Early-Arriving Syp1p and Ede1p Function in Endocytic Site Placement and Formation in Budding Yeast

Helen E. M. Stimpson,* Christopher P. Toret,*† Aaron T. Cheng, Barbara S. Pauly, and David G. Drubin

Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA 94720-3202

Submitted May 27, 2009; Revised August 17, 2009; Accepted September 15, 2009
Monitoring Editor: Sandra L. Schmid

Recent studies have revealed the detailed timing of protein recruitment to endocytic sites in budding yeast. However, little is understood about the early stages of their formation. Here we identify the septin-associated protein Syp1p as a component of the machinery that drives clathrin-mediated endocytosis in budding yeast. Syp1p arrives at endocytic sites early in their formation and shares unique dynamics with the EH-domain protein Ede1p. We find that Syp1p is related in amino acid sequence to several mammalian proteins one of which, SGIP1- α , is an endocytic component that binds the Ede1p homolog Eps15. Like Syp1p, SGIP1- α arrives early at sites of clathrin-mediated endocytosis, suggesting that Syp1p/Ede1p and SGIP1- α /Eps15 may have a conserved function. In yeast, both Syp1p and Ede1p play important roles in the rate of endocytic site turnover. Additionally, Ede1p is important for endocytic site formation, whereas Syp1p acts as a polarized factor that recruits both Ede1p and endocytic sites to the necks of emerging buds. Thus Ede1p and Syp1p are conserved, early-arriving endocytic proteins with roles in the formation and placement of endocytic sites, respectively.

INTRODUCTION

The dynamics of protein recruitment to sites of clathrin-mediated endocytosis have been revealed by live-cell microscopy in budding yeast and mammalian cells (Merrifield *et al.*, 2002; Kaksonen *et al.*, 2003; Kaksonen *et al.*, 2005). These studies have identified numerous proteins that sequentially assemble at endocytic sites and have shown that actin polymerization can power internalization. It is now evident that the dynamic recruitment and disappearance of endocytic proteins are precisely coordinated for productive internalization and that each protein has defined dynamics at endocytic sites. In *Saccharomyces cerevisiae* four endocytic modules have been defined that each contain proteins with similar dynamics: the coat, WASP/myo, amphiphysin, and actin modules (Kaksonen *et al.*, 2005).

Despite detailed knowledge of events at endocytic sites, little is understood about the early stages of their formation. The best candidates for proteins that initiate endocytic site formation are those that arrive earliest. In mammalian cells the classical coat protein clathrin marks the earliest known stage of endocytic site formation (Merrifield *et al.*, 2002). Additionally, the adapter AP-2, is critical for site formation and has similar dynamic behavior to clathrin and so is thought to arrive early (Hinrichsen *et al.*, 2003; Motley *et al.*, 2003; Ehrlich *et al.*, 2004; Keyel *et al.*, 2004). In yeast, the role

of AP-2 is unclear, but clathrin, a coat module component, marks the first stages of endocytosis, and its deletion causes severe defects in the number of sites formed (Huang *et al.*, 1999; Kaksonen *et al.*, 2005; Newpher *et al.*, 2005; Newpher and Lemmon, 2006). An additional yeast protein, Ede1p, arrives early and plays a role in endocytic site formation, although its dynamics and function have yet to be fully investigated (Kaksonen *et al.*, 2005; Toshima *et al.*, 2006). Ede1p is a homologue of the mammalian adapter Eps15, which localizes to endocytic sites but has as yet undefined dynamics (Tebar *et al.*, 1996; van Delft *et al.*, 1997). Intriguingly, Ede1p exhibits distinct dynamics to clathrin, suggesting that there are two different activities in endocytic site formation (Kaksonen *et al.*, 2005; Toshima *et al.*, 2006). Further analysis of early-arriving proteins will be key in determining how assembly of endocytic sites is initiated.

The initiation of endocytic sites is closely linked to their placement on the plasma membrane. The plasma membrane is highly organized and the composition of distinct regions is controlled in part through endocytosis and exocytosis. Mechanisms must, therefore, exist to target components involved in endocytic site initiation to specific regions of the plasma membrane. In agreement with this conclusion, budding yeast actin patches (which are known to be endocytic sites) are polarized to regions of cell surface growth. Specifically, endocytosis is targeted to: 1) the site on the mother from which a bud will emerge in late G1; 2) the necks of small-to-medium-sized buds and bud tips during apical growth; 3) the entire daughter during isotropic growth; and 4) the bud neck before, during, and after cytokinesis (Pruyne and Bretscher, 2000a,b). The importance of this cell cycle-coupled distribution is illustrated by the observation that many endocytic mutants have defects in polarized growth (Pruyne and Bretscher, 2000a), but the underlying reasons for endocytic polarization remain unknown. Understanding how yeast select sites for endocytosis is fundamental in

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E09-05-0429>) on September 23, 2009.

* These authors contributed equally to this work.

† Present address: Department of Biological Sciences, Stanford University, Stanford, CA 94305.

Address correspondence to: David G. Drubin (drubin@berkeley.edu).

Abbreviations used: TIRF, total internal reflection; WASP, Wiscott-Aldrich syndrome protein.

understanding their formation and may prove relevant to cell polarity in more complex cells.

The *S. cerevisiae* protein Syp1p was recently found to be central in a protein interaction network involved in polarized cell growth (Tarassov *et al.*, 2008) and to associate with the septin ring and play a role in its turnover (Qiu *et al.*, 2008). Deletion of *SYPI* has no consequences for cell growth or morphology (Marcoux *et al.*, 2000; Qiu *et al.*, 2008). However, support for a role of Syp1p in cell polarity comes from the observation that its overexpression can rescue growth and budding defects in cells lacking profilin (Pfy1p) or Arf3p (Marcoux *et al.*, 2000; Lambert *et al.*, 2007). In addition to being implicated in cell polarity, Arf3p is implicated in endocytosis, to which Syp1p is connected through global interaction studies. Several genome-wide studies have identified physical interactions between Syp1p and endocytic components, including Sla1p, Ent2p, Las17p, Myo5p, Vrp1p, and Ede1p (Gavin *et al.*, 2002; Krogan *et al.*, 2006; Collins *et al.*, 2007; Tarassov *et al.*, 2008; Yu *et al.*, 2008). Notable among these endocytic interactions is Ede1p, which has been shown to interact with Syp1p in multiple analyses (Gavin *et al.*, 2002; Krogan *et al.*, 2006; Collins *et al.*, 2007; Yu *et al.*, 2008). We used protein sequence analysis and live-cell imaging to reveal that Syp1p is a conserved endocytic protein that shares dynamics with Ede1p and plays a role in the polarized distribution of endocytic sites, whereas Ede1p modulates endocytic site formation.

MATERIALS AND METHODS

Yeast Media and Strains

Yeast strains were grown at 25°C in rich medium (YPD) or synthetic medium (SD) supplemented with appropriate amino acids. C-terminal green fluorescent protein (GFP) tags were integrated chromosomally as described previously (Longtine *et al.*, 1998). All strains expressing GFP-tagged proteins had growth properties similar to control strains. The presence of Syp1-GFP or Ede1-GFP did not affect lifetimes of Sla1-mCherry at endocytic patches when compared with wild-type cells (data not shown), suggesting that the two GFP-tagged proteins are functional and do not interfere with endocytic site dynamics. For gene deletions a *Candida glabrata* *LEU2* selection cassette was integrated in place of the open reading frame. Yeast strains are listed in Supplemental Table S1. For microscopy, cells were grown to log phase in SD without tryptophan and attached to concanavalin A-coated coverslips, which were sealed to slides with vacuum grease (Dow Corning, Midland, MI).

Mammalian Cell Culture and Transfection

Swiss 3T3 cells stably expressing DsRed-mLca (mouse clathrin light chain a; described hereafter as DsRed-clathrin) were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 2 mM GlutaMAX-I (Invitrogen). Forty-eight hours before imaging, cells were transiently transfected with a plasmid encoding SGIP1- α -GFP (as previously described in Engqvist-Goldstein *et al.*, 1999; Uezu *et al.*, 2007). Cells were transfected using Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's protocol. Twenty-four hours before imaging, cells were reseeded onto uncoated glass coverslips. For microscopy the coverslips were transferred to medium containing DMEM containing 10% FBS and 10 mM HEPES.

Live-Cell Imaging

Imaging was carried out using an Olympus IX71 or IX81 microscope (Melville, NY) equipped with a 100 $\times$ /NA1.4 objective and Orca II camera (Hamamatsu, Bridgewater, NJ). For imaging of Swiss 3T3 cells, the stage temperature was maintained between 35 and 37°C using a heated blower. Total internal reflection fluorescence (TIRF) microscopy was performed using an Olympus IX81 microscope equipped with a 100 $\times$ /NA1.65 or 60 $\times$ /NA1.45 objective (for yeast and Swiss3T3 cells, respectively). A 488-nm Argon ion laser (Melles Griot, Rochester, NY) was used to excite GFP and dsRed, and a mercury lamp filtered through a 575/20-nm filter was used to excite monomeric red fluorescent protein (mRFP). Excitation intensity was regulated by neutral density filters. Simultaneous two-color TIRF imaging was performed using an image splitter (Optical Insights, Tucson, AZ) to separate red and green emission signals to two sides of the camera sensor using a 565-nm dichroic mirror and 530/30-nm and 630/50-nm emission filters. The excitation beams from the two light sources were combined using a beam splitter.

After each experiment, images of microbeads were captured and used to align images.

For movies of Syp1-GFP and Ede1-RFP throughout an entire cell cycle (see Figure 4D), cells were kept on concanavalin A-coated coverslips in a media chamber, and red and green images were captured immediately after one another using a mercury lamp and filter wheel with GFP and RFP filter sets. Exposure times were 0.5–1 s for yeast and 1 s for Swiss 3T3 cells. Frame rates are noted in the text. Images were acquired using Metamorph (Universal Imaging, West Chester, PA), and were analyzed and manipulated using Image J (<http://rsb.info.nih.gov/ij/>) and Adobe Photoshop (Adobe Systems, San Jose, CA). Movies were adjusted for photobleaching over time.

Endocytosis Assays

For FM4-64 labeling, the dye was solubilized in DMSO, diluted to 8 μ M in SD, and perfused into a flow chamber containing cells. Imaging was performed at the indicated time points after addition of the dye. Lucifer yellow uptake assays were performed as described previously (Belmont and Drubin, 1998). To assay Can1-GFP uptake, cells were grown to log phase, and endocytosis was induced by the addition of cyclohexamide to a final concentration of 100 μ g/ml. Cells were imaged at the indicated time points after addition. Ste2-GFP and GFP-Snc1p were expressed from centromeric plasmids as previously described (Stefan and Blumer, 1999; Lewis *et al.*, 2000), and their localization was assessed at steady state.

Protein Sequence Analysis

Protein sequence similarities, domain structures, and predicted secondary structures were assessed using HHPred, NCBI BLAST, PFAM, and PSIPRED (Altschul *et al.*, 1997; McGuffin *et al.*, 2000; Soding *et al.*, 2005; Finn *et al.*, 2008). Sequence identity was calculated using EMBOSS Needle (Rice *et al.*, 2000). Alignment for presentation was created using Multalign (Corpet, 1988).

RESULTS

Syp1p and Ede1p Show Novel Endocytic Dynamics

Genome-wide studies have identified physical interactions between the septin-associated protein Syp1p and a number of endocytic proteins, suggesting that Syp1p might have an endocytic role (Gavin *et al.*, 2002; Krogan *et al.*, 2006; Collins *et al.*, 2007; Tarassov *et al.*, 2008; Yu *et al.*, 2008). In cells expressing Syp1-GFP, we found that in addition to the bud neck localization reported by Qiu *et al.* (2008), cortical foci were visible (Figure 1A). The distribution of Syp1p was strongly reminiscent of endocytic sites. Individual Syp1-GFP foci clearly colocalized with the endocytic marker Ede1-RFP, indicating that Syp1p foci are endocytic "patches" (Figure 1B).

We next analyzed the dynamic behavior of Syp1p at endocytic sites. Endocytic dynamics cannot be determined in polarized regions of the cell, such as the bud neck, because of the high density of sites in these regions. For this reason, all dynamic analysis in this study was performed on patches outside these regions of the cell. We first coexpressed Syp1-GFP with the endocytic marker Abp1-mRFP and examined their cell surface colocalization by TIRF microscopy. Abp1p-mRFP serves as a reporter for actin polymerization in the final stages of endocytic internalization and is an established reference for endocytic protein dynamics (Kaksonen *et al.*, 2003, 2005). Syp1-GFP patches were transient and colocalized with Abp1p-mRFP for $\sim$ 5 s before their dissipation (Figure 1C). Because the overlap between Syp1p and Abp1p was short, we next coexpressed Syp1-GFP with Sla1-mCherry, a marker of the endocytic coat module that internalizes with Abp1p, but which has a longer lifetime of $\sim$ 30 s (Sun *et al.*, 2007). In cells coexpressing Syp1-GFP and Sla1-mCherry, 93% of Syp1-GFP patches accumulated Sla1p ($n = 40$), and 90% of Sla1-mCherry patches arose from Syp1p patches ($n = 40$), indicating that Syp1p is present at essentially all endocytic sites around the cell, in addition to being intensely concentrated at the bud neck. In individual patches Syp1p and Sla1p overlapped temporally, with Syp1p accumulating before Sla1p, and Sla1p disappearing after Syp1p (Figure 1D): Syp1-GFP patches were present at the cortex from 2 s to 128 s before Sla1-mCherry accumula-

Figure 1. Syp1p and Ede1p dynamics at endocytic sites. (A) Epifluorescence images of live wild-type cells expressing Syp1-GFP or Ede1-GFP (strains DDY3865 and -3866). (B) Epifluorescence images of a single cell coexpressing Syp1-GFP and Ede1-RFP (strain DDY3871). (C) Dynamic cell surface localization of Syp1-GFP and Ede1-GFP (viewed by TIRF microscopy) in reference to Abp1-RFP (viewed by epifluorescence microscopy). Time series shows individual patches from two-color movies. Frame interval, 2 s. The proteins were coexpressed in strains DDY3867 and -3868. (D) Dynamic cell surface localization of Syp1-GFP and Ede1-GFP (viewed by TIRF microscopy) in reference to Sla1-mCherry (viewed by epifluorescence microscopy). Time series shows individual patches from two-color movies. Frame interval, 2 s. The proteins were coexpressed in DDY3899 and -3900. (E) Average lifetimes of Syp1-GFP and Ede1-GFP patches $\pm$ SD, $n = 60$. Data taken from 4-min TIRF microscopy movies with 2-s frame intervals. (F) Distribution of Syp1-GFP and Ede1-GFP patch lifetimes from the same dataset as in C. (G) Kymographs of representative Syp1-GFP and Ede1-GFP patches from epifluorescence movies. Patches were oriented so the outside of the cell is on top. (H) Quantification of fluorescence intensity for individual Syp1-GFP and Ede1-GFP patches over time. Each curve represents data from one patch. Fluorescence intensity was corrected for photobleaching. Movies were taken with 2-s frame intervals. All scale bars, 2 μ m.

tion and the two proteins colocalized for an average of 24.3 ± 13.1 s ($n = 30$, mean $\pm$ SD). Sla1-mCherry patches

were visible for an average of 5.2 ± 4.5 s, after Syp1-GFP disappeared. Thus Syp1p arrives early at endocytic sites,

before Sla1p, and disappears shortly after actin assembly commences.

Uniquely among the endocytic proteins with which Syp1p is reported to interact, Ede1p arrives early at endocytic sites (Toshima *et al.*, 2006). Endocytic dynamics can indicate the stage at which an endocytic protein functions, and so given Syp1p's and Ede1p's common early arrival, we decided to investigate the dynamics of both proteins in detail to determine if they were similar. Ede1p dynamics relative to Abp1p have yet to be analyzed, but unlike clathrin, the only other known early-arriving yeast endocytic component, Ede1p remains at the cortex and disappears before coat module internalization (Toshima *et al.*, 2006). Like Syp1p, Ede1-GFP was found in cortical patches around the cell, as well as strongly concentrated to the bud neck (Figure 1A), and disappeared from patches within ~5 s of Abp1p appearance (Figure 1C). Ede1p dynamics relative to Sla1p were also similar to Syp1p's (Figure 1D): Ede1-GFP patches were present from 12 s to 218 s before arrival of Sla1-mCherry, and the two proteins colocalized for 26.0 ± 8.7 s. Sla1-mCherry patches persisted for an average of 3.5 ± 2.6 s after Ede1p disappeared. Both Syp1p and Ede1p patches were long-lived: in cells expressing Syp1p-GFP or Ede1-GFP, cortical patches had mean lifetimes of 80.6 ± 45.6 and 88.36 ± 52.6 s, respectively ($n = 60$, Figure 1E). The high SD reflects a wide range of lifetimes—from 34 s to 4 min (Figure 1F)—making Syp1p and Ede1p two of the earliest arriving endocytic components.

Imaging in medial focal planes revealed that like Ede1-GFP, and in contrast to components of the coat module, Syp1-GFP remained at the cortex throughout its lifetime (Figure 1G). Thus, Syp1p and Ede1p have similar endocytic dynamics, which are unlike those of other endocytic components: both remain at the cortex and have long and variable patch lifetimes. Analysis of fluorescence intensity over time did, however, reveal that Ede1p patch intensities steadily increased until dissipation, whereas Syp1p patch intensities were more irregular, suggesting that Syp1p may associate less stably with endocytic sites (Figure 1H). Before this work, Ede1p was the sole endocytic protein that did not share dynamics with other endocytic components, thus Syp1p is a novel early-arriving endocytic component that shares unique dynamics with Ede1p.

Syp1p Is a Conserved Endocytic Protein

Having established that Syp1p is an endocytic protein, we carried out sequence analysis to search for functional domains and related proteins. Similarity searching with the Syp1p sequence produced hits against a number of conserved metazoan proteins, including the endocytic protein SGIP1- α , the putative endocytic F-BAR protein FCHO1, and the F-BAR protein FCHO2 (Figure 2A; Henne *et al.*, 2007; Sakaushi *et al.*, 2007; Uezu *et al.*, 2007).

Sequence identity is relatively low between Syp1p and SGIP1- α or FCHO1/2 (between 17 and 20%), but all four proteins have a similar domain arrangement (Figure 2B). Both FCHO1 and FCHO2 have N-terminal lipid binding F-BAR domains, and SGIP1- α has an experimentally confirmed N-terminal lipid-binding domain (Uezu *et al.*, 2007). A search in PFAM for conserved domains in Syp1p revealed similarity at the N-terminus to the FCH domain, which is part of the F-BAR domain (Itoh *et al.*, 2005; Tsujita *et al.*, 2006). In an F-BAR domain, the FCH domain is followed by a helical region: immediately following the putative FCH domain in Syp1p is a region predicted to have a similar arrangement of helices to the N-terminus of FCHO1/2 (McGuffin *et al.*, 2000). The FCH domain similarity in Syp1p,

together with the predicted arrangement of helices in its N-terminus and similarity to FCHO1/2, strongly suggest that Syp1p has an F-BAR domain. In contrast, SGIP1- α does not have F-BAR domain similarity, suggesting that SGIP1- α may bind membranes by a different mechanism. In agreement with this observation, most similarity between Syp1p and SGIP1- α is outside the N-terminus: Syp1p and SGIP1- α have 17% sequence identity overall, but only 7% identity in the first 100 amino acids, where the SGIP1- α lipid-binding domain and Syp1p FCH domain lie. In addition to lipid-binding domains, all four proteins have a central region rich in prolines and a predicted (but as yet uncharacterized) SAFF domain. Following the SAFF domain is a C-terminal region with some conservation, particularly between the mammalian proteins (identity in this region is 53% between FCHO1 and FCHO2, 35% between FCHO1 and SGIP1- α , and 17–19% between Syp1p and the three mammalian proteins). The overall similarity in domain structure between these proteins, together with the fact that Syp1p, FCHO1 and SGIP1- α are all present at endocytic sites, suggests that Syp1p is part of a family of endocytic proteins.

Of the three mammalian proteins with similarity to Syp1p, SGIP1- α is most clearly implicated in endocytosis. SGIP1- α is known to localize to clathrin-coated pits and to bind Eps15, a homolog of Ede1p (Uezu *et al.*, 2007). The observation that a protein with similarity to Syp1p binds an Ede1p homolog was of particular interest given the shared dynamics and reported interaction of Syp1p and Ede1p. To determine if there is conservation in the dynamic behavior of Syp1p and SGIP1- α , we next analyzed SGIP1- α at endocytic sites in real-time. Swiss 3T3 cells stably expressing DsRed-clathrin were transfected with SGIP1- α -GFP, and the cell surface of live cells was examined by TIRF microscopy (Figure 2C). We found that 98.7% of clathrin punctae contained SGIP1- α ($n = 850$), and 99.9% of SGIP1- α punctae contained clathrin ($n = 850$), indicating that essentially all SGIP1- α and clathrin at the cell surface are colocalized. RT-PCR confirmed that SGIP1- α is expressed in these cells, and so our data are likely to be relevant to this cell type (data not shown). Intriguingly, we noted that in cells expressing SGIP1- α -GFP the turnover of clathrin was decreased: mean clathrin lifetimes were approximately two and a half times higher than in cells expressing only DsRed-clathrin, and the number of punctae that turned over in the time frame of our analysis decreased (from 66.5% in cells expressing only DsRed-clathrin to 40% in cells coexpressing SGIP1- α -GFP). Examining the dynamics of SGIP1- α -GFP at individual dynamic sites revealed that it accumulated at a time similar to that of clathrin (representative events are shown in Figure 2D): in all of 75 newly appearing punctae SGIP1- α appeared within 4 s of clathrin. SGIP1- α and clathrin also dissipated within 10 s of one another in foci that disappeared. Like yeast clathrin, mammalian clathrin arrives early at endocytic sites (Merrifield *et al.*, 2002), and so these data suggest that SGIP1- α is an early-arriving component of the endocytic site. Thus both Syp1p and SGIP1- α are early-arriving endocytic components that are reported to bind to the homologs Ede1p/Eps15.

The Role of Syp1p and Ede1p in Endocytosis

Because little is currently known about how early-arriving proteins influence endocytic site formation, we further investigated the roles of Syp1p and Ede1p in clathrin-mediated endocytosis in the context of the well-defined endocytic pathway of budding yeast. Syp1p and Ede1p share unique endocytic dynamics, and so we reasoned that they might act in a similar aspect of internalization. For this reason, we

Figure 2. SGIP1- α is a Syp1p homolog. (A) Sequence alignment of Syp1p, SGIP1- α (*Mus musculus*, NP_659155.1), FCHO1 (*H. sapiens*, NP_055937.1), and FCHO2 (*H. sapiens*, NP_620137). Key to alignment: red, complete consensus; blue, consensus of two or three sequences; #, conserved as D/E.; and !, conserved as I/V. (B) Predicted domain structures of Syp1p, SGIP1- α , FCHO1, and FCHO2. MP, membrane phospholipid-binding domain; SAFF, a PFAM predicted domain. (C) Colocalization of SGIP1- α -GFP and DsRed-clathrin (mouse clathrin light chain a) at the cell surface by TIRF microscopy in Swiss 3T3 cells. Scale bar, 2 μ m. (D) Dynamic cell surface localization of SGIP1- α -GFP and DsRed-clathrin by TIRF microscopy. Three representative endocytic site assembly (top) and disassembly (bottom) events are shown. For each event, a montage of DsRed-clathrin and SGIP1- α -GFP recruitment to endocytic sites is shown (frame interval, 12 s), as well as quantification of fluorescence intensity over time (frame interval, 2 s). The region of the graph corresponding to the montage is outlined in gray.

analyzed the effects of *syp1 Δ ede1 Δ* double mutants as well as the two single mutants on endocytosis.

We first analyzed the importance of Syp1p and Ede1p for endocytic uptake. Ede1p is reported to play a role in both

fluid phase endocytosis and uptake of protein cargo (Gagny *et al.*, 2000). In our strain background, however, the fluid phase defect of *ede1Δ* cells was limited, and there was no detectable defect in either process when *SYPI* was deleted alone or in combination with *EDE1* (Supplemental Figure S1). Previously, the detection of defects in endocytic dynamics has uncovered core endocytic roles for proteins without detectable uptake defects (Kaksonen *et al.*, 2003; Gheorghe *et al.*, 2008; Robertson *et al.*, 2009), and so we next analyzed the effects of Syp1p and Ede1p on individual endocytic sites.

To ensure that we assayed global changes in the endocytic machinery and not defects in recruitment of individual proteins, we analyzed lifetimes of several patch proteins in *syp1Δ* and *ede1Δ* cells. Dynamics were analyzed outside polarized regions because of the difficulties in imaging individual patches in densely-packed areas of the cell. The proteins analyzed were Las17-GFP, Sla2-GFP, Ent2-GFP, Ent1-GFP, and Sla1-GFP. The mammalian homologues of these proteins are N-WASP (Las17p), HIP1r (Sla2p), and the epsins (Ent1/2p). We measured a decrease in Sla1-GFP and Las17-GFP patch lifetimes in *ede1Δ* cells relative to wild-type cells of 30% and 32%, respectively ($p < 0.0001$, $n = 30$, Figure 3A). This effect is in agreement with earlier observations of Sla1-GFP in *ede1Δ* cells (Kaksonen *et al.*, 2005). Similar trends were seen for Sla2-GFP and Ent2-GFP, indicating a general role for Ede1p in endocytic site turnover.

Similar patterns were seen in *syp1Δ* cells: the mean Sla1-GFP lifetime decreased by 20% relative to wild-type cells and the Las17-GFP lifetime by 18% ($p < 0.0001$, $n = 30$, Figure 3A). The effects of *ede1Δ* and *syp1Δ* were additive: lifetimes further decreased by 17 and 14% in *syp1Δ ede1Δ* double mutants relative to *ede1Δ* cells ($p < 0.0001$ for Sla1p, $p = 0.0013$ for Las17p, $n = 30$), a decrease of 43 and 42% from the mean wild-type lifetimes. Again, similar trends were seen for Ent2-GFP and Sla2-GFP.

In the final stages of its lifetime, actin polymerization drives a 200-nm movement of the endocytic patch away from the cortex. This movement is thought to represent membrane invagination (Kaksonen *et al.*, 2003, 2005). Despite their effects on endocytic site lifetime, deletion of *EDE1* and *SYPI* did not affect internalization (Figure 3B): more than 94% of Sla1p and Sla2p patches and more than 80% of Ent2p patches moved inward before disappearing in wild-type, *syp1Δ*, *ede1Δ*, and *syp1Δ ede1Δ* cells (the lower percentage of internalizing Ent2-GFP patches may result from our limited ability to detect this protein's weak fluorescence). In contrast to other markers analyzed, Las17p remains at the surface throughout its lifetime (Kaksonen *et al.*, 2003), and in line with this more than 96% of Las17p patches remained at the surface in wild-type, *syp1Δ*, *ede1Δ*, and *syp1Δ ede1Δ* cells. Thus deletion of *SYPI* or *EDE1* causes similar and marked defects in the lifetimes of endocytic sites, but does not prevent their internalization. Together, these data suggest that Syp1p and Ede1p may moderate endocytic site turnover. The effect of Syp1p on endocytic site lifetime is less severe than that of Ede1p, which may explain why its absence does not cause a bulk endocytic defect. That the effects of Syp1p and Ede1p are additive suggests that, though the two proteins interact and share dynamics, they each provide some distinct functionality.

The final endocytic component we analyzed was Ent1-GFP. Similarly to other components of the endocytic machinery the mean Ent1-GFP patch lifetime in *syp1Δ* cells decreased relative to wild-type cells by 24% ($p < 0.0001$, $n = 30$, Figure 3A). Deletion of *SYPI* did not affect internalization of Ent1-GFP patches (Figure 3B): more than 96% of Ent1-GFP patches internalized in wild-type and *syp1Δ* cells.

Figure 3. The endocytic role of Syp1p and Ede1p. (A) Patch lifetimes of Las17-GFP, Sla1-GFP, Sla2-GFP, Ent1-GFP, and Ent2-GFP in wild-type, *syp1Δ*, *ede1Δ*, and *syp1Δ ede1Δ* cells (strains DDY2736, -3696, -3697, -3700, -3701, -3798, and -3872–3885). $n = 30$ patches for each strain. Error bars, SD. Frame rate was 1 frame per second. (B) Kymographs of representative Las17-GFP, Sla1-GFP, Sla2-GFP, Ent1-GFP, and Ent2-GFP patches from epifluorescence movies of wild-type, *syp1Δ*, *ede1Δ*, and *syp1Δ ede1Δ* cells (the same strains listed in A). Patches are oriented so the outside of the cell is on top. (C) Epifluorescence images of live wild-type *syp1Δ*, *ede1Δ*, and *syp1Δ ede1Δ* cells expressing Ent1-GFP or Ent2-GFP (strains DDY3696, -3697, and -3880–3885). Top, small-budded cells; bottom, large-budded cells. Scale bars, 2 μ m.

In *ede1Δ* cells, however, patch localization outside the neck was largely lost, and fluorescence was distributed diffusely around the cortex and concentrated strongly at the neck of small-budded cells (Figure 3C). Curiously, in *syp1Δ ede1Δ* cells, Ent1-GFP again localized to patches outside the neck,

Figure 4. Localization interdependence of Syp1p and Ede1p. (A) Epifluorescence images of wild-type and *ede1Δ* cells expressing Syp1-GFP (strains DDY3865 and -3869). (B) Dynamic surface localization of Syp1-GFP in wild-type and *ede1Δ* cells viewed by TIRF microscopy. Left, single frames from movies; Right, kymograph representations of the same movies. Movies were taken with 1-s frame intervals for 2 min. (C) Epifluorescence images of small- (top), medium- (middle), and large-budded (bottom) wild-type and *syp1Δ* cells expressing Ede1-GFP (strains DDY3866 and -3870). Arrowheads, the bud neck region affected by *SYP1* deletion. (D) Cell cycle distribution of Syp1-GFP and Ede1-RFP in wild-type cells (strain DDY3871). Individual cells were imaged every 10 min for 2 h, and one image is shown for every 20 min (the full sequence can be seen in Supplemental Movie S2); green and red images were acquired immediately after one another. White arrowheads, the first signs of neck localization during bud emergence (a second cell cycle has begun at 120 min); red arrowheads, localization to the bud neck at cytokinesis. Scale bars, 2 μ m throughout.

albeit more faintly than in wild-type cells. Similarly to other patch markers, Ent1-GFP lifetimes further decreased in *syp1Δ ede1Δ* cells, by 46% relative to wild-type cells, without any change in their internalization (Figure 3, A and B). Null alleles of *ENT1* and *ENT2* are synthetically lethal, and the two proteins are believed to be functionally redundant (Wendland *et al.*, 1999; Toret *et al.*, 2008). Intriguingly, however, Ent2p remained in patches in all strains (Figure 3C). Thus Syp1p and Ede1p have a specific role in Ent1p localization at the bud neck in addition to Syp1p's more general role in Ent1p turnover at endocytic sites. These results highlight a novel difference between the yeast epsins Ent1p and Ent2p.

Distribution of Syp1p and Ede1p

That the endocytic defects in Syp1p and Ede1p cells were additive suggested that though they have similar dynamics, the two proteins have some separable activity. We next

tested if their localization was interdependent. We first analyzed the role of Ede1p in Syp1p localization. In *ede1Δ* cells, as in wild-type cells, Syp1-GFP was strongly concentrated at the necks of small- medium-sized buds (Figure 4A; small-medium buds were defined as those in which the ratio of the daughter:mother cell radius < 0.5). However, distinct Syp1-GFP patches were not visible around the cell. Instead, cortical Syp1-GFP was diffusely localized with polarization toward the daughter cell. TIRF microscopy revealed that Syp1-GFP formed dynamic foci at the cortex of *ede1Δ* cells, but that these foci were unstable, disappearing within 3 s of appearance (Figure 4B, Supplemental Movie S1). Thus, Ede1p is not required for recruitment of Syp1p to the plasma membrane, but is required for its stable association with endocytic sites.

We next examined Ede1p localization in *syp1Δ* cells. Like Syp1p, Ede1p patches are strongly concentrated at the necks

of cells with small-medium-sized buds (Figure 1A). In *syp1Δ* cells this intense concentration was lost, and Ede1-GFP was only poorly recruited to the neck (arrowheads, Figure 4C). Ede1p was, however, efficiently recruited into endocytic sites in other regions of the cell, and other aspects of endocytic polarization remained unchanged (Figure 4C). These data indicate that Syp1p affects Ede1p recruitment, but only to the bud neck of cells with small-medium-sized buds.

In an attempt to better understand how Syp1p affects polarization of Ede1p to the neck of small-medium-sized buds specifically, we decided to more closely examine Syp1p and Ede1p distribution throughout the cell cycle. We imaged wild-type cells coexpressing Syp1-GFP and Ede1-RFP for 2 h. Both Syp1p and Ede1p patches were concentrated at the neck of small-medium-sized buds, and toward the daughter cell (Figure 4D and Supplemental Movie S2). There was, however, a clear difference in distribution of the two proteins in large-budded cells: the concentration of patches at the bud neck before and after cytokinesis was clearly evident for Ede1-GFP, but was faint for Syp1-RFP. Thus Syp1p is not evenly distributed across endocytic sites and recruits Ede1p to the bud neck region to which it is most intensely concentrated. This is the first report of a yeast endocytic protein that is not uniformly distributed among endocytic sites.

Ede1p and Syp1p Direct Endocytic Site Formation and Distribution

Previously, *ede1Δ* cells were shown to form fewer Sla1p-labeled endocytic sites than wild-type cells (Kaksonen *et al.*, 2005). Together with our observation that Syp1p targets a pool of Ede1p to necks of small-budded cells, these observations suggested roles for Ede1p and Syp1p in endocytic site formation and placement, respectively. To test this hypothesis, we next analyzed the effects of Syp1p and Ede1p on the number and placement of endocytic sites.

We first assessed the number of endocytic sites in *syp1Δ* and *ede1Δ* cells. Maximum intensity Z-projections of live cells were analyzed, as previously described, to determine the average number of patches per surface area (Kaksonen *et al.*, 2005). Several endocytic components were analyzed, and patch densities were quantified only in unpolarized cells in which individual patches are distinguishable (representative Sla1-GFP images are shown in Figure 5A; others are in Supplemental Figure S2). In *ede1Δ* cells there was a 46% decrease in the number of Sla1-GFP patches per surface area relative to wild-type cells (Figure 5B). A similar decrease was seen for Las17-GFP-, Ent2-GFP-, and Sla2-GFP-labeled patches. Ent1-GFP patch densities were not quantified because of its altered distribution in *ede1Δ* cells (Figure 3B). These data reveal that Ede1p has a general role in endocytic site formation. In contrast, patch density in *syp1Δ* or *syp1Δ ede1Δ* cells was unchanged relative to wild-type or *ede1Δ* cells, respectively.

Having already observed that Syp1p recruits Ede1p to necks of small-medium-sized buds, we decided to determine if Syp1p also recruited other patch components to this region of the cell. In maximum intensity Z-projections of wild-type cells, a concentration of endocytic sites was evident at the base of the small-medium-sized buds (Figure 5C). In *syp1Δ* cells, however, there was a decrease in patch density in this region for all markers (Figure 5C, arrowheads). To quantify this effect, we scored polarization of patches in medial focal planes, where the ring of endocytic sites at the bud neck is seen as a patch at either side of the neck (or a bar across the neck if fluorescence is intense). Cells with small-medium-sized buds (in which the ratio of the

daughter:mother cell radius < 0.5) were scored as polarized if at least one patch was present at either side of the neck. In *syp1Δ* cells we measured a consistent decrease in patch polarization for all markers (Figure 5D): the average proportion of cells with Sla1p and Las17p patches at the small bud neck decreased by 32% and 29%, respectively. Thus Syp1p has a general effect on recruitment of endocytic proteins to the small bud neck, rather than a defect only in recruitment of Ede1p. A decrease in bud neck polarization was also observed in *ede1Δ* cells, which is likely a result of the global decrease in patch number. Similar trends were seen for Ent2p, Ent1p, and Sla2p in all strains, although the percentage decreases were lower (Figure 5D). Because polarization of endocytic sites is often described as actin patch polarization (Pruyne and Bretscher, 2000a,b), we additionally analyzed Abp1-GFP (which marks endocytic actin) and found its distribution to be similarly affected, with a 29% average decrease in the proportion of cells with endocytic sites at the small bud neck (Figure 5D). These data indicate that Syp1p has a role not only in Ede1p distribution, but in placement of endocytic sites in general, to the necks of cells with small-medium-sized buds.

DISCUSSION

Here we describe Syp1p as a conserved early-arriving endocytic protein that shares novel dynamics with Ede1p. Syp1p and Ede1p play roles in the formation and placement, respectively, of sites of clathrin-mediated endocytosis in budding yeast.

Syp1p and Ede1p: The Early Module

Previously, Syp1p was shown to localize to the bud neck in a septin-dependent manner (Qiu *et al.*, 2008). We find that bud neck Syp1p colocalizes with the endocytic protein Ede1p and that Syp1p is present in endocytic sites throughout the cell. Based on their dynamics, yeast endocytic proteins can be considered as belonging to distinct modules (Figure 6A). By analyzing endocytic sites in unpolarized regions of the cell, we found that Syp1p and Ede1p share novel endocytic dynamics. Both Syp1p and Ede1p arrive early at endocytic sites, 30 s to 210 s before initiation of the actin nucleation that powers internalization (Figure 6A). The only other yeast protein with similar endocytic timing is clathrin, which has similarly long and variable lifetimes (Kaksonen *et al.*, 2005). However, Syp1p and Ede1p remain at the cortex throughout their lifetime and disappear shortly after actin assembly begins, whereas clathrin persists and internalizes with the vesicle. Clathrin has been considered a component of the coat module, but its early arrival suggests it may link the coat module to the early stages of endocytic site formation marked by Syp1p and Ede1p (Figure 6A). Before this work, Ede1p did not share dynamics with known endocytic components and so did not fall into the defined modules. Based on their unique shared dynamics, we define Syp1p and Ede1p as components of an “early” endocytic module (Figure 6A).

In addition to shared dynamics, Syp1p and Ede1p have partially interdependent localizations. Ede1p is required for Syp1p's stable recruitment to endocytic sites, although Syp1p is still at the cortex in *ede1Δ* cells. This result implies that Syp1p reaches the cortex independently of Ede1p, possibly through Syp1p's putative F-BAR domain. How Ede1p recognizes the cortex is unknown, but in *syp1Δ* cells, Ede1p—along with other endocytic proteins—is less efficiently recruited to the neck of the emerging bud. Because Syp1p affects general recruitment of endocytic sites to necks

Figure 5. The early module regulates endocytic site formation and distribution. (A) Maximum intensity projections of Z-stacks of wild-type, *syp1*Δ, *ede1*Δ, and *syp1*Δ *ede1*Δ cells expressing Sla1-GFP (strains DDY3700, -3798, -3875, and -3876). Z-stacks were acquired through the entire cell at 0.15- μm intervals. Scale bar, 2 μm . (B) Patch number per cell surface area (μm^2) in wild-type, *syp1*Δ, *ede1*Δ, and *syp1*Δ *ede1*Δ cells expressing the indicated GFP-tagged markers (strains DDY2736, -3696, -3697, -3700, -3701, -3798, and -3872-3885). $n = 20$ cells for each strain; error bars, SD. Patches were counted in approximately spherical unbudded or large-budded cells; cell surface area was estimated as an average of sphere surface areas calculated from four diameters measured from the maximum intensity projections. (C) Maximum intensity projections of Z-stacks of small-budded wild-type, *syp1*Δ, *ede1*Δ, and *syp1*Δ *ede1*Δ cells expressing the indicated GFP-tagged markers (strains DDY2736, -3696, -3697, -3700, -3701, -3798, and -3872-3889). Z-stacks were acquired through the entire cell with 0.15- μm intervals. Arrowheads indicate the bud neck region affected by *SYP1* deletion. Scale bar, 2 μm . (D) Percentage of wild-type, *syp1*Δ, *ede1*Δ, and *syp1*Δ *ede1*Δ cells expressing the indicated GFP-tagged markers with patches polarized to the small-budded neck (using the same strains listed in C). Mean averages from three separate experiments are shown ($n = 60$ cells for each strain in each experiment; error bars, SD). Cells were scored as described in the text.

of emerging buds, we cannot determine if its effects on Ede1p are specific. However, given their reported interac-

tion and the unlikelihood of later-arriving components recruiting Ede1p, it may be that Syp1p directly recruits Ede1p

Figure 6. Model for the role of the early module in endocytic site formation. (A) Temporal relationship of the endocytic modules including the newly defined early module. (B) Roles and interactions for the early module in endocytic site organization. In our model, Ede1p and Syp1p interact with the endocytic and polarity machinery, respectively, to initiate endocytic site formation and control spatial distribution.

to the bud neck (Figure 6B). It is clear, however, that Syp1p and Ede1p cannot have an obligate interaction, as Syp1p is not distributed evenly across endocytic sites with Ede1p.

We found proteins with similarity to Syp1p throughout metazoans, one of which, SGIP1- α , is an established component of clathrin-coated pits (Uezu *et al.*, 2007). Real-time analysis showed that SGIP1- α arrives early at endocytic sites together with clathrin. Furthermore, SGIP1- α was previously shown to bind to the endocytic adapter Eps15, an Ede1p homologue (Polo *et al.*, 2003), suggesting that the early module is conserved. Our data reveal new complexity in the early stages of endocytic site formation in mammals and highlight unrecognized similarities between the yeast and mammalian systems.

Ede1p and Syp1p Modulate Endocytic Site Formation and Spatial Distribution

We observed a severe decrease in the number of endocytic sites formed in *ede1 Δ* cells that agrees with earlier reports for Sla1p-labeled sites (Kaksonen *et al.*, 2005). Deletion of clathrin subunits also decreases endocytic site formation (Kaksonen *et al.*, 2005). Whether clathrin and Ede1p operate redundantly remains to be explored, but there is evidence that their roles are different. Deletion of clathrin impairs formation of Sla1p and Las17p patches, but not Sla2p patches (Kaksonen *et al.*, 2005; Newpher and Lemmon, 2006). In contrast, we found that deletion of *EDE1* reduced patch number for all proteins tested, suggesting that Ede1p has a more central role than clathrin in endocytic site formation.

The only patch formation defect in *syp1 Δ* cells was at the neck of the emerging bud, where Syp1p is intensely concentrated. In *syp1 Δ* cells the intense bar of Ede1-GFP fluorescence at the small-budded neck was lost and Ede1p was present only in fainter patches. A defect in recruitment to the neck was also seen for late-arriving markers, showing that like Ede1p, Syp1p affects a broad spectrum of endocytic proteins. Deletion of *SYP1* resulted in a 30% reduction in the

number of cells with late-arriving endocytic markers at the small-budded neck. Our quantification may underestimate penetrance of the defect (because of challenges in discerning whether patches are at or near the bud neck and because some endocytic sites are likely to form at the neck by chance even without targeting), but Syp1p has a clear role in endocytic site formation at the bud neck. Thus the early module components help direct endocytic site formation and placement: two processes expected to be regulated early in the life of the endocytic site.

A Specific Role for Syp1p at the Bud Neck

Endocytic polarization in yeast has long been observed and is known to lie downstream of the Rho GTPase Cdc42p (Adams *et al.*, 1990; Lechler *et al.*, 2001). However, the reasons for endocytic polarization and the means by which it is specified are unknown. As an endocytic component that interacts with the cell polarity machinery (Tarassov *et al.*, 2008), Syp1p is a candidate to link the two processes. The septins recruit Syp1p to the bud neck (Qiu *et al.*, 2008), where in turn we find that Syp1p plays a role in endocytic site recruitment. Syp1p's endocytic role provides a potential explanation for the recently reported *syp1 Δ* septin turnover defect (Qiu *et al.*, 2008). Syp1p could act as an adapter, recognizing the septin ring and directing endocytic site formation to the bud neck. Syp1p would then promote endocytosis at the bud neck, which might allow turnover of membrane components that regulate septin stability.

Because Ede1p is a major factor in endocytic site formation and because of its reported interaction with Syp1p and their similar dynamics, we suggest that Syp1p may stimulate bud neck endocytic sites formation through recruitment of Ede1p (Figure 6B). Although we cannot determine if the effects of Syp1p on Ede1p at the bud neck are specific, the unlikelihood of later-arriving proteins recruiting Ede1p makes this a plausible model. Of course, we do not rule out the possibility that Syp1p acts through other mechanisms. In *ede1* null cells, there are fewer bud neck endocytic sites than

in wild-type cells, despite Syp1p's intense concentration at the neck: although this result must be interpreted with caution—because there are fewer endocytic sites throughout *ede1* null cells—it is clear that Syp1p cannot mediate normal bud neck endocytic site formation in the absence of Ede1p. Unfortunately, individual sites cannot be distinguished in the bud neck, and so we cannot analyze the specific effects of Syp1p or Ede1p on individual endocytic site dynamics in this region. That some Ede1p is still present at the bud neck in *syp1Δ* cells suggests that another mechanism acts redundantly with Syp1p in Ede1p recruitment to the neck. That morphological defects are not observed in *syp1Δ* cells (this work; Marcoux *et al.*, 2000; Qiu *et al.*, 2008) reinforces the idea that Ede1p or other components must help initiate endocytosis at the bud neck.

In line with its role in patch polarization, Syp1p is unevenly distributed across endocytic sites—its fluorescence at necks is intense in small-budded cells, and faint during cytokinesis, when other endocytic components are highly concentrated. Syp1p is the only endocytic component so far described to be enriched at a subset of actin patches (Pruyne and Bretscher, 2000a,b), providing the first indication that all yeast endocytic sites are not identical, and the first indication of how spatial regulation of endocytic sites might be achieved. Whether other early-arriving proteins localize endocytic sites to other regions of the cell or if this Syp1p function is conserved remains to be determined.

The Early Module Influences Endocytic Site Lifetime

By analyzing endocytic sites outside the neck and small bud, we determined that Ede1p and Syp1p influence endocytic site turnover around the cell in addition to their roles in endocytic site formation and placement. Clathrin, Syp1p, and Ede1p are all present during an early phase of the endocytic site, in which their lifetimes are variable. Endocytic sites transition to a phase of regular duration upon arrival of the WASP/myo module proteins (Figure 6A; Kaksonen *et al.*, 2005). Deletion of *EDE1* or *SYP1* shortened lifetimes of Sla1p, Las17p, Ent2p, and Sla2p, all markers of the regular phase. Thus, at least in unpolarized areas of the cell, Syp1p and Ede1p decrease turnover of proteins in the coat and WASP/myo modules. These data reveal that Syp1p and Ede1p both have dual roles: one in endocytic site turnover around the cell and another in site formation (Ede1p) or placement (Syp1p).

Deletion of *SYP1* and *EDE1* did not affect the patch internalization that is thought to represent vesicle invagination (Kaksonen *et al.*, 2003, 2005), making it unlikely that shortened patch lifetimes are caused by instability or a failure in vesicle formation. Instead, the time from onset of the regular phase to the onset of internalization is reduced, suggesting that Syp1p and Ede1p moderate site maturation before invagination. Mutation of clathrin also reduces lifetimes of Sla1p and Las17p patches without affecting internalization (Kaksonen *et al.*, 2005), suggesting a general role for the early endocytic machinery in modulating site turnover. Interestingly, knockdown of the Ede1p homolog Eps15, decreases turnover of endocytic sites in animal cells (Mettlen *et al.*, 2010). A greater understanding of the differences between the two systems will be required to understand why Eps15 and Ede1p have seemingly opposite effects.

The epsin, Ent1p, is affected similarly to other endocytic markers by *SYP1* deletion, but is mislocalized in *ede1Δ* cells. This mislocalization is reversed by additional *SYP1* deletion. A possible explanation for these results is an Ent1p–Syp1p interaction that is usually masked by Ede1p. In the absence of Ede1p, Syp1p, and Ent1p are localized similarly: if Syp1p

were responsible for Ent1p mislocalization, this would explain its reversal upon *SYP1* deletion. Ent1p binds the lipid PIP₂ (Aguilar *et al.*, 2003), which may explain the weak patch localization of Ent1p in the absence of both Syp1p and Ede1p. This finding shows a difference between the redundantly lethal epsins Ent1p and Ent2p.

Despite defects in endocytic dynamics, and unlike *EDE1* deletion, *SYP1* deletion did not impair endocytic uptake. Syp1p is thus a member of the growing number of yeast proteins considered core components of the endocytic machinery solely because of their effects on patch dynamics (e.g., Abp1p and capping protein; Kaksonen *et al.*, 2005). That Syp1p does not have the same effects on endocytic uptake as Ede1p might reflect the more modest effects of Syp1p on endocytic lifetimes, or a cell cycle- or cargo-specific role at the bud neck. Deletion of *SYP1* does not affect recruitment of Ede1p outside the neck, and so it is unsurprising that it does not perturb endocytic uptake via Ede1p. In support of a potential cargo selective role for Syp1p, knockdown of its homolog SGIP1- α in mammalian cells affects uptake of transferrin but not EGF, even though both are cargos for clathrin-mediated endocytosis (Uezu *et al.*, 2007). A general role may exist for cargo binding in regulating transition to the regular phase of vesicle formation, similar to the cargo-stabilizing phenomenon that acts early in endocytic site formation in mammals (Ehrlich *et al.*, 2004; Loerke *et al.*, 2009). In support of this possibility the budding yeast endocytic cargo alpha factor accumulates at Ede1p patches before actin assembly (Toshima *et al.*, 2006). Further studies of early-arriving endocytic proteins will illuminate the principles governing endocytic site initiation and turnover.

ACKNOWLEDGMENTS

We thank H. Nakanishi (Kumamoto University, Kumamoto, Japan) for the gift of SGIP1- α expression plasmids and W. Almers (Vollum Institute, Oregon Health & Science University, Portland, OR) for the Swiss 3T3 cells expressing DsRed-clathrin. We also thank members of the Drubin and Barnes labs for advice, reagents, and helpful discussions, particularly Voytek Okreglak, Yidi Sun, and Susheela Carroll for critical reading of the manuscript. This work was supported by National Institutes of Health Grants GM50399 and GM65462 to D.G.D., and B.S.P. was supported by a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft.

REFERENCES

- Adams, A. E., Johnson, D. I., Longnecker, R. M., Sloat, B. F., and Pringle, J. R. (1990). CDC42 and CDC43, two additional genes involved in budding and the establishment of cell polarity in the yeast *Saccharomyces cerevisiae*. *J. Cell Biol.* 111, 131–142.
- Aguilar, R. C., Watson, H. A., and Wendland, B. (2003). The yeast Epsin Ent1 is recruited to membranes through multiple independent interactions. *J. Biol. Chem.* 278, 10737–10743.
- Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25, 3389–3402.
- Belmont, L. D., and Drubin, D. G. (1998). The yeast V159N actin mutant reveals roles for actin dynamics in vivo. *J. Cell Biol.* 142, 1289–1299.
- Collins, S. R., Kemmeren, P., Zhao, X. C., Greenblatt, J. F., Spencer, F., Holstege, F. C., Weissman, J. S., and Krogan, N. J. (2007). Toward a comprehensive atlas of the physical interactome of *Saccharomyces cerevisiae*. *Mol. Cell Proteom.* 6, 439–450.
- Corpet, F. (1988). Multiple sequence alignment with hierarchical clustering. *Nucleic Acids Res.* 16, 10881–10890.
- Ehrlich, M., Boll, W., Van Oijen, A., Hariharan, R., Chandran, K., Nibert, M. L., and Kirchhausen, T. (2004). Endocytosis by random initiation and stabilization of clathrin-coated pits. *Cell* 118, 591–605.
- Engqvist-Goldstein, A. E., Kessels, M. M., Chopra, V. S., Hayden, M. R., and Drubin, D. G. (1999). An actin-binding protein of the Sla2/Huntingtin inter-

- acting protein 1 family is a novel component of clathrin-coated pits and vesicles. *J. Cell Biol.* 147, 1503–1518.
- Finn, R. D., Tate, J., Mistry, J., Coghill, P. C., Sammut, S. J., Hotz, H. R., Ceric, G., Forslund, K., Eddy, S. R., Sonnhammer, E. L., and Bateman, A. (2008). The Pfam protein families database. *Nucleic Acids Res.* 36, D281–288.
- Gagny, B., Wiederkehr, A., Dumoulin, P., Winsor, B., Riezman, H., and Haguenauer-Tsapis, R. (2000). A novel EH domain protein of *Saccharomyces cerevisiae*, Ede1p, involved in endocytosis. *J. Cell Sci.* 113(Pt 18), 3309–3319.
- Gavin, A. C., et al. (2002). Functional organization of the yeast proteome by systematic analysis of protein complexes. *Nature* 415, 141–147.
- Gheorghe, D. M., Aghamohammadzadeh, S., Smaczynska-de, R., II, Allwood, E. G., Winder, S. J., and Ayscough, K. R. (2008). Interactions between the yeast SM22 homologue Scp1 and actin demonstrate the importance of actin bundling in endocytosis. *J. Biol. Chem.* 283, 15037–15046.
- Henne, W. M., Kent, H. M., Ford, M. G., Hegde, B. G., Daumke, O., Butler, P. J., Mittal, R., Langen, R., Evans, P. R., and McMahon, H. T. (2007). Structure and analysis of FCHO2 F-BAR domain: a dimerizing and membrane recruitment module that effects membrane curvature. *Structure* 15, 839–852.
- Hinrichsen, L., Harborth, J., Andrees, L., Weber, K., and Ungewickell, E. J. (2003). Effect of clathrin heavy chain- and alpha-adaptin-specific small inhibitory RNAs on endocytic accessory proteins and receptor trafficking in HeLa cells. *J. Biol. Chem.* 278, 45160–45170.
- Huang, K. M., D'Hondt, K., Riezman, H., and Lemmon, S. K. (1999). Clathrin functions in the absence of heterotetrameric adaptors and AP180-related proteins in yeast. *EMBO J.* 18, 3897–3908.
- 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.
- 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.
- Keyel, P. A., Watkins, S. C., and Traub, L. M. (2004). Endocytic adaptor molecules reveal an endosomal population of clathrin by total internal reflection fluorescence microscopy. *J. Biol. Chem.* 279, 13190–13204.
- Krogan, N. J., et al. (2006). Global landscape of protein complexes in the yeast *Saccharomyces cerevisiae*. *Nature* 440, 637–643.
- Lambert, A. A., Perron, M. P., Lavoie, E., and Pallotta, D. (2007). The *Saccharomyces cerevisiae* Arf3 protein is involved in actin cable and cortical patch formation. *FEMS Yeast Res.* 7, 782–795.
- 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.
- Lewis, M. J., Nichols, B. J., Prescianotto-Baschong, C., Riezman, H., and Pelham, H. R. (2000). Specific retrieval of the exocytic SNARE Snc1p from early yeast endosomes. *Mol. Biol. Cell* 11, 23–38.
- Loerke, D., Mettlen, M., Yarar, D., Jaqaman, K., Jaqaman, H., Danuser, G., and Schmid, S. L. (2009). Cargo and dynamin regulate clathrin-coated pit maturation. *PLoS Biol.* 7, e57.
- Longtine, M. S., McKenzie, A., 3rd, Demarini, D. J., Shah, N. G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J. R. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. *Yeast* 14, 953–961.
- Marcoux, N., Cloutier, S., Zakrzewska, E., Charest, P. M., Bourbonnais, Y., and Pallotta, D. (2000). Suppression of the profilin-deficient phenotype by the RHO2 signaling pathway in *Saccharomyces cerevisiae*. *Genetics* 156, 579–592.
- McGuffin, L. J., Bryson, K., and Jones, D. T. (2000). The PSIPRED protein structure prediction server. *Bioinformatics* 16, 404–405.
- 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.
- Mettlen, M., Stoeber, M., Loerke, D., Antonescu, C. N., Danuser, G., and Schmid, S. L. (2009). Endocytic accessory proteins are functionally distinguished by their differential effects on the maturation of clathrin-coated pits. *Mol. Biol. Cell* 20, 3251–3260.
- Motley, A., Bright, N. A., Seaman, M. N., and Robinson, M. S. (2003). Clathrin-mediated endocytosis in AP-2-depleted cells. *J. Cell Biol.* 162, 909–918.
- Newpher, T. M., and Lemmon, S. K. (2006). Clathrin is important for normal actin dynamics and progression of Sla2p-containing patches during endocytosis in yeast. *Traffic* 7, 574–588.
- 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.
- Polo, S., Confalonieri, S., Salcini, A. E., and Di Fiore, P. P. (2003). EH and UIM: endocytosis and more. *Sci. STKE* 2003, re17.
- Pruyne, D., and Bretscher, A. (2000a). Polarization of cell growth in yeast. *J. Cell Sci.* 113(Pt 4), 571–585.
- Pruyne, D., and Bretscher, A. (2000b). Polarization of cell growth in yeast. I. Establishment and maintenance of polarity states. *J. Cell Sci.* 113(Pt 3), 365–375.
- Qiu, W., Neo, S. P., Yu, X., and Cai, M. (2008). A novel septin-associated protein, Syp1p, is required for normal cell cycle-dependent septin cytoskeleton dynamics in yeast. *Genetics* 180, 1445–1457.
- Rice, P., Longden, I., and Bleasby, A. (2000). EMBOSS: The European Molecular Biology Open Software Suite. *Trends Genet.* 16, 276–277.
- Robertson, A. S., Allwood, E. G., Smith, A. P., Gardiner, F. C., Costa, R., Winder, S. J., and Ayscough, K. R. (2009). The WASP homologue Las17 activates the novel actin-regulatory activity of Ysc84 to promote endocytosis in yeast. *Mol. Biol. Cell* 20, 1618–1628.
- Sakaushi, S., Inoue, K., Zushi, H., Senda-Murata, K., Fukada, T., Oka, S., and Sugimoto, K. (2007). Dynamic behavior of FCHO1 revealed by live-cell imaging microscopy: its possible involvement in clathrin-coated vesicle formation. *Biosci. Biotechnol. Biochem.* 71, 1764–1768.
- Soding, J., Biegert, A., and Lupas, A. N. (2005). The HHpred interactive server for protein homology detection and structure prediction. *Nucleic Acids Res.* 33, W244–248.
- Stefan, C. J., and Blumer, K. J. (1999). A syntaxin homolog encoded by VAM3 mediates down-regulation of a yeast G protein-coupled receptor. *J. Biol. Chem.* 274, 1835–1841.
- Sun, Y., Carroll, S., Kaksonen, M., Toshima, J. Y., and Drubin, D. G. (2007). PtdIns(4,5)P2 turnover is required for multiple stages during clathrin- and actin-dependent endocytic internalization. *J. Cell Biol.* 177, 355–367.
- Tarassov, K., Messier, V., Landry, C. R., Radinovic, S., Serna Molina, M. M., Shames, I., Malitskaya, Y., Vogel, J., Bussey, H., and Michnick, S. W. (2008). An in vivo map of the yeast protein interactome. *Science* 320, 1465–1470.
- Tebar, F., Sorkina, T., Sorkin, A., Ericsson, M., and Kirchhausen, T. (1996). Eps15 is a component of clathrin-coated pits and vesicles and is located at the rim of coated pits. *J. Biol. Chem.* 271, 28727–28730.
- Toret, C. P., Lee, L., Sekiya-Kawasaki, M., and Drubin, D. G. (2008). Multiple pathways regulate endocytic coat disassembly in *Saccharomyces cerevisiae* for optimal downstream trafficking. *Traffic* 9, 848–859.
- Toshima, J. Y., Toshima, J., Kaksonen, M., Martin, A. C., King, D. S., and Drubin, D. G. (2006). Spatial dynamics of receptor-mediated endocytic trafficking in budding yeast revealed by using fluorescent alpha-factor derivatives. *Proc. Natl. Acad. Sci. USA* 103, 5793–5798.
- 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.
- Uezu, A., et al. (2007). SGIP1alpha is an endocytic protein that directly interacts with phospholipids and Eps15. *J. Biol. Chem.* 282, 26481–26489.
- van Delft, S., Schumacher, C., Hage, W., Verkleij, A. J., and van Bergen en Henegouwen, P. M. (1997). Association and colocalization of Eps15 with adaptor protein-2 and clathrin. *J. Cell Biol.* 136, 811–821.
- Wendland, B., Steece, K. E., and Emr, S. D. (1999). Yeast epsins contain an essential N-terminal ENTH domain, bind clathrin and are required for endocytosis. *EMBO J.* 18, 4383–4393.
- Yu, H., et al. (2008). High-quality binary protein interaction map of the yeast interactome network. *Science* 322, 104–110.