

Analysis of yeast endocytic site formation and maturation through a regulatory transition point

Susheela Y. Carroll, Helen E. M. Stimpson, Jasper Weinberg, Christopher P. Toret, Yidi Sun, and David G. Drubin

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

ABSTRACT The earliest stages of endocytic site formation and the regulation of endocytic site maturation are not well understood. Here we analyzed the order in which the earliest proteins are detectable at endocytic sites in budding yeast and found that an uncharacterized protein, Pal1p/Ydr348cp, is also present at the initial stages of endocytosis. Because Ede1p (homologue of Eps15) and clathrin are the early-arriving proteins most important for cargo uptake, their roles during the early stages of endocytosis were examined more comprehensively. Ede1p is necessary for efficient recruitment of most early-arriving proteins, but not for the recruitment of the adaptor protein Yap1802p, to endocytic sites. The early-arriving proteins, as well as the later-arriving proteins Sla2p and Ent1/2p (homologues of Hip1R and epsins), were found to have longer lifetimes in *CLC1*-knockout yeast, which indicates that clathrin light chain facilitates the transition from the intermediate to late coat stages. Cargo also arrives during the early stages of endocytosis, and therefore its effect on endocytic machinery dynamics was investigated. Our results are consistent with a role for cargo in regulating the transition of endocytic sites from the early stages of formation to the late stages during which vesicle formation occurs.

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INTRODUCTION

Endocytosis is the process by which cells internalize proteins and lipids from the plasma membrane and molecules from the surrounding environment. Live-cell fluorescence microscopy in *Saccharomyces cerevisiae* and mammalian cells has revealed dynamics of the machinery that drives endocytosis (Gaidarov *et al.*, 1999; Merrifield *et al.*, 2002, 2005; Kaksonen *et al.*, 2003; Rappoport and Simon, 2003; Rappoport *et al.*, 2005; Ehrlich *et al.*, 2004; Keyel *et al.*, 2004; Yasar *et al.*, 2005; Henne *et al.*, 2010; reviewed in Perrais and Merrifield, 2005). Protein recruitment to yeast endocytic sites occurs in a highly predictable order, and in mammalian and yeast cells recruitment signatures of groups of endocytic proteins have also been identified (Kaksonen *et al.*, 2005; Taylor *et al.*, 2011). Knowledge of

endocytic protein spatiotemporal dynamics has provided valuable insight into the mechanisms underlying endocytic vesicle formation in yeast and mammalian cells (Merrifield *et al.*, 2005; Newpher *et al.*, 2005; Sun *et al.*, 2006; Barker *et al.*, 2007; Ferguson *et al.*, 2009; Liu *et al.*, 2009; Smaczynska-de *et al.*, 2010). In budding yeast, >40 proteins have been shown to form “patches” at endocytic sites. These proteins have been classified into the early, coat, WASP/Myo, amphiphysin, or actin modules based on when the proteins arrive at endocytic sites, the length of time they are detectable at the cell surface (termed patch “lifetime”), and their spatial dynamics at endocytic sites (Kaksonen *et al.*, 2005; Carroll *et al.*, 2009; Stimpson *et al.*, 2009; summarized in Tonikian *et al.*, 2009).

There has recently been much interest in the early stages of endocytic site formation in both yeast and mammalian cells, specifically focusing on proteins that are likely to be involved in site establishment (Boettner *et al.*, 2009; Reider *et al.*, 2009; Stimpson *et al.*, 2009; Henne *et al.*, 2010). In mammalian cells, the proteins Fer/Cip4 homology domain–only proteins 1 and 2 (FCHO1/2), Eps15, and intersectin are present during the earliest known stages of clathrin coat assembly and are believed to be important in the nucleation of endocytic sites (Henne *et al.*, 2010). In comparison, Ede1p (an Eps15 homologue), Syp1p (an FCHO1/2 homologue), clathrin, and the AP2 complex are among the first proteins to appear at nascent

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

Abbreviations used: CCP, clathrin-coated pit; FCHO1/2, Fer/Cip4 homology domain–only proteins 1 and 2; TIRF, total internal reflection fluorescence.

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endocytic sites in budding yeast (Kaksonen *et al.*, 2005; Newpher *et al.*, 2005; Toshima *et al.*, 2006; Boettner *et al.*, 2009; Carroll *et al.*, 2009; Reider *et al.*, 2009; Stimpson *et al.*, 2009). However, the order in which these proteins arrive at endocytic sites with respect to each other, and their roles in early site formation, have not been thoroughly studied. An exploration of the early endocytic stages in budding yeast will elucidate mechanisms that regulate endocytic site establishment.

Proteins that arrive early during endocytic site formation have variable patch lifetimes that range from 30 s to 4 min, whereas later-arriving proteins have highly regular patch lifetimes that generally last 10–40 s (Kaksonen *et al.*, 2005; Newpher *et al.*, 2005; Carroll *et al.*, 2009; Stimpson *et al.*, 2009). This observation suggests that a transition point exists past which endocytic vesicle formation cannot progress until some event triggers recruitment and/or activation of later-acting components of the endocytic machinery that drive the process to completion. The transition point in yeast endocytosis may be similar to a proposed endocytic checkpoint acting during clathrin-coated pit (CCP) maturation in mammalian cells (Loerke *et al.*, 2009). Studies in mammalian cells have shown that various endocytic cargo molecules and their adaptors have differing effects on CCP maturation and therefore might be influencing progression past an endocytic checkpoint (Ehrlich *et al.*, 2004; Loerke *et al.*, 2009; Mettlen *et al.*, 2009, 2010). Because in yeast endocytic cargoes are believed to arrive during the early phase of endocytosis (Toshima *et al.*, 2006), they may also have a role in promoting early endocytic-site progression. Furthermore, the absence of clathrin in yeast cells causes some later-arriving proteins to have shorter patch lifetimes and others to have extended lifetimes, suggesting that clathrin has a role in endocytic site maturation (Kaksonen *et al.*, 2005; Newpher and Lemmon, 2006; Newpher *et al.*, 2006). This complex role for clathrin is also evident in animal cells in which knockdowns of clathrin light chain *a* and *b* isoforms have different effects on CCP dynamics (Mettlen *et al.*, 2009). A more comprehensive study of the roles of the early-arriving proteins and endocytic cargo in regulating early endocytic stages promises to provide insight into the initiation stage of endocytosis and how endocytic sites progress through the transition point.

Here we find that the previously unstudied protein Pal1p/Ydr348cp and the adaptor protein Yap1802p arrive together with Ede1p, Syp1p, clathrin, and the AP2 complex during the earliest stage of endocytic site formation. Whereas Ede1p is important for recruitment of most early-arriving proteins to endocytic sites, clathrin light chain is important for transitioning endocytic sites out of the intermediate coat stage marked by Sla2p and Ent1/2p and into the late stages of endocytic site internalization. We also provide evidence that cargo may regulate yeast endocytic site maturation.

RESULTS

Arrival of proteins at the yeast early endocytic site

Ede1p, Syp1p, clathrin, and the AP2 complex are known to arrive at endocytic sites early in the pathway (Kaksonen *et al.*, 2005; Newpher *et al.*, 2005; Boettner *et al.*, 2009; Carroll *et al.*, 2009; Stimpson *et al.*, 2009; Reider *et al.*, 2009; Toshima *et al.*, 2006). We predicted that the uncharacterized protein Ydr348cp might also be an endocytic protein because high-throughput studies have found that it has physical interactions with Ede1p, and Ydr348c-GFP localizes to the cell periphery and bud neck (Gavin *et al.*, 2002, 2006; Huh *et al.*, 2003). Furthermore, Ydr348cp is considered an orthologue of the *Schizosaccharomyces pombe* protein Pal1p, and both proteins share 37% identity over a conserved “Pal1” domain (Ge *et al.*, 2005; Penkett *et al.*, 2006; Finn *et al.*, 2008). Pal1p is implicated in endo-

cytosis since it is located at the cell periphery and has been shown to physically interact with the endocytic protein Sla2p (Ge *et al.*, 2005). On the basis of this information, we here name *S. cerevisiae* Ydr348cp as “Pal1p.” We C-terminally tagged Pal1p with GFP and were able to confirm its interaction with a C-terminally 13Myc tagged-Ede1p by coimmunoprecipitation (Figure 1A). In live cells, Pal1-green fluorescent protein (GFP) forms dynamic patches at the cell surface (Figure 1B, left) that have an average lifetime of 65 ± 35 s ($n = 50$). When the cells are imaged in a medial focal plane, it is clear that these patches internalize from the cortex before disassembling (94%, $n = 50$), which is a characteristic of endocytic coat proteins (Kaksonen *et al.*, 2005). A total of 94% of Sla1-mCherry patches contained Pal1-GFP ($n = 50$), indicating that Pal1-GFP is an endocytic protein present at essentially all endocytic sites (Figure 1C). Pal1-GFP also arrives at endocytic sites before Sla1-mCherry (94%, $n = 47$) (Figure 1E). On the basis of the length of time that Pal1p is present at endocytic sites, Pal1p can be considered as an early-arriving endocytic coat protein.

Yap1801p and Yap1802p, homologues of mammalian AP180, are believed to recruit clathrin to the plasma membrane (Newpher *et al.*, 2005) and therefore may act as early-arriving endocytic proteins. The dynamics of Yap1802p was analyzed to determine whether the yeast AP180s arrive early at endocytic sites. Yap1802-GFP patches have an average lifetime of 63 ± 26 s ($n = 50$) and, like Pal1p, move off the cortex before disassembly (98%, $n = 50$; Figure 1B, right). In a two-color analysis, Yap1802-GFP was present at 96% of Sla1-mCherry patches ($n = 25$), and Yap1802-GFP arrived at endocytic sites before Sla1-mCherry (100%, $n = 24$; Figure 1, D and E). Together these data place Yap1802p early in the coat module.

Dynamics and recruitment of many later-arriving endocytic proteins have been described (Kaksonen *et al.*, 2003, 2005), which has been pivotal in our understanding of endocytic mechanisms. However, a detailed analysis of the arrival of early endocytic proteins, which could aid our understanding of the mechanism of site establishment and progression, is lacking. Two-color total internal reflection fluorescence (TIRF) microscopy was used to determine whether any regular order of recruitment of early proteins to endocytic sites could be detected relative to Ede1-red fluorescent protein (RFP) recruitment. Clathrin light chain (Clc1p), Apl1p, and Yap1802p were used as markers for clathrin, the AP2 complex, and the yeast AP180s, respectively. We found that Syp1-GFP, Clc1-GFP, Apl1-3XGFP, Yap1802-GFP, and Pal1-GFP appear at a similar time as Ede1-RFP in instances when both proteins were clearly visible ($n = 20$ patches for each strain; Figure 1F). There was no consistent pattern in whether the GFP-tagged proteins were detected before or after Ede1-RFP was detected; instead, our results suggest the early endocytic proteins arrive at the nascent endocytic site at approximately the same time (usually within 4 s; Figure 1, F and G). However, the fluorescence signal from these proteins is quite dim and the patches tend to fluctuate in intensity until their disassembly, precluding a definitive statement on whether there is any regular order of arrival. On the basis of these observations, we define a new early coat module comprised of clathrin, the AP2 complex, the yeast AP180s, and Pal1p (Figure 2). The early coat module proteins internalize with the vesicle before disassembly, which is distinct from the early module proteins (Ede1p and Syp1p) that disassemble before coat internalization (Stimpson *et al.*, 2009).

Analysis of the roles of early-arriving proteins in endocytosis

A more comprehensive analysis of the function of the early-arriving proteins is needed to better understand endocytic site formation and progression to later stages. Because the function of Pal1p in

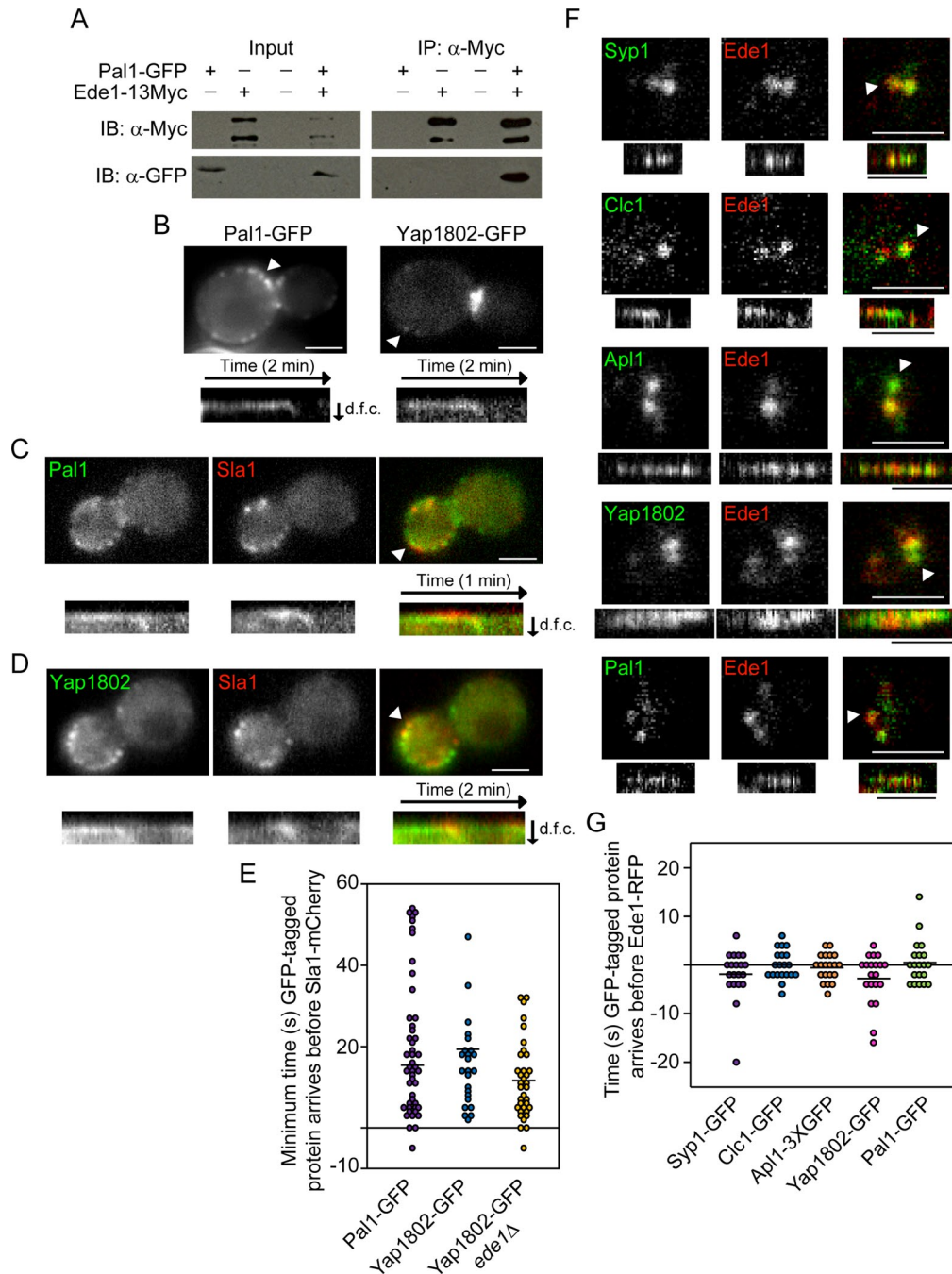


FIGURE 1: Analysis of the yeast early endocytic site. (A) Protein lysates from yeast expressing Pal1-GFP and/or Ede1-13Myc were incubated with beads conjugated to an anti-Myc antibody. Pal1-GFP copurifies with Ede1-13Myc. IB, immunoblot; IP, immunoprecipitation. (B) Epifluorescence images of yeast expressing Pal1-GFP or Yap1802-GFP. Kymographs are of the patches indicated by the white arrowheads. (C) Epifluorescence images of a yeast cell expressing Sla1-mCherry and Pal1-GFP. Kymographs of the patch indicated by the white arrowhead are from two-color movies acquired at a rate of one frame per second. (D) Epifluorescence images of a yeast cell expressing Sla1-mCherry and Yap1802-GFP. Kymographs of the patch indicated by the white arrowhead are from two-color movies acquired at a rate of two frames per second. (E). Minimum amount of time the indicated GFP-tagged protein arrived at endocytic sites before Sla1-mCherry (Pal1-GFP, $n = 47$; Yap1802-GFP, $n = 24$; Yap1802-GFP *ede1* Δ , $n = 35$). In some instances, the GFP-tagged protein was present in the first frame of the movie, so the exact amount of time it was present before Sla1-mCherry arrived is unknown. Negative time represents instances when Sla1-mCherry arrived before the GFP-tagged protein. Horizontal bars represent average time for a group. (F) Two-color TIRF microscopy images of yeast expressing Ede1-RFP and Syp1-GFP, Clc1-GFP, Apl1-3XGFP, Yap1802-GFP, or Pal1-GFP. Kymographs of the patches indicated by the white arrowheads are from two-color movies. Black time bars, 30 s. (G) Amount of time the indicated GFP-tagged protein was detected at sites before Ede1-RFP was detected ($n = 20$ patches for each strain). Negative time represents instances when Ede1-RFP was detected before the GFP-tagged protein. GFP and RFP tagged proteins were first detected within 4 s of each other $\geq 80\%$ of the time ($n = 20$ patches for each strain). Horizontal bars represent average time for a group. All white scale bars, 2 μ m. d.f.c., distance from cortex.

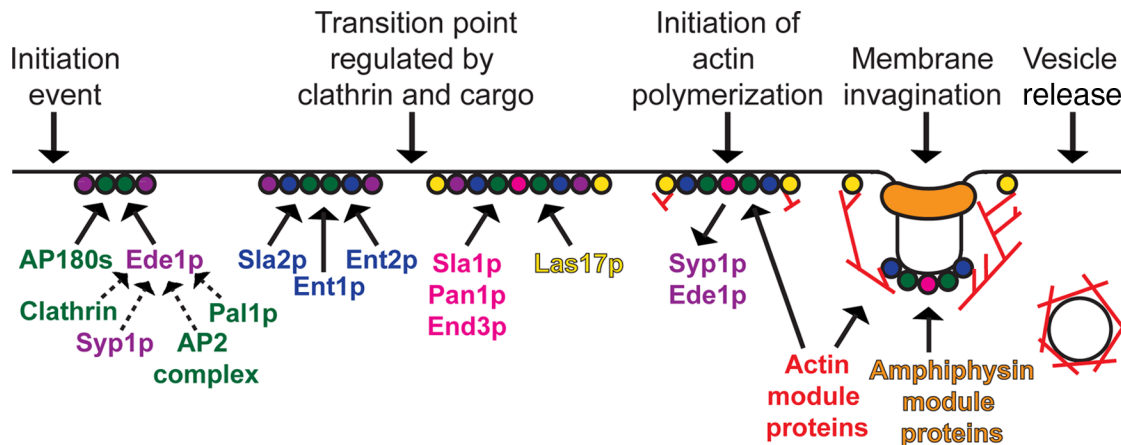


FIGURE 2: Model for the temporal recruitment of endocytic proteins. The early module proteins (Ede1p and Syp1p; purple) and the early coat module proteins (clathrin, the AP2 complex, the yeast AP180s, and Pal1p; green) arrive earliest during endocytic site formation. Ede1p is important for the recruitment of Syp1p, clathrin, the AP2 complex, and Pal1p to endocytic sites. The dashed lines indicate that Ede1p may be involved directly or indirectly in endocytic protein recruitment. Next, the intermediate coat module proteins (Sla2p, Ent1p, and Ent2p; blue) arrive at endocytic sites. Endocytic sites seem to mature through a transition point regulated by clathrin and cargo before the late coat module proteins (Sla1p, Pan1p, and End3p; pink) and Las17p (a WASP/Myo module protein; yellow) are recruited to sites. Ede1p and Syp1p disassemble from endocytic sites at the start of actin polymerization. During membrane invagination, Sla2p, Ent1p, and Ent2p may be involved in connecting the actin network to the vesicle coat and/or plasma membrane.

endocytosis has not been analyzed, the role of Pal1p in fluid-phase and receptor-mediated endocytosis was examined. Similar to what was observed in wild-type cells, *pal1Δ* yeast are able to internalize FM 4-64 and clear fluorescently labeled α -factor from the plasma membrane (Figure 3A). These results show that Pal1p is not required for bulk or receptor-mediated endocytosis. The dynamics of Sla1-GFP was examined in *pal1Δ* yeast to determine how Pal1p affects other components of the endocytic machinery. Unlike *ede1Δ* yeast, in which Sla1-GFP lifetimes and numbers of patches are known to decrease (Kaksonen *et al.*, 2005; Stimpson *et al.*, 2009), the average lifetimes ($n = 50$ patches) and numbers of Sla1-GFP patches ($n = 20$ cells) remain unchanged in *pal1Δ* yeast (Figure 3B).

Deleting *SYP1*, AP2 complex subunits, and *YAP1801/2* does not cause readily detectable defects in endocytic dynamics, but these proteins are important for the internalization of certain cargoes (Huang *et al.*, 1999; Kaksonen *et al.*, 2005; Burston *et al.*, 2009; Carroll *et al.*, 2009; Reider *et al.*, 2009; Stimpson *et al.*, 2009). Ede1p and clathrin, however, are important for proper dynamics of later-arriving endocytic proteins and for efficient cargo uptake (Chu *et al.*, 1996; Gagny *et al.*, 2000; Kaksonen *et al.*, 2005; Newpher and Lemmon, 2006; Stimpson *et al.*, 2009). Therefore we decided to more thoroughly investigate the roles of Ede1p and clathrin in the initiation and maturation of early endocytic sites by assessing the localization and dynamics of early-arriving proteins in *ede1Δ* and *clc1Δ* yeast.

Clc1-GFP, Apl1-3XGFP, and Pal1-GFP all failed to form distinct, stable cortical patches in *ede1Δ* yeast (Figure 3C). This phenotype is similar to the behavior observed for Syp1-GFP in the absence of Ede1p (Reider *et al.*, 2009; Stimpson *et al.*, 2009). It is surprising that Yap1802-GFP forms patches in *ede1Δ* yeast (Figure 3C) and Yap1802-GFP still arrives before Sla1-mCherry in these cells (91%, $n = 35$; Figures 1E and 3D). Thus, Ede1p is important for the recruitment of most early-arriving proteins to endocytic sites, although Yap1802p can localize correctly in the absence of Ede1p. Of interest, Ede1-GFP patch lifetimes are unchanged in *syp1Δ*, *apl1Δ*, *yap1802Δ*, and *pal1Δ* yeast ($n = 50$ patches for each strain; results not shown).

The role of clathrin in early endocytic site establishment and maturation was studied using a clathrin light chain-deletion mutant. Ede1-GFP, Apl1-3XGFP, Yap1802-GFP, and Pal1-GFP are able to form cortical patches in *clc1Δ* yeast, indicating that clathrin light chain is dispensable for endocytic site establishment (Figure 3E). Although previous studies found that Syp1-GFP patch localization was reduced in *clc1Δ* yeast (Boettner *et al.*, 2009), we observe that Syp1-GFP is able to form cortical patches in *clc1Δ* yeast (Figure 3E). However, the dynamics of Ede1-GFP, Syp1-GFP, Apl1-3XGFP, Yap1802-GFP, and Pal1-GFP patches are perturbed in clathrin light chain mutants: very few “turnover” patches (patches that assemble and disassemble within a 4-min period and are likely to represent productive endocytic events) are observed when compared with wild-type cells (Figure 3, E and F). There is also an increase in the number of “persistent” patches (patches that persist for >4 min) in *clc1Δ* yeast for each of the early-arriving endocytic proteins ($n = 50$ patches for each strain). Patches that only assemble (but do not disassemble) or that only disassemble (but do not assemble) during a 4-min movie were termed “partial” patches.

Clathrin light chain is important for progression through the intermediate coat stage

The increase in numbers of persistent patches formed by early-arriving endocytic proteins in *clc1Δ* yeast is similar to the phenotype previously observed for the later-arriving protein Sla2p, a Hip1R homologue, in clathrin knockouts (Newpher and Lemmon, 2006; Newpher *et al.*, 2006). Therefore the recruitment and dynamics of later-arriving endocytic proteins were analyzed in *clc1Δ* yeast. Previous studies found that Sla2-GFP forms patches with extended lifetimes in *clc1Δ* yeast (Newpher and Lemmon, 2006; Newpher *et al.*, 2006). We were able to repeat these findings ($n = 50$; Figure 4A). Of interest, we found that Ent1-GFP and Ent2-GFP also form more persistent patches in *clc1Δ* yeast ($n = 50$ patches for each strain; Figure 4A). In contrast to this result, Sla1-GFP and Las17-GFP were reported to have shorter lifetimes in *clc1Δ* yeast (Kaksonen *et al.*, 2005; Newpher and Lemmon, 2006). We were able to reproduce

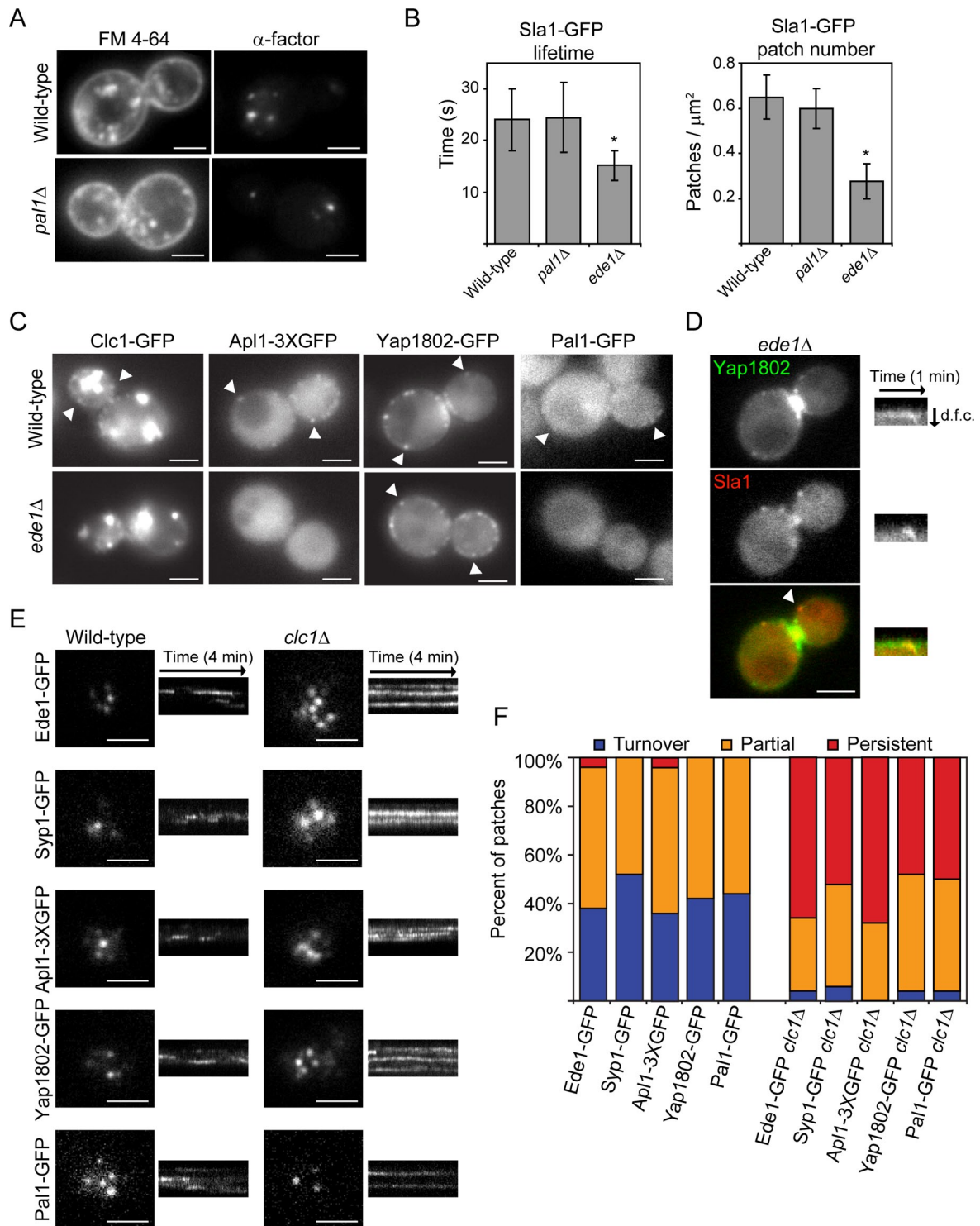


FIGURE 3: Analysis of the roles of Pal1p, Ede1p, and Clc1p in endocytosis. (A) FM 4-64 uptake (left) and fluorescent α -factor uptake (right) in the indicated strains after 10 min. (B) Lifetimes of Sla1-GFP patches \pm SD ($n = 50$ patches; left) and Sla1-GFP patch number per cell surface area (μm^2) \pm SD ($n = 20$ cells; right) for the indicated yeast. Patch number was counted from maximum-intensity Z-projections of unbudded or large-budded cells. Movies used to generate lifetime data were acquired at a rate of one frame per second. Asterisk indicates a statistically significant decrease compared with wild-type ($p < 0.0001$). (C) Images of wild-type and *ede1* Δ yeast expressing Clc1-GFP, Apl1-3XGFP, Yap1802-GFP, or Pal1-GFP. White arrowheads indicate examples of cortical patches. (D) Images of an *ede1* Δ yeast cell expressing Yap1802-GFP and Sla1-mCherry. Kymographs of the patch indicated by the white arrowhead are from two-color movies. d.f.c., distance from cortex. (E) TIRF microscopy images of wild-type and *clc1* Δ yeast cell expressing Ede1-GFP, Syp1-GFP, Apl1-3XGFP, Yap1802-GFP, or Pal1-GFP. Kymographs are taken from 4-min movies. (F) Percentage of patches in the indicated strains that assemble and disassemble within a 4-min interval (turnover), are present throughout the TIRF microscopy movie (persistent), or are present in either the first or last frames of the movie (partial; $n = 50$ patches). All white scale bars, 2 μm .

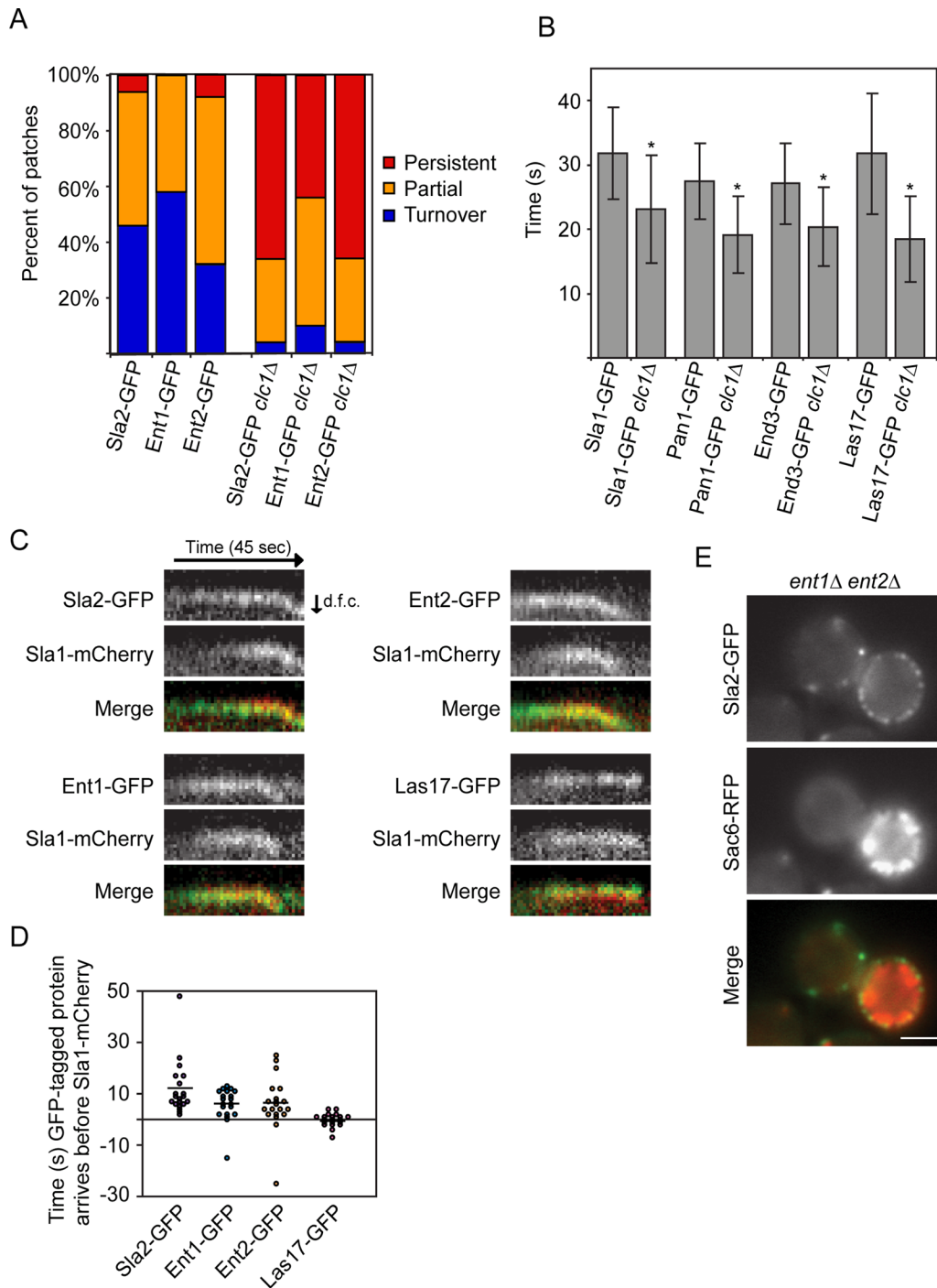


FIGURE 4: Sla2p, Ent1p, and Ent2p behave similarly in *clc1Δ* yeast, have similar localization dynamics, and display similar knockout phenotypes. (A) Percentage of patches in the indicated strains that assemble and disassemble within a 4-min interval (turnover), are present throughout the TIRF microscopy movie (persistent), or are present in either the first or last frames of the movie (partial; $n = 50$ patches). (B) Lifetime of Sla1-GFP, Pan1-GFP, End3-GFP, and Las17-GFP patches \pm SD in wild-type or *clc1Δ* yeast ($n = 50$ patches). Movies used to generate lifetime data were acquired at a rate of one frame per second. Asterisk indicates a statistically significant decrease compared with wild type ($p < 0.0001$). (C) Kymographs from epifluorescence two-color movies of yeast expressing Sla1-mCherry and Sla2-GFP, Ent1-GFP, Ent2-GFP, or Las17-GFP. Movies were acquired at a rate of one frame per second. d.f.c., distance from cortex. (D) Amount of time the indicated GFP-tagged protein arrived before Sla1-mCherry ($n = 20$ patches for each strain). Negative time represents instances when Sla1-mCherry arrived before the GFP-tagged protein. Horizontal bars represent average time for a group. (E) Fluorescence images of an *ent1Δ ent2Δ* yeast cell expressing Sla2-GFP and Sac6-RFP. White scale bars, 2 μ m.

this observation and furthermore found that Pan1-GFP and End3-GFP patches also have shorter lifetimes in clathrin light chain mutants ($n = 50$ patches for each strain; $p < 0.0001$ for each strain;

Figure 4B). It should be noted, however, that Pan1-GFP and End3-GFP form a few persistent patches in *clc1Δ* yeast that are present throughout 90-s movies. Together these results show that clathrin

light chain is important for the proper disassembly of Sla2p, Ent1p, and Ent2p.

Owing to their similar phenotypes in *clc1Δ* yeast, we examined Sla2p, Ent1p, and Ent2p more carefully. Previous studies found that Sla2-GFP arrives at endocytic sites before Sla1-mCherry (Newpher and Lemmon, 2006), and we were able to reproduce these results (100%, $n = 20$; Figure 4, C and D). Moreover, we now find that Ent1-GFP and Ent2-GFP also arrive at endocytic sites before Sla1-mCherry (90% for both, $n = 20$ patches for each strain; Figure 4, C and D). However, Sla1-mCherry and Las17-GFP arrive at endocytic sites at a similar time point ($n = 20$ patches for each strain; Figure 4, C and D). After finding that Sla2p, Ent1p, and Ent2p have a unique localization dynamics, we investigated whether these proteins also have similar functions in endocytosis. In our strain background *ent1Δ ent2Δ* yeast are viable, but we found that they form abnormal actin structures, marked by Sac6-RFP, that are similar to those previously reported in *ent1Δ ent2Δ* yeast expressing an ENTH^{Y100R} domain from Ent1p (Figure 4E) (Aguilar et al., 2006). The actin structures in *ent1Δ ent2Δ* yeast are also very similar to the elongated actin tails formed in *sla2Δ* yeast (Kaksonen et al., 2003). However, this phenotype is not caused by a lack of recruitment of Sla2p to endocytic sites in *ent1Δ ent2Δ* yeast (Figure 4E). These data suggest that Sla2p, Ent1p, and Ent2p act at a similar stage of endocytosis and should be considered components of a new, intermediate coat module (Figure 2).

Cargo may play a role in progression through a regulatory transition point controlling endocytic site maturation

After establishing how early-arriving proteins affect endocytic site formation and maturation, we next sought to investigate the role of cargo in this process, since cargo also arrives early at endocytic sites (Toshima et al., 2006). The dynamics of the endocytic machinery was monitored in a secretion mutant, *sec18-1^{ts}*, in which cell surface levels of endocytic cargo can be reduced. The endocytic cargo protein GFP-Snc1, which cycles continuously between the plasma membrane and endosomal compartments (Lewis et al., 2000), disappears from the plasma membrane of *sec18-1^{ts}* yeast within 20 min of shifting to 37°C (Figure 5A). This indicates that within the first 20 min of shifting *sec18-1^{ts}* yeast to 37°C, nearly all of the GFP-Snc1 is endocytosed from the plasma membrane and further secretion to the plasma membrane is blocked. After the yeast are incubated for a longer period of time at 37°C (30 min), the number of persistent Ede1-GFP, Clc1-GFP, and Sla2-GFP patches increases in *sec18-1^{ts}* mutants when compared with wild-type cells ($n = 50$ patches for each strain; Figure 5B). The decrease in the number of dynamic Ede1-GFP patches in *sec18-1^{ts}* mutants could be reversed by returning the yeast to 25°C for 20 min, thereby indicating that the cells remained viable ($n = 50$; Figure 5B). Furthermore, the number of Sla1-GFP patches in *sec18-1^{ts}* mutants decreases when the yeast are incubated for 45 min at 37°C ($n = 20$ cells; $p < 0.0001$; Figure 5H). These results imply that endocytic sites present in *sec18-1^{ts}* mutants do not properly mature when the yeast are shifted to 37°C for at least 30 min. This defect in endocytic patch dynamics in *sec18-1^{ts}* mutants at the restrictive temperature is accompanied by a defect in FM 4-64 uptake (Figure 5C). The endocytic defects in the *sec18-1^{ts}* mutant are likely not caused by a depletion of plasma membrane phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) because there is no detectable change in the distribution of GFP-2XPH(PLCδ), which is a marker for this lipid (Figure 5D; Stefan et al., 2002).

To determine whether other secretion mutants also have endocytic defects, we examined endocytosis in *sec1-1^{ts}* mutants

(Novick et al., 1980). The endocytic cargo GFP-Snc1 clears from the plasma membrane of *sec1-1^{ts}* yeast within 45 min of shifting to 37°C (Supplemental Figure S1A). This result indicates that GFP-Snc1 is endocytosed from the plasma membrane and further secretion is blocked within the first 45 min of shifting *sec1-1^{ts}* yeast to 37°C. There is a following increase in the number of persistent Ede1-GFP patches in *sec1-1^{ts}* mutants when the yeast are incubated at 37°C for a longer period of time (90 min; $n = 50$; Supplemental Figure S1B). The *sec1-1^{ts}* mutants also have defects in Lucifer yellow uptake after an extended incubation at the restrictive temperature (Supplemental Figure S1C). Together, these results show that *sec18-1^{ts}* and *sec1-1^{ts}* mutants have defects in endocytosis.

In *sec18-1^{ts}* and *sec1-1^{ts}* yeast at the restrictive temperature, constitutively recycled cargoes are endocytosed from the plasma membrane and are not replaced. The decrease in endocytic cargo concentration at the plasma membrane might influence endocytic site progression through a regulatory transition point. To test this hypothesis, the amino acid permease Gap1-RFP was first overexpressed in *sec18-1^{ts}* yeast. Gap1p is expressed at the cell surface when wild-type or *sec18-1^{ts}* yeast are grown on a poor nitrogen source (Figure 5E, i, ii, iv, and v). However, Gap1p is cleared from the plasma membrane and trafficked to the vacuole in the presence of a preferred nitrogen source, such as glutamate (Roberg et al., 1997; Soetens et al., 2001). Within 14 min of adding 0.1% glutamate, plasma membrane Gap1-RFP levels decrease in wild-type cells and in *sec18-1^{ts}* cells incubated at the restrictive temperature (Figure 5E, iii and vi). Gap1-RFP does not accumulate in the vacuole when glutamate is added to *sec18-1^{ts}* yeast, likely due to the mutant's trafficking defects (Hicke et al., 1997). Although there is a decrease in plasma membrane Gap1-RFP levels over time in *sec18-1^{ts}* mutants, there is a greater decrease in plasma membrane Gap1-RFP levels in *sec18-1^{ts}* cells treated with glutamate compared with untreated *sec18-1^{ts}* cells ($n = 20$ cells; $p < 0.0001$; Figure 5, E, insets, and F). The increase in persistent Ede1-GFP patches ($n = 50$ patches) and the decrease in number of Sla1-GFP patches ($n = 20$ cells) at 37°C in *sec18-1^{ts}* cells are partially rescued when Gap1-RFP is targeted for endocytosis by the addition of glutamate (Figure 5, G and H). The defect in Ede1-GFP dynamics at 37°C in *sec1-1^{ts}* yeast is also rescued when Gap1-RFP internalization is induced by adding glutamate ($n = 50$; Supplemental Figure S1, D and E). The defects in Ede1p patch dynamics ($n = 50$) and number of Sla1p patches ($n = 20$ cells) at 37°C in *sec18-1^{ts}* yeast are not rescued when glutamate is added to yeast that are also lacking Gap1p, indicating that the Gap1p cargo is mediating this effect (results not shown). The lifetime of Ede1-GFP patches and the number of Sla1-GFP patches in wild-type cells overexpressing Gap1-RFP does not change when glutamate is added (data not shown). Our results indicate that endocytic defects of *sec18-1^{ts}* and *sec1-1^{ts}* yeast can be partially rescued by inducing internalization of an endocytic cargo.

To further investigate this cargo-dependent effect, we added cycloheximide to yeast, which is another method reported to induce internalization of multispan plasma membrane proteins (Galan and Haguenaer-Tsapis, 1997; Lin et al., 2008). Cells incubated for 2 h in 50 μg/ml cycloheximide clear RFP-Snc1 and Fur4-GFP from the plasma membrane (data not shown). Cycloheximide-induced depletion of membrane cargo also results in an increase in the number of persistent Ede1-GFP patches (data not shown). Admittedly, cycloheximide effects on cell physiology are rather global, but this observation nonetheless is consistent with a role for cargo in modulating dynamics of the endocytic pathway.

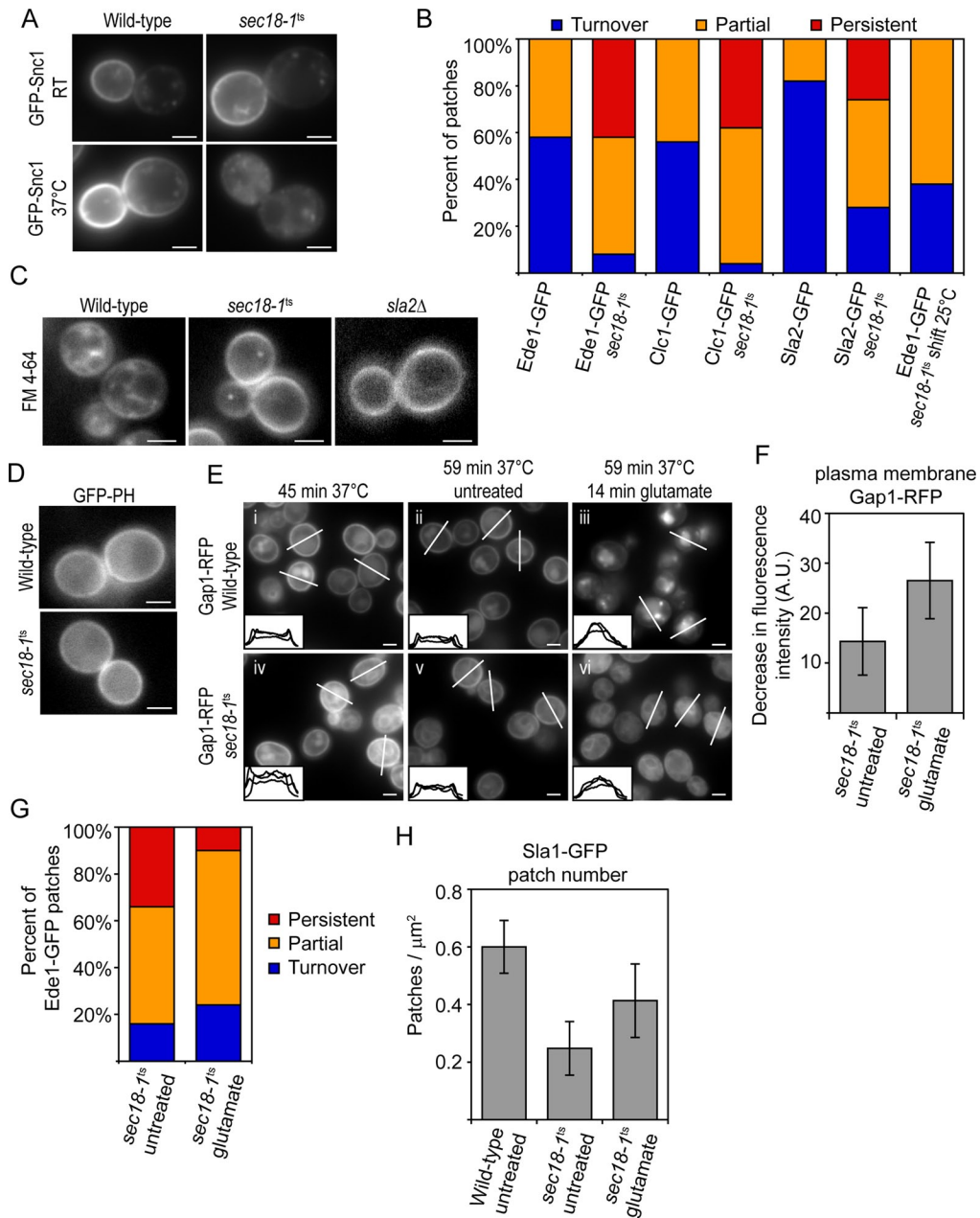


FIGURE 5: Endocytic defects in *sec18-1^{ts}* yeast. (A) Images of wild-type or *sec18-1^{ts}* yeast expressing GFP-Snc1. Yeast were imaged at room temperature (RT) or incubated at 37°C for 20 min before imaging. (B) Percentage of patches in the indicated strains that assemble and disassemble within a 4-min interval (turnover), are present throughout the TIRF microscopy movie (persistent), or are present in either the first or last frames of the movie (partial; $n = 50$ patches). Wild-type and mutant strains were incubated at 37°C for 30 min before imaging, except for yeast incubated at 37°C for 30 min and then shifted to 25°C for 20 min before imaging (Ede1-GFP *sec18-1^{ts}* shift 25°C). (C) FM 4-64 uptake in strains incubated at 37°C for 30 min and then labeled with FM 4-64 for 20 min at 37°C before imaging. (D) Images of wild-type and *sec18-1^{ts}* yeast expressing GFP-2XPH(PLC δ), which is a marker for PtdIns(4,5)P $_2$. Yeast were incubated at 37°C for 30 min before imaging. (E) Images of Gap1-RFP overexpressed in wild-type or *sec18-1^{ts}* yeast. Cells were incubated at 37°C for 45 min (i, iv) or 59 min (ii, v), or cells were incubated at 37°C for 45 min and then treated with 0.1% glutamate (wt/vol) for 14 min at 37°C (iii, vi). Insets are line scans of fluorescence intensity (y-axis; arbitrary units) along the three white lines shown. (F) Decrease in the fluorescence intensity (arbitrary units) \pm SE of the mean ($n = 20$ cells) of plasma membrane-localized Gap1-RFP in *sec18-1^{ts}* yeast incubated at 37°C. Gap1-RFP levels were measured at a 45-min and a 65-min time point in cells that were incubated with (glutamate) or without (untreated) 0.1% glutamate (wt/vol) during this time period. (G) Percentage of Ede1-GFP patches in *sec18-1^{ts}* yeast overexpressing Gap1-RFP that are turnover, persistent, or partial patches (4-min TIRF microscopy movie; $n = 50$ patches). Yeast were incubated at 37°C for 55 min (untreated) or incubated at 37°C for 45 min and treated with 0.1% glutamate for 10 min at 37°C (glutamate) before imaging. (H) Sla1-GFP patch number per cell surface area (μm^2) \pm SD ($n = 20$ cells) for wild-type and *sec18-1^{ts}* yeast overexpressing Gap1-RFP. Yeast were incubated at 37°C for 55 min (untreated) or incubated at 37°C for 45 min and treated with 0.1% glutamate for 10 min at 37°C (glutamate) before imaging. Patch number was counted from maximum-intensity Z-projections of unbudded or large-budded cells. All white scale bars, 2 μm .

DISCUSSION

In this study, we identified and analyzed the relative timing of recruitment of the earliest-arriving endocytic proteins and investigated the roles of these early proteins and cargo in endocytic dynamics.

Assembly of proteins at the early endocytic site

This work expanded the number of proteins known to arrive at the earliest stages of yeast endocytic site formation to include Yap1802p and the previously uncharacterized protein Pal1p. On the basis of their patch lifetimes and dynamics, Pal1p and Yap1802p have been classified as components of the early coat module.

Insight into the earliest stages of endocytosis was gained by defining which proteins are first detected at endocytic sites. We find that Ede1p, Syp1p, clathrin light chain, the AP2 complex, Yap1802p, and Pal1p appear at endocytic sites at about the same time. It is possible that a signal at the plasma membrane simultaneously recruits the early-arriving endocytic proteins and cooperative binding aids their stabilization at endocytic sites. It is also possible that some early-arriving endocytic proteins physically interact in the cytosol prior to associating with the plasma membrane, which has been observed for other endocytic proteins (Lundmark and Carlsson, 2004). Because the homologues of Ede1p and Syp1p (Eps15 and FCHO1/2, respectively) are also among the first proteins to arrive during CCP formation in mammalian cells (Henne *et al.*, 2010), further investigation of the initial steps of endocytosis in both systems might reveal additional similarities.

Role of the early-arriving proteins in endocytosis

This work provided insight into how the early-arriving proteins contribute to endocytic site formation. *PAL1* deletion mutants did not have detectable endocytic defects, which is consistent with the observation that many of the early-arriving proteins function in a cargo-specific manner (Burston *et al.*, 2009; Carroll *et al.*, 2009; Reider *et al.*, 2009; Stimpson *et al.*, 2009). Pal1p may also be a cargo-specific adaptor or it could act redundantly with other endocytic proteins.

Ede1p was hypothesized to be involved in endocytic site establishment because it affects the formation of productive endocytic sites (Kaksonen *et al.*, 2005; Stimpson *et al.*, 2009). However, previous studies only examined the dynamics of later-arriving proteins and therefore did not reveal the role of Ede1p in early stages of site formation. We reported here that clathrin light chain, the AP2 complex, and Pal1p do not stably associate with endocytic sites in *ede1Δ* yeast. In contrast, Yap1802p was recruited to sites in the absence of Ede1p, and Yap1802p alone was unable to recruit and stabilize the other early-arriving proteins at endocytic sites. Yap1802p might localize to endocytic sites in the absence of Ede1p because it has an AP180 N-terminal homology domain (Legendre-Guillemain *et al.*, 2004; Maldonado-Baez *et al.*, 2008), which may bind PtdIns(4,5)P₂ on the plasma membrane. Conflicting reports exist over whether Syp1p is recruited to cortical patches in *ede1Δ* yeast (Boettner *et al.*, 2009; Reider *et al.*, 2009; Stimpson *et al.*, 2009). It is unclear why some early-arriving endocytic proteins are able to form patches in *ede1Δ* yeast, but our results confirm that Ede1p is an important factor in normal endocytic site initiation. Although knockdown of Eps15 in mammalian cells increased productive CCP lifetimes (Mettlen *et al.*, 2009), which is a different phenotype than that observed in *ede1Δ* yeast, results from these experiments suggest that Eps15 and Ede1p are both important components of the endocytic machinery.

We find that all of the early-arriving endocytic proteins are able to form cortical patches in *clc1Δ* yeast. Clathrin light chain has been

proposed to promote yeast endocytic site progression, possibly through its interaction with Sla2p's coiled-coil domain (Newpher and Lemmon, 2006; Newpher *et al.*, 2006). This study confirms that clathrin light chain is important for site maturation and disassembly, and we find that it specifically regulates the transition from the intermediate to late coat stages (Figure 2). In mammalian cells, knockdown of the clathrin light chain b isoform increased the lifetimes of late abortive CCPs and decreased the lifetimes of productive CCPs (Mettlen *et al.*, 2009). It is possible that yeast Clc1p may be more similar to the mammalian clathrin light chain b isoform because there is an increase in early endocytic protein lifetimes, as well as a decrease in late coat module protein lifetimes, in *clc1Δ* yeast. Although clathrin light chain is believed to be important for efficient heavy chain trimerization (Chu *et al.*, 1996), it is important to note that small amounts of clathrin heavy chain may be present and functioning at endocytic sites in *clc1Δ* yeast. More research is needed to elucidate the roles of the different clathrin subunits in endocytic site regulation.

Comparing the phenotypes of *clc1Δ* and *ede1Δ* yeast reveals that the lack of clathrin at endocytic sites in *ede1Δ* yeast does not result in the same phenotypes observed in *clc1Δ* yeast. For example, we find that Sla2p and Ent2p form more persistent patches in *clc1Δ* yeast, but the lifetimes of these proteins are shortened in *ede1Δ* yeast (Stimpson *et al.*, 2009). It is possible that small amounts of clathrin might localize to and promote maturation of endocytic sites in *ede1Δ* yeast. Alternatively, endocytic proteins that normally inhibit endocytic site maturation might not be recruited to endocytic sites, or they might be regulated differently, in *ede1Δ* yeast.

Formation of an intermediate coat module

Sla2p, Ent1p, and Ent2p arrive at endocytic sites before Sla1p, and *ent1Δ ent2Δ* yeast have similar phenotypes to *sla2Δ* yeast. These results have now led us to propose the existence of an intermediate coat module separate from the late coat module containing the Pan1 complex (Pan1p, Sla1p, and End3p; Figure 2; Kaksonen *et al.*, 2003). The intermediate coat module may serve to link the early proteins involved in endocytic site formation to the later-arriving proteins that drive membrane invagination. Sla2p has been hypothesized to connect the force of actin polymerization to membrane invagination (Kaksonen *et al.*, 2006), so Ent1p and Ent2p may also function in this step of endocytosis.

Secretion and cargo may regulate progression of endocytic events

Previous studies found that several secretion mutants have defects in endocytosis (Riezman, 1985; Hicke *et al.*, 1997), but how the two cellular processes are connected is not understood. We now find that endocytic sites are formed in *sec18-1^{ts}* yeast, which contain the early-arriving and intermediate coat module proteins, but the sites do not progress to completion. We speculate that when cargoes are depleted from the yeast cell surface in a secretion mutant or by the addition of cycloheximide, the lack of cargo may activate a checkpoint, preventing the endocytic site from maturing past the intermediate coat stage (Figure 2). In further support of this model, we found that Ede1p lifetimes are shorter in yeast buds, which contain higher concentrations of cargo than mother cells (Layton *et al.*, 2011). This model is also consistent with the observation that cargo normally arrives during the early stages of endocytosis (Toshima *et al.*, 2006).

Depletion of endocytic cargo may not be the only reason why endocytosis is defective in *sec18-1^{ts}* yeast. Adding glutamate to induce internalization of the cargo Gap1p in *sec18-1^{ts}* yeast did not

fully rescue the endocytic defects, suggesting that other factors may be involved in creating this phenotype. Although the distribution of PtdIns(4,5)P₂ did not seem to change in *sec18-1^{ts}* yeast, the lipid environment at the plasma membrane may be altered in secretion mutants (Yakir-Tamang and Gerst, 2009). Trafficking is perturbed in secretion mutants, and important machinery needed for endocytosis might not be recycled or delivered to the plasma membrane. It is also possible that in yeast exocytic events are linked to endocytic events directly. In fact, in mammalian cells compensatory endocytosis follows an exocytic event in some cells, and mechanisms linking exocytosis and endocytosis have also been proposed (Gundelfinger *et al.*, 2003; Yao *et al.*, 2009; Bai *et al.*, 2010; Pechstein *et al.*, 2010). Another interesting factor to consider is that signaling mechanisms could be contributing to the partial rescue of the endocytic defects observed in *sec18-1^{ts}* yeast when Gap1p internalization is induced. For example, the addition of glutamate may cause the cargo Gap1p to activate a signaling pathway that alters the dynamics of endocytic proteins.

Studies in mammalian cells suggested that cargoes, along with other factors, regulate a checkpoint in clathrin-mediated endocytosis (Ehrlich *et al.*, 2004; Loerke *et al.*, 2009; Mettlen *et al.*, 2009; Mettlen *et al.*, 2010). For example, nascent CCPs may abort if cargoes have not been captured, and the capture of certain cargo/adaptor complexes can increase efficiency of CCP maturation (Ehrlich *et al.*, 2004; Loerke *et al.*, 2009; Mettlen *et al.*, 2009). A slightly different mechanism may exist in yeast, in which endocytic sites may arrest at the intermediate coat stage if cargo and/or clathrin are not available to promote progression through an endocytic restriction point. In mammalian cells, different cargo proteins use different endocytic adaptors, and these factors may contribute to the heterogeneity in CCP dynamics and progression through the proposed endocytic checkpoint (Loerke *et al.*, 2009; Mettlen *et al.*, 2010). Furthermore, the clustering of transferrin receptors has been shown to increase CCP initiation (Liu *et al.*, 2010). Perhaps future studies will establish whether other endocytic cargoes in yeast have regulatory capabilities and whether their different adaptor proteins are involved in endocytic site maturation. The results presented here increase our overall understanding of endocytic sites as we continue to discover similarities between yeast and mammalian endocytic machinery.

MATERIALS AND METHODS

Strains

Yeast strains used in this study are listed in Supplemental Table S1. Single C-terminal GFP and 13Myc tags were integrated into the chromosome as previously described (Longtine *et al.*, 1998). C-terminal 3XGFP tags were created as previously described (Sun *et al.*, 2007). Gene deletions were generated by replacing the gene open reading frame with *Candida glabrata* *LEU2*, *URA3*, or *HIS3* cassettes. Yeast expressing GFP- and RFP-tagged Snc1p were transformed with the plasmids pRS416-*GFP-SNC1* and pRS416-*RFP-SNC1*. Yeast were transformed with the plasmid pRS426-*GFP-2XPH(PLCδ)* to monitor PtdIns(4,5)P₂ levels (Stefan *et al.*, 2002).

Coimmunoprecipitation and immunoblotting

Coimmunoprecipitation was performed essentially as described previously (Peng and Weisman, 2008). Briefly, cell lysates were incubated with mouse anti-Myc antibody for 2 h and then incubated with protein G-Sepharose beads (GE Healthcare, Piscataway, NJ) for 2 h. The beads were washed with lysis buffer containing 10% glycerol, and then the proteins were eluted. Coimmunoprecipitation was assayed by immunoblot. To detect Pal1-GFP, the mem-

brane was probed with 1:2000 rabbit anti-GFP antibody (Torrey Pines Biolabs, Secaucus, NJ). To detect Ede1-13Myc, the membrane was probed with 1:4000 mouse anti-Myc 9E10.

Microscopy

Yeast strains used for imaging were grown to log phase at 25°C in synthetic media lacking tryptophan (imaging media) and immobilized on concanavalin A-coated coverslips. The *clc1Δ* yeast and *ent1Δ ent2Δ* yeast strains were maintained as heterozygous diploids, which were sporulated and dissected before use. The spores were grown overnight in yeast extract/peptone/dextrose (YPD) and were then grown for 4 h in imaging media prior to imaging. Because *sla2Δ* yeast have growth defects, this strain was also grown overnight in YPD and then grown for 4 h in imaging media. The *sec18-1^{ts}* and *sec1-1^{ts}* yeast were grown overnight at 25°C and then incubated at 37°C for the time indicated and imaged in a 37°C temperature-controlled chamber. Yeast expressing Gap1-RFP were grown in synthetic media composed of yeast nitrogen base without amino acids and without ammonium sulfate (Difco, BD Biosciences, Franklin Lakes, NJ), 2% glucose, and 0.5% ammonium sulfate. The yeast were then incubated at 37°C for the time indicated before imaging. To induce Gap1-RFP uptake, the media was replaced with prewarmed imaging media supplemented with 0.1% glutamate (wt/vol) for the time indicated.

Olympus IX71 and IX81 microscopes with 100×/numerical aperture (NA) 1.4 objectives, Orca cameras (Hamamatsu, Hamamatsu, Japan), appropriate filter sets, and neutral density filters were used to image live yeast cells. Simultaneous two-color imaging was performed as described previously using a 488-nm argon-ion laser (CVI Melles Griot, Albuquerque, NM) to excite GFP and either a mercury lamp filtered through a 575/20-nm filter (Figures 1, C and D, 3D, and 4, C and E) or a 561-nm argon-ion laser (CVI Melles Griot; Figure 1F) to excite RFP or mCherry (Stimpson *et al.*, 2009). TIRF microscopy was performed using an IX81 microscope with a 100×/1.65 NA objective and an adjustable angle laser beam, which was lowered to reduce background signal. Two-color TIRF microscopy in Figure 1F was performed by lowering the angle of both the 488- and 561-nm laser beams separately to reduce background signal. Movies were acquired at a rate of one frame every 2 s, unless indicated otherwise. Images were collected using MetaMorph software (Molecular Devices, Sunnyvale, CA) and processed using ImageJ (National Institutes of Health, Bethesda, MD). Patch lifetimes were calculated from patches that assembled and disassembled during the movie (turnover patches). Patch number was counted from maximum-intensity Z-projections of unbudded or large-budded cells. Z-stacks were acquired through the entire cell at 0.15-μm intervals. Gap1-RFP fluorescence intensity was calculated by averaging the pixel intensity at four locations around the cell cortex.

FM 4-64, fluorescent α-factor, and Lucifer yellow uptake assays

For FM 4-64 staining, cells were incubated with 8 μM FM 4-64 in imaging media for the time indicated and then imaged. HiLyte Fluor 488 α-factor (AnaSpec, Fremont, CA) uptake experiments were performed essentially as described previously (Toshima *et al.*, 2006). Cells were grown to log phase in imaging media. The cells were pelleted and then resuspended to ~40 OD/ml in imaging media and incubated with 200 ng of fluorescent α-factor on ice for 1 h. The cells were washed three times in ice-cold imaging media, warmed to room temperature, and imaged after the time indicated. Lucifer yellow uptake assays were performed as described previously (Baggett *et al.*, 2003), but with some modifications. Cells were first

grown to early log phase in YPD. Approximately 1×10^7 cells were pelleted and then resuspended in 90 μ l of YPD. After incubation of the cells for 90 min at 37°C, 10 μ l of 40 mg/ml Lucifer yellow CH dilithium salt (Invitrogen, Carlsbad, CA) was added. Cells were incubated for 90 min at 37°C and then washed four times in ice-cold 50 mM potassium phosphate buffer, pH 7.4, containing 10 mM Na₂S₂O₈ and 10 mM NaF. Cells were imaged in the wash buffer at room temperature.

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