Developmental Cell *11*, 33–46, July, 2006 ©2006 Elsevier Inc. DOI 10.1016/j.devcel.2006.05.008

Endocytic Internalization in Budding Yeast Requires Coordinated Actin Nucleation and Myosin Motor Activity

Yidi Sun,[1,2] Adam C. Martin,[1,2] and David G. Drubin[1,*]
[1]16 Barker Hall
Department of Molecular and Cell Biology
University of California, Berkeley
Berkeley, California 94720

Summary

Actin polymerization essential for endocytic internalization in budding yeast is controlled by four nucleation promoting factors (NPFs) that each exhibits a unique dynamic behavior at endocytic sites. How each NPF functions and is regulated to restrict actin assembly to late stages of endocytic internalization is not known. Quantitative analysis of NPF biochemical activities, and genetic analysis of recruitment and regulatory mechanisms, defined a linear pathway in which protein composition changes at endocytic sites control actin assembly and function. We show that yeast WASP initiates actin assembly at endocytic sites and that this assembly and the recruitment of a yeast WIP-like protein by WASP recruit a type I myosin with both NPF and motor activities. Importantly, type I myosin motor and NPF activities are separable, and both contribute to endocytic coat inward movement, which likely represents membrane invagination. These results reveal a mechanism in which actin nucleation and myosin motor activity cooperate to promote endocytic internalization.

Introduction

Endocytosis is an intricate process, involving the ordered recruitment of endocytic adaptors, clathrin, and actin cytoskeletal proteins to the plasma membrane in both yeast and mammalian cells (Engqvist-Goldstein and Drubin, 2003; Merrifield, 2004). Arp2/3 complex-mediated actin filament nucleation underlies a transient burst of actin polymerization, which coincides with endocytic membrane invagination and vesicle scission (Benesch et al., 2005; Kaksonen et al., 2003; Martin et al., 2005; Merrifield et al., 2002, 2004, 2005; Yarar et al., 2005). In yeast, actin polymerization and organization of actin filaments into a meshwork are required for endocytic internalization (Engqvist-Goldstein and Drubin, 2003). However, little is known about the mechanism by which actin assembly facilitates endocytic vesicle formation. Furthermore, the Arp2/3 actin nucleation machinery and type I myosins both localize to yeast endocytic sites (Jonsdottir and Li, 2004; Kaksonen et al., 2003), but the relative contributions of nucleation and myosin motor activity to endocytosis have yet to be determined.

The Arp2/3 complex is activated by NPFs (Pollard and Borisy, 2003). In yeast, at least three NPFs, Las17p (WASP), Pan1p (Eps15), and Abp1p (mAbp1), perform endocytosis-related functions (Figure 1A) (Engqvist-Goldstein and Drubin, 2003). In addition, Vrp1p (WIP) stimulates type I myosin NPF activity in vitro (Geli et al., 2000; Lechler et al., 2001; Sirotkin et al., 2005). Live-cell imaging demonstrated that each of these NPFs exhibits unique timing of appearance and dynamic behavior at cortical patches (Jonsdottir and Li, 2004; Kaksonen et al., 2003; Sirotkin et al., 2005), which are endocytic sites in budding yeast (Huckaba et al., 2004; Kaksonen et al., 2003, 2005; Newpher et al., 2005). Las17p and Pan1p are recruited to cortical patches early, arriving ~20 s before actin is detected (Kaksonen et al., 2003). In contrast, Abp1p and Myo5p are late cortical patch components, arriving around the same time as actin and the Arp2/3 complex (Jonsdottir and Li, 2004; Kaksonen et al., 2003). Las17p and Myo5p remain associated with the plasma membrane during endocytic internalization, whereas Abp1p and Pan1p are internalized. Despite differences in NPF behavior, genetic evidence suggested redundant functions for NPFs (Duncan et al., 2001; Evangelista et al., 2000; Lechler et al., 2000; Toshima et al., 2005). Therefore, it is important to understand why such a high level of complexity in Arp2/3 regulation is necessary for endocytosis. Furthermore, because several NPFs arrive before actin assembly is initiated, it is essential to determine how actin assembly is restricted to the late stages of the endocytic internalization pathway.

Yeast type I myosins (Myo3p and Myo5p) localize to endocytic sites and are essential for endocytic internalization (Geli and Riezman, 1996; Goodson et al., 1996; Jonsdottir and Li, 2004). Myo3p and Myo5p contain an amino-terminal ATPase motor domain and a carboxy-terminal Arp2/3 binding "CA" domain like those found in NPFs (Figure 1A), suggesting that these myosins may function in both myosin-based and nucleation-based force generation. However, a predicted Myo3p rigor mutant blocked cortical actin polymerization in permeabilized cells (Lechler et al., 2000). Thus, it was doubtful that the relative importance of motor function and actin nucleation could be evaluated by separation-of-function mutants. Analysis of purified, full-length Myo3/5p is required to determine whether type I myosin motor activity and NPF activity are functionally coupled.

In this study, we use biochemistry in parallel with live-cell imaging and genetics to examine how individual NPFs, and a type I myosin motor, function to control endocytic actin assembly and to drive endocytic internalization.

Results

Quantitative Biochemical Analysis of the NPF Activities of Four Yeast Endocytic Proteins

We sought to directly compare the NPF activities of the four yeast NPFs, but we first needed to establish that budding yeast type I myosin has NPF activity. Vrp1p stimulates the NPF activity of type I myosin fragments containing the Arp2/3 binding "CA" domain in both

*Correspondence: drubin@socrates.berkeley.edu
[2]These authors contributed equally to this work.

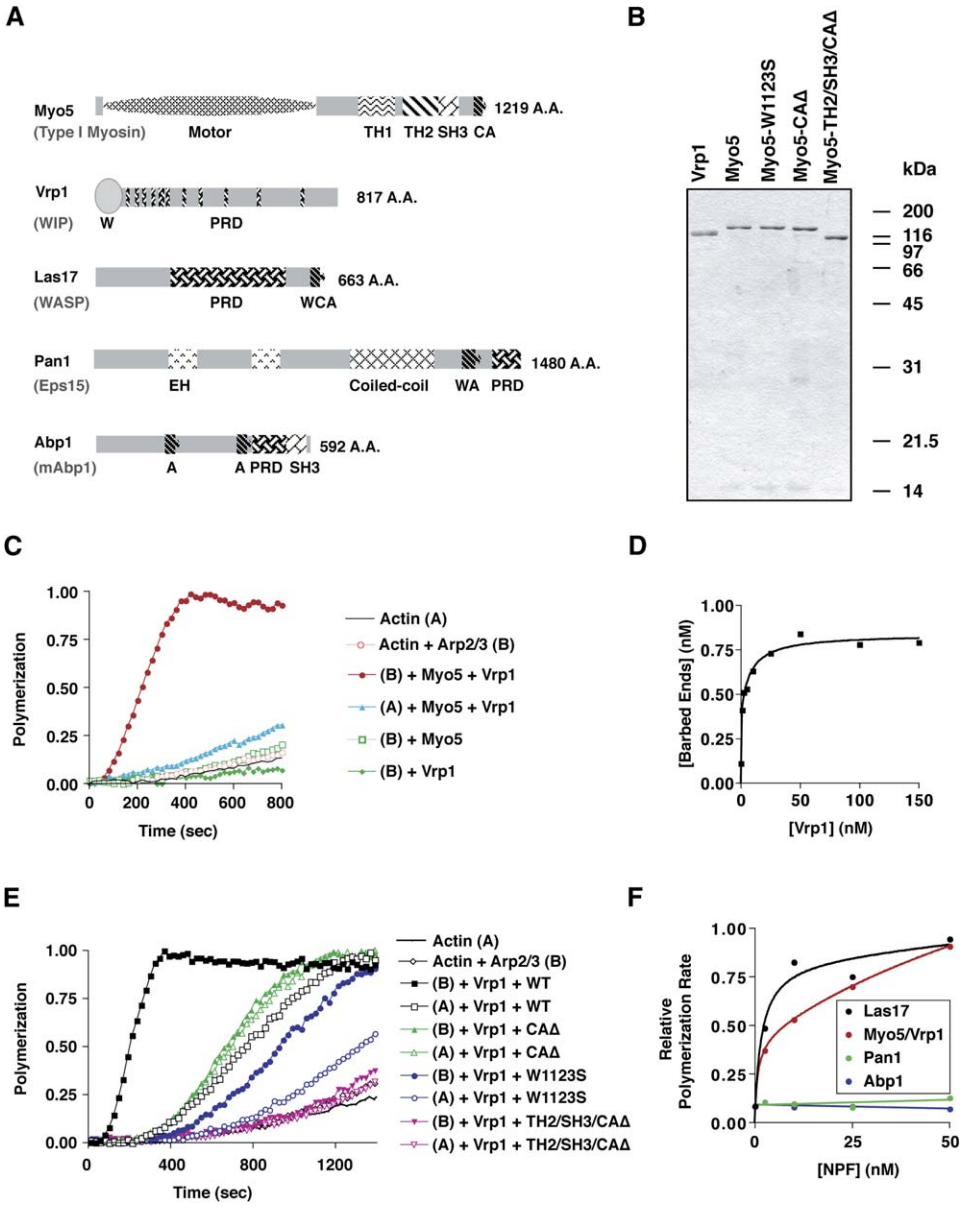

Figure 1. Vrp1p Stimulates Arp2/3 Complex Activation by Myo5p

(A) Domain structure of yeast NPFs. TH1 and TH2, tail homology 1 and 2; SH3, Src homology 3; W, WASP homology 2; C, Central; A, acidic; PRD, proline-rich domain; EH, Eps15 homology.

(B) Coomassie blue-stained gel of purified full-length Vrp1p, Myo5p, and Myo5p mutants.

(C–F) NPF activity of the Myo5p/Vrp1p complex. (C) Actin (1.5 μM, 5% pyrene labeled) was polymerized with 5 nM Arp2/3 complex, 15 nM Myo5p, and 50 nM Vrp1p, when indicated. (D) Barbed end concentrations were calculated as described in the Experimental Procedures. Actin (1.5 μM), 5 nM Arp2/3, and 10 nM Myo5p were used with various Vrp1p concentrations. (E) Actin (1.5 μM, 10% pyrene labeled) was polymerized with 10 nM Arp2/3 complex, 25 nM Vrp1p, and 15 nM Myo5p or Myo5p mutants, when indicated. (F) Yeast actin (1.5 μM, 5% pyrene-labeled rabbit muscle actin) was polymerized with 2.5 nM Arp2/3 complex and the indicated NPF. Vrp1p (50 nM) was used with Myo5p. The slopes of polymerization curves were measured at 30% polymerization and were normalized to the maximal rate of Las17p-induced polymerization.

budding and fission yeast (Geli et al., 2000; Lechler et al., 2001; Sirotkin et al., 2005). However, full-length type I myosin containing both a motor and Arp2/3 binding domain has never been purified. Because NPF truncations can have considerably different activity from the full-length protein (Rodal et al., 2003; Rohatgi et al., 1999), we purified full-length Myo5p and Vrp1p (Figure 1B). Although full-length Myo5p was inefficient at activating the Arp2/3 complex in a pyrene-actin assembly assay, addition of Vrp1p resulted in a 10-fold increase in the concentration of nucleated barbed ends, saturating at ~50 nM Vrp1p (Figures 1C and 1D). A Myo5p mutant lacking the Arp2/3 binding domain (Myo5-CAΔp) failed to activate the Arp2/3 complex in the presence of Vrp1p, demonstrating that increased actin nucleation is due to Arp2/3 complex activation by Myo5p/Vrp1p (Figure 1E). In contrast to the TH2-SH3-CA domain of fission yeast type I myosin (Sirotkin et al., 2005), full-length Myo5p/Vrp1p promoted the formation of stable Arp2/3-dependent y-branches (Movie S1), demonstrating that

Myo5p/Vrp1p generates a branched actin array, similar to Las17p (data not shown).

We next determined whether Vrp1p and Myo5p must bind to each other for full NPF activity. Mutation of a conserved tryptophan in the Src homology 3 (SH3) domain of Myo5p (W1123S) abolishes Vrp1p binding (Geli et al., 2000). We purified Myo5-W1123Sp and found that direct binding of Vrp1p to Myo5p is required for Myo5p's full NPF activity (Figure 1E). In addition, Myo5-W1123Sp and Vrp1p accelerated actin assembly less than wild-type Myo5p and Vrp1p in the absence of the Arp2/3 complex (Figure 1E). Deletion of both the SH3 and CA domains (Myo5-TH2/SH3/CAΔp) eliminated Myo5p's ability to increase actin assembly in either the presence or absence of the Arp2/3 complex, further suggesting that both the SH3 and CA domains promote actin assembly (Figure 1E).

Having established that Myo5p/Vrp1p functions as an NPF in budding yeast, we next compared Myo5p/Vrp1p NPF activity to that of other yeast NPFs with a homologous system of NPFs, actin, and Arp2/3 complex isolated from yeast. We used 2.5 nM Arp2/3 complex to accentuate differences between these NPFs. Under these conditions, Pan1p and Abp1p had no detectable NPF activity, whereas both Las17p and Myo5p/Vrp1p efficiently activated the Arp2/3 complex (Figure 1F and Figure S1). When we used a higher Arp2/3 complex concentration, Pan1p and Abp1p had detectable NPF activity (Figure S1), as reported previously (Duncan et al., 2001; Goode et al., 2001; Toshima et al., 2005). Thus, Las17p and Myo5p/Vrp1p have high NPF activity relative to Pan1p and Abp1p. The fact that the most potent NPFs, Las17p and Myo5p, are not internalized during endocytosis strongly suggests that actin filaments are oriented primarily with their barbed ends facing the plasma membrane during endocytic internalization, as predicted by photobleaching studies (Kaksonen et al., 2003, 2005).

A Linear Pathway of NPF Recruitment Precedes Endocytic Vesicle Release

Budding yeast NPFs exhibit distinct dynamic behaviors at endocytic sites (Jonsdottir and Li, 2004; Kaksonen et al., 2003). Vrp1-GFP also had distinct dynamics (Figure 2A), exhibiting a lifetime of ~20 s, longer than both Myo5-GFP (~10 s) and actin (~15 s), but shorter than Las17p and Pan1p (~30–40 s). Similar to Las17p, but unlike Pan1p or Abp1p, Vrp1-GFP remained nonmotile throughout its lifetime (Figure 2A and Movie S2).

We next examined the spatiotemporal relationships of all NPFs by using Myo5p as a reference (Figure 2B and Movie S3). As summarized in Figure 2C, Las17p and Pan1p appear at cortical patches first, followed by Abp1p, Myo5p, and the Arp2/3 complex (Arc15p) (Jonsdottir and Li, 2004; Kaksonen et al., 2003). Vrp1p appeared at patches after Las17p but before Myo5p and Abp1p (Figures 2B and 2C). Because extensive protein-protein interactions link Las17p, Myo5p, and Vrp1p (Goode and Rodal, 2001), we tested whether these proteins recruit each other to cortical patches. In *las17Δ* cells, Vrp1-GFP no longer assembled into cortical patches, demonstrating that Vrp1p localization requires Las17p (Figure 2D), consistent with results in fission yeast (Sirotkin et al., 2005). In *vrp1Δ* cells, Las17p still localized to patches (Figure S2A); however, Myo5p cortical patch intensity was greatly reduced (Figure 2E). Vrp1p appeared to recruit Myo5p via Myo5p's SH3 domain, since Myo5-W1123S-GFP exhibited similar partial mislocalization in the presence of wild-type Vrp1p (Figure 2E). Because the timing of Myo5-GFP recruitment coincided with the initiation of actin assembly, it was possible that actin filaments contribute to Myo5p localization. Indeed, inhibition of actin assembly by Latrunculin A (LatA) resulted in partial loss of Myo5p from cortical patches in wild-type cells (Figure S2B) (Anderson et al., 1998) and complete loss of Myo5p in *vrp1Δ* cells (Figure 2F). Overall, these results suggest a functional hierarchy of protein recruitment to cortical patches in which Las17p recruits Vrp1p, which, together with actin filaments, recruits Myo5p. Because binding to Vrp1p is required for Myo5p NPF activity, Myo5p is likely activated upon its recruitment to endocytic sites.

Myo5-GFP fluorescence was previously reported to appear around the same time as Arp2-DsRed and to peak immediately before the transition of Arp2-DsRed from its slow-to-fast movement phases (Jonsdottir and Li, 2004). Because actin patch fast movement likely represents movement of released endocytic vesicles (Kaksonen et al., 2003, 2005), Myo5p was proposed to play a role in vesicle scission (Jonsdottir and Li, 2004). However, DsRed is an obligate tetramer (Baird et al., 2000), thus the DsRed moiety may have altered actin and Arp2/3 complex dynamics in those studies. Abp1p tagged with monomeric RFP (Abp1-RFP) is a well-validated marker for actin (Kaksonen et al., 2005). Therefore, to determine at what stage of endocytic internalization Myo5p might function, we simultaneously imaged Myo5-GFP and Abp1-RFP with a high time resolution of 1 s. Analysis of patch intensity demonstrated that Myo5p peaked before Abp1p and the Arp2/3 complex (Arc15p) and was no longer detectable during the transition from slow to fast movement (Figures 2B and 2C and Movie S3). Recently, the peak fluorescence intensity for a GFP-tagged yeast amphiphysin homolog, Rvs167p, was shown to coincide with its one-step, inward movement into the cytoplasm, possibly representing a vesicle scission event (Kaksonen et al., 2005). Importantly, Myo5p/Vrp1p, Las17p, and Pan1p all peaked before the Rvs167p peak (Figures 2B and 2C), suggesting that these NPFs function prior to vesicle scission.

To determine the stoichiometry of NPFs and their binding partners in cortical patches, we used GFP-fluorescence intensity to compare their local concentrations (Figure 2G, Movies S5 and S6, and Figure S2C). Because these proteins are dynamic, we measured the peak intensity of each fusion protein at endocytic sites. Abp1p was the most abundant cortical patch protein, appearing to be 2-fold more abundant than the Arp2/3 complex (Arc15p) and 6-fold more abundant than Las17p. Vrp1p had a similar intensity to Las17p, consistent with its localization being dependent on Las17p. There was half as much Vrp1p as Myo5p, suggesting that at most, only half of the Myo5p is activated by Vrp1p at a given time in vivo. This fraction is likely to be even lower since we did not account for Myo3p, which also binds to Vrp1p (Anderson et al., 1998; Evangelista et al., 2000). These stoichiometries agree with the observation that Myo5p is only partially dependent on Vrp1p for cortical patch

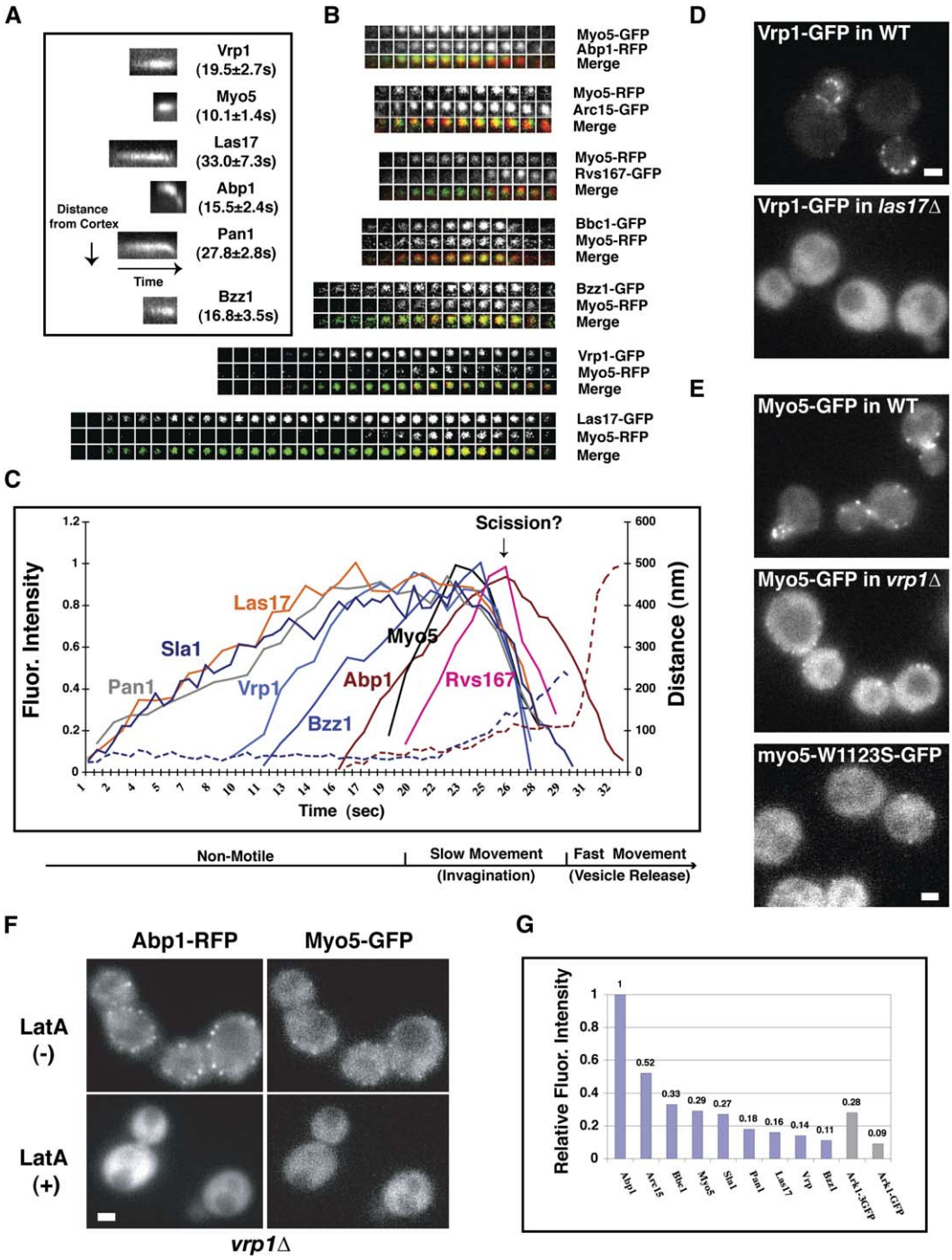

Figure 2. Ordered Recruitment of NPFs and Their Regulators to Endocytic Sites

(A) Kymographs of single patches from movies of cells expressing the indicated GFP-tagged protein (one frame/s). Lifetimes ± standard deviations (SD) are indicated in parentheses.

(B) Montages, single channel or merged images, of single patches from two-color movies of cells expressing GFP-tagged and RFP-tagged proteins. Time lapse between frames is 1 s.

(C) Alignment of averaged patch intensity measurements of NPFs and their regulators. The data were averaged from at least three patches with one-color movies of GFP-tagged endocytic proteins. Traces were aligned by time separating intensity peaks in two-color movies for 15 patches. Dotted lines show the distance of Sla1p (blue) and Abp1p (red) movement.

(D) Vrp1-GFP localization depends on Las17p. Vrp1-GFP was expressed in wild-type and *las17Δ* cells.

(E) Myo5-GFP localization depends partially on Vrp1p. Myo5-GFP was expressed in wild-type and *vrp1Δ* cells. Myo5-W1123S-GFP was expressed in otherwise wild-type cells.

(F) Myo5-GFP localization requires both Vrp1p and F-actin. Abp1-RFP (left) and Myo5-GFP (right) were coexpressed in *vrp1Δ* cells, and either DMSO (top) or 200 μM LatA (bottom) was added for 10 min at 25ºC.

(G) Cortical patch protein stoichiometries. Peak GFP fluorescence from at least 15 patches was averaged for each fusion protein. Fluorescence was normalized to Abp1-GFP fluorescence. See Figure S2 for further details. All scale bars are 1 μm.

recruitment. Because the total NPF concentration is three times greater than that of the Arp2/3 complex, only a fraction of these NPFs can interact with the Arp2/3 complex at a given time.

Distinct NPF Functions during Endocytic Internalization

To dissect the functions of individual NPFs in the endocytic pathway, we created precise mutants of the Arp2/3 complex binding domains of Las17p (*las17-WCA⊿*), Pan1p (*pan1-WA⊿*), and Myo5p (*myo5-CA⊿*). These mutants exhibited wild-type protein expression levels and localization (data not shown). Because Myo3p and Myo5p function redundantly (Geli and Riezman, 1996; Goodson et al., 1996), analysis of *myo5-CA⊿* was performed in a *myo3⊿* background. Mutant cells expressing a component of the endocytic coat module, Sla1-GFP, and a marker for actin, Abp1-RFP, were imaged to visualize endocytosis in real time. In wild-type cells, Sla1-GFP containing patches are joined by Abp1-RFP shortly before both of these proteins disappear (Kaksonen et al., 2003). NPF mutations had specific effects on the timing of Abp1p recruitment. The *las17-WCA⊿* mutant delayed actin polymerization at endocytic sites, represented by the delay in Abp1-RFP recruitment (Figure 3A and Movie S7). Neither *pan1-WA⊿*, *myo5-CA⊿ myo3⊿*, nor *pan1-WA⊿ myo5-CA⊿ myo3⊿* delayed actin polymerization, suggesting that Las17p is sufficient to initiate actin polymerization at endocytic sites (Figure 3A and Movies S7 and S8). However, when combined with *las17-WCA⊿*, both *pan1-WA⊿* and *myo5-CA⊿ myo3⊿* further delayed actin assembly initiation (Figure 3A). Thus, Pan1p and Myo3/5p NPF activity are important for initiating actin assembly only in the absence of Las17p.

In wild-type cells, Abp1p recruitment accompanies the slow inward movement of both Sla1p and Abp1p away from the plasma membrane (Kaksonen et al., 2003). This slow Sla1-GFP movement likely reflects endocytic membrane invagination (Kaksonen et al., 2003, 2005). Surprisingly, although *las17-WCA⊿* caused a significant actin assembly delay, the frequency of Sla1-GFP inward movement was only slightly lower than that in wild-type cells (Figure 3A and Movie S7). Furthermore, most Sla1-GFP patches in *pan1-WA⊿* and *pan1-WA⊿ las17-WCA⊿* mutant cells showed inward movement (Figure 3A), consistent with the finding that a double mutant defective in Las17p and Pan1p NPF activity shows only a modest defect in α-factor internalization (Toshima et al., 2005). In contrast, the frequency of Sla1-GFP inward movement was significantly reduced in the *myo5-CA⊿ myo3⊿* mutant (Figure 3A). The *myo3⊿ myo5⊿* and *vrp1⊿* mutants blocked Sla1-GFP inward movement (Figure 3A and Movie S9), further supporting the conclusion that Myo5p/Vrp1p NPF activity is important for endocytic internalization. Strikingly, the *las17-WCA⊿ myo5-CA⊿ myo3⊿* triple mutant was completely defective in Sla1-GFP inward movement (Figure 3A and Movie S8), demonstrating that although Myo5p/Vrp1p is most important, Las17p and Myo5p/Vrp1p NPF activities both contribute to endocytic internalization. The endocytic defect of the *las17-WCA⊿ myo5-CA⊿ myo3⊿* triple mutant likely resulted from a defect in actin nucleation since Abp1-RFP fluorescence was greatly reduced at endocytic sites (Figure 3A and Movie S8). The timing of actin assembly in the *las17-WCA⊿ myo5-CA⊿ myo3⊿* mutant was indistinguishable from that in the *las17⊿* mutant (Figure 3A). This is probably because the *las17⊿* mutant causes Vrp1p mislocalization, which subsequently affects Myo5p localization and NPF activity. Together, these data demonstrate that the two most potent NPFs, Las17p and Myo5p/Vrp1p, are most important for endocytic coat inward movement/membrane invagination.

Regulation of Actin Nucleation Activity during Endocytosis

As shown above, Las17p initiates actin assembly at endocytic sites. However, Las17p is present in cortical patches for ~20 s before actin is detected (Kaksonen et al., 2003). Sla1p may inactivate Las17p until the appropriate activator arrives (Rodal et al., 2003). Therefore, we tested whether other SH3-containing proteins can activate Las17p NPF activity in the presence of Sla1p. We found that purified Bzz1p (Figure S3), a syndapin-like protein with two SH3 domains, relieved Las17p inhibition by Sla1-SH3p in a dose-dependent manner (Figures 3B and 3C). Relief of Sla1p's inhibition of Las17p required Bzz1p's SH3 domains (data not shown). Therefore, this result reveals a novel mechanism for the activation of a WASP-family protein, in which inhibition of NPF activity by one SH3-containing protein is relieved by another SH3-containing protein. To investigate whether Bzz1p regulates Las17p NPF activity in vivo, we examined Bzz1-GFP dynamics in live cells. Bzz1-GFP remained associated with the plasma membrane during endocytic internalization, similar to Las17p, but exhibited a lifetime of ~17 s (Figure 2A and Movie S4). Two-color imaging demonstrated that Bzz1-GFP was recruited to cortical patches immediately before actin polymerization was initiated (Figures 2B and 2C and Movie S3), consistent with Bzz1p relieving Las17p inhibition by Sla1p. Although initiation of actin polymerization was normal in the *bzz1⊿* mutant, actin polymerization was delayed in *bzz1⊿ vrp1⊿* cells (data not shown), suggesting that Bzz1p contributes to actin assembly initiation in vivo. One or several of four uncharacterized SH3-containing Las17p interacting proteins may also function with Bzz1p to activate Las17p in vivo (Madania et al., 1999; Tong et al., 2002). In total, these data suggest that Las17p activity is regulated by the protein composition of the patch, which changes dynamically.

Once actin assembly is initiated, it must be moderated to prevent excessive polymerization, which may impair endocytosis and deplete the pool of actin and its binding proteins (Sekiya-Kawasaki et al., 2003; Toshima et al., 2005). Bbc1p inhibits Las17p NPF activity in vitro (Rodal et al., 2003), and *bbc1⊿* cells exhibit exaggerated Sla1p and Abp1p inward movement, with distances of up to four times as far into the cytoplasm (Kaksonen et al., 2005). Because Bbc1p dynamics were indistinguishable from those of Myo5p (Figure 2B), and Myo5p/Vrp1p NPF activity appeared most important for Sla1-GFP slow movement, we determined whether Bbc1p also inhibits Myo5p/Vrp1p Arp2/3 complex activation. Bbc1p inhibited Myo5p/Vrp1p activity in a dose-dependent manner, similar to Las17p (Figure 3D). To determine whether the enhanced Sla1-GFP inward movement observed in

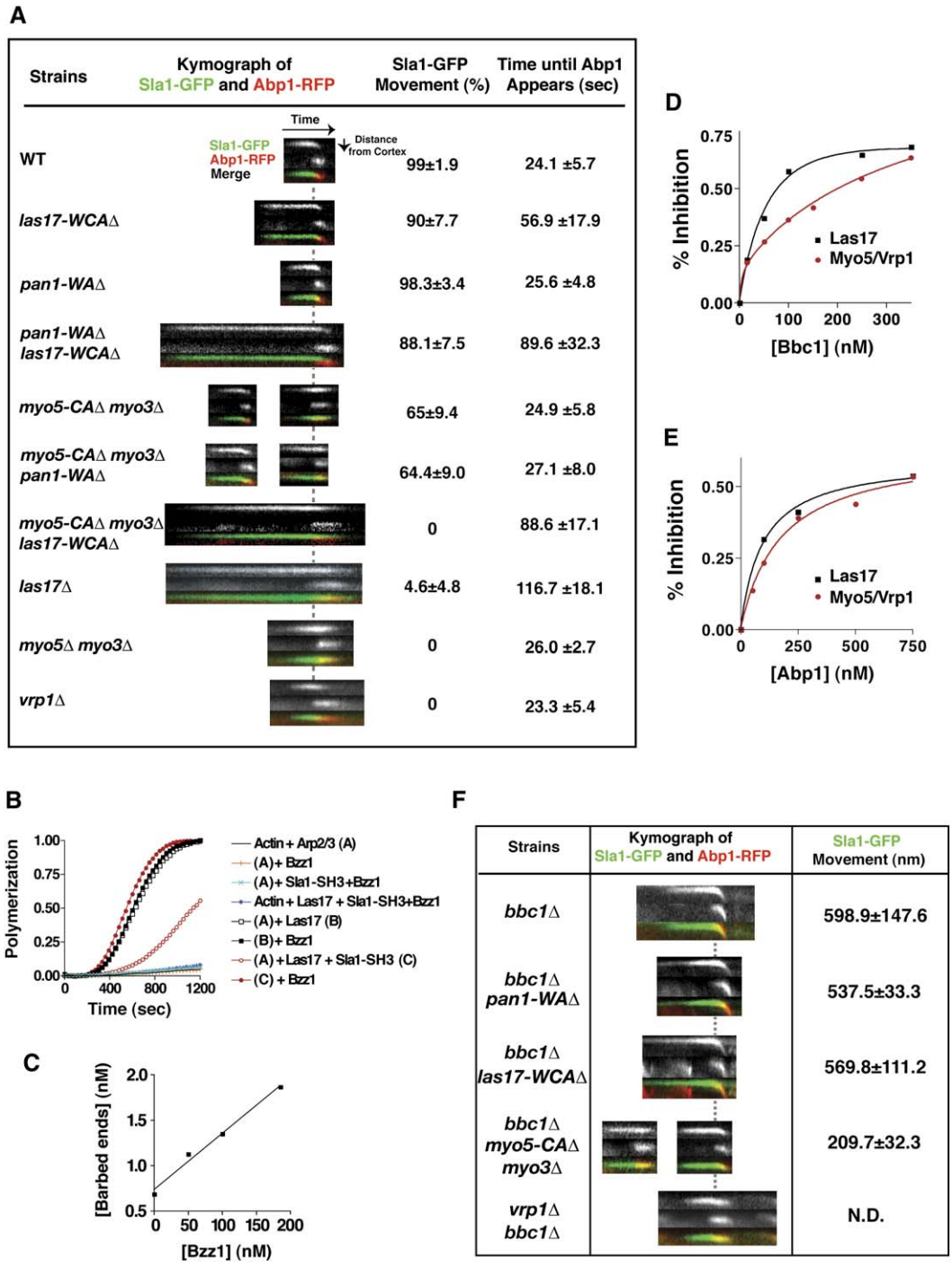

Figure 3. NPF Functions during Endocytic Internalization

(A) Kymographs of individual patches from two-color movies of cells expressing Sla1-GFP, Abp1-RFP, and the indicated NPF mutant(s). Kymographs were aligned with Abp1-RFP appearance (dotted line). Means of the frequency of Sla1-GFP inward movement (%) and Sla1-GFP lifetime before Abp1-RFP appearance (s) ± SD are indicated to the right. At least 45 patches from three cells were analyzed to calculate the frequency of Sla1-GFP inward movement.

(B) Bzz1p relieves Las17p inhibition by Sla1-SH3p. Actin (1 μM, 5% pyrene labeled) was polymerized with 10 nM Arp2/3 complex, 10 nM Las17p, 250 nM Sla1-SH3p, and 150 nM Bzz1p, when indicated.

(C) Dose dependence of Bzz1p activation of Las17p. Actin (2 μM, 5% pyrene labeled), 20 nM Arp2/3 complex, 20 nM Las17p, and 250 nM Sla1-SH3p were used.

(D) Bbc1p inhibits Myo5p/Vrp1p induced actin assembly. Percent inhibition was calculated with the reduction in the concentration of nucleated barbed ends. Actin (1.5 μM) was polymerized with 10 nM Arp2/3, 15 nM Myo5p, and 25 nM Vrp1p or 5 nM Arp2/3 and 10 nM Las17p.

(E) Abp1p inhibits Myo5p/Vrp1p induced actin assembly. Actin (1.5 μM) was polymerized with 5 nM Arp2/3, 5 nM Myo5p, and 25 nM Vrp1p or 2.5 nM Arp2/3 and 5 nM Las17p.

(F) Kymographs from two-color movies of *bbc1Δ* cells expressing Sla1-GFP, Abp1-RFP, and the indicated NPF mutant(s). The mean distance of Sla1-GFP inward movement ± SD was calculated from five patches for each strain.

bbc1Δ cells resulted from misregulation of either Las17p or Myo5p/Vrp1p NPF activity, we crossed the *bbc1Δ* mutant with the NPF mutants. The enhanced Sla1-GFP inward movement in *bbc1Δ* cells was specifically suppressed by *myo5-CAΔ myo3Δ* and *vrp1Δ*, but not by *las17-WCAΔ* or *pan1-WAΔ* (Figure 3F and Movie S10). These results suggest that misregulation of Myo5p/Vrp1p NPF activity contributes to the exaggerated Sla1-GFP slow movement observed in *bbc1Δ* cells. Therefore, Myo5p/Vrp1p regulation by Bbc1p appears to affect the duration of endocytic coat inward movement.

In addition to Bbc1p, Abp1p can also inhibit Arp2/3 complex activation by Las17p in vitro (D'Agostino and Goode, 2005). This inhibition was not specific to Las17p, as Abp1p also inhibited Myo5p/Vrp1p activity in a dose-dependent manner (Figure 3E). Therefore, rather than an Arp2/3 complex activator, Abp1p appears to function as a general NPF inhibitor, possibly limiting the level of nucleation that occurs at an endocytic patch.

Myo5p's Motor Activity Functions Independently from Its NPF Activity

So far, our study has focused on the importance of actin nucleation during endocytosis. The type I myosin motor is another "force generator" that may also contribute to endocytic internalization. Because Myo3/5p motor activity was proposed to affect actin polymerization in permeabilized cells (Lechler et al., 2000), a critical question is whether Myo5p motor activity can be distinguished from its actin assembly activity. To address this question, we first purified full-length Myo5p and Myo5p motor mutants to test their motor activities in vitro (Figure 4A). Mutation of glycine 132 to arginine (G132R) changes a conserved residue of the nucleotide binding region and is predicted to be a rigor mutant (Lechler et al., 2000). Phosphorylation by p21 activated kinases (PAKs) is proposed to regulate yeast type I myosin motor activity (Wu et al., 1996). Therefore we also mutated the phosporylated serine (S357) of the PAK consensus site in Myo5p to either alanine (inactive) or aspartate (active). In addition, we purified Myo5p that completely lacked the motor domain (motorΔ).

Using an actin filament gliding assay, we assessed Myo5p motor activity by measuring the speed of actin filament translocation across a Myo5p-coated coverslip (Figure 4B and Movie S11). Wild-type Myo5p moved rabbit actin filaments at a speed of 0.15 ± 0.03 μm/s, similar to *Acanthamoeba* type I myosin (Zot et al., 1992). Similar gliding speeds were obtained with yeast actin filaments (0.17 ± 0.04 μm/s). These observations demonstrate that yeast type I myosin has motor activity. Actin filament translocation was dependent on the motor domain as Myo5-motorΔp and the Myo5-G132Rp mutants failed to move actin filaments (Figure 4B). In addition, Myo5-S357Ap and Myo5-S357Dp reduced the speed of actin filament movement by 80% and 60%, respectively (Figure 4B). Because both the S357A and S357D mutants were defective in actin filament gliding, it is not clear whether the S357D mutation fully mimics phosphorylation.

To determine whether Myo5p motor activity is required for its NPF activity, we investigated whether Myo5p motor mutants activate the Arp2/3 complex with the pyrene-actin assembly assay. All Myo5p motor mutants, including the motor deletion, showed indistinguishable NPF activity from the wild-type protein (Figure 4C). Furthermore, Myo5-CAΔp exhibited normal motor activity (Figure 4B), demonstrating that the Arp2/3 binding region of Myo5p is not required for motor function. Therefore, the motor and NPF functions of Myo5p are separable.

Myo5p Motor Activity Is Required for Endocytic Coat Inward Movement

We next determined whether Myo5p motor activity is required for endocytosis. We integrated the *myo5-G132R*, *myo5-S357A*, and *myo5-S357D* mutations into the yeast genome, replacing the endogenous copy of *MYO5*. Myo5p motor mutant proteins were expressed at wild-type levels (data not shown). Although *myo5-G132R*, *myo5-S357A*, and *myo5-S357D* grew normally in the presence of *MYO3*, *myo5-G132R*, *myo5-S337A*, and to a lesser extent, *myo5-S357D*, growth was temperature sensitive when combined with the *myo3Δ* mutant (Figure S4). Examination of GFP-tagged Myo5p mutants demonstrated that Myo5-G132R was recruited to cortical patches with similar efficiency to the wild-type protein (Figure 5A and Movie S12). In contrast, Myo5-S357A and Myo5-S357D were present in cortical patches at reduced levels (Figure 5A and Movie S12). Because F-actin is important for Myo5p patch localization, these mutants may decrease Myo5p affinity for F-actin. Myo5p lifetime at patches was increased for all three mutants, with Myo5-G132R being the longest lived and Myo5-S357D being the shortest lived (Figure 5B). Thus, Myo5p lifetimes are inversely correlated with the in vitro motor activities of these mutants (Figure 4B).

To determine how Myo5p motor activity affects endocytic internalization, we analyzed Sla1-GFP and Abp1-RFP dynamics in *myo5-G132R myo3Δ*, *myo5-S357A myo3Δ*, and *myo5-S357D myo3Δ* mutant cells. Sla1-GFP inward movement was defective in Myo5p motor mutants, suggesting that Myo5p motor activity is essential for plasma membrane invagination (Figure 5C and Movie S13). In addition, internalization of a marker for fluid phase endocytosis, Lucifer yellow, was defective in all three motor mutants, similar to the *myo5Δ myo3Δ* mutant (Figure 5D). Mislocalization of Myo5-S357Ap, and Myo5-S357Dp, is unlikely to be solely responsible for their endocytic defects because the *myo5-W1123S* mutant, which was also partially mislocalized, was only partially defective in Sla1-GFP and Lucifer yellow internalization (Figures 5A–5D). Furthermore, Myo5-G132R is present at normal levels in cortical patches. Importantly, Abp1-RFP is efficiently recruited to Sla1-GFP patches in the motor mutants, providing strong evidence that Myo5p motor activity is not required for cortical actin polymerization in vivo (Figure 5C). Together, these data suggest that Myo5p motor activity, independent of Myo5p actin nucleation activity, is required for endocytic internalization.

To determine whether the elevated NPF activity present in a *bbc1Δ* mutant can suppress the Myo5p motor mutant internalization defect, we combined Myo5-G132R-GFP with the *myo3Δ* and *bbc1Δ* mutants and examined Abp1-RFP inward movement. Myo5-G132R-GFP mutant cells were completely defective in the

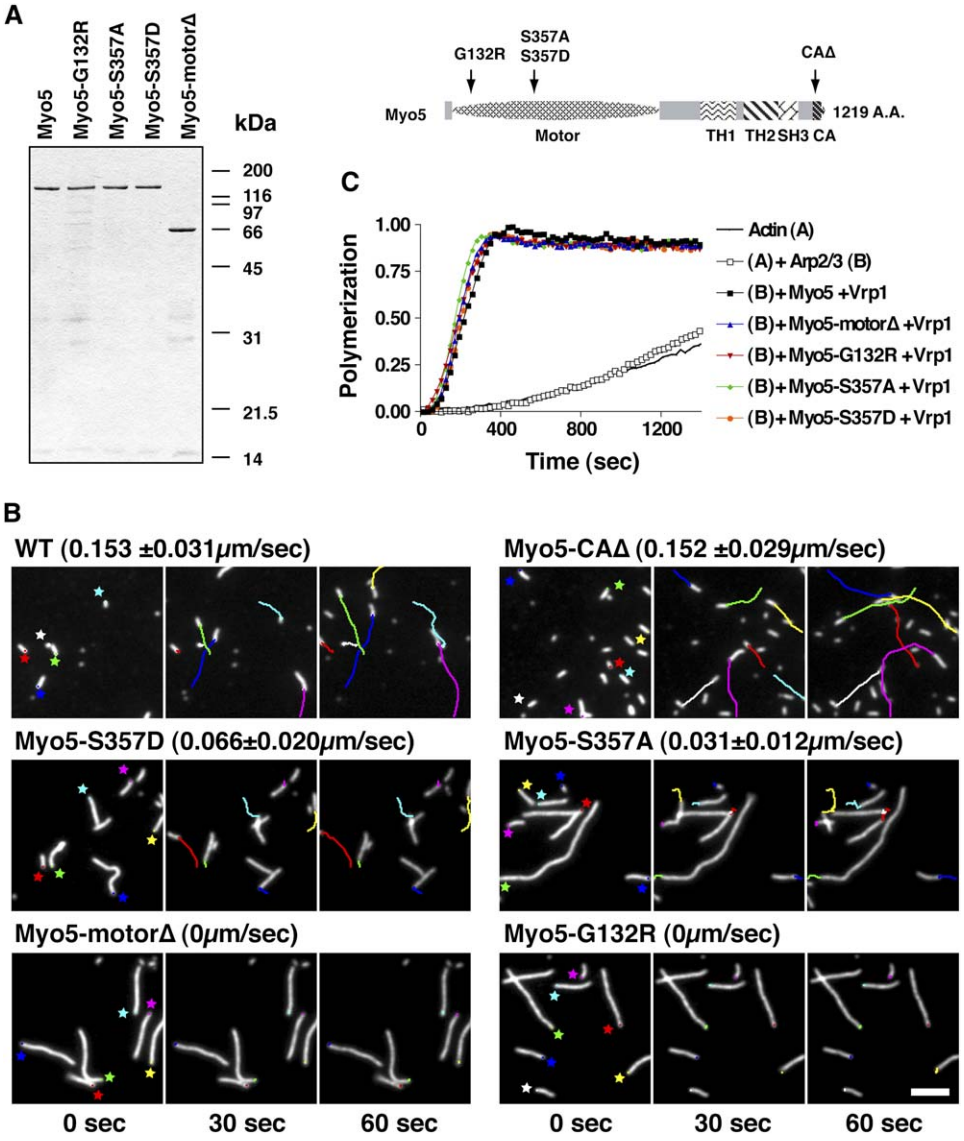

Figure 4. Myo5p Functions as a Molecular Motor

(A) Coomassie blue-stained gel of purified full-length Myo5p and Myo5p motor mutants.

(B) Actin filament gliding assay demonstrating Myo5p motor activity. Rhodamine phalloidin-labeled actin filaments were bound to Myo5p coated coverslips, and the gliding reaction was initiated with ATP. Single frames from a 1 min movie are presented (two frames/s). Stars indicate the starting positions of individual filament ends. Paths of individual actin filaments are shown in color. The mean speed ± SD is shown above each mutant. Scale bar = 2.5 μm.

(C) Myo5p motor activity is independent of Myo5p/Vrp1p NPF activity. Actin (1.5 μM, 10% pyrene labeled) was polymerized with 10 nM Arp2/3 complex, 25 nM Vrp1p, and 15 nM Myo5p or Myo5p mutants, when indicated.

inward movement of Abp1-RFP patches, which normally move 948.2 ± 132.7 nm (n = 10) in *bbc1Δ* cells (Figure 5E and Movie S14). Thus, even in the presence of elevated NPF activity, Myo5p motor activity is required for internalization. This result further suggests that Myo5p motor activity is not required for actin polymerization, but perhaps plays a direct role promoting membrane invagination.

Myo5p Motor Activity Is Required for Actin Network Retrograde Flow

Las17p and Myo5p/Vrp1p NPF activity and Myo5p motor activity are both required for endocytic internalization (Figure 6A). The mechanism by which these activities promote internalization needs to be investigated. Wild-type actin patches are smaller than the resolving power of a fluorescence microscope, making it impossible to observe the structure, organization, and orientation of actin filaments by this technique. However, deletion of Sla2p, a protein essential for endocytosis, results in elongated actin tails that originate from a stable complex of NPFs, endocytic adaptors, and clathrin (Kaksonen et al., 2003; Newpher et al., 2005). Photobleaching studies indicated that actin subunits are added near the plasma membrane and are treadmilled back into the cytoplasm (Kaksonen et al., 2003), as is expected to occur in wild-type patches. Therefore, we used the *sla2Δ* mutant as a model in vivo system to

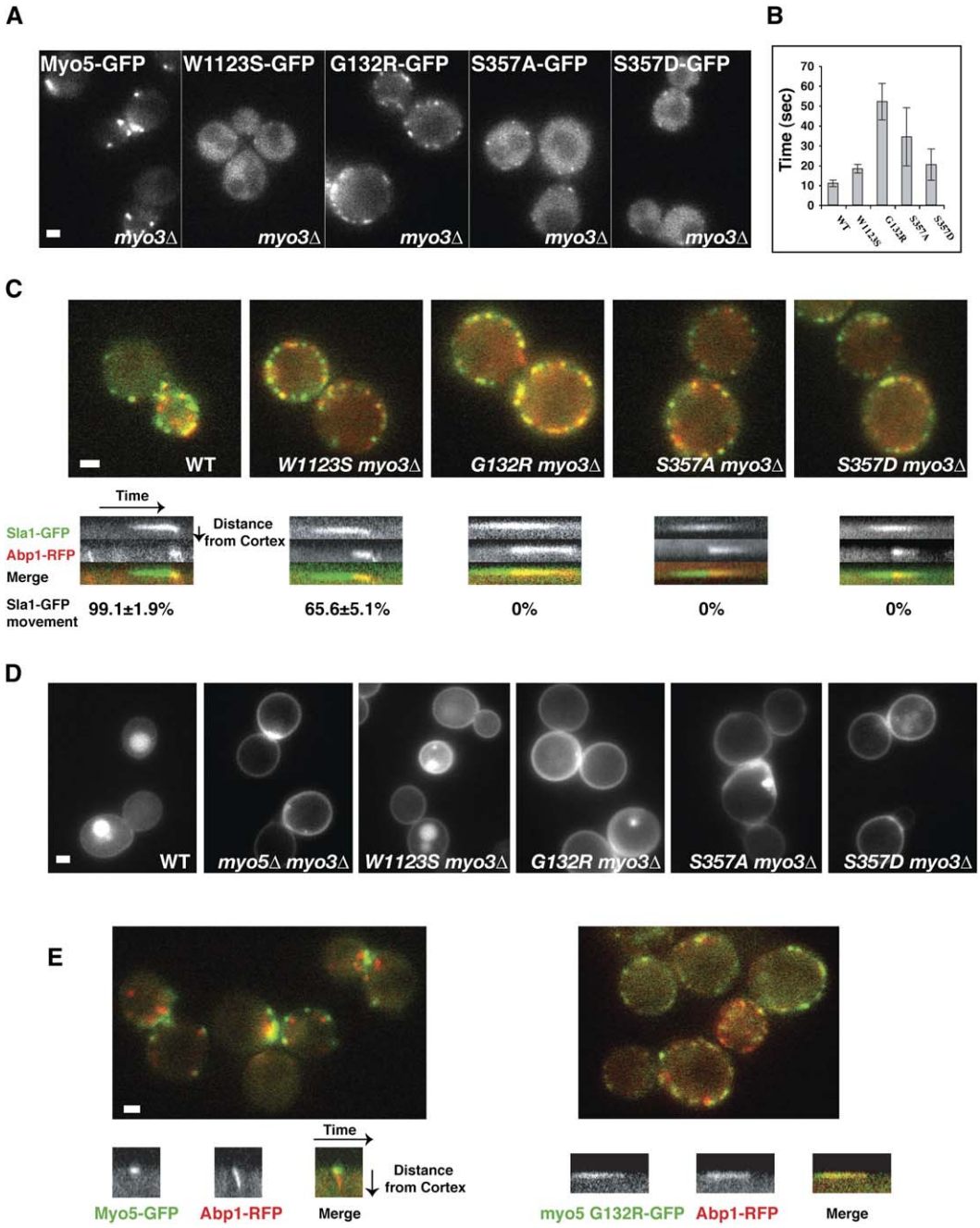

Figure 5. Myo5p Motor Activity Is Required for Endocytic Coat Inward Movement

(A) Cells expressing GFP-tagged Myo5p and Myo5p mutants.

(B) Myo5p mutant lifetime in a *myo3Δ* mutant background. Error bars represent SD.

(C) First image (top) and kymograph representation (bottom) from two-color movies of cells expressing Sla1-GFP, Abp1-RFP, and the indicated Myo5p mutant. Means for the frequency of Sla1-GFP inward movement ± SD were calculated from at least 45 patches from three cells.

(D) Fluorescence microscopy analysis of Lucifer yellow endocytic uptake. Cells were incubated with Lucifer yellow for 2 hr at 25ºC.

(E) First image (top) and kymograph representation (bottom) from two-color movies of *bbc1Δ* cells expressing either Myo5-GFP or Myo5-G132R-GFP and Abp1-RFP in wild-type or a *myo3Δ* mutant background, respectively. All scale bars are 1 μm.

examine how NPF and Myo5p motor activities affect actin dynamics.

We expressed either Myo5-GFP, Myo5-CAΔ-GFP (in a *myo3Δ*), or Las17-WCAΔ-GFP in *sla2Δ* mutant cells also expressing Abp1-RFP to label actin tails. Similar to Las17-GFP, Myo5-GFP localized to the heads of actin comet tails (Figure 6B). Interestingly, actin tails still formed in both the Myo5-CAΔ-GFP and Las17-WCAΔ-GFP mutants, albeit they were less continuous (Figure 6B and Movie S15). A similar phenotype for *sla2Δ* tails was observed with Arp2/3 complex mutants that significantly lower nucleation activity and impair endocytic internalization (Martin et al., 2005). Therefore, reducing actin polymerization efficiency does not block the formation of

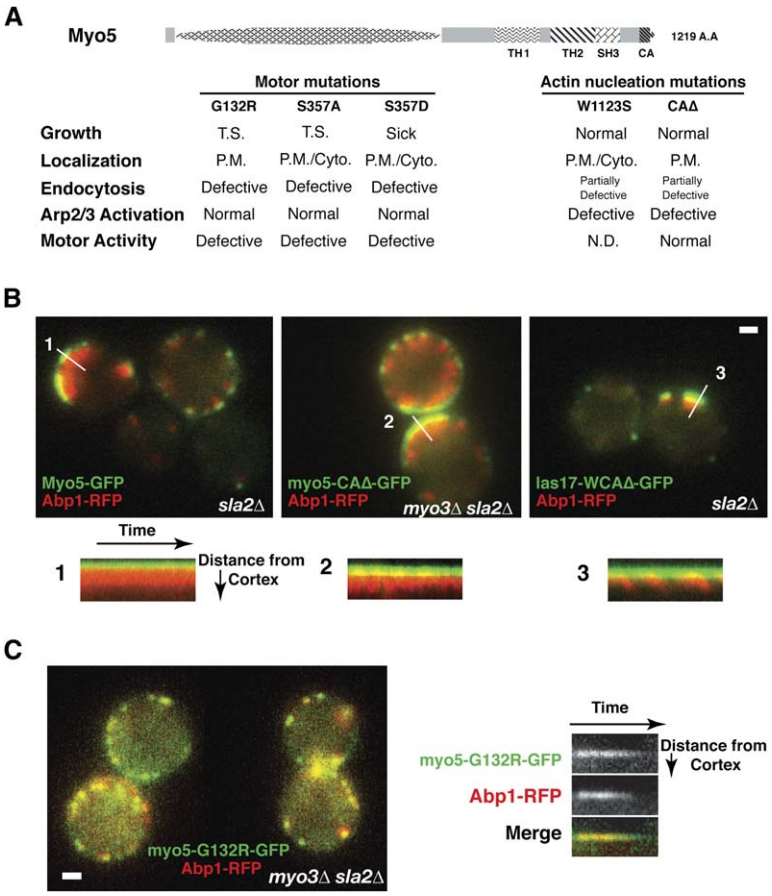

Figure 6. Myo5p Motor Activity Is Required for Actin Network Retrograde Flow

(A) Summary of in vivo and in vitro phenotypes of Myo5p mutants. T.S., temperature sensitive; P.M., plasma membrane; Cyto., cytoplasmic.

(B) Actin tail formation in *sla2Δ* cells expressing Abp1-RFP and either Myo5-GFP, Myo5-CAΔ-GFP, or Las17-CAΔ-GFP. First image (top) and kymograph representation (bottom) from two-color movies (one frame/s).

(C) First image (left) and kymograph representation (right) from a two-color movie of *sla2Δ myo3Δ* cells expressing Abp1-RFP and Myo5-G132R-GFP. All scale bars are 1 μm.

sla2Δ actin tails. In contrast, Myo5-G132R-GFP (in a *myo3Δ*) resulted in a striking block in actin tail formation in *sla2Δ* cells (Figure 6C and Movie S16). Actin polymerization still occurred at Myo5-G132R-GFP sites; however, instead of actin filaments flowing away from the plasma membrane in tails that project into the cytoplasm, patch-like actin structures that colocalized with Myo5-G132R-GFP were formed (Figure 6C). Similar results were also obtained with the Myo5-S357A mutant (data not shown). These results suggest that Myo5p motor activity is required for actin filament retrograde flow away from the plasma membrane.

Discussion

A Pathway for Endocytic Actin Assembly and Force Production

Actin and actin-associated proteins arrive at endocytic sites in a highly defined temporal order in both yeast and mammalian cells (Merrifield, 2004). Although budding yeast NPFs are recruited to endocytic sites with distinct timing and have distinct motility behaviors, when they are active and how they function during endocytosis was not clear. Combining live-cell imaging of endocytosis with genetics and biochemical experiments using full-length proteins has enabled us to propose the following model for how NPFs and their binding partners function and how they are recruited to, and regulated at, endocytic sites in budding yeast (Figure 7). Las17p (WASP) arrives at endocytic sites ~20 s before actin assembly is initiated (Kaksonen et al., 2003). Las17p serves as a scaffold that assembles key components of the actin machinery for endocytic internalization. Initially, Sla1p, and possibly other early endocytic proteins, maintain Las17p in an inactive state (Rodal et al., 2003). Vrp1p (WIP) and Bzz1p (syndapin) are subsequently recruited to endocytic sites by Las17p (this study; Sirotkin et al., 2005; Soulard et al., 2002). Bzz1p, and possibly other SH3-containing proteins, relieves Las17p inhibition by Sla1p, initiating actin nucleation at endocytic sites. Vrp1p and F-actin then recruit Myo5p (type I myosin) to endocytic sites, and Vrp1p activates Myo5p NPF activity. Las17p augments productive membrane invagination, which is most dependent on Myo5p/Vrp1p NPF activity. Concurrent with membrane invagination, Abp1p and Bbc1p negatively regulate Las17p and Myo5p/Vrp1p NPF activity (this study; D'Agostino and Goode, 2005; Rodal et al., 2003). In particular, Bbc1p inhibition of Myo5p/Vrp1p NPF activity regulates the duration of coat protein inward movement. This regulation presumably fine-tunes the level of actin nucleation at endocytic sites, ensuring the proper size and architecture of the actin meshwork.

Las17p and Myo5p/Vrp1p NPF activity and Myo5p motor activity are required for endocytic internalization. We speculate that actin nucleation and myosin motor activity cooperate to generate forces that drive membrane invagination. We showed that the NPFs that remain nonmotile at the plasma membrane, Las17p and Myo5p/Vrp1p, have the highest NPF activity, which

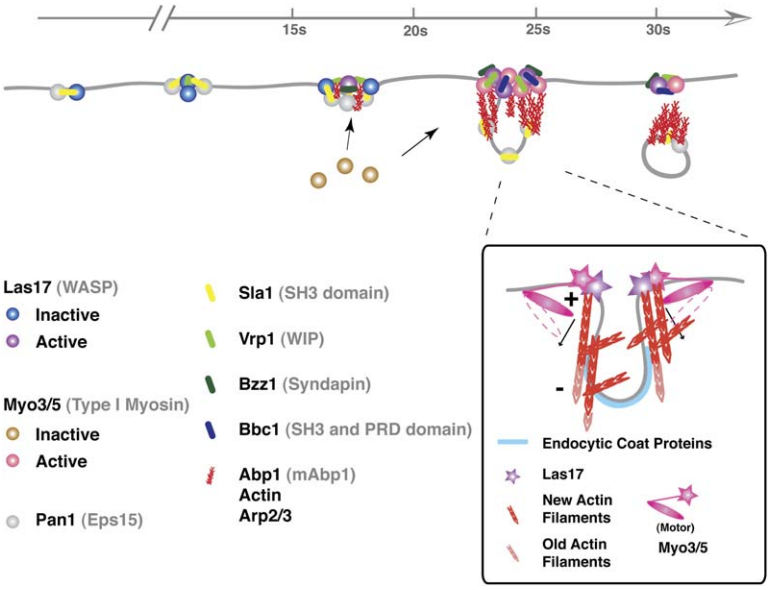

Figure 7. A Model for How Actin Assembly Is Regulated and Produces Force during Endocytic Internalization

See Discussion for description.

further supports the conclusion based on photobleaching studies that actin nucleation occurs preferentially at the plasma membrane in wild-type cells (Kaksonen et al., 2003, 2005). The likely actin filament orientation at endocytic sites indicates that a type I myosin associated with these filaments would walk toward the plasma membrane. Myo5p motor activity may contribute to membrane invagination by translocating actin filaments associated with endocytic coat proteins away from the plasma membrane into the cytosol. Sla2p and Pan1p may link actin filaments to the endocytic coat (Baggett et al., 2003; Henry et al., 2002; Sun et al., 2005; Toshima et al., 2005; Wendland and Emr, 1998). This model for actin-mediated endocytic membrane invagination is supported by the observation that Myo5p mutants defective in motor activity fail to translocate actin filaments off the plasma membrane in *sla2Δ* cells. In addition, type I myosin motor activity may be required for the proper organization and/or orientation of actin filaments at endocytic sites such that actin polymerization effectively generates force. Further investigation is needed to clarify the force generating mechanisms that facilitate endocytic internalization.

Clathrin-mediated endocytosis in yeast and mammalian cells is far more similar than had previously been appreciated. Components of the mammalian endocytic actin machinery, including N-WASP, WIP, and syndapin, share homology to yeast endocytic proteins, and in both instances, these proteins play a role in clathrin-mediated endocytosis (Kaksonen et al., 2005; Merrifield, 2004; Newpher et al., 2005). Type I myosins are also important for endocytosis and other membrane-related processes in a variety of organisms (Osherov and May, 2000). Thus, the mechanisms proposed here are likely to be widely conserved.

Distinct Roles for NPFs during Endocytosis

Our biochemical studies showed that Las17p and Myo5p/Vrp1p have significantly higher NPF activity than Pan1p and Abp1p, suggesting that the different NPFs may have distinct roles during endocytosis. The difference in NPF activities may reflect the fact that Pan1p and Abp1p lack typical WH2 (W) and Central (C) domains, which are important for Arp2/3 complex activation by WASP (Pollard and Borisy, 2003).

Each yeast NPF is a modular protein. Our analysis of NPF-related endocytic function therefore depended on the use of precise mutations targeted to the Arp2/3 binding domains. NPFs with the greatest NPF activity in vitro were the most important for endocytic function in vivo. Importantly, we identified unique endocytic functions for the NPFs, including those with similarly potent NPF activities. Las17p NPF activity is required to efficiently initiate actin assembly at endocytic sites. Myo5p/Vrp1p NPF activity is most important during endocytic coat inward movement. NPFs also appeared to have partially overlapping functions. Myo5p/Vrp1p and Pan1p could help initiate endocytic actin polymerization in the absence of Las17p. In addition, Las17p and Myo5p/Vrp1p NPF activities cooperated to promote endocytic coat inward movement, which likely represents membrane invagination. Because blocking invagination prevents the analysis of subsequent endocytic steps, new strategies will be needed to determine whether actin nucleation also plays a role during vesicle scission.

In contrast to the other yeast NPFs, deletion of Abp1p by itself results in only a slight defect in endocytic events (Kaksonen et al., 2005). The Abp1p "A" domains, which bind to the Arp2/3 complex, have been shown to be required for Las17p inhibition (D'Agostino and Goode, 2005). This suggests that Abp1p may functionally compete with other NPFs for Arp2/3 complex binding. Because Abp1p localization at endocytic sites is dependent on F-actin, Abp1p is possibly involved in a negative feedback loop to restrain actin nucleation after actin assembly is initiated.

Regulation of Actin Assembly by Dynamic Changes in Patch Composition

Yeast NPFs contain SH3 or proline-rich domains that mediate a complex array of protein-protein interactions (Goode and Rodal, 2001). Our results suggest that

dynamic changes in the protein composition of endocytic sites, mediated by SH3/proline-rich domain interactions, play a key role in spatio-temporal regulation of actin assembly.

We identified a critical mechanism for the activation of a WASP-family protein in which the SH3 domains of Bzz1p (a yeast syndapin-like protein) relieve Las17p inhibition by the SH3 domains of Sla1p. Consistent with this activity, Bzz1p appears at endocytic sites immediately before actin polymerization is detected. Bzz1p also contains an N-terminal F-BAR/EFC domain that tubulates membranes in mammalian cells (Itoh et al., 2005; Tsujita et al., 2006). Therefore, Bzz1p is a conserved endocytic component that may link actin assembly to membrane curvature. Not all SH3-containing proteins are antagonistic with Sla1p because another SH3-containing protein, Bbc1p, further inhibits Las17p in the presence of Sla1-SH3p (Rodal et al., 2003). In addition to Bzz1p, Bbc1p, and Sla1p, at least four other uncharacterized SH3-containing proteins interact with Las17p (Madania et al., 1999; Tong et al., 2002). Analysis of their combinatorial effects on Las17p nucleation activity in vitro, as well as their appearance at endocytic sites in vivo, will be required to fully elucidate the mechanisms of Las17p regulation.

In contrast to Las17p, it appears that Myo5p's NPF activity is activated upon its recruitment to endocytic sites. Vrp1p cooperates with actin filaments to recruit Myo5p to cortical patches via its SH3 domain. Furthermore, Vrp1p binding to Myo5p's SH3 domain activates Myo5p NPF activity in vitro. The proline-rich domain of Bbc1p also binds to Myo5p's SH3 domain (Mochida et al., 2002) and inhibits Myo5p/Vrp1p NPF activity. We found that Myo5p/Vrp1p NPF activity, but not Las17p activity, is required for the exaggerated movement of endocytic structures in a *bbc1Δ* mutant. Because Bbc1p and Vrp1p both bind to Myo5p's SH3 domain, Bbc1p possibly regulates Myo5p NPF activity by competing with Vrp1p for Myo5p binding. Based on our quantitative analysis of the relative abundance of these proteins in cortical patches, Bbc1p and Myo5p are present in similar amounts, whereas Vrp1p is present at about half the concentration of these proteins. The *bbc1Δ* mutant may result in a higher proportion of Myo3/5p being activated by Vrp1p. Bbc1p may cooperate with Abp1p to restrain actin nucleation at endocytic sites. The *abp1Δ* and *bbc1Δ* mutants exhibit similar profiles of genetic interactions, suggesting that they share a common function that is important for efficient endocytic internalization (Mochida et al., 2002).

Type I Myosin Motor Activity Is Required for Endocytic Internalization but Not for Cortical Actin Polymerization

Previously, Myo3/5p motor activity was implicated in cortical actin assembly in permeabilized yeast cells (Lechler et al., 2000). Therefore, whether Myo5p's motor activity could be functionally separated from actin nucleation was not clear. We conclusively demonstrated that the Myo5p motor and Arp2/3 complex activation activities are separable in vitro. Furthermore, actin polymerization efficiently occurred at endocytic sites in motor mutants in vivo and motor mutant defects could not be rescued by increasing NPF activity with the *bbc1Δ* mutant. Significantly, Myo5p motor mutants blocked endocytic coat inward movement, the same step blocked by NPF mutants defective in Arp2/3 activation. Therefore, we propose that motor and NPF activities function together to promote endocytic membrane invagination.

Experimental Procedures

Plasmids and Strains

Plasmids and yeast strains used in this study are listed in Tables S1 and S2, respectively. C-terminal GFP and RFP tags were integrated by homologous recombination, as described previously (Kaksonen et al., 2005). Growth of Myo5-GFP *myo3Δ*, Vrp1-GFP, and wild-type strains were indistinguishable, demonstrating that the tagged proteins are functional. Myo5 mutants were generated as described in the Supplemental Experimental Procedures. All strains were grown in standard rich media (YPD) at 25ºC, unless otherwise noted.

Protein Purification

Myo5p, Vrp1p, Las17p, Sla1-SH3p, Bzz1p, Bbc1p, and Abp1p were overexpressed and purified by previously described procedures (Rodal et al., 2003), with the following exceptions. Full-length Myo5p was purified in a buffer containing 20 mM HEPES (pH 7.5), 1 mM EGTA, 5 mM $MgCl_2$, 1 mM ATP, and 100 mM KCl. Myc-tagged Myo5p bound to anti-myc-coated beads was washed with buffer containing 1 M KCl to remove contaminating Las17p. Cmd1p, Myo5p's light chain, copurified with Myo5p, as detected by Western blotting (data not shown). Myo5p was stored at −80ºC in 20 mM HEPES (pH 7.5), 1 mM EGTA, 0.1 mM $MgCl_2$, 0.1 mM ATP, 50 mM KCl, and 5% glycerol. To purify Vrp1p, a Mono Q column (Amersham Pharmacia) was used to remove contaminating Las17p and actin. HA-tagged Arp2/3 complex, TAP-tagged Pan1p, rabbit skeletal muscle actin, and yeast actin were purified as described previously (Martin et al., 2005; Toshima et al., 2005). Protein concentrations were determined by using SYPRO dye (Molecular Probes) with BSA as a standard.

Nucleation Assays

Actin assembly was induced with 50 mM KCl, 1 mM EGTA, and 1 mM $MgCl_2$ (final concentration). Pyrene-actin assembly was monitored with a Fluoromax 2 fluorometer (Jobin-Yvon Horiba) or a Victor 3 plate reader (Perkin Elmer) with 355 nm and 410 nm filters. Barbed end concentrations nucleated by the Arp2/3 complex were calculated with the rate of actin polymerization at 50% polymerization and a k_+ of 8.7 $\mu M^{-1}s^{-1}$, as described previously (Rodal et al., 2003). Rabbit skeletal muscle actin was used unless otherwise noted.

Microscopy

The Lucifer yellow uptake assay (Duncan et al., 2001) and live-cell imaging (Kaksonen et al., 2005) were performed as described previously. All imaging studies were performed at approximately 25ºC. Image analysis was performed with ImageJ (http://rsb.info.nih.gov/ij/) (Kaksonen et al., 2003).

Myosin Gliding Assay

Myo5p (200 nM) was bound to a nitrocellulose-coated coverslip for 4 min, and the coverslip was subsequently blocked with 0.5% BSA for 1 min. Rhodamine-phalloidin (Molecular Probes) stabilized actin filaments (160 nM) were bound to Myo5p on the coverslip in the absence of ATP. The motility reaction was initiated by adding ATP (2 mM) and was immediately visualized by using an Olympus IX-71 fluorescence microscope, with a 100×/NA 1.4 objective, and an Orca-ER camera. The reaction buffer contained 25 mM Imidazole (pH 7.0), 25 mM KCl, 4 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 18 μg/ml catalase, 0.1 mg/ml glucose oxidase, 3 mg/ml glucose, and 2 mM ATP.

Supplemental Data

Supplemental Data include additional Experimental Procedures, figures, tables, and movies and are available at http://www.developmentalcell.com/cgi/content/full/11/1/33/DC1/.

Acknowledgments

We thank A. Rodal, J. Toshima, M. Sekiya-Kawasaki, S. Carroll, C. Toret, M. Kaksonen, and L. Lee for providing strains and plasmids used in this study. We also thank J. Toshima for purified Pan1p and yeast actin. Finally, we thank M. Welch, L. Lee, V. Okreglak, C. Toret, C.X. Zhang, I. LeBlanc, and M. Kaksonen for critically reading the manuscript. This work was supported by National Institutes of Health grants GM42759 and GM50399 to D.G.D. and National Institutes of Health Shared Instrumentation Grant S10-RR019290 to D.G.D.

Received: November 29, 2005
Revised: May 3, 2006
Accepted: May 16, 2006
Published: July 10, 2006

References

Anderson, B.L., Boldogh, I., Evangelista, M., Boone, C., Greene, L.A., and Pon, L.A. (1998). The Src homology domain 3 (SH3) of a yeast type I myosin, Myo5p, binds to verprolin and is required for targeting to sites of actin polarization. J. Cell Biol. *141*, 1357–1370.

Baggett, J.J., D'Aquino, K.E., and Wendland, B. (2003). The Sla2p talin domain plays a role in endocytosis in *Saccharomyces cerevisiae*. Genetics *165*, 1661–1674.

Baird, G.S., Zacharias, D.A., and Tsien, R.Y. (2000). Biochemistry, mutagenesis, and oligomerization of DsRed, a red fluorescent protein from coral. Proc. Natl. Acad. Sci. USA *97*, 11984–11989.

Benesch, S., Polo, S., Lai, F.P., Anderson, K.I., Stradal, T.E., Wehland, J., and Rottner, K. (2005). N-WASP deficiency impairs EGF internalization and actin assembly at clathrin-coated pits. J. Cell Sci. *118*, 3103–3115.

D'Agostino, J.L., and Goode, B.L. (2005). Dissection of Arp2/3 complex actin nucleation mechanism and distinct roles for its nucleation-promoting factors in *Saccharomyces cerevisiae*. Genetics *171*, 35–47.

Duncan, M.C., Cope, M.J., Goode, B.L., Wendland, B., and Drubin, D.G. (2001). Yeast Eps15-like endocytic protein, Pan1p, activates the Arp2/3 complex. Nat. Cell Biol. *3*, 687–690.

Engqvist-Goldstein, A.E., and Drubin, D.G. (2003). Actin assembly and endocytosis: from yeast to mammals. Annu. Rev. Cell Dev. Biol. *19*, 287–332.

Evangelista, M., Klebl, B.M., Tong, A.H., Webb, B.A., Leeuw, T., Leberer, E., Whiteway, M., Thomas, D.Y., and Boone, C. (2000). A role for myosin-I in actin assembly through interactions with Vrp1p, Bee1p, and the Arp2/3 complex. J. Cell Biol. *148*, 353–362.

Geli, M.I., and Riezman, H. (1996). Role of type I myosins in receptor-mediated endocytosis in yeast. Science *272*, 533–535.

Geli, M.I., Lombardi, R., Schmelzl, B., and Riezman, H. (2000). An intact SH3 domain is required for myosin I-induced actin polymerization. EMBO J. *19*, 4281–4291.

Goode, B.L., and Rodal, A.A. (2001). Modular complexes that regulate actin assembly in budding yeast. Curr. Opin. Microbiol. *4*, 703–712.

Goode, B.L., Rodal, A.A., Barnes, G., and Drubin, D.G. (2001). Activation of the Arp2/3 complex by the actin filament binding protein Abp1p. J. Cell Biol. *153*, 627–634.

Goodson, H.V., Anderson, B.L., Warrick, H.M., Pon, L.A., and Spudich, J.A. (1996). Synthetic lethality screen identifies a novel yeast myosin I gene (MYO5): myosin I proteins are required for polarization of the actin cytoskeleton. J. Cell Biol. *133*, 1277–1291.

Henry, K.R., D'Hondt, K., Chang, J., Newpher, T., Huang, K., Hudson, R.T., Riezman, H., and Lemmon, S.K. (2002). Scd5p and clathrin function are important for cortical actin organization, endocytosis, and localization of sla2p in yeast. Mol. Biol. Cell *13*, 2607–2625.

Huckaba, T.M., Gay, A.C., Pantalena, L.F., Yang, H.C., and Pon, L.A. (2004). Live cell imaging of the assembly, disassembly, and actin cable-dependent movement of endosomes and actin patches in the budding yeast, *Saccharomyces cerevisiae*. J. Cell Biol. *167*, 519–530.

Itoh, T., Erdmann, K.S., Roux, A., Habermann, B., Werner, H., and De Camilli, P. (2005). Dynamin and the actin cytoskeleton cooperatively regulate plasma membrane invagination by BAR and F-BAR proteins. Dev. Cell *9*, 791–804.

Jonsdottir, G.A., and Li, R. (2004). Dynamics of yeast Myosin I: evidence for a possible role in scission of endocytic vesicles. Curr. Biol. *14*, 1604–1609.

Kaksonen, M., Sun, Y., and Drubin, D.G. (2003). A pathway for association of receptors, adaptors, and actin during endocytic internalization. Cell *115*, 475–487.

Kaksonen, M., Toret, C.P., and Drubin, D.G. (2005). A modular design for the clathrin- and actin-mediated endocytosis machinery. Cell *123*, 305–320.

Lechler, T., Shevchenko, A., and Li, R. (2000). Direct involvement of yeast type I myosins in Cdc42-dependent actin polymerization. J. Cell Biol. *148*, 363–373.

Lechler, T., Jonsdottir, G.A., Klee, S.K., Pellman, D., and Li, R. (2001). A two-tiered mechanism by which Cdc42 controls the localization and activation of an Arp2/3-activating motor complex in yeast. J. Cell Biol. *155*, 261–270.

Madania, A., Dumoulin, P., Grava, S., Kitamoto, H., Scharer-Brodbeck, C., Soulard, A., Moreau, V., and Winsor, B. (1999). The *Saccharomyces cerevisiae* homologue of human Wiskott-Aldrich syndrome protein Las17p interacts with the Arp2/3 complex. Mol. Biol. Cell *10*, 3521–3538.

Martin, A.C., Xu, X.P., Rouiller, I., Kaksonen, M., Sun, Y., Belmont, L., Volkmann, N., Hanein, D., Welch, M., and Drubin, D.G. (2005). Effects of Arp2 and Arp3 nucleotide-binding pocket mutations on Arp2/3 complex function. J. Cell Biol. *168*, 315–328.

Merrifield, C.J. (2004). Seeing is believing: imaging actin dynamics at single sites of endocytosis. Trends Cell Biol. *14*, 352–358.

Merrifield, C.J., Feldman, M.E., Wan, L., and Almers, W. (2002). Imaging actin and dynamin recruitment during invagination of single clathrin-coated pits. Nat. Cell Biol. *4*, 691–698.

Merrifield, C.J., Qualmann, B., Kessels, M.M., and Almers, W. (2004). Neural Wiskott Aldrich Syndrome Protein (N-WASP) and the Arp2/3 complex are recruited to sites of clathrin-mediated endocytosis in cultured fibroblasts. Eur. J. Cell Biol. *83*, 13–18.

Merrifield, C.J., Perrais, D., and Zenisek, D. (2005). Coupling between clathrin-coated-pit invagination, cortactin recruitment, and membrane scission observed in live cells. Cell *121*, 593–606.

Mochida, J., Yamamoto, T., Fujimura-Kamada, K., and Tanaka, K. (2002). The novel adaptor protein, Mti1p, and Vrp1p, a homolog of Wiskott-Aldrich syndrome protein-interacting protein (WIP), may antagonistically regulate type I myosins in *Saccharomyces cerevisiae*. Genetics *160*, 923–934.

Newpher, T.M., Smith, R.P., Lemmon, V., and Lemmon, S.K. (2005). In vivo dynamics of clathrin and its adaptor-dependent recruitment to the actin-based endocytic machinery in yeast. Dev. Cell *9*, 87–98.

Osherov, N., and May, G.S. (2000). In vivo function of class I myosins. Cell Motil. Cytoskeleton *47*, 163–173.

Pollard, T.D., and Borisy, G.G. (2003). Cellular motility driven by assembly and disassembly of actin filaments. Cell *112*, 453–465.

Rodal, A.A., Manning, A.L., Goode, B.L., and Drubin, D.G. (2003). Negative regulation of yeast WASp by two SH3 domain-containing proteins. Curr. Biol. *13*, 1000–1008.

Rohatgi, R., Ma, L., Miki, H., Lopez, M., Kirchhausen, T., Takenawa, T., and Kirschner, M.W. (1999). The interaction between N-WASP and the Arp2/3 complex links Cdc42-dependent signals to actin assembly. Cell *97*, 221–231.

Sekiya-Kawasaki, M., Groen, A.C., Cope, M.J., Kaksonen, M., Watson, H.A., Zhang, C., Shokat, K.M., Wendland, B., McDonald, K.L., McCaffery, J.M., and Drubin, D.G. (2003). Dynamic phosphoregulation of the cortical actin cytoskeleton and endocytic machinery revealed by real-time chemical genetic analysis. J. Cell Biol. *162*, 765–772.

Sirotkin, V., Beltzner, C.C., Marchand, J.B., and Pollard, T.D. (2005). Interactions of WASp, myosin-I, and verprolin with Arp2/3 complex

during actin patch assembly in fission yeast. J. Cell Biol. *170*, 637–648.

Soulard, A., Lechler, T., Spiridonov, V., Shevchenko, A., Li, R., and Winsor, B. (2002). *Saccharomyces cerevisiae* Bzz1p is implicated with type I myosins in actin patch polarization and is able to recruit actin-polymerizing machinery in vitro. Mol. Cell. Biol. *22*, 7889–7906.

Sun, Y., Kaksonen, M., Madden, D.T., Schekman, R., and Drubin, D.G. (2005). Interaction of Sla2p's ANTH domain with PtdIns(4,5)P2 is important for actin-dependent endocytic internalization. Mol. Biol. Cell *16*, 717–730.

Tong, A.H., Drees, B., Nardelli, G., Bader, G.D., Brannetti, B., Castagnoli, L., Evangelista, M., Ferracuti, S., Nelson, B., Paoluzi, S., et al. (2002). A combined experimental and computational strategy to define protein interaction networks for peptide recognition modules. Science *295*, 321–324.

Toshima, J., Toshima, J.Y., Martin, A.C., and Drubin, D.G. (2005). Phosphoregulation of Arp2/3-dependent actin assembly during receptor-mediated endocytosis. Nat. Cell Biol. *7*, 246–254.

Tsujita, K., Suetsugu, S., Sasaki, N., Furutani, M., Oikawa, T., and Takenawa, T. (2006). Coordination between the actin cytoskeleton and membrane deformation by a novel membrane tubulation domain of PCH proteins is involved in endocytosis. J. Cell Biol. *172*, 269–279.

Wendland, B., and Emr, S.D. (1998). Pan1p, yeast eps15, functions as a multivalent adaptor that coordinates protein-protein interactions essential for endocytosis. J. Cell Biol. *141*, 71–84.

Wu, C., Lee, S.F., Furmaniak-Kazmierczak, E., Cote, G.P., Thomas, D.Y., and Leberer, E. (1996). Activation of myosin-I by members of the Ste20p protein kinase family. J. Biol. Chem. *271*, 31787–31790.

Yarar, D., Waterman-Storer, C.M., and Schmid, S.L. (2005). A dynamic actin cytoskeleton functions at multiple stages of clathrin-mediated endocytosis. Mol. Biol. Cell *16*, 964–975.

Zot, H.G., Doberstein, S.K., and Pollard, T.D. (1992). Myosin-I moves actin filaments on a phospholipid substrate: implications for membrane targeting. J. Cell Biol. *116*, 367–376.