Wild-type haploid cells expressing Abp1p-HcRed and Abp140p-GFP from the chromosomal loci were grown to mid-log phase in lactate medium and were imaged in a single cortical focal plane using simultaneous two-color imaging as for Fig. 1. Images shown are still frames from a time-lapse series showing Abp140p-GFP–labeled actin cables in the top row, Abp1p-HcRed–labeled actin patches in the middle row, and a merged image showing Abp140p-GFP in green and Abp1p-HcRed in red in the bottom row. The cell shown is an unbudded cell in which the presumptive bud site is at the top of the cell. Arrows in the merged images mark an Abp1p-HcRed–labeled actin patch that undergoes linear, retrograde movement along an Abp140p-GFP–labeled actin cable. Bar, 2 μm.

Strain	Frequency of movement			Velocity of linear movement
	Cortical	Linear	n	
	%	%		µm/s
arp2-1 (RT)	60.3	13.7	525	0.39 ± 0.09
arp2-1 (37°C)	3.6	4.4	417	0.40 ± 0.10
ABP1 (wt)	72.2	23.1	761	0.39 ± 0.11
abp1Δ	71.5	21.6	803	0.36 ± 0.10

Actin patch motility in the temperature-sensitive *arp2-1* mutant strain was analyzed using Abp1p-GFP at permissive temperature (23°C) and after incubation at restrictive temperature (37°C) for 30 min. Actin patch motility was measured in an *ABP1* deletion mutant (*abp1Δ*) and the corresponding wild-type strain (*ABP1*) using Sac6p-GFP. Time-lapse imaging was performed at 0.2-s intervals in a single plane of focus over a 40-s imaging period. Movement of every detectable actin patch per cell was determined. Movements were defined as linear if they were linear for four consecutive frames during the imaging period. Movements were defined as cortical if they were nonlinear and occurred at the cortex of the bud or mother cell. Some actin patches did not move in the time interval analyzed. The frequencies of movement are the percentage of actin patches that displayed cortical or linear movement. Velocities of linear movement are the mean velocity ± SD. The number of GFP-labeled actin patches analyzed per condition is shown under the "*n*" column heading.

taining F-actin, Arp3, and capping protein (Schafer et al., 1998), it is possible that endosomes in budding yeast may be similar to those present in other eukaryotes. Moreover, our findings support the notion that F-actin and actin patch proteins are released from the endosome surface when endosomes interact (and possibly fuse) with FM4-64–labeled internal compartments. This interpretation is supported by the observations that Abp1p-GFP and Sac6p-GFP disassemble from actin patches after they interact with FM4-64–labeled internal compartments.

Previous reports indicate that FM4-64 accumulates in endosomal sorting compartments after 10 min of staining (Vida and Emr, 1995; Holthuis et al., 1998). The conditions that we used to label internal compartments with FM4-64 were similar to those used to stain structures identified as endosomal sorting compartments. Therefore, it is likely that the FM4-64–labeled internal compartments observed in this paper are endosomal sorting compartments.

Mechanism of actin patch/endosome movement

Early characterizations revealed that actin patches contain different subsets of proteins, and exhibited different motility patterns (Warren et al., 2002). Consequently, it was suggested that different actin patches performed diverse functions. An elegant study by Kaksonen et al. (2003) revealed that proteins are recruited to actin patches in a strictly regulated temporal fashion. Thus, proteins that are recruited early in the life span of an actin patch show motility patterns that are restricted to the plasma membrane, whereas those that are recruited late in the life of an actin patch show a more complex motility pattern, including a primary movement at the cortex, followed by a secondary movement away from the membrane that is often linear and long-range. Moreover, they showed that Abp1p is recruited late in the life cycle of an actin patch, shortly before the transition from cortical motility to linear, long-range movement. Here, we showed that accumulation of Abp1p in an actin patch occurs <1 s before accumulation of the lipophilic endocytic marker, FM4-64. Thus, the endocytic event appears to happen at the end of the cortical phase of the actin patch life cycle.

Other reports showed that the Arp2/3 complex and Arp2/3 complex activators (e.g., Abp1p, the WASp homologue, Las17p/Bee1p, and type I myosins, Myo3p and Myo5p) localize to actin patches and are required for actin patch assembly, the internalization step of endocytosis, and actin patch movement (Moreau et al., 1997; Winter et al., 1997; Anderson et al., 1998; Goode et al., 2001; Lechler et al., 2001). Because there are multiple Arp2/3 complex activators in actin patches, it is possible that the Arp2/3 complex and actin polymerization may contribute directly to each of these events. We find that the Arp2/3 complex does not generate forces for linear, retrograde actin cable movements, which occur during transport of endosomes to FM4-64–labeled internal compartments. Instead, our findings support a role for the Arp2/3 complex in cortical, nonlinear endosome movements that are either required for or occur during endosome internal-
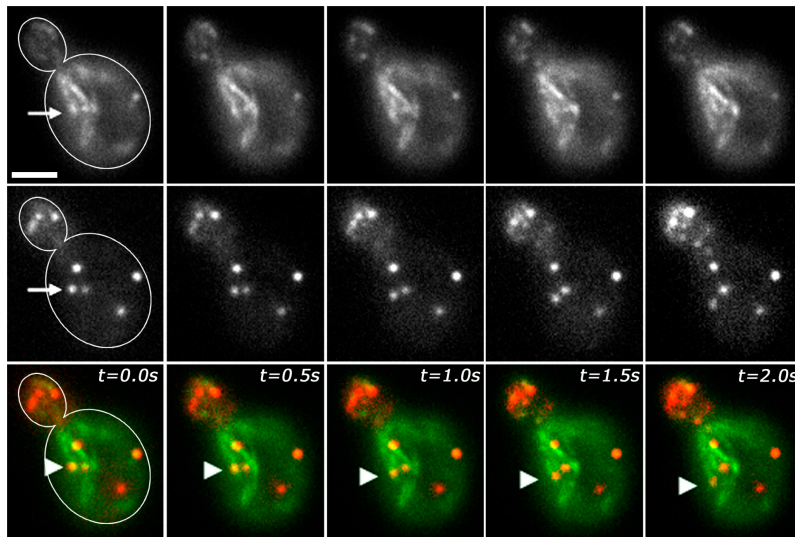

Figure 10. **An actin patch undergoing retrograde movement remains associated with an elongating actin cable at a fixed point.** Mid-log phase yeast expressing Abp1p-HcRed and Abp140p-GFP were studied using simultaneous two-color imaging as for Fig. 4. Images shown are still frames from a time-lapse series showing Abp140p-GFP–labeled actin cables in the top row, Abp1p-HcRed–labeled actin patches in the middle row, and a merged image showing Abp140p-GFP in green and Abp1p-HcRed in red in the bottom row. Arrowheads in the merged images show the change in position of an actin patch that is associated with the tip of an elongating actin cable as both of these structures undergo linear, retrograde movement. Arrows mark the position of the tip of the actin cable at t = 0 (top row), and the position of the actin patch at t = 0 (middle row). Bar, 2 µm.

ization. Interestingly, we find that deletion of Abp1p, the Arp2/3 complex activator that assembles onto actin patches immediately before internalization, had no effect on linear or nonlinear actin patch movement.

Here, we provide evidence for a new mechanism for linear retrograde actin patch movement that is dependent on actin cables, bundles of actin filaments that undergo assembly- and elongation-driven retrograde flow from buds to mother cells. According to this model, actin patches use actin cables as "conveyor belts" for long-distance retrograde movement from the bud to FM4-64–labeled internal compartments. This model is based on the findings that (1) actin patches require actin cables for retrograde movement; (2) actin patches colocalize with actin cables as they undergo retrograde movement; (3) the velocity of retrograde actin cable movement is similar to that of retrograde actin cable flow; and (4) actin patches make no net movement along the length of actin cables during retrograde flow.

Previous work in fission yeast indicated that actin patches undergo linear, retrograde movement along actin cables (Pelham and Chang, 2001). They showed that mutation of an Arp2/3 complex subunit or treatment with low levels of a drug that dampens actin dynamics (latrunculin-A) reduced the velocity of actin patch movement. This led to the conclusion that the Arp2/3 complex and actin polymerization generate the force for movement of actin patches using actin cables as tracks. This mechanism is similar to that observed for actin cable–dependent anterograde movement of mitochondria during inheritance in budding yeast (Boldogh et al., 2001). However, it is different from the conveyor belt model for actin cable–driven retrograde movement of actin patches/ endosomes proposed here. In the absence of information on the movement of actin patches relative to actin cables in *Schizosaccharomyces pombe*, it is difficult to draw conclusions regarding the mechanism underlying this process. Indeed, because the Arp2/3 complex is required for assembly and cortical movement of actin patches and for the internalization step in endocytosis, the reduced velocity of actin patch movement observed in Arp3 mutants in *S. pombe* may be due to defects in actin patch assembly or motility events that preclude association of actin patches with actin cables. Moreover, dampening actin dynamics by treatment with low levels of latrunculin-A blocks actin cable assembly and retrograde movement in budding yeast (Yang and Pon, 2002). Thus, dampening of actin dynamics by latrunculin-A treatment or Arp2/3 complex mutations could inhibit actin patch movement in *S. pombe* through effects on actin cable assembly and elongation.

In summary, our studies support the model that F-actin and actin patch proteins are on the surface of endosomes during endosome formation, fission, and movement, and that F-actin and actin patch proteins are released from the endosome surface when the endosome interacts (and possibly fuses) with FM4-64–labeled internal compartments. Moreover, we propose a mechanism for endosome motility in which endosomes bind to elongating actin cables, and use the forces of actin cable extension and movement to drive their long-distance, retrograde movement to endosomal sorting compartments.

One fundamental question that is raised by these studies is the function of these retrograde endosome movements in a cell that is undergoing growth by polarized secretion. In cells that are specialized for secretion, endocytosis is required to recycle lipids and proteins from the plasma membrane (for review see Gundelfinger et al., 2003). For example, secretion of cortical granules in sea urchin eggs after fertilization, which produces a barrier around the egg to prevent polyspermy, is followed by endocytic membrane retrieval (Whalley et al., 1995). Similarly, in endocrine cells, including pancreatic β-cells and adrenal chromaffin cells, there are two forms of compensatory endocytic recycling that are stimulated by secretion; the form used appears to depend on the length and intensity of the secretion signal (Artalejo et al., 2002; Hoy et al., 2002). Finally, one of the best-characterized examples of compensatory endocytosis occurs in neurons, where recovery of membrane and the secretion apparatus occurs in specialized regions at the nerve terminus (for review see Murthy and De Camilli, 2003). In each of these situations, endocytosis is required to recycle constituents that are necessary for further rounds of secretion. In light of this, uptake of endosomes from the site of polarized secretion in budding yeast and retrograde movement to endosomal sorting compartments may contribute to membrane recycling events that are necessary for polarized secretion. Ongoing studies are designed to address this issue.

Table II. **Strains used in this work**

Strain	Genotype	Source
BY4741	MAT**a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Research Genetics
YCY027	MAT**a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ABP140:GFP:KanMX6*	This work
YCY031	MAT**a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ABP140:GFP:KanMX6 ABP1:HcRed:HIS3*	This work
Y4133	MATα *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 bni1-11::URA3 bnr1Δ::KanMX6*	Evangelista et al., 2002
AGY001	MATα *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 bni1-11::URA3 bnr1Δ::KanMX6 ABP1:HcRed:HIS3*	This work
THY150	MAT**a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ABP1:HcRed:HIS3*	This work
THY157	MAT**a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ABP1:GFP:KanMX6*	This work
THY166	MAT**a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SAC6:GFP:HIS3 abp1Δ::KanMX6*	This work
THY168	MAT**a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SAC6:GFP:HIS3*	This work
THY169	MAT**a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ABP1:GFP:KanMX6 SAC6:HcRed:HIS3*	This work
YMW81	MAT**a** *ade2-101 his3Δ200 leu2Δ1 lys2-801 trp1Δ63 ura3-52 arp2-1*	Moreau et al., 1997
THY170	MAT**a** *ade2-101 his3Δ200 leu2Δ1 lys2-801 trp1Δ63 ura3-52 arp2-1 ABP1:GFP:HIS3*	This work

Materials and methods

Yeast strains and fluorescent protein tagging
Yeast strains used in this work are listed in Table II. Yeast cell growth and manipulations were performed according to Sherman (2002). To visualize actin cables in living cells, the COOH terminus of Abp140p was tagged with GFP(S65T) using PCR-based insertion of the GFP gene into the chromosomal copy of *ABP140* as described previously (Yang and Pon, 2002). To visualize actin patches in living cells, the COOH terminus of Abp1p was tagged with either GFP(S65T) or HcRed using PCR-based insertion of the gene into the chromosomal copy of *ABP1*. For GFP and HcRed, the plasmids pFA6a-GFP(S65T)-kanMX6 (Longtine et al., 1998) and pCY17, respectively, were amplified with the primers ABP1f1 (5'-AAAAGGTCT-CTTCCCCAGCAATTATGTGTCTTTGGGCAACCGGATCCCCGGGTTAAT-TAA-3') and ABP1r1 (3'-ACGTAAGAATAATATAATAGCATGACGCTG-ACGTGTGATTGAATTCGAGCTCGTTTAAAC-3'), where the underlined sequences correspond to the sequences flanking the stop codon of *ABP1*. Likewise, we tagged the chromosomal copy of *SAC6* with GFP and HcRed in the same manner using the primers SAC6f1 (5'-AATTATTACTTTTATCG-CTTCGTTAATGACTTTGAACAAACGGATCCCCGGGTTAATTAA-3') and SAC6r1 (5'-AAGCTGAGTAGAAAACAGGTTACGAAAGTTGTTTGTTGGC-GAATTCGAGCTCGTTTAAAC-3'), where the underlined sequences correspond to the sequences flanking the stop codon of *SAC6*. To construct pCY17, the HcRed gene from pHcRed1 (CLONTECH Laboratories, Inc.) was amplified with primers containing the cut sites for PacI and AscI, digested, and ligated into the corresponding sites in pFA6a-GFP(S65T)-HIS3MX6, functionally replacing the GFP(S65T) gene with HcRed.

Yeast cells were transformed with PCR products using the lithium acetate method (Gietz et al., 1995). Transformants, which were positive for integration at the target locus, were validated by PCR, and the tagged constructs were visualized in cells by fluorescence microscopy (see below). The tags had no obvious effect on cell growth, actin organization, or function.

Microscopy and image analysis
For time-lapse fluorescence imaging, cells were grown in lactate medium as described previously (Yang and Pon, 2002) until mid-log phase at 25°C. For labeling of endocytic vesicles and endosomal compartments, FM4-64 (Molecular Probes, Inc.) was added to cell cultures at a final concentration of 10 μM for variable amounts of time as described in the figure legends; cells were then washed twice with lactate medium. 3 μl of cell suspension was applied to a microscope slide and covered with a coverslip. Images were acquired using a microscope (model E600; Nikon) equipped with a Plan-Apo 100×/1.4 NA objective, a cooled charge-coupled device camera (Orca-ER; Hamamatsu), and a Dual-View image splitter (Optical Insights) for simultaneous two-color imaging. The temperature of the objective lens was controlled by an objective heater (Bioptechs). To acquire 3D images over time, optical sections were obtained at 0.4-μm steps via a piezoelectric focus motor mounted on the objective lens (Polytech PI). Images were collected and analyzed using Openlab 3.1.5 software (Improvision) and ImageJ 1.30, respectively. QuickTime movies were made from time-lapse images using Volocity 2.6 (Improvision). For determination of the velocity of actin patches and elongating actin cables, the fluorescent movements of actin patches or fiduciary marks on elongating cables were measured as a function of time, as described previously (Smith et al., 2001; Yang and Pon, 2002).

Online supplemental material
Wild-type haploid cells expressing Abp1p-HcRed and Abp140p-GFP from the chromosomal loci were grown to mid-log phase in lactate medium at RT. 3 μl of cell suspension was applied to a microscope slide and covered with a coverslip. Images were acquired using a microscope (model E600; Nikon) equipped with a Plan-Apo 100×/1.4 NA objective, a cooled charge-coupled device camera (Orca-ER; Hamamatsu), and a Dual-View image splitter (Optical Insights) for simultaneous two-color imaging. Images were collected and analyzed using Openlab 3.1.5 software (Improvision) and ImageJ 1.30, respectively. QuickTime movies were made from time-lapse images using Volocity 2.6 (Improvision). Online supplemental material available at http://www.jcb.org/cgi/content/full/jcb.200404173/DC1.

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