Regulation of Clathrin-Mediated Endocytosis by Dynamic Ubiquitination/Deubiquitination of Ede1

by

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ABSTRACT

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Clathrin-mediated endocytosis in budding yeast requires the regulated recruitment and disassociation of over 60 proteins at discreet plasma membrane puncta. Post-translational modifications, such as phosphorylation and ubiquitination, may play important regulatory roles in this highly processive and ordered process. However, while ubiquitination plays an important role in cargo selection, functions for ubiquitination of the endocytic machinery are not known. We identified the deubiquitinase (DUB) Ubp7p as a late arriving endocytic protein. Deletion of the DUBs Ubp2p and Ubp7p resulted in elongation of the lifetimes of endocytic coat proteins at the plasma membrane and recruitment of endocytic proteins to internal membranes. These phenotypes could be replicated by expressing a permanently ubiquitinated version of the early endocytic adaptor Ede1p, the yeast Eps15 homolog, which is implicated in the initiation of endocytic sites. However, Ede1p absence did not fully suppress the deubiquitinase deletion phenotype, suggesting that Ede1p is not the only endocytic target whose deubiquitination affects coat formation and disassembly.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ANTH: AP180 N-terminal homology
DUB: deubiquitinase
EH-NPF: Interaction between Eps15 homology domain and asparagine-proline-phenylalanine motif
HECT: homologous to the E6-AP carboxyl terminus
RFP: red fluorescent protein
SH3: SRC homology 3 domain
TIRFM: total internal reflection microscopy
UCH: ubiquitin carboxyl-terminal hydrolase
UIM: ubiquitin-interacting motif
WASP: Wiskott-Aldrich Syndrome Protein.

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CHAPTER 1
GENERAL INTRODUCTION
ENDOCYTOSIS IN YEAST: PAST, PRESENT AND FUTURE

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The yeast endocytosis field stretches back decades. Striking parallels between yeast and mammalian endocytosis allow discoveries in one system to be applied to the other. Yeast endocytosis occurs at sites on the plasma membrane marked by the ordered recruitment of endocytic proteins, culminating in the rapid accumulation of actin directly before the scission event. These cortical actin patches were first visualized by immunofluorescence and rhodamine-phalloidin staining (Adams and Pringle, 1984; Kilmartin and Adams, 1984) in budding yeast, where they are polarized to the growing bud (Figure 1.1). A growing body of work suggested the involvement of actin in endocytosis (Kubler and Riezman, 1993; Mulholland et al., 1994).

The spread of simultaneous two-color imaging and total internal reflection microscopy resulted in rapid advances in the study of endocytosis. These new techniques were used to demonstrate that the previously observed partial colocalization of actin and endocytic proteins was a result of taking snapshots of a highly dynamic process. Upon full spatiotemporal visualization, endocytosis clearly involved the ordered arrival and dissociation of many proteins. The excellent genetics of *S. cerevisiae* and the existence of tools such as GFP tagged libraries, combined with modern microscopy, enabled researchers to rapidly screen for proteins that localize to endocytic sites and to observe their dynamics in mutant yeast strains (Kaksonen et al., 2003; Kaksonen et al., 2005). The first definitive demonstration that actin patches are sites of endocytosis was actin patches with internalizing alpha-factor, a historically important model cargo, and attained when two-color live-cell fluorescence microscopy demonstrated colocalization of FM4-64, a fluorescent lipid marker (Huckaba et al., 2004; Toshima et al., 2006).

Studies of endocytosis in yeast continue to make important contributions even after several decades of intense research. Newly developed tools and techniques continue to drive the field forward. Mass spectrometry enables sensitive detection of post-translational modifications and low-affinity binding partners (Toshima et al., 2007). Synthetic genetic arrays and other genomic approaches expand the knowledge of the complex network of interactions and identify novel interacting proteins (Friesen et al., 2006; Tonikian et al., 2009). Studies of post-translational modifications have benefited from the development of analog-sensitive kinases, which allow for a rapid, isothermic, reversible and precise inhibition (Sekiya-Kawasaki et al., 2003). These approaches all augment the techniques used to launch the field, including two-color fluorescence microscopy, co-immunoprecipitation and double-mutant analysis. These technological advances allow researchers to identify potential endocytic proteins via interactions revealed by genomic screens, and to then rapidly identify binding partners and potential regulatory modifications by mass spectrometry, and to perform sensitive localization

Figure 1.1

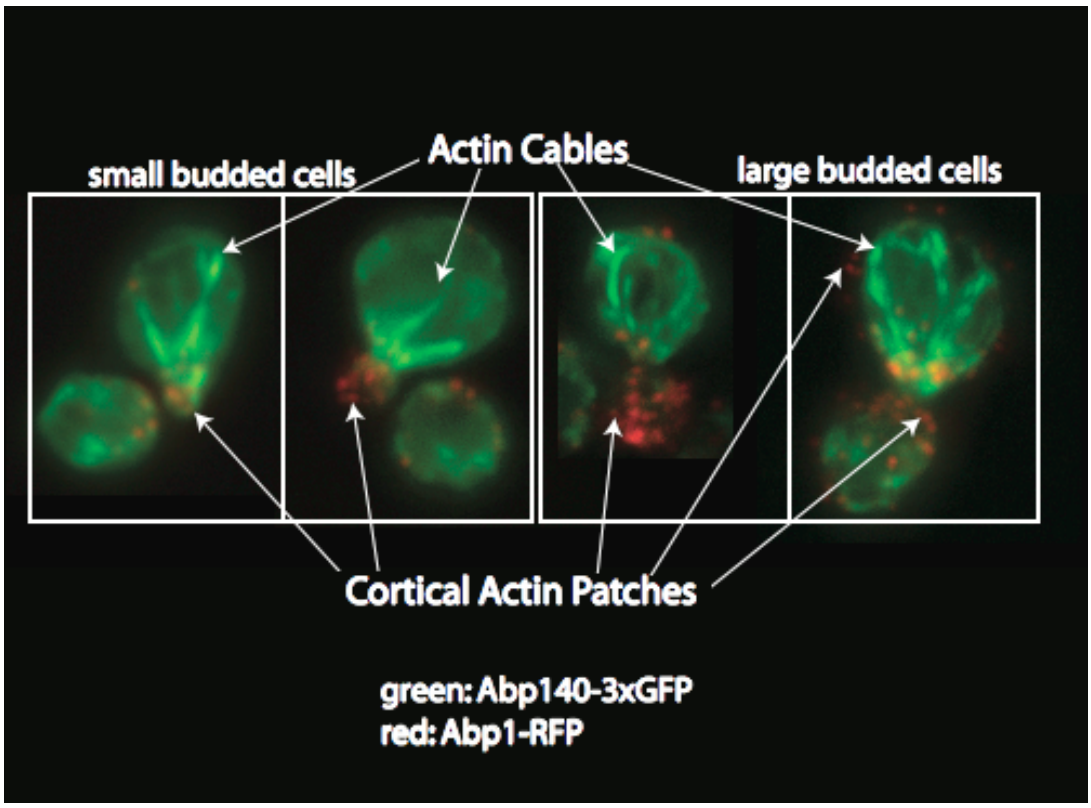

small budded cells
Actin Cables
large budded cells
Cortical Actin Patches
green: Abp140-3xGFP
red: Abp1-RFP

Figure 1.1 Actin structures in budding yeast

In budding yeast actin forms three structures: the cytokinetic ring (not shown) responsible for separating the mother and daughter cell cytoplasm, actin cables, which are parallel bundles of short actin filaments running the length of the cell, nucleated by formins and serving as tracks for myosin-based motility (green), and cortical actin patches, composed of branched filaments nucleated by the Arp2/3 complex, and which are the sites of endocytosis (red). Cables and patches are visualized by imaging Abp140-3xGFP and Abp1-RFP respectively.

assays using fluorescently tagged proteins under normal conditions and in mutants of interacting proteins.

While the field has advanced in great leaps and bounds there are still many questions to be answered in the coming years. Active fields of study include determining the detailed mechanisms behind endocytic site selection, cargo recruitment, actin assembly, scission and uncoating, and the role of lipid and post-translational protein modifications in regulating the timing of recruitment, activation and dissociation of the endocytic machinery.

This review will cover recent advances in the study of budding yeast endocytosis, starting from the early stages of coat protein and cargo recruitment, through the maturation of the patch to the polymerization of actin and scission. The advances provided through ultrastructural analysis and mathematical modeling will be discussed along with modes of regulation and the relationship between yeast and mammalian endocytosis.

EARLY STAGES OF ENDOCYTOSIS, INCLUDING CARGO SELECTION

How endocytic sites are initiated is a mystery. Mutations that partially disturb cellular actin affect the polarization of endocytic patches in daughter cells, but this observation is a bit mysterious as actin does not appear at the endocytic site until late in the pathway, after the endocytic site has been selected (Wong et al., 2010). Perhaps the polarization of endocytic sites to daughter cells is facilitated by actin cables (Figure 1.1), or perhaps actin is present at an undetectable level at patches before the rapid polymerization late in the pathway (discussed below).

Among the earliest arriving proteins detected at yeast endocytic sites are the ubiquitin-binding protein Ede1p (Eps15), which is important for the recruitment of many later arriving proteins, and the F-BAR protein Syp1p (FCHo1/SGIP1) (Figure 1.2) (Reider et al., 2009; Stimpson et al., 2009). (Throughout this article, the first mention of a yeast protein will be followed in parentheses by the mammalian homologue, when known. For a complete listing, see Table I.) Syp1p is known to promote the formation of endocytic sites in a polarized fashion at the bud neck, but deletion of *SYP1* does not prevent patch formation in buds, while *EDE1* deletion reduces the number of endocytic sites but does not change their polarization (Stimpson et al., 2009). Clearly some additional factors are involved in the selection and initiation of endocytic sites

The ability of both Syp1p and Ede1p to bind to membranes - Syp1p via its F-BAR domain and Ede1p in a ubiquitin-dependent manner (Aguilar et al., 2003) - suggests that patch initiation may occur via a lipid signal, although no such signal has been identified in yeast. While PIP2 may be important for initiation in mammalian endocytosis via FCHo proteins (Henne et al.), temperature-sensitive mutations of the PIP2 phosphatase Mss4p do not inhibit actin patch initiation (Homma et al., 1998). While the F-BAR domain of Syp1p can bind and tubulate membranes (Reider et al., 2009), electron microscopy (EM) suggests that the membrane remains relatively flat during the initial stages of the endocytic pathway (C. Buser and D. Drubin, unpublished) (Idrissi et al., 2008), indicating that Syp1p may be binding without tubulation.

Figure 1.2

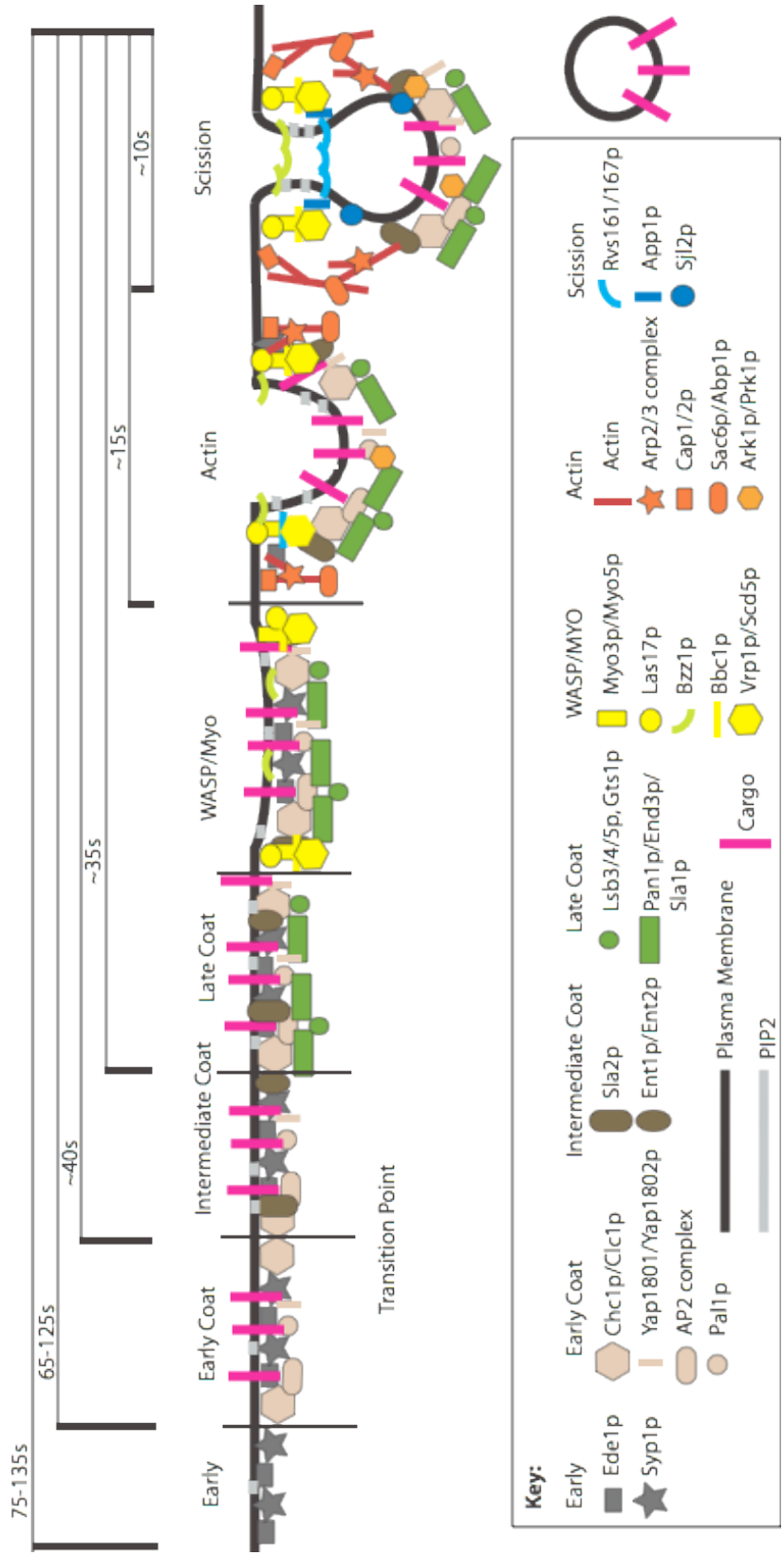

75-135s
65-125s
~40s
~35s
~15s
~10s
Early
Early Coat
Intermediate Coat
Late Coat
WASP/Myo
Actin
Scission
Transition Point
Key:
Early
Ede1p
Syp1p
Early Coat
Chc1p/Clc1p
Yap1801/Yap1802p
AP2 complex
Pal1p
Intermediate Coat
Sla2p
Ent1p/Ent2p
Late Coat
Lsb3/4/5p, Gts1p
Pan1p/End3p/
Sla1p
WASP/MYO
Myo3p/Myo5p
Las17p
Bzz1p
Bbc1p
Vrp1p/Scd5p
Actin
Actin
Arp2/3 complex
Cap1/2p
Sac6p/Abp1p
Ark1p/Prk1p
Scission
Rvs161/167p
App1p
Sjl2p
Plasma Membrane
PIP2
Cargo

Figure 1.2. Timeline for endocytic vesicle formation and modular organization of proteins.

Endocytic proteins are dynamically recruited in a highly predictable order. The “early” and “early coat” proteins are present at the cell surface for a variable amount of time. After a transition point, possibly defined by cargo recruitment, the lifetimes of endocytic proteins are quite regular. Actin polymerization and BAR domain proteins bend the membrane into an extended tubule, which is pinched off by the combined actions of BAR domain proteins, actin polymerization and possibly by a lipid-phase separation. The newly formed vesicle is uncoated by the combined actions of the Ark1p/Prk1p kinases, the Sjl2p lipid-phosphatase and the actions of Arf3p/Gts1p/Lsb5p.

Table I *S. cerevisiae* endocytic proteins and their mammalian homologs

Yeast Protein	Mammalian Protein
EARLY	
Ede1p	Eps15
Syp1p	FCho1/2
EARLY COAT	
Chc1p	Clathrin heavy chain
Clc1p	Clathrin light chain
Yap1801/2p	AP180
Pal1p	Fungi only
Apl1p	AP2 complex beta subunit
Apl3p	AP2 complex alpha subunit
Apm4p	AP2 complex mu subunit
Aps2p	AP2 complex sigma subunit
INTERMEDIATE COAT	
Sla2p	Hip1R
Ent1/2p	Epsin
LATE COAT	
Pan1p	Intersectin
Sla1p	Intersectin/CIN85
End3p	Eps15*
Lsb3p	SH3YL1*
Lsb4p	SH3YL1*
Lsb5p	Fungi Only
Gts1p	SMAP2 (small ArfGAP2)*
WASP/MYO	
Las17p	WASP/N-WASP
Vrp1p	WIP/WIRE
Bzz1p	syndapin
Scd5p	Saccharomycetales only
Myo3p	myosin-1E (Type 1 Myosin)
Myo5p	myosin-1E (Type 1 Myosin)
Bbc1p	Fungi only
Aim21p	Saccharomycetales only
ACTIN	

Yeast Protein	Mammalian Protein
Act1p	Actin
Arc15/18/19/35/40p & Arp2/3p	Arp2/3 complex
Abp1p	ABP1
Cap1p	Capping protein alpha
Cap2p	Capping protein beta
Sac6p	fimbrin
Scp1p	transgelin
Twf1p	twinfilin
Crn1p	coronin
Ark1p/Prk1p/Akl1p	BMP2 inducible kinase/AP2 associated kinase 1/AAK1
Cof1p	cofilin
Aip1p	Aip1
Bsp1p	no related proteins identified
Pfy1p	profilin
Aim3p	Saccharomycetales only
SCISSION	
Rvs161p	amphiphysin
Rvs167p	amphiphysin/endophilin
App1p	Fungi Only
Sjl2p	synaptojanin-1

*Physical homology, but no functional homology identified.

The arrival of the early proteins is quickly followed by the accumulation of cargo molecules at the nascent patch (Figure 1.2). Historically, the difficulties of imaging cargo accumulating in patches prevented solving the long-standing chicken and egg question of the endocytosis field: does the clustering of cargo recruit the endocytic machinery, or is cargo concentrated at a pre-formed endocytic site? Studies using a fluorescent derivative of alpha factor demonstrated that cargo arrives after the appearance of Ede1p, but before the appearance of Sla1p (intersectin/CIN85) (Figure 1.2) (Toshima et al., 2006). This timing and apparent differences in bud vs mother cell endocytic patch dynamics (Layton et al., 2011) support the hypothesis that the variable lifetimes of the early proteins (Ede1p, Syp1p, Clc1p (clathrin light chain), Chc1p (clathrin heavy chain), Yap1801/2p (AP180), Pal1p, AP2 complex) compared to the regular lifetimes of later proteins like Sla1p might be due to a cargo checkpoint that pauses the pathway after the arrival of early proteins until cargo loading is complete, and that the very regular timing of the later events of the pathway is the result of an endocytic site being fully loaded with cargo (Figure 1.2). Such a checkpoint also has been postulated for mammalian cells (Loerke et al., 2009). While alpha factor arrives after early proteins, it is possible that other cargos may be recruited later, after the arrival of intermediate or late coat, or may cluster before being recognized by the endocytic machinery (Di Pietro et al.).

In mammalian cells, the AP2 complex is an important cargo adaptor whose knockdown impairs internalization of several cargos including the well-studied transferrin receptor (Motley et al., 2003). In yeast, no endocytic phenotype had been observed in AP2 subunit knockouts until studies on the yeast killer toxin K28 demonstrated that AP2 knockouts are resistant to the toxin, and thus AP2 is likely involved in K28 internalization (Carroll et al., 2009). Other reported cargo-specific adaptors include Yap1801/2p as an adaptor for Snc1p (Burston et al., 2009), and Syp1p as an adaptor for Mid2p (Reider et al., 2009).

Ubiquitin has long been recognized as a signal for internalization of membrane proteins, and several early and coat proteins, including Ede1p, Sla1p and Ent1/2p (epsin), have ubiquitin-binding domains (Aguilar et al., 2003; Hicke and Riezman, 1996) (Table II). It has become clear, however, that while these ubiquitin-binding domains may be important for cargo binding, they also are likely important for regulating protein-protein interactions within the endocytic network. Extensive analysis of mutations in the ubiquitin-binding domains of Ent1/2p and Ede1p demonstrate that in the absence of these domains both ubiquitinated and non-ubiquitinated cargos are internalized equally, though at a reduced rate compared to wild-type, suggesting that the UIMs regulate a general, rather than a ubiquitinated-receptor specific, step in endocytosis (Dores et al., 2010). Because many endocytic proteins are likely to be ubiquitinated (Table II), the ubiquitin-binding domains, along with the EH-NPF interactions, are likely to stabilize interactions between some of the ~60 proteins that localize to endocytic patches. Similar mutational analyses define a role for the Yap1801/2p endocytic adaptors as functioning redundantly with Ent1/2p, likely by stabilizing the endocytic protein network via NPF-EH and UIM-ubiquitin interactions (Maldonado-Baez et al., 2008).

Table II Ubiquitin binding and ubiquitinated endocytic proteins

	Ubiquitin binding (Aguilar et al., 2003; Costa et al., 2005; Dores et al., 2010; Stamenova et al., 2007)	**Ubiquitinated by Rsp5p** (Dores et al., 2010; Gupta et al., 2007; Stamenova et al., 2004)	**Ubiquitinated *in vivo*** (Peng et al., 2003)
EARLY			
Ede1p	+	+	+
EARLY COAT			
Clc1p	n.d.	—	+
INTERMEDIATE COAT			
Ent1p	+	—	+
Ent2p	+	+	+
Sla2p	n.d.	—	+
LATE COAT			
Lsb5p	+	—	-
Sla1p	+	—	+
WASP/MYO			
Myo3p	n.d.	—	+
ACTIN			
Abp1p	n.d.	—	+
Arc18p	n.d.	—	+
Arc35p	n.d.	—	+
Arc40p	n.d.	—	+
Arp2p	n.d.	—	+
Arp3p	n.d.	—	+
Sac6p	n.d.	—	+
SCISSION			
Rvs167p	n.d.	+	+

In vivo ubiquitination data comes from genomic screens and may not be relevant for endocytosis. Negative signs for “Ubiquitinated by Rsp5p” indicate “not detected,” n.d. indicates “not done”.

Early arriving endocytic proteins are candidates to both regulate and facilitate the early steps of patch initiation, patch polarization, and cargo recruitment. They may also regulate later steps, however. In addition to its adaptor and endocytic site-specification roles (Reider et al., 2009; Stimpson et al., 2009), Syp1p is implicated as a regulator of Arp2/3 activity late in the endocytic pathway, just before Syp1p leaves the patch (Boettner et al., 2009). Many endocytic proteins are large, multi-domain proteins with multiple separable functions, and may therefore act at multiple steps during endocytosis.

The early arriving endocytic proteins are integral for, but are not the only players involved in, defining how many endocytic sites are formed, and where they form. They bind to cargo and to each other, forming the nascent endocytic protein network. These are the proteins that act in an early temporally variable phase before an apparent cargo-triggered transition point that marks the beginning of the extremely temporally regular later stages of endocytosis (Figure 1.2).

COAT MATURATION

After the proposed cargo-triggered transition point, other coat proteins join the endocytic site in regular temporal fashion. The next set of proteins to be recruited to the endocytic site, Sla2p (Hip1R) and Ent1/2p, have domains that specifically bind to phosphatidylinositol 4,5-bisphosphate (PIP2) (Aguilar et al., 2003; Sun et al., 2005). The ubiquitin-interaction motifs of Ent1/2p were thought to bind ubiquitinated cargo, but recent work suggests they are more important for promoting protein-protein interactions within the endocytic machinery, as discussed above (Dores et al., 2010; Maldonado-Baez et al., 2008).

The Pan1p (intersectin)/End3p (Eps15)/Sla1p complex is related to, and may serve a similar function as, the mammalian endocytic protein intersectin (Kaksonen et al., 2006). While Pan1p has nucleation promoting factor (NPF) activity, its most important role may be to serve as a scaffold to recruit other proteins to endocytic sites. The stability of the Pan1p/End3p/Sla1p complex is regulated by the Ark1p/Prk1p kinases (AAK1), which phosphorylate the complex, promoting disassembly, as discussed below (Toret et al., 2008; Zeng et al., 2001).

The other proteins in the late coat module arrive along with members of the WASP/Myo module, but are grouped as coat proteins because, in contrast to the WASP/Myo proteins, they are internalized with the newly formed vesicle (Figure 1.2). Lsb5p and Gts1p (small ArfGAP2) have roles in uncoating (Toret et al., 2008), while Lsb3p (SH3YL1) and Lsb4p/Ysc84p (SH3YL1) promote actin polymerization and bundling (Robertson et al., 2009).

The later arriving components of the endocytic coat are likely involved in linking together the early coat, which recruits cargo and begins to shape the membrane, with the actin and scission machinery.

WASP/MYOSIN RECRUITMENT

Las17p (WASP/N-WASP) and Myo3/5p (myosin-1E) are the most important NPFs *in vivo*, based on patch internalization movements in mutants; specific mutation of their NPF domains results in patches that fail to internalize (Sun et al., 2006), similar to what is observed in latrunculin A treated cells (Kaksonen et al., 2003). Abp1p (ABP1)

and Pan1p can stimulate Arp2/3 activity *in vitro*, but removal of their NPF domains *in vivo* causes less severe phenotypes than similar mutations in either Las17p or Myo3/5p, demonstrating that they play lesser roles in Arp2/3-mediated actin polymerization during endocytosis (Galletta et al., 2008; Sun et al., 2006).

The yeast Arp2/3 complex has a higher basal activity than its mammalian counterpart, although NPFs still strongly stimulate its nucleation activity. The myosin activator Vrp1p (WIP/WIRE) arrives after Las17p, and is followed by Myo3/5p, which arrive just before actin is detected (Figure 1.2) (Sun et al., 2006). Regulation of Las17p NPF activity is not fully understood. Las17p arrives ~20s before actin is detectable (Figure 1.2) (Kaksonen et al., 2005). During this time, the Arp2/3 complex might be held in an inactive state by the earlier arriving proteins Syp1p and Sla1p (Boettner et al., 2009; Rodal et al., 2003). At the onset of internalization, Syp1p departs and Sla1p moves inward with the coat, while Las17p remains closer to the plasma membrane, which may relieve the inhibition of the Arp2/3 complex (Sun et al., 2006). As Sla1p internalizes, the Las17p inhibitor Bbc1p localizes to the patch (Kaksonen et al., 2005; Rodal et al., 2003). How Bbc1p contributes to Las17p regulation is not understood. Combining null mutants of the inhibitors Sla1p and Bbc1p results in long actin tails associated with deep membrane invaginations (Figure 1.3A) (Kaksonen et al., 2005). The F-BAR protein Bzz1p (syndapin) can stimulate activity of Las17p/Sla1p and it arrives slightly before actin (Sun et al., 2006). It is possible that the Bzz1p F-BAR domain might recognize curvature induced by Syp1p, also an F-BAR protein, and thus be recruited to help relieve Sla1p inhibition at endocytic sites. Las17p has many interacting partners that bind to its proline-rich regions via SH3 domains, but the exact roles of many of these proteins are poorly understood (Madania et al., 1999). The role of Ysc84p as a Las17p activating protein was recently reported while its homolog Lsb3p remains relatively uncharacterized (Robertson et al., 2009), so keys to WASP/Myo activation and repression may reside in interactions of NPFs with relatively unstudied proteins.

Particle-tracking of fluorescently tagged members of the WASP/Myo module reveal that these proteins do not internalize with the newly formed vesicle but rather stay at the plasma membrane (Figure 1.2) (Kaksonen et al., 2005). Immuno-EM studies revealed a second population of Myo5p at the invagination tip, as well as Las17p localized on the sides of tubules (Idrissi et al., 2008). Fluorescence recovery after photobleaching (FRAP) analysis of actin tails in *sla2Δ* cells (Figure 1.3B) suggests that actin is polymerized at the plasma membrane and moves inwards (Kaksonen et al., 2003), so what is the role of Myo5p at the tip of an invagination? The myosin could be acting as a bridge by simultaneously binding to actin filaments and coat proteins, it could be nucleating filaments for a reason that is presently obscure, or it could be acting as a motor generating force through contact with actin filaments. The latter possibility is supported by the observation that motor domain mutants prevent inward movement, but do not prevent actin polymerization, which suggests that the NPF and motor functions are separable, but that both are important (Sun et al., 2006).

Despite the identification of a large number of associated players, how the Arp2/3 complex is regulated during endocytosis remains incompletely understood. Additional

Figure 1.3

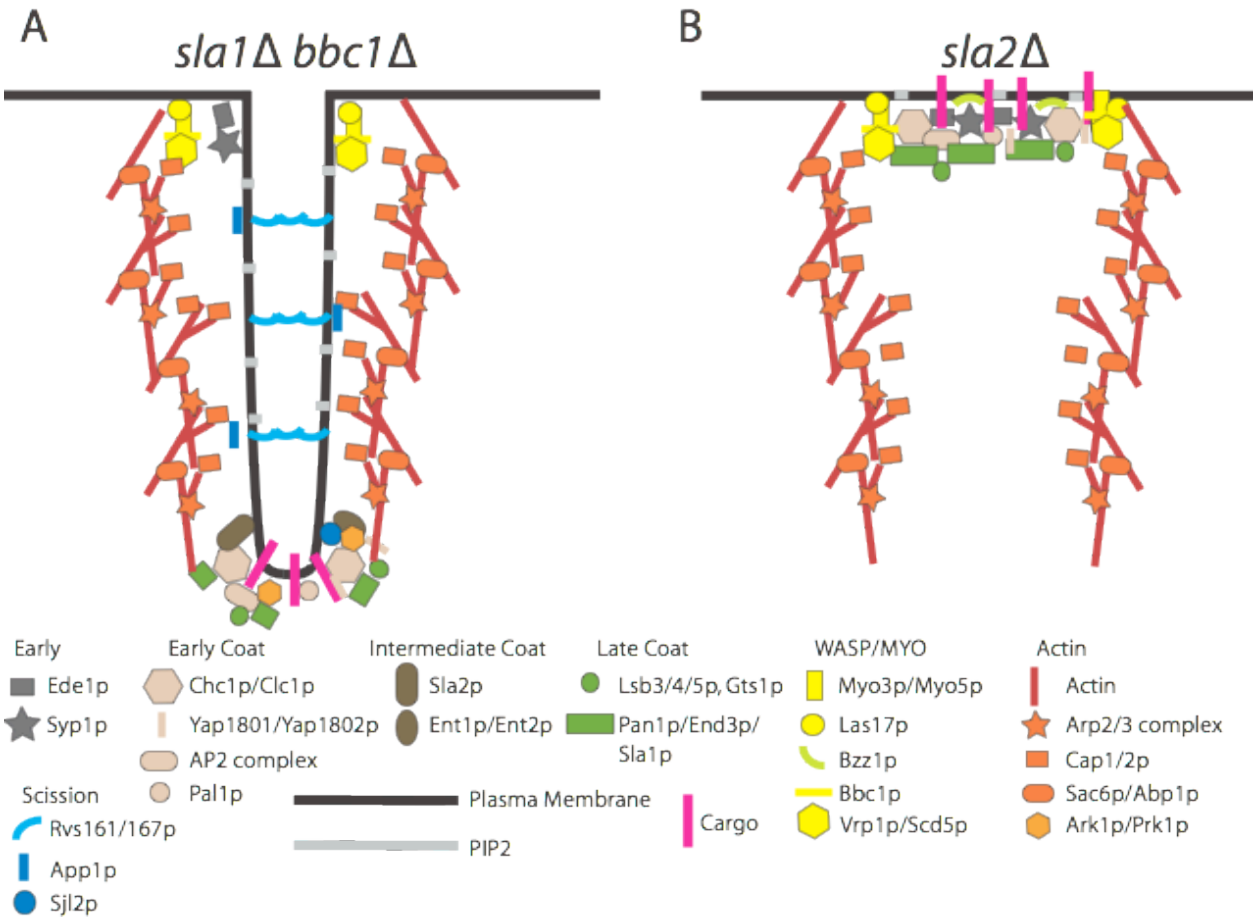

A
sla1Δ bbc1Δ
B
sla2Δ
Early
Ede1p
Syp1p
Early Coat
Chc1p/Clc1p
Yap1801/Yap1802p
AP2 complex
Pal1p
Intermediate Coat
Sla2p
Ent1p/Ent2p
Late Coat
Lsb3/4/5p, Gts1p
Pan1p/End3p/
Sla1p
WASP/MYO
Myo3p/Myo5p
Las17p
Bzz1p
Bbc1p
Vrp1p/Scd5p
Actin
Actin
Arp2/3 complex
Cap1/2p
Sac6p/Abp1p
Ark1p/Prk1p
Scission
Rvs161/167p
App1p
Sjl2p
Plasma Membrane
PIP2
Cargo

Figure 1.3. Endocytic mutants affecting actin polymerization

a. In *sla1Δ bbc1Δ* mutants, Las17p inhibition is greatly reduced, resulting in excessive actin polymerization. The connection between actin and the endocytic coat is intact, so deep invaginations are formed.

b. In *sla2Δ* mutants the connection between actin and the endocytic coat is missing, resulting in long, treadmilling actin tails that continuously assemble proximal to the plasma membrane, and flat membranes. Coat proteins and NPFs remain at the plasma membrane while all the actin-associated proteins normally associated with endocytosis localize to the comet tails.

factors, perhaps unidentified proteins or post-translational and lipid modifications, are likely to act as switches for activating the NPFs in order to promote actin polymerization, membrane invagination and ultimately vesicle scission. Interestingly, it was recently suggested that in fission yeast, actin filaments from one endocytic structure can activate actin assembly at another endocytic site by contacting it (Basu and Chang, 2011). The authors speculate that pre-existing actin filaments in the first structure trigger autocatalytic Arp2/3 activation. This phenomenon has also been observed *in vitro* on NPF-coated beads and rods where actin polymerization does not occur until a short F-actin primer makes contact with the NFP-coated surface (Achard et al., 2010). These results suggest the possibility that endocytic sites are primed to assemble actin and await initiation of the autocatalytic assembly process.

ACTIN POLYMERIZATION

Actin assembly is required for yeast endocytosis. This requirement can be partially overcome by providing osmotic support, which suggests that actin polymerization is needed to counter the turgor pressure at the plasma membrane (Aghamohammadzadeh and Ayscough, 2009). A similar phenomenon in mammalian cells may explain discrepancies in the reported dependency on actin polymerization of mammalian clathrin-dependent endocytosis (Boulant et al.). Severing the attachment between the actin network and the endocytic membrane in *sla2Δ* cells or inhibiting polymerization by latrunculin A sequestration of monomers, stops endocytosis (Kaksonen et al., 2003). The organization and physical properties of the actin network are also important for force generation; deletion of bundling proteins Sac6p (fimbrin) and Scp1p (transgelin) results in nonproductive endocytic sites, although actin can still polymerize at the plasma membrane (Goodman et al., 2003; Kaksonen et al., 2005).

In order to provide force to pull a vesicle out from the planar plasma membrane, the expanding actin network must be tightly connected to the internalizing invagination. In *sla2Δ* cells, which may lack this connection, actin polymerizes into long “comet tails” that continue to undergo flux while endocytosis is unproductive (Figure 1.3B) (Kaksonen et al., 2003). Fluorescence microscopy reveals that while coat proteins remain at the plasma membrane and are not internalized, actin-associated proteins are recruited to the long tails in *sla2Δ* cells (Kaksonen et al., 2003). Electron microscopy confirmed this conclusion as these mutant cells have very few invaginations (C. Buser and D. Drubin, unpublished). Together, these data suggest that Sla2p may be integral in connecting the coat to the polymerizing actin. Sla2p can bind to PIP2 in membranes via its ANTH domain and can interact with other coat proteins including Clc1p, Ede1p and Sla1p, as well as with actin, Las17p, and the Arp2/3 complex (Boettner et al.; Gavin et al., 2002; Gourlay et al., 2003; McCann and Craig, 1999; Newpher et al., 2006; Newpher and Lemmon, 2006; Sun et al., 2005). These many interactions suggest that Sla2p may act as an adaptor, connecting the clathrin coat and plasma membrane to actin filaments, which lets the growing network exert force on the membrane, deforming the membrane and ultimately helping to pinch off a vesicle.

The roles of the Arp2/3 complex and its NPF, Las17p, have been investigated using Las17p-coated microbeads added to yeast extract (Michelot et al., 2010). These beads stimulate actin polymerization and recruit a large number of endocytic proteins. A

cloud of polymerized actin forms and a symmetry-breaking event often follows, resulting in bead motility. Mass spectrometry of the bead-associated actin networks identified actin regulating proteins (e.g., Cap1/2p (capping protein alpha/beta), Sac6p, the Arp2/3 complex) and endocytic specific proteins (e.g., Sla1p and Syp1p). These results demonstrate that an NPF (Las17p) is sufficient to form an actin network of biologically relevant composition. The exact mechanism for creating a branched dendritic network, like that found at endocytic patches (Rodal et al., 2005; Young et al., 2004), versus a parallel bundled network, as exists in cables, is not known, but recent reports from *S. pombe* implicate fimbrin (Sac6p) as an important factor for excluding tropomyosin from endocytic actin filaments via fimbrin's actin binding activity, yet also confirm that its crosslinking activity is important for endocytic function; a truncated fimbrin mutant, which lacks actin bundling activity while preserving actin binding activity, localizes to endocytic sites and excludes tropomyosin, but otherwise phenocopies a fimbrin null (Skau et al., 2011; Skau and Kovar, 2010).

As polymerization is turned off, disassembly of the actin network ensues, and the action of cofilin/Aip1/coronin (Cof1p/Aip1p/Crn1p) becomes dominant. Cof1p binds to older, ADP-bound actin filaments with the aid of Crn1p while being inhibited from binding to younger ATP/ADP+Pi-actin by Crn1p (Gandhi et al., 2009). The Cof1p induces a twist, causing filaments to break apart [55]. Aip1p is important in breaking down short actin filaments produced by Cof1p into monomers (Okreglak and Drubin, 2010). Intriguingly, actin oligomer-based polymerization can occur, and this oligomer assembly pathway is enhanced by the loss of Aip1p, suggesting that oligomer annealing occurs *in vivo* at actin patches (Okreglak and Drubin, 2010).

Roles for actin after uncoating have been suggested. There are reports that vesicles appear to move along actin cables (Huckaba et al., 2004; Toshima et al., 2006). Some imaging suggests that endosomes move into close proximity with internalizing vesicles, facilitating fusion (Toshima et al., 2006). Observing actin cables in EM is very difficult, but immuno-EM results support the existence of a link between cables and endocytic sites (Mulholland et al., 1994).

The importance of actin in yeast endocytosis is unquestionable. Actin assembly provides force necessary for membrane deformation and scission, and likely plays a role in moving the newly formed vesicles away from the plasma membrane. Open questions remain about the regulation of actin assembly, the signals that control the NPFs and initiate polymerization as well as the signals that turn off polymerization to allow for disassembly and the eventual uncoating of the vesicle. However, many of the key players seem to have been identified.

SCISSION AND UNCOATING

In the final steps of endocytosis, the vesicle pinches off from the plasma membrane, moves inward toward the cell center, and the endocytic coat disassembles from the newly formed vesicle. While in mammalian cells the GTPase dynamin is essential for scission of clathrin-coated vesicles, the role of dynamin in yeast endocytosis is unsettled. While at least one study suggested that dynamin does not participate, recent reports have suggested that the dynamin Vps1p might be involved in endocytosis (Nannapaneni et al., 2010; Smaczynska-de et al., 2010; Yu and Cai, 2004).

However, the involvement of Vps1p in multiple important intracellular trafficking events has prevented definitive conclusions from being drawn.

In order for scission to occur, the two membrane bilayers must be brought into close proximity, at a distance of ~10 nm or less, at which point they can fuse and a vesicle can form (Liu et al., 2006). To perform this action, yeast take advantage of the membrane tubulating activities of the BAR and F-BAR proteins. While the Rvs161/167p (amphiphysin) heterodimeric BAR protein complex is capable of deforming membranes, this activity, combined with the force derived from actin polymerization, may not be sufficient to pinch off the membrane (Liu et al., 2006). Where does the rest of the force come from? One possible source is a line tension generated by lipid phase separation, as discussed below. The combination of the line tension, tubulation from BAR proteins and actin polymerization pushing on coat proteins at the bud and perhaps squeezing the tubule are proposed to provide the scission force (Liu et al., 2009; Youn et al.).

This model provides a plausible explanation for why the lipid phosphatase synaptojanin is important for scission (Sun et al., 2007). Along with creating a proposed lipid phase separation by having higher activity on the bud then on the tubule, Sjl2p (synaptojanin) has a role in vesicle uncoating. This is not surprising as a number of endocytic coat proteins that are internalized with the forming vesicle have PIP2 binding domains (Sla2p, Ent1/2p), and thus the action of Sjl2p reduces their affinity for the vesicle as it destroys the PIP2 (Toret et al., 2008). At the same time, the regulatory kinases Ark1p and Prk1p are responsible for phosphorylating a variety of coat proteins including Pan1p, Sla1p and Ent1/2p, and enabling their disassembly (Figure 1.4). Arf3p/Lsb5p/Gts1p also promote disassembly of the Pan1p/Sla1p/End3p complex, yet at the same time *arf3* mutants provide evidence for an Arf3p role in modulation of PIP2 levels (Costa et al., 2005; Smaczynska-de et al., 2008). These results suggest that a wide variety of factors contribute to the dissociation of coat proteins in a coordinated fashion in which multiple pathways converge to dissociate certain key proteins leading to the disassembly of the entire coat. Weakening of the coat-actin connection is expected to briefly relax tension on the membrane, which may be required for the final vesicle scission event (Liu et al., 2009).

While a number of uncoating factors are known, other proteins specific for removing different endocytic proteins, or shutting off actin polymerization, may exist. The signals that turn on or recruit uncoating factors are likewise unclear, but membrane curvature, actin filaments and lipid composition are likely candidates.

PHOSPHORYLATION, UBIQUITINATION AND LIPID MODIFICATION

Endocytosis is regulated by a variety of signals including phosphorylation, ubiquitination and lipid modification. The plasma membrane is a complex sea of proteins and lipids of various types including: phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositols, phosphatidylserine, sphingolipids and ergosterol (van der Rest et al., 1995). These lipids play a poorly understood role in regulating endocytosis. Adding another layer of complexity, the inner and outer leaflets of the plasma membrane are asymmetric, with the inner leaflet being enriched for phosphatidylserine and PIP2. Fluorescent probes combining GFP and lipid-binding protein domains, mutants of enzymes responsible for producing or modifying lipids, and the ability to directly grow

Figure 1.4

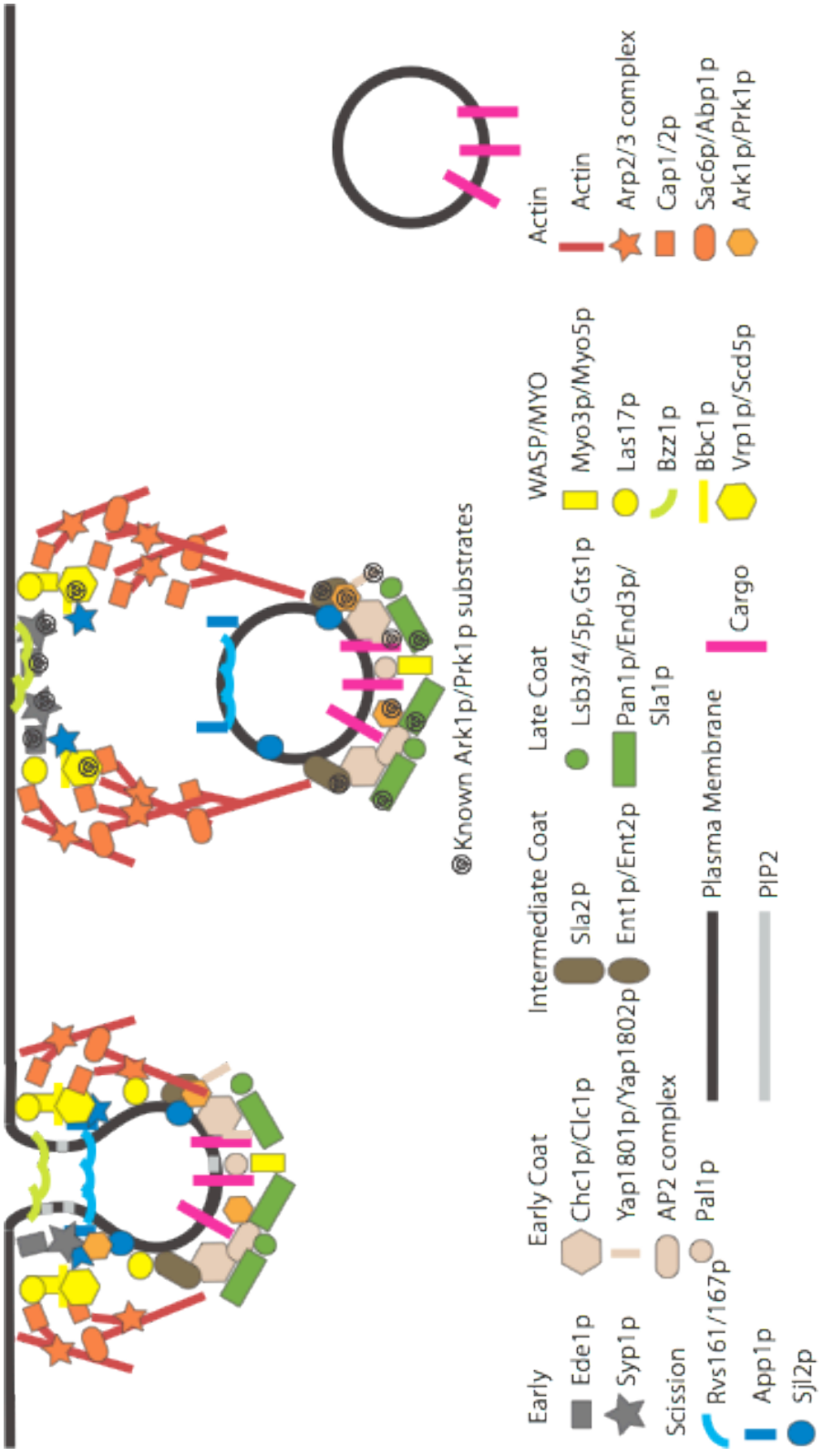

Known Ark1p/Prk1p substrates
Early
Ede1p
Syp1p
Scission
Rvs161/167p
App1p
Sjl2p
Early Coat
Chc1p/Clc1p
Yap1801p/Yap1802p
AP2 complex
Pal1p
Intermediate Coat
Sla2p
Ent1p/Ent2p
Late Coat
Lsb3/4/5p, Gts1p
Pan1p/End3p/
Sla1p
Plasma Membrane
PIP2
Cargo
WASP/MYO
Myo3p/Myo5p
Las17p
Bzz1p
Bbc1p
Vrp1p/Scd5p
Actin
Actin
Arp2/3 complex
Cap1/2p
Sac6p/Abp1p
Ark1p/Prk1p

Figure 1.4. Scission and uncoating of endocytic vesicles

Scission is accomplished by constriction of the bud neck, driven by BAR protein driven membrane deformation, actin generated force and the proposed Sjl2p-imposed line tension created by lipid phase separation. After scission, the Pan1 complex proteins Sla1p, Pan1p and End3p are phosphorylated by Ark1p/Prk1p resulting in their dissociation. Ark1p/Prk1p are also responsible for turning off actin polymerization, allowing Cof1p/Aip1p/Crn1p to disassemble the actin filaments. Sjl2p is responsible for dephosphorylating PIP2, reducing the affinity of Sla2p and Ent1/2p for the vesicles. Arf3p, along with Gts1p and Lsb5p, are also involved in the dissociation of the Pan1 complex.

yeast in or on media containing exogenous lipids has facilitated studies of how lipids contribute to endocytic regulation.

The importance of PIP2 in yeast endocytosis has been demonstrated genetically; mutants of Mss4p, which produces PIP2 from PIP, and of Sjl1p and Sjl2p, the PIP2 phosphatases, have endocytic defects (Singer-Kruger et al., 1998; Sun et al., 2007). Using the PIP2-binding ANTH domain of Sla2p, it was demonstrated that PIP2 is concentrated at endocytic sites as the endocytic coat assembles, and disappears upon scission, soon after Sjl2p is recruited (Sun et al., 2007). It should be noted that while PIP2 appears to be concentrated at endocytic sites, Mss4p is more broadly distributed on the plasma membrane, which suggests that PIP2 may be concentrated by PIP2-binding coat proteins such as Sla2p, Ent1/2p and Rvs161/167p.

The phenotypes of synaptojanin mutants suggest multiple roles for PIP2 in endocytosis. Disassembly of PIP2-binding proteins upon vesicle formation is delayed in these mutants, suggesting that Sjl2p hydrolysis of PIP2 is important for uncoating of Sla2p and Ent1/2p (Sun et al., 2007; Toret et al., 2008). Secondly, long extended invaginations with coat proteins at the tips form in *sjl1Δ sjl2Δ* cells suggesting a role for PIP2 regulation in scission (Sun et al., 2007). Thirdly, these mutant cells inappropriately initiate invaginations on invaginations, suggesting a role for PIP2 as a signal for formation of endocytic sites (Sun et al., 2007). Mathematical modeling led to the proposal that PIP2's scission role might be in development of a line tension between an area of low PIP2 concentration - the tip of the invagination where Sjl2p is active - and the extended tubule where PIP2 is protected from Sjl2p by the BAR domain proteins (Liu et al., 2009; Sun et al., 2007). The observed phenotype fits this hypothesis; when Sjl2p is missing, a concentration gradient cannot form, and so scission does not occur. Experimental support for this model was provided by studies in mammalian cells on dynamin-independent scission (Romer et al.).

While the role of PIP2 in endocytosis may be best studied among the lipids, genetic analyses suggest that sterols and sphingolipids may also be important for endocytosis (Friant et al., 2001; Munn et al., 1999; Zanolari et al., 2000). Deletions of ergosterol biosynthetic enzymes *ERG2* or *ERG6* result in endocytic defects that are exacerbated when combined with each other or with deletions of *ERG3* (Heese-Peck et al., 2002; Munn et al., 1999). Temperature-sensitive mutations in *LCB1/END8*, whose product catalyzes the first step in sphingolipid synthesis, are deficient in endocytosis, and this block can be overcome by addition of exogenous sphingoid bases (Zanolari et al., 2000). As new tools for the detection and modulation of other specific lipids are developed, our understanding of their contributions to endocytosis will be expanded.

Ubiquitination and phosphorylation have long studied roles in regulating the internalization of transmembrane cargo molecules. In the canonical model, a receptor gets phosphorylated upon ligand binding, the phosphorylation signals for ubiquitination, which can be in the form of mono-, multiple mono-, or K63-linked poly-ubiquitinations, which leads to internalization mediated by ubiquitin-binding coat molecules. Ubiquitination of cargo molecules is carried out by the WW domain HECT-type ubiquitin ligase Rsp5p assisted by a number of adaptor proteins including the recently described family of arrestins (Lin et al., 2008). Mutations in Rsp5p's individual WW domains, which

mediate protein-protein interactions, affect the internalization of specific cargos, while temperature-sensitive mutations in this protein generally reduce fluid-phase endocytosis (Gajewska et al., 2001).

It is possible that Rsp5p also regulates the endocytic machinery directly. Mammalian homologs of Ede1p and Ent1/2p - Eps15 and epsin respectively - are ubiquitinated *in vivo*, and are thought to be regulated by binding to their own modifications (Oldham et al., 2002). Rsp5p also may regulate endocytosis via effects on the actin cytoskeleton (Kaminska et al., 2002). The BAR domain protein Rvs167p has been reported to be ubiquitinated by Rsp5p, but no phenotype was observed in the K->R mutant, in which the lysine proposed to be ubiquitinated is mutated to arginine (Table II) (Stamenova et al., 2004).

Phosphorylation of Pan1p by Prk1p prevents binding of Pan1p to F-actin, a prerequisite for its activation of the Arp2/3 complex (Toshima et al., 2005). A role for Ark1p and Prk1p as negative regulators of endocytic actin assembly is supported by the observation that *ark1Δ prk1Δ* cells have large clumps of actin, presumably due to uncontrolled actin polymerization (Cope et al., 1999; Sekiya-Kawasaki et al., 2003). These data suggest that Prk1p activity is important both for uncoating of internalized vesicles and cessation of actin polymerization. Prk1p autophosphorylation provides a mechanism for regulation of its kinase activity. Prk1p phosphorylates multiple targets, including Sla1p. These phosphoproteins then stimulate Prk1p autophosphorylation which reduces its kinase activity (Huang et al., 2009). This mechanism may both prevent hyperphosphorylation of targets and turn off Prk1p activity.

Despite recent advances, many questions about how yeast endocytosis is regulated still need to be addressed: Do lipids in addition to PIP2 play important regulatory roles? What is the nature of the signal that specifies endocytic site selection? Is the endocytic machinery ubiquitinated as a regulatory mechanism? Does phosphorylation only regulate the endocytic machinery during late steps in endocytosis? Dissection of regulatory mechanisms is an exciting and fertile area of current research.

ULTRASTRUCTURAL ANALYSIS

Fluorescence microscopy has proven to be an invaluable tool for studying endocytosis in yeast. With a practical resolution limit of ~200nm, conventional fluorescence microscopy cannot resolve important details such as the shape of the invagination, where proteins localize along the invagination, or the orientation and organization of the actin network. These details can only be resolved using electron microscopy and possibly one day by super-resolution fluorescence microscopy. Unfortunately, there are several complications to studying endocytosis via electron microscopy. Firstly, as endocytosis is a highly dynamic process and EM is performed on fixed cells, one can only capture snapshots and it may be unclear exactly what step in the process an image reveals. Secondly, the more highly curved the cell, the harder it is to section perpendicularly to the plasma membrane so that the invagination is in the section and is oriented properly. Sites of endocytosis are concentrated in the more highly curved small bud (Figure 1). Thirdly, the cytoplasm of yeast is especially dense and filled with ribosomes, making it suboptimal for viewing actin filaments, which are notoriously hard to view by electron microscopy in the first place.

Immuno-EM was used to demonstrate precise localization of various endocytic proteins (Idrissi et al., 2008). These results validated many of the proposed localizations of proteins based on fluorescence microscopy studies, such as the BAR domain protein Rvs167p localizing to the sides of tubules. However, the appearance of Myo5p not only at the base but also at the tips of invaginations was an unexpected result, as was the localization of Las17p at both the base of the invagination and partway up the tubule (Idrissi et al., 2008; Kaksonen et al., 2003; Sun et al., 2006). These observations demonstrate the importance of ultrastructural analysis in providing critical information on the localization of proteins that could not be acquired by fluorescence microscopy alone (Idrissi et al., 2008; Mulholland et al., 1994).

Recent innovative use of correlative light-electron microscopy has allowed researchers to tackle the problem of determining which transient stage of endocytosis has been captured in an electron micrograph. An elegant correlative technique was developed in which fixed samples are imaged first by fluorescence microscopy, and then by electron microscopy (Kukulski et al., 2011). Using fluorescent microbeads as fiduciary marks, the authors could image the same endocytic sites using both techniques. By using two fluorescent markers that localize to endocytic sites at different times, the authors classified the sites into two groups, those with Abp1-RFP only and those with both Abp1-RFP and Rvs167-GFP, a scission protein. Since approximately half of the double-labeled sites had vesicles and half had intact invaginations, the authors concluded that scission must occur halfway through the ~10s period during which the two proteins colocalize. This technique holds promise for such applications as determining exactly when invaginations begin to form relative to recruitment of specific proteins and which proteins are first involved in constricting the neck of an invagination (Kukulski et al., 2011).

For this correlative light-electron microscopy study, high-pressure freezing with freeze-substitution was used, which is best able to preserve the fine structure of dynamic cellular features such as endocytic invaginations. In the future, combining high-pressure freezing/freeze-substitution with immunostaining promises to both faithfully preserve structural features and provide precise localization of specific proteins. Modulating the stains used in the freeze-substitution steps can enhance the contrast between membranes and ribosome-rich yeast cytoplasm creating even clearer images (Mobius, 2009).

One crucial challenge for ultrastructural analysis is to elucidate the exact geometry of the actin network that provides much of the force that drives invagination and scission. Important unanswered questions that could potentially be addressed by ultrastructural analysis include: Where do the filaments attach to the invagination and with what orientation? Where do they contact the plasma membrane? Does the network enclose the entire invagination or is the tip left exposed? Where exactly are the assembly nucleators? Are they all at the base of the invagination or are some at the tip or on the sides? Further technical innovation will be required to tackle the crucial but very challenging problem of reliably visualizing actin in EM of yeast. Analysis of unroofed yeast cells is one promising avenue to overcoming the challenge presented by the dense cytoplasm of yeast (Rodal et al., 2005).

THEORETICAL MODELING

Mathematical modeling is an important tool for exploring the mechanical and physical aspects of biological processes. If experimentalists can provide theoreticians with sufficient high quality quantitative data, theoretical models can be developed. Such models allow researchers to understand how the process can and cannot work, they provide testable hypotheses, and they identify areas in which our understanding is incomplete, stimulating further experimentation, which in turn inspires new iterations of the models.

In an attempt to develop a comprehensive model for the endocytic pathway, over 20 measured parameters from a variety of studies were used to model the recruitment of endocytic proteins, membrane deformation and scission (Liu et al., 2009). The most important notion introduced in this model is that membrane curvature is a signal source for biochemical reactions involved in membrane scission. The model accurately predicted phenotypes of some mutations, such as the retraction seen in BAR domain knockouts, validating the model's predictive powers (Liu et al., 2009).

The model also established that much of the scission force could be generated by a lipid phase separation. The calculated force of actin polymerization was found to be too small to drive membrane deformation and scission to completion. The authors speculated that scission might be driven by an interfacial force from lipid phase separation created when PIP2 is concentrated in the tubule and depleted in the bud. The model proposed that a pair of feedback loops drive this process (Figure 1.5). First, binding of BAR domain proteins on the tubule deforms the membrane, thus promoting recruitment of additional BAR domain proteins. These BAR proteins were proposed to protect PIP2 from dephosphorylation by the synaptojanin Sjl2p, the phosphatase responsible for PIP2 dephosphorylation. Second, BAR protein-induced curvature along the tubule was predicted to drive recruitment and activation of Sjl2p at the unprotected tubule-bud interface. As PIP2 is hydrolyzed, the phase boundary becomes more pronounced, and a line tension at the interface increases curvature, further increasing Sjl2p's recruitment and activity. The model proposes that these two positive feedback loops rapidly generate the force necessary to drive scission (Liu et al., 2009).

The notion that Sjl2p's activity might be stimulated by increasing membrane curvature recently gained experimental support when it was shown in a cell-free system that synaptojanin-1 is more efficient at dephosphorylating PIP2 in more highly curved membranes, especially when a BAR domain protein is added to create very highly curved membranes (Chang-Ileto et al., 2011). This observation validates a major requirement of the model.

This model highlights one of the main differences between mammalian and yeast clathrin-mediated endocytosis: the apparent lack in yeast of dynamin participation in scission. It was long assumed that the requirement for actin in yeast endocytosis indicated that the primary scission force was provided by actin polymerization, but this model suggests that the phase separation of PIP2 also contributes necessary force.

Another recent mathematical model focused on force generation by actin polymerization during fission yeast endocytosis (Berro et al., 2010). Using constants

Figure 1.5

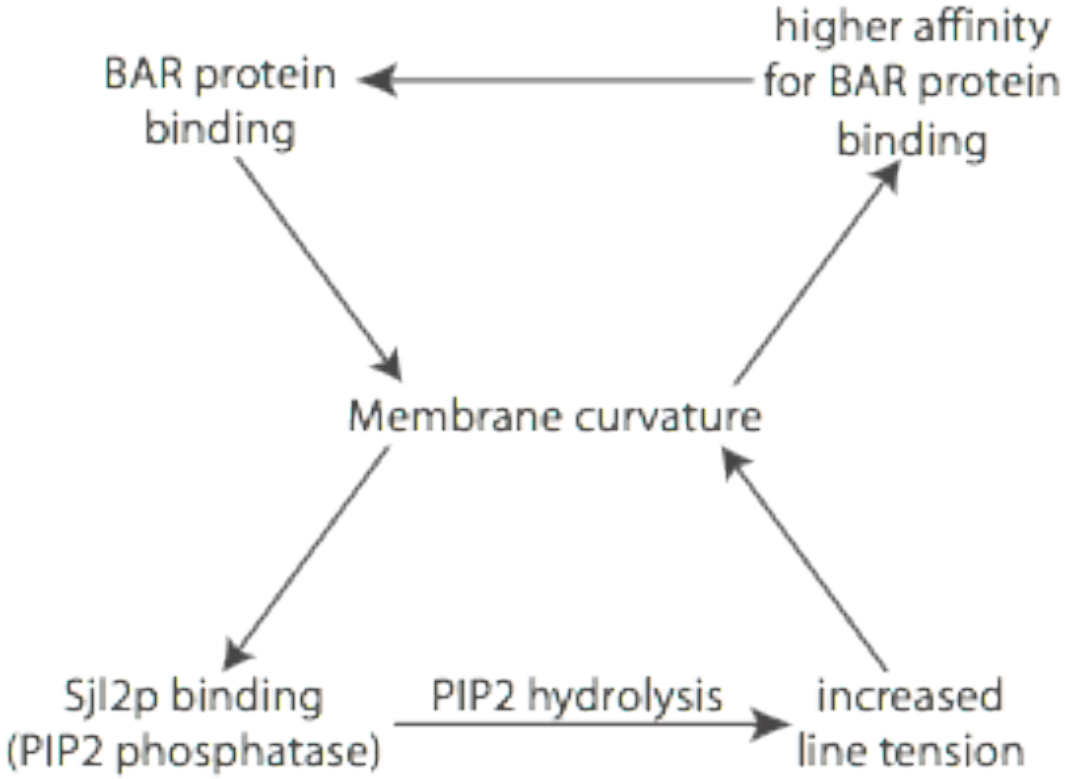

higher affinity
for BAR protein
binding
BAR protein
binding
Membrane curvature
Sjl2p binding
(PIP2 phosphatase)
PIP2 hydrolysis
increased
line tension

Figure 1.5. Positive feedback loops involved in scission

During scission, two positive feedback loops are proposed to drive the increase in membrane curvature that leads to pinching off of the vesicle. Firstly, BAR domain proteins bind to areas of high membrane curvature generated by actin polymerization forces, and through their binding enhance the membrane curvature, recruiting more BAR domain proteins. Secondly, Sjl2p, the PIP2 phosphatase, is more active on curved membranes [83]. The activity of Sjl2p on the curved neck decreases levels of PIP2 on the unprotected bud while the PIP2 of the neck is shielded by the BAR domain proteins. The lipid-phase separation produces a line tension, which further increases membrane curvature, enhancing the activity of Sjl2p.

derived from *in vitro* biochemical studies and quantification of local protein concentrations by fluorescence microscopy *in vivo*, the authors developed a model focused on actin polymerization and depolymerization at endocytic sites. Among the novel conclusions drawn from this study were: (i) *in vivo* binding of capping protein and Arp2/3 complex are faster than *in vitro* data suggest, and (ii) disassembly happens faster than can be explained solely by pointed-end depolymerization, which suggests that severing proteins may produce small fragments of actin that diffuse away and depolymerize away from the patch. *In vitro,* Aip1p enhances the depolymerization of short fragments produced by cofilin severing (Okreglak and Drubin, 2010).

RELATIONSHIP TO MAMMALIAN ENDOCYTOSIS

Of the ~60 yeast proteins known to be localized to sites of endocytosis, ~85% have homology to mammalian proteins also involved in endocytosis (Table I). There are several striking examples wherein a single, modular mammalian protein has homology to several yeast proteins which each contain a subset of the modules. For example, mammalian intersectin has two Eps15 homology domains and 5 SH3 domains and is believed to play a similar role to the yeast proteins Pan1p and Sla1p. Together, these proteins have two Eps15 homology domains (Pan1p) and three SH3 domains (Sla1p) and are known to function in a complex together with End3p, which has another two Eps15 homology domains. The functional and domain homology suggests that while individual proteins may not be perfectly conserved, the network of interactions that hold the endocytic machinery together is conserved (Taylor et al., 2011).

The dynamic, ordered recruitment of endocytic proteins is similar between yeast and mammals (Taylor et al., 2011). Clathrin and adaptor proteins arrive early, followed by additional coat proteins, then actin and scission factors, and ending with uncoating proteins. Although several publications have emphasized differences in the dynamics of endocytosis in yeast and mammals, to what extent apparent differences were due to how the process was being studied was not clear. For yeast, but not mammalian cell studies, fluorescent endocytic proteins are typically expressed from their native promoters without overexpression. Using zinc-finger nucleases to edit the genomes of mammalian cell lines, tagged clathrin light chain and dynamin were expressed at endogenous levels and the observed dynamics of mammalian endocytosis were described to be more similar to those of yeast than had previously been appreciated (Doyon et al., 2011).

Interestingly, in contrast to mammalian cells, endocytosis in yeast still occurs in both *clc1Δ* and *chc1Δ* cells, establishing that while important, clathrin is not essential for yeast endocytosis (Newpher et al., 2005; Tan et al., 1993). While clathrin-mediated endocytosis is conserved from yeast to mammals, no yeast counterparts for mammalian clathrin-independent pathways such as caveolin-mediated endocytosis have been identified. This relative simplicity enhances the power of yeast genetics because it is possible to focus on a single pathway without the confounding endocytosis of cargo by multiple pathways.

One potential major difference between yeast and mammalian endocytosis concerns the function of dynamin during scission. The yeast genome encodes three dynamin-related GTPases. Two are associated with mitochondria while the third, Vps1p,

is crucial for intracellular trafficking events, but is not essential for endocytosis. Strikingly, the physical appearance of clathrin-coated pits in dynamin knockout mouse cells examined by electron microscopy strongly resemble the tubular invaginations present in yeast cells. Like yeast cells, the mammalian tubular invaginations were formed in an actin-dependent manner (Ferguson et al., 2009).

A third major difference is that mammalian but not yeast endocytosis relies heavily on the AP2 complex as a cargo adaptor; several well-studied cargos including the transferrin receptor show reduced internalization in AP2 knockdowns (Motley et al., 2003). In yeast, the only known role for the AP2 complex is in endocytosis of the killer toxin K28; internalization of most cargo molecules is not affected in cells lacking the AP2 complex (Carroll et al., 2009).

Because of the extensive homology between proteins and dynamics of yeast and mammalian clathrin-mediated endocytosis, findings in one system are often applicable in the other. Comparisons provide an avenue toward distilling fundamental mechanisms of endocytosis.

CONCLUDING REMARKS

The combination of traditional genetic approaches with the introduction of two-color, live-cell fluorescence microscopy, modern genomic techniques and mass spectrometry has resulted in a plethora of new discoveries about yeast endocytosis. Nearly 60 proteins are known to localize to sites of endocytosis and functions have been identified for many of these proteins. Yet open questions exist for all steps of the endocytic process. How sites are initiated, how activities of the ~60 proteins are coordinated, and how actin polymerization and uncoating are regulated are examples of active areas of research, which promise to further our understanding of this vital process in the coming years.

One of the most active and fertile areas of research is investigation of the role of post-translational modifications in regulating the endocytic machinery. The role of ubiquitination of the machinery has been studied far less than the role of phosphorylation, yet mutations in deubiquitinases and the E3 ligase Rsp5p have clear endocytic phenotypes.

CHAPTER 2
Regulation of clathrin-mediated endocytosis by dynamic ubiquitination and deubiquitination

INTRODUCTION

Clathrin-mediated endocytosis in yeast or mammals requires the coordinated actions of over 60 different proteins at distinct plasma membrane foci containing filamentous actin (Kaksonen et al., 2003; Kaksonen et al., 2005; Taylor et al., 2011). While the order of arrival and departure of these many proteins has been described with high precision, the role of post-translational modifications in regulating the ordered arrival and disassembly of the endocytic machinery has been less fully explored. A role for the Ark1p/Prk1p kinases has been established (Chi et al., 2012; Cope et al., 1999; Sekiya-Kawasaki et al., 2003; Toret et al., 2008; Zeng et al., 2001), yet how dynamic ubiquitination and deubiquitination might regulate the endocytic machinery is not known.

A role for ubiquitin as a signal molecule for internalization of cargo molecules is well established (Egner and Kuchler, 1996; Hicke and Riezman, 1996; Kolling and Hollenberg, 1994). The role of arrestins and other endocytic proteins as specific adaptors that promote ubiquitination and internalization of specific cargos has also been explored (Lin et al., 2008). However, while there is some evidence that ubiquitination and ubiquitin-binding domains may help hold components of the endocytic machinery together (Dores et al., 2010), how ubiquitination affects the dynamics and function of the endocytic machinery has not been well studied. A mutant of Rvs167p that lacks a ubiquitinated lysine was reported to have no phenotype (Stamenova et al., 2004), and although ubiquitination of Eps15 (homologous to yeast Ede1p) has been proposed to control an intramolecular interaction, this interesting hypothesis lacks supporting evidence (Hoeller et al., 2006; Woelk et al., 2006).

Since the yeast genome encodes 43 E3 ubiquitin ligases and 19 deubiquitinases (DUBs)(Cherry et al., 2012), assigning endocytic functions to any given enzyme can be complicated as a result of compensatory effects from other enzymes when one is mutated, as well as participation of enzymes in multiple processes. The yeast Nedd4 homologue, Rsp5p, a HECT domain E3 ligase, is known to ubiquitinate endocytic proteins and endocytic cargos (Dores et al., 2010; Gupta et al., 2007; Stamenova et al., 2004). Rsp5p can bind to lipids via its C2 domain and to proteins via its WW domains, and it interacts with adaptor proteins such as the arrestins (Dunn et al., 2004; Gajewska et al., 2001; Lin et al., 2008). Temperature-sensitive mutations in Rsp5p have fluid-phase endocytosis defects (Gajewska et al., 2001). Rsp5p performs a coupled monoubiquitination of Vps9p wherein the substrate binds to a ubiquitin modification on the ligase before being ubiquitinated, and is thought to likewise ubiquitinate Ede1p after it binds to the modified ligase (Woelk et al., 2006). Rsp5p is an essential protein also involved in RNA export, regulation of fatty acid biosynthesis, mitochondrial organization, and DNA repair (Fisk and Yaffe, 1999; Jeong et al., 2005; Kaliszewski et al., 2008; Neumann et al., 2003).

The DUB Ubp2p is known to physically associate with Rsp5p and is specific for K63 linkages, which are known to be important for endocytosis, making it a strong candidate for regulating ubiquitination of the endocytic machinery (Kee et al., 2005; Kee

et al., 2006). The DUB Ubp7p was identified via phage display as an interaction partner for the SH3 domains of multiple endocytic proteins (Tonikian et al., 2009), and it appears in actin tails polymerized by Las17p (yeast WASP)-coated beads (Michelot et al., 2010).

RESULTS AND DISCUSSION

Ubp7p localizes to endocytic sites and Rsp5p can be trapped at the plasma membrane

A genomic N-terminal fusion of the GFP coding sequence to the Ubp7p coding sequence, driven by the endogenous promoter, encodes a protein that localizes to dynamic plasma membrane puncta, as does a C-terminal fusion that truncates the UCH domain (Figure 2.1A, Figure 2.2). The dynamics and internalization of the tagged actin-binding protein Abp1-RFP are normal in both strains (data not shown). Ubp7ΔUCH-GFP puncta have a short lifetime of ~13 seconds and do not internalize with the nascent vesicle, but remain at the plasma membrane (Figure 2.1C). The latter behavior was exhibited by several proteins involved in actin polymerization, such as Las17p and Myo3/5p, but was not exhibited by coat proteins such as Sla1p, which internalize with the vesicle (Kaksonen et al., 2005).

Ubp7ΔUCH-GFP puncta colocalize with Abp1-RFP (Figure 2.1B). In striking contrast, in *sla2Δ* cells, which have a strong endocytic block and form long actin tails due a disruption of the link between the endocytic coat and actin filaments (Skruzny et al., 2012), Ubp7p localizes only at the plasma membrane, as do coat proteins, and not to the actin tails, where actin-regulating proteins localize (Figure 2.1D). Similarly, in cells treated with the actin monomer sequestering drug latrunculin A, Ubp7ΔUCH-GFP localizes to cortical puncta even after actin has depolymerized (data not shown), a behavior shared with coat proteins. The proline rich region of Ubp7p interacts with the SH3 domains of endocytic proteins (Tonikian et al., 2009). Surprisingly, single deletion mutants of any of several of these SH3-containing endocytic proteins, greatly reduces Ubp7ΔUCH-GFP cortical localization (Figure 2.1F, Figure 2.3) suggesting that Ubp7p is recruited to the plasma membrane by SH3-containing endocytic proteins. Since many of these SH3 domain proteins arrive long before Ubp7p is detectable at endocytic patches, they may bind to proline-rich regions of other proteins. Perhaps Ubp7p is recruited when other proteins with proline-rich regions, such as Gts1p, Scd5p and Aim21p begin to leave the patch (Tonikian et al., 2009). Once recruited, Ubp7p presumably removes ubiquitin from endocytic proteins.

C-terminal fusions of Rsp5p are lethal (Lam et al., 2009; Salvat et al., 2004), but N-terminal fusions as the sole source of Rsp5p in the cell allow for normal growth (Kee et al., 2005). N-terminal GFP fusions localize diffusely to the cytoplasm and to small fast moving internal puncta and larger slower moving puncta (Supplementary Movie 1). There was no detectable colocalization of GFP-Rsp5 with Abp1-RFP or Sac6-RFP by epifluorescence. Moreover, there was no detectable colocalization with Sac6-RFP by TIRFM. We therefore hypothesized that Rsp5p's interactions with substrate endocytic proteins may be transient and thus difficult to visualize. Endocytic mutants *sla2Δ* and *ark1Δ prk1Δ* cause strong endocytic blocks, so we imaged GFP-Rsp5 in these mutants to test whether it gets trapped at the cell cortex. We observed a strong cortical

Figure 2.1

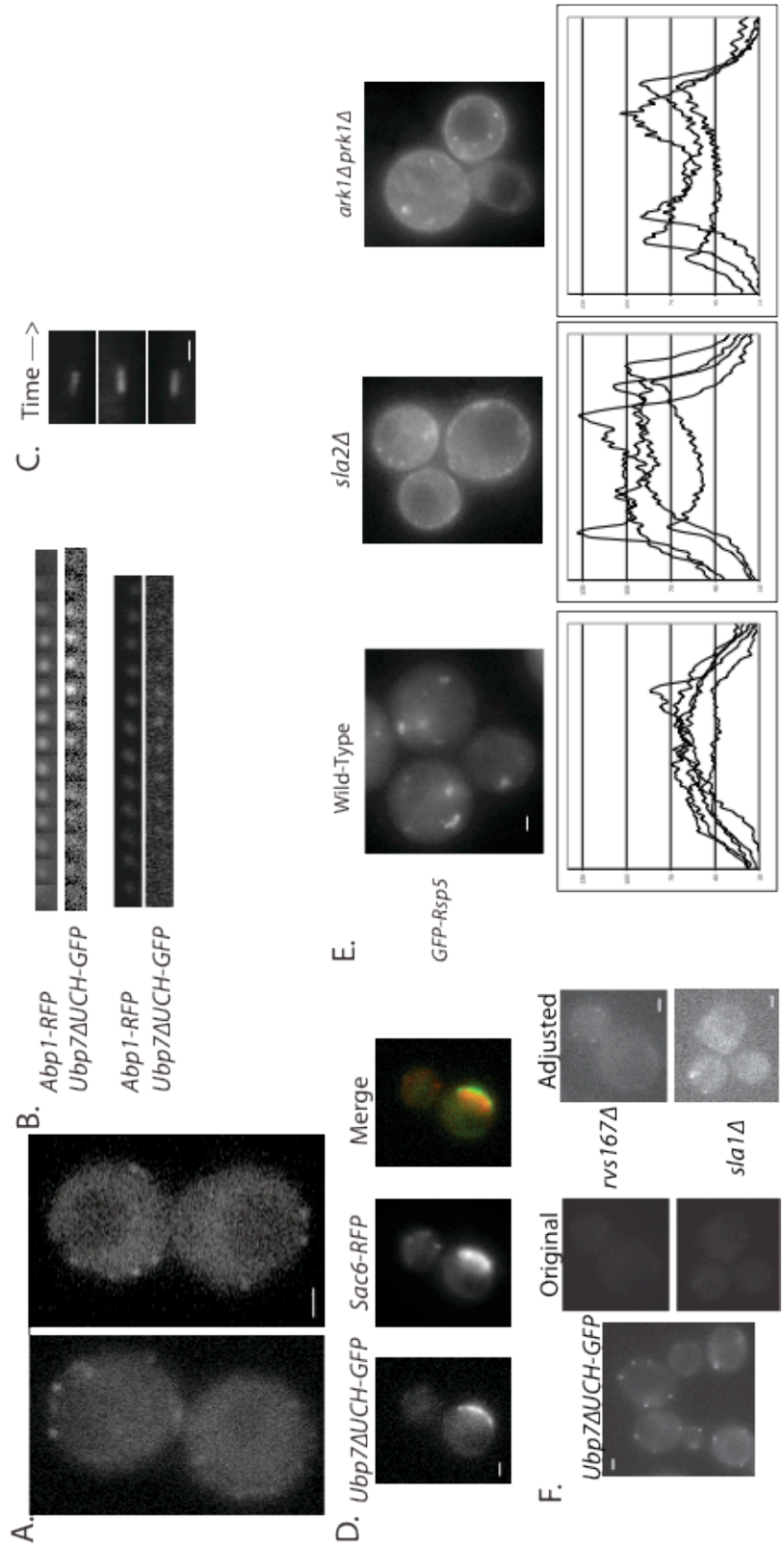
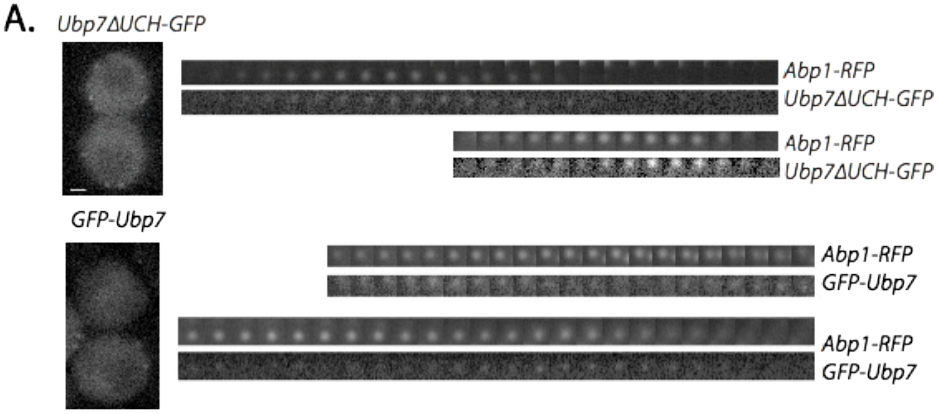
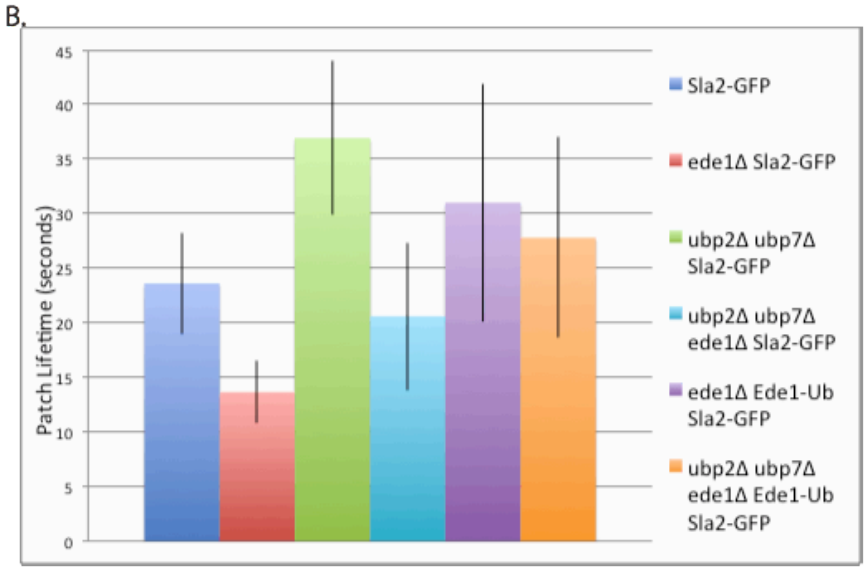

A.
B.
Abp1-RFP
Ubp7ΔUCH-GFP
Abp1-RFP
Ubp7ΔUCH-GFP
C.
Time —>
D.
Ubp7ΔUCH-GFP
Sac6-RFP
Merge
E.
Wild-Type
sla2Δ
ark1Δprk1Δ
GFP-Rsp5
F.
Ubp7ΔUCH-GFP
Original
Adjusted
rvs167Δ
sla1Δ

Figure 2.1: Localization of ubiquitination and deubiquitination machinery (A) GFP tagged Ubp7p localizes to cortical puncta. (B) Dual-color microscopy reveals that Ubp7ΔUCH-GFP colocalizes with Abp1-RFP at endocytic sites and arrives late in the process after actin begins assembling. (C.) Kymographs of Ubp7ΔUCH-GFP suggest that Ubp7p localizes to the plasma membrane for a short time (~13 seconds) and does not internalize with the budding vesicle. (D) In an *sla2Δ* mutant endocytosis is blocked and large actin tails are formed inside the cell as actin subunits continue to flux through the network. Actin binding proteins such as Sac6p decorate the tail while Ubp7ΔUCH-GFP is confined to the plasma membrane similar to endocytic coat proteins. (E) GFP-Rsp5 normally localizes to internal puncta, but in endocytic mutants such as *sla2Δ* and *ark1Δ prk1Δ*, which have strong endocytic blocks, GFP-Rsp5 also appears on the plasma membrane. Line scans of wild-type cells show an even background with internal puncta, while *sla2Δ* and *ark1Δ prk1Δ* line scans show strong peaks at the edges of cells, consistent with a plasma membrane localization (F) Ubp7ΔUCH-GFP cortical localization is reduced in cells missing SH3 domain proteins, original and brightness/contrast adjusted images are shown. Scale bars are 1μm in A and D-F, 200nm in C.

Figure 2.2


A.
Ubp7ΔUCH-GFP
Abp1-RFP
Ubp7ΔUCH-GFP
Abp1-RFP
Ubp7ΔUCH-GFP
GFP-Ubp7
Abp1-RFP
GFP-Ubp7
Abp1-RFP
GFP-Ubp7


B.
Patch Lifetime (seconds)
0
5
10
15
20
25
30
35
40
45
Sla2-GFP
ede1Δ Sla2-GFP
ubp2Δ ubp7Δ Sla2-GFP
ubp2Δ ubp7Δ ede1Δ Sla2-GFP
ede1Δ Ede1-Ub Sla2-GFP
ubp2Δ ubp7Δ ede1Δ Ede1-Ub Sla2-GFP

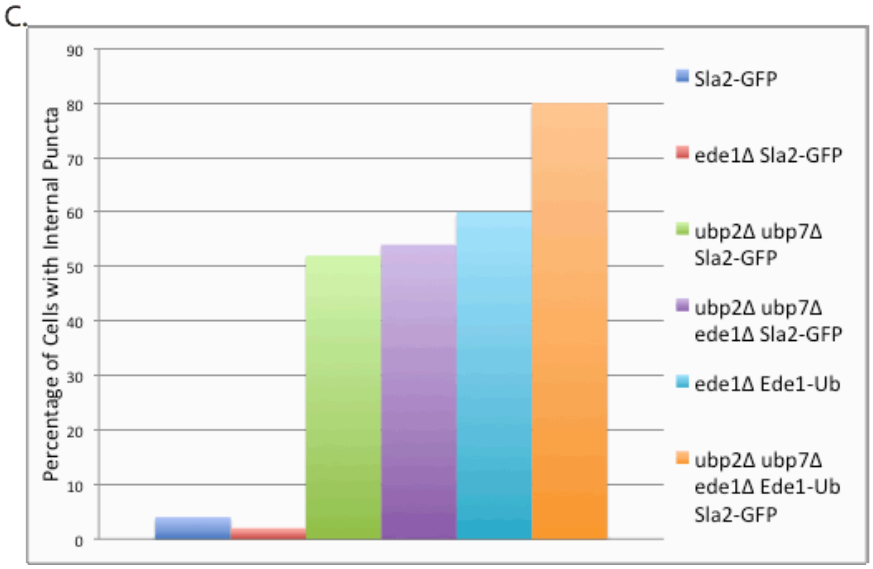

C.
Percentage of Cells with Internal Puncta
0
10
20
30
40
50
60
70
80
90
Sla2-GFP
ede1Δ Sla2-GFP
ubp2Δ ubp7Δ Sla2-GFP
ubp2Δ ubp7Δ ede1Δ Sla2-GFP
ede1Δ Ede1-Ub
ubp2Δ ubp7Δ ede1Δ Ede1-Ub Sla2-GFP

Figure 2.2 N- and C-terminal GFP tags of Ubp7p behave with similar dynamics
(A) GFP-Ubp7 and Ubp7ΔUCH-GFP both localize to cortical puncta and colocalize with Abp1-RFP, arriving after Abp1p, departing at a similar time, remaining at the cell surface, and not internalizing with the nascent vesicle. Scale bar is 1μm. (B) Deletion of *EDE1* results in a decrease in patch lifetime while deletion of *UBP2* and *UBP7* results in an increase in patch lifetime. Patch lifetimes in triple mutants are close to normal, while expression of Ede1-Ub results in a slightly longer than normal patch lifetime (n>100 patches). Error bars are ± 1 standard deviation. (C.) The number of cells with internal patches is minimal in wild-type and *ede1Δ* cells, elevated in *ubp2Δ ubp7Δ*, Ede1-Ub, and *ubp2Δ ubp7Δ ede1Δ* cells, and highest in *ubp2Δ ubp7Δ ede1Δ* Ede1-Ub cells (n≥50 cells).

Figure 2.3

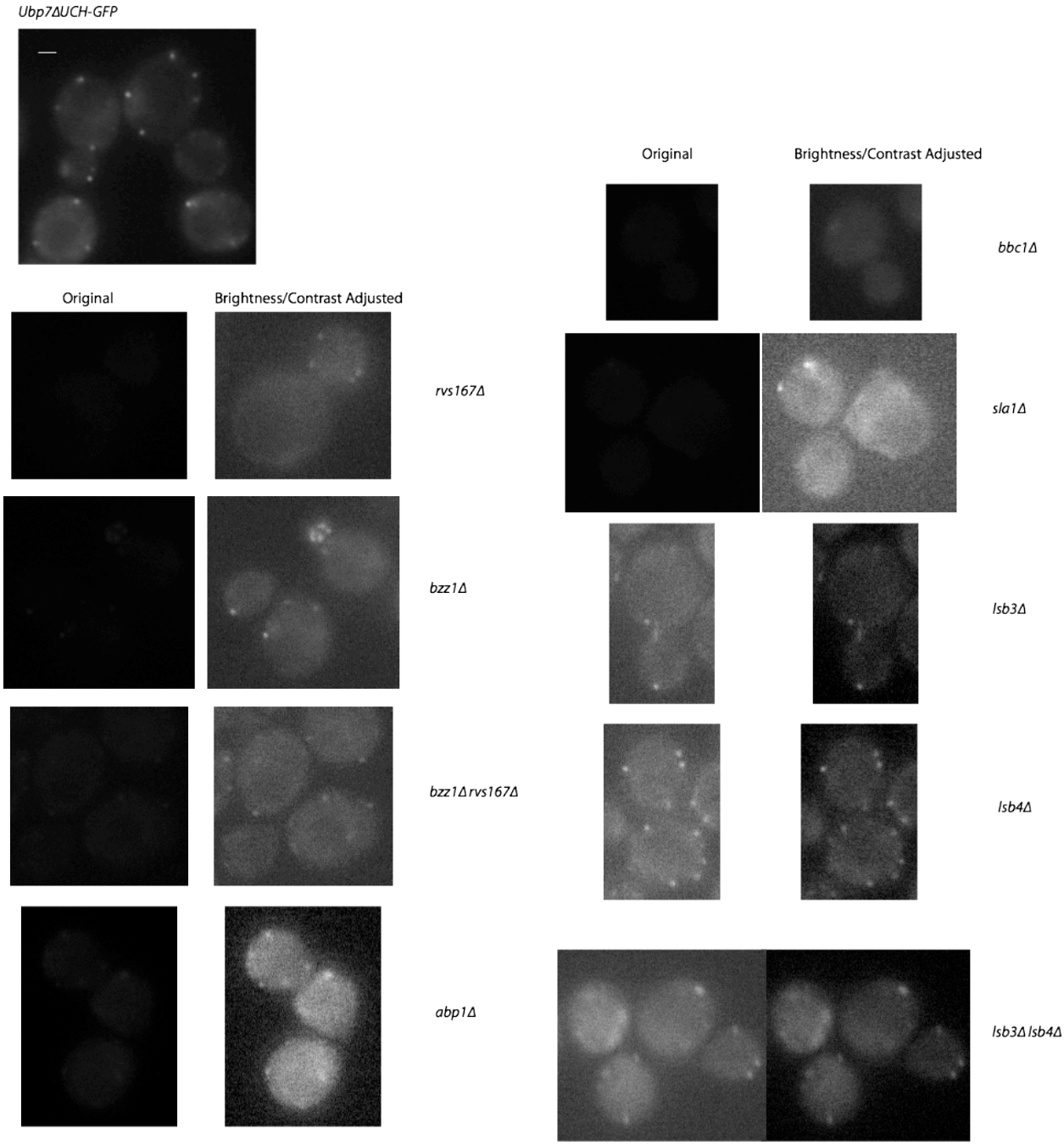

Ubp7ΔUCH-GFP
Original
Brightness/Contrast Adjusted
rvs167Δ
bzz1Δ
bzz1Δrvs167Δ
abp1Δ
Original
Brightness/Contrast Adjusted
bbc1Δ
sla1Δ
lsb3Δ
lsb4Δ
lsb3Δlsb4Δ

Figure 2.3. Cortical localization of Ubp7ΔUCH-GFP is reduced in cells deleted for SH3 domain endocytic proteins

Ubp7ΔUCH-GFP is present at cortical patches in wild-type cells. Ubp7p localization is reduced but not completely eliminated in deletions of several SH3 domain containing proteins. Scale bar is 1μm.

fluorescent signal in these mutants (Figure 2.1E) suggesting that GFP-Rsp5 is trapped at the plasma membrane when endocytosis is blocked. While definitive proof of the presence of an E3 ubiquitin ligase at endocytic sites remains elusive, the fact that Rsp5p accumulates at the plasma membrane when endocytosis is blocked suggests that it normally spends time at the plasma membrane and is removed by endocytosis. These results demonstrate that both the ubiquitination and the deubiquitination machinery associate with the plasma membrane, indicating that these enzymes are well positioned to regulate the endocytic machinery. Evidence that Rsp5p performs an endocytic function comes from endocytic defects observed in *rsp5* mutants (Gajewska et al., 2001; Kaminska et al., 2002).

Deletion of DUBs extends cortical coat lifetimes and causes recruitment of endocytic proteins to early endosomes

Next we examined the effects on endocytosis of deleting the candidate deubiquitinases. The described phenotypes were observed in both *ubp2Δ* and *ubp7Δ* single mutant cells, while the double mutants had stronger phenotypes than either single mutant (data not shown). The cortical lifetimes of GFP or RFP labeled endocytic proteins were measured in wild-type and *ubp2Δ ubp7Δ* cells (Figure 2.4A). Lifetimes were extended for many endocytic proteins, especially those in the coat module, and to a lesser extent, proteins in the WASP/Myo and Scission modules, which arrive later (see Figure 1.2). The fluorescently labeled coat proteins internalize normally (Figure 2.4B) even when their lifetimes are significantly extended ($p \leq 0.05$).

In addition to showing extended cortical lifetimes, endocytic proteins appeared on ectopic cytoplasmic patches in *ubp2Δ ubp7Δ* cells (Figure 2.5A). Under normal circumstances endocytic proteins appear at cortical patches and disappear upon uncoating after traveling a few hundred nanometers into the cell (Kaksonen et al., 2005). In *ubp2Δ ubp7Δ* cells, endocytic proteins localize to cytoplasmic puncta. These puncta are stained by FM4-64 within three minutes of addition of the lipid dye, suggesting that they assemble on early endosomes (Figure 2.5C). All of the labeled endocytic proteins tested, including early (Ede1p), coat (Sla2p, Ent2p) and actin (Sac6p) module proteins, appeared on the internal patches. These internal patches generally move rapidly within the cytoplasm and then disappear, either by uncoating or by moving out of the plane of focus.

In cells expressing both Sla1-GFP and Abp1-RFP, internal puncta that remain in the plane of focus are first labeled by Sla1-GFP, and then by Abp1-RFP. Then they both disappear (Figure 2.5B). This is the same temporal order that is seen at productive endocytic sites on the plasma membrane, suggesting that the defect caused by deleting the two DUBs causes endocytic sites to be assembled inappropriately on early endosomes, and that these sites at least partially proceed through the normal sequence of endocytic events. We hypothesize that deletion of the DUBs leaves behind ubiquitin as a signal on some protein, which results in aberrant initiation of endocytosis on early endosomes. This signal could either be ubiquitination of the endocytic machinery or ubiquitination of transmembrane cargo molecules.

Deletion of genes encoding two DUBs, Ubp7p, which localizes to endocytic sites, and Ubp2p, which associates with Rsp5p, significantly increases the lifetimes of early

Figure 2.4

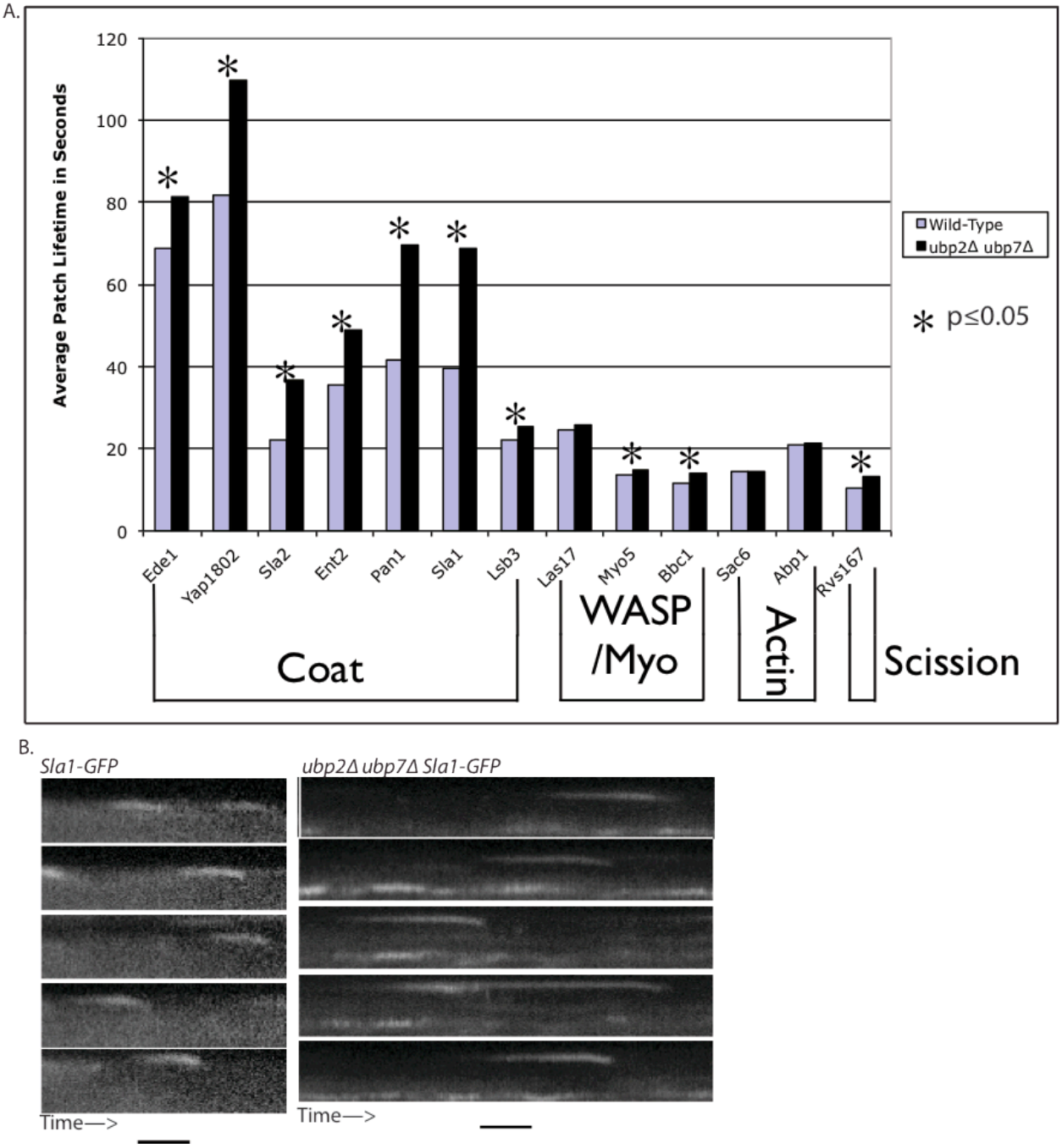

A.
Average Patch Lifetime in Seconds
120
100
80
60
40
20
0
Wild-Type
ubp2Δ ubp7Δ
* p≤0.05
Ede1
Yap1802
Sla2
Ent2
Pan1
Sla1
Lsb3
Las17
Myo5
Bbc1
Sac6
Abp1
Rvs167
Coat
WASP /Myo
Actin
Scission
B.
Sla1-GFP
ubp2Δ ubp7Δ Sla1-GFP
Time—>
Time—>

Figure 2.4: *ubp2Δ ubp7Δ* double mutants have extended endocytic coat lifetimes
Double mutant cells have significantly ($p \leq 0.05$, T-test) extended patch lifetimes for many endocytic proteins, especially proteins in the coat module, but show only modest effects for proteins in later-arriving modules ($n > 100$ patches). Error bars are ± 1 standard deviation. (B) Example kymographs of Sla1-GFP in wild-type and double mutant cells. Mutant cells have longer patch lifetimes. Time bar is 20s.

Figure 2.5

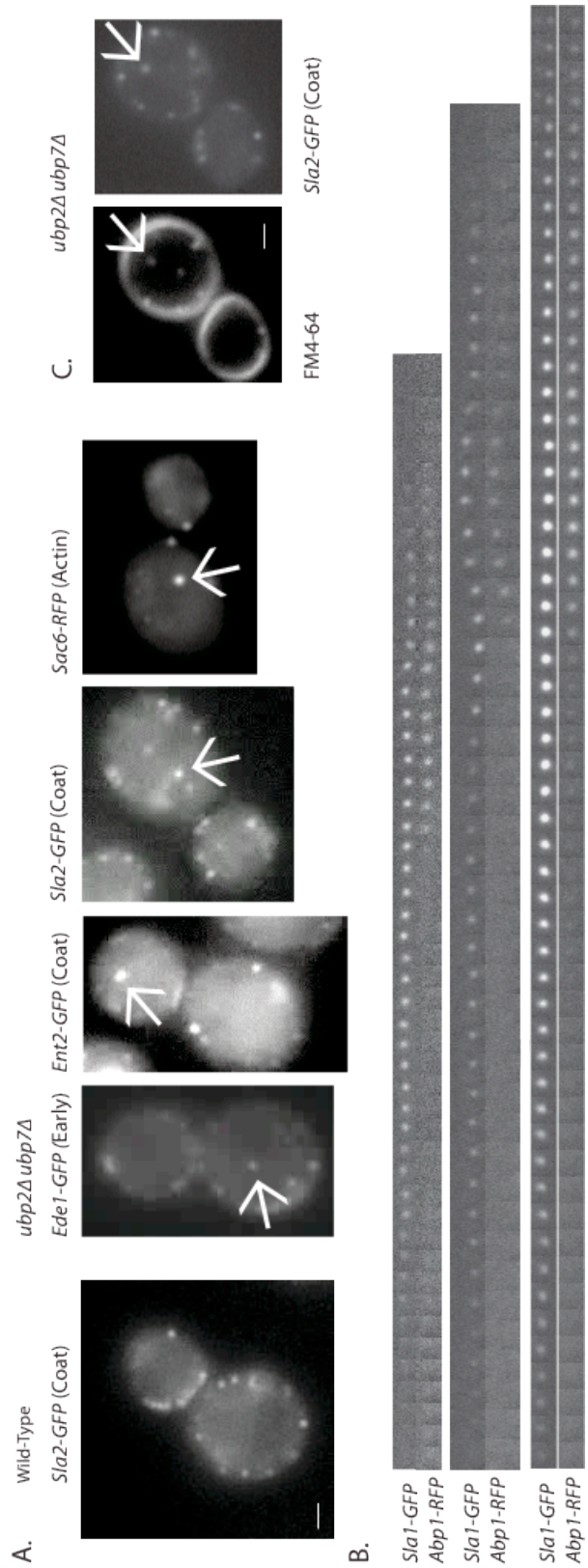

A.
Wild-Type
ubp2Δ ubp7Δ
Sla2-GFP (Coat)
Ede1-GFP (Early)
Ent2-GFP (Coat)
Sla2-GFP (Coat)
Sac6-RFP (Actin)
B.
Sla1-GFP
Abp1-RFP
Sla1-GFP
Abp1-RFP
Sla1-GFP
Abp1-RFP
C.
ubp2Δ ubp7Δ
FM4-64
Sla2-GFP (Coat)

Figure 2.5: *ubp2Δ ubp7Δ* double mutants have cytoplasmic patches (A) Double mutant cells have internal patches, to which multiple different endocytic proteins from several different modules get recruited in the normal sequence. These patches are dynamic and mobile, appearing and disappearing and moving around inside the cell. Scale bar is 1μm. (B) Dual-color microscopy of endocytic patches demonstrates that they behave similarly to cortical actin patches. Sla1p appears first, followed by Abp1p, which marks actin, followed by the disappearance of the patch. (C) Within 3 minutes of addition of FM4-64, colocalization is observed between FM4-64 puncta and cytoplasmic puncta marked by Sla2-GFP. Scale bar is 1μm.

and coat proteins and causes endocytic proteins to be recruited to internal membrane structures. Rsp5p is trapped at the plasma membrane in endocytic mutants, suggesting that it normally associates with the plasma membrane and is removed by endocytosis.

Ubiquitination of Ede1p is increased in ubp2Δ ubp7Δ cells

We identified the early endocytic protein Ede1p as a strong candidate for regulation by ubiquitination. Ede1p is known to be ubiquitinated by Rsp5p, and it binds intramolecularly to its own ubiquitin modification (Dores et al., 2010). Additionally, Ede1p arrives early in the endocytic pathway, and it is important for endocytic site initiation (Stimpson et al., 2009), a process that may be perturbed in *ubp2Δ ubp7Δ* cells as evidenced by the formation of ectopic cytoplasmic puncta.

In order to test whether deletion of the two DUB genes changes the ubiquitination state of Ede1p, we performed nickel affinity purifications of His6-Ub proteins from wild-type and DUB deleted strains that had all endogenous copies of ubiquitin deleted and His6-myc-ubiquitin as the sole source of ubiquitin, and probed immuno-blots for endocytic proteins. Post-translational modifications were well preserved by the protocol as seen by multiple Ede1p bands (Figure 2.6). Notably, more high-molecular weight Ede1p was recovered from the *ubp2Δ ubp7Δ* strain than from the wild-type strain. Similarly, more of the highest molecular weight Rvs167p species is recovered from the *ubp2Δ ubp7Δ* strain.

These data suggest that Ubp2p and Ubp7p are responsible for the deubiquitination of Ede1p and Rvs167p, and thus that the phenotypes observed in the *ubp2Δ ubp7Δ* cells might be, at least in part, due to excessive ubiquitination of Ede1p and/or Rvs167p.

Expression of an Ede1-ubiquitin fusion protein phenocopies ubp2Δ ubp7Δ cells

Similar to *ubp2Δ ubp7Δ* cells, expression of Ede1p carrying a C-terminal ubiquitin fusion, in the absence of endogenous Ede1p, caused appearance of cytoplasmic puncta labeled by tagged endocytic proteins, and extended lifetimes for coat proteins at plasma membrane-associated endocytic sites (Figure 2.7A, 2.2B). While expression of the Ede1p-ubiquitin fusion was sufficient to cause both phenotypes, Ede1p was not necessary for the cytoplasmic patch phenotype in *ubp2Δ ubp7Δ* cells since deletion of *EDE1* did not suppress the appearance of internal patches (Figure 2.7A, 2.2C). However, deletion of *EDE1* did suppress the extended coat protein lifetime phenotype (Figure 2.2B). This result is consistent with the observation that deletion of *EDE1* reduces the cortical lifetime of patch proteins (Stimpson et al., 2009). The endocytic coat protein lifetime increase seen in Ede1-Ub cells is not as strong as that seen in *ubp2Δ ubp7Δ* cells, further suggesting that Ede1p is not the only endocytic target of the DUBs. Nevertheless, expression of Pan1-Ub, another yeast homolog of mammalian Eps15 (Duncan et al., 2001; Toshima et al., 2007; Wendland et al., 1996), did not replicate either phenotype (data not shown).

We hypothesize that the extended coat protein lifetimes observed in *ubp2Δ ubp7Δ* cells might be due to a defect in uncoating. An alternative explanation is that failure to remove ubiquitin from transmembrane cargo molecules results in continued signaling for coat protein recruitment and maintenance. However, the ability of the Ede1-Ub fusion protein to cause both extended coat lifetimes and cytoplasmic endocytic

Figure 2.6

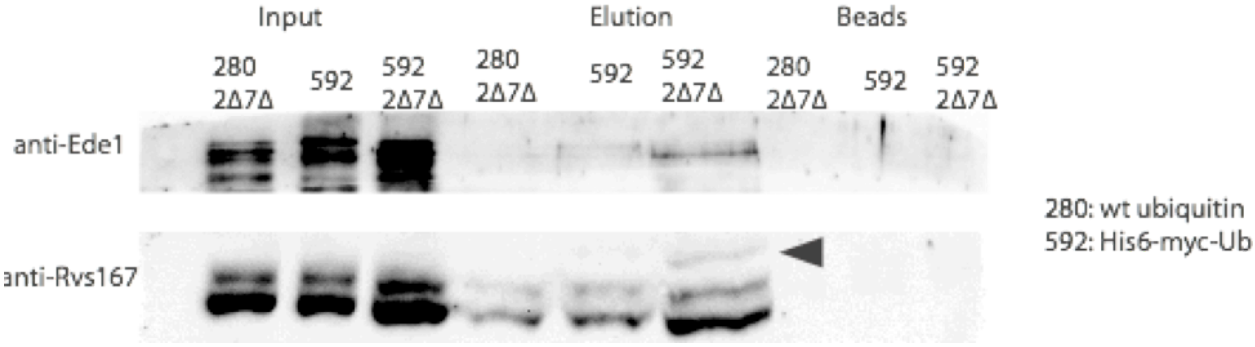

Input
Elution
Beads
280 2Δ7Δ
592
592 2Δ7Δ
280 2Δ7Δ
592
592 2Δ7Δ
280 2Δ7Δ
592
592 2Δ7Δ
anti-Ede1
anti-Rvs167
280: wt ubiquitin
592: His6-myc-Ub

Figure 2.6: Ede1p is more highly ubiquitinated in *ubp2Δ ubp7Δ* cells His6-Ubiquitinated proteins were purified from *UBP2 UBP7* and *ubp2Δ ubp7Δ* cells and the proteins eluted from nickel columns were blotted for Ede1p and Rvs167p. Only upper bands for Ede1p were detected in the eluted fractions. More Ede1p was purified in *ubp2Δ ubp7Δ* cells suggesting that Ede1p is more highly ubiquitinated in the mutant cells. This experiment was repeated three times with similar results.

Figure 2.7

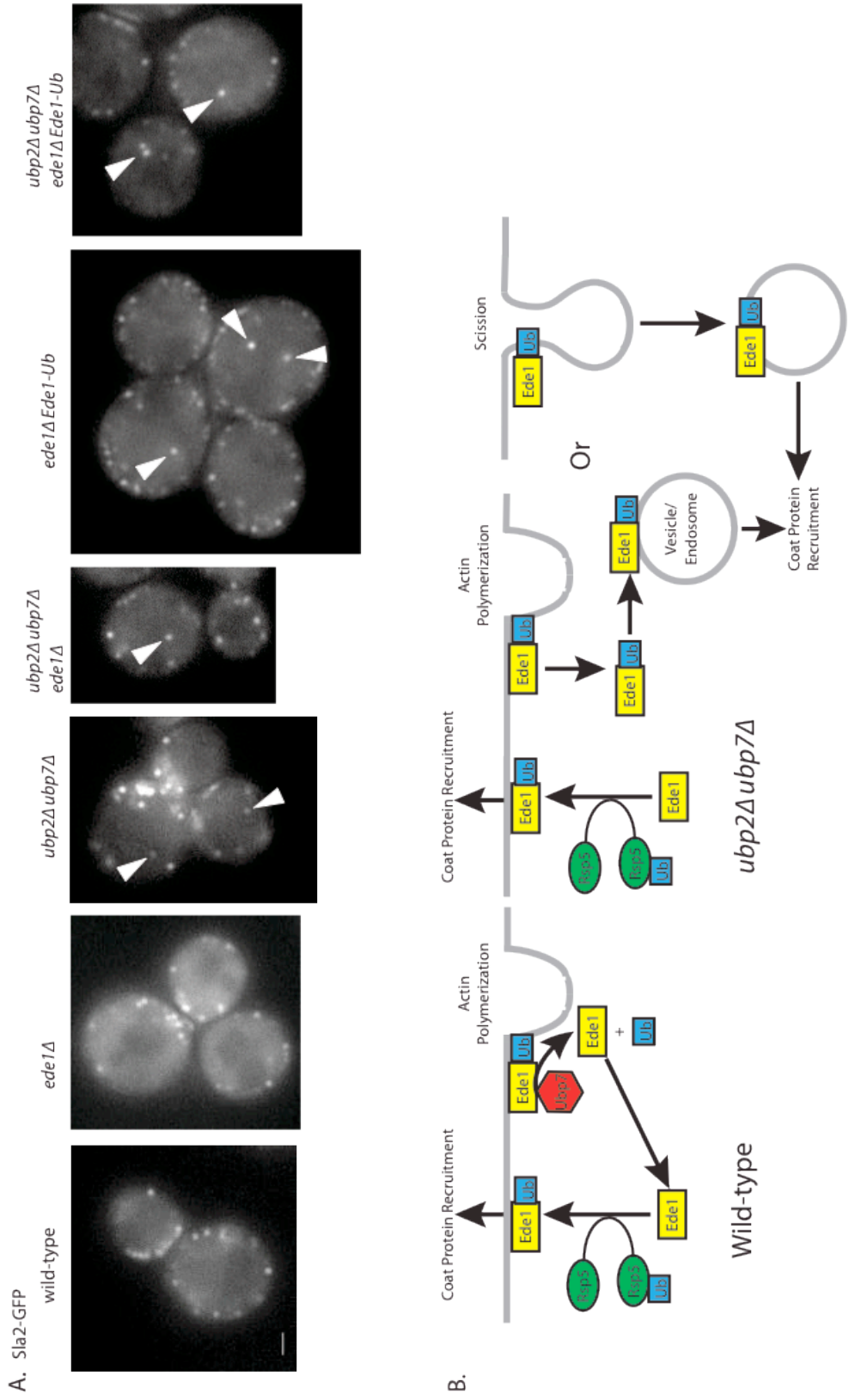

A. Sla2-GFP
wild-type
ede1Δ
ubp2Δ ubp7Δ
ubp2Δ ubp7Δ ede1Δ
ede1Δ Ede1-Ub
ubp2Δ ubp7Δ ede1Δ Ede1-Ub
B.
Coat Protein Recruitment
Actin Polymerization
Scission
Ede1
Ub
Usp3
Usp15
Or
Vesicle/ Endosome
Coat Protein Recruitment
Wild-type
ubp2Δ ubp7Δ

Figure 2.7: Permanently ubiquitinated Ede1p produces internal patches
(A)Expression of Ede1-Ub results in internal patches similar to those found in *ubp2Δ ubp7Δ* double mutant cells. Scale bar is 1μm. (B) Model of regulation of endocytic patch dynamics by ubiquitination/deubiquitination. Ede1p gets ubiquitinated by Rsp5p, which facilitates endocytic site initiation and/or coat protein recruitment, and is deubiquitinated late in the process, just before scission, by Ubp2/7p, which facilitates disassembly of the coat. In *ubp2Δ ubp7Δ* cells or Ede1-Ub cells, Ede1p retains the ubiquitin and is capable of inappropriately initiating sites and/or promoting recruitment of endocytic coat proteins on early endosomes.

protein puncta, implicates Ede1p as a functional target of the DUBs for coat regulation (Figure 2.6B). This conclusion is consistent with previous reports that Ede1p is ubiquitinated by Rsp5p (Dores et al., 2010), with our detection of multiple Ede1p species on immunoblots, and our recovery of more of the high molecular weight Ede1p species from *ubp2Δ ubp7Δ* cells.

The data presented here suggest that dynamic ubiquitination of the endocytic machinery regulates endocytic coat formation and disassembly. The recruitment of a DUB during the late stages of clathrin-mediated endocytosis implicates ubiquitin removal in a late process such as endocytic coat disassembly, while appearance of cytoplasmic puncta containing endocytic proteins in *ubp2Δ ubp7Δ* cells suggests that ubiquitin could be a signal for coat assembly or endocytic site initiation (Figure 2.6B).

Increased coat lifetimes in the absence of increased actin lifetimes when endocytic ubiquitination and deubiquitination are perturbed suggests that actin polymerization is regulated independently from coat formation and progression. The appearance of cytoplasmic puncta that mimic the normal ordered arrival and departure of endocytic proteins supports the idea that the DUBs remove ubiquitins that signal coat formation and progression of the endocytic pathway.

While the specific mechanism for the regulatory effect of ubiquitination on coat protein dynamics is presently not known, it has been hypothesized that Ede1p may act similarly to its mammalian homolog Eps15 and bind to its own ubiquitin via the C-terminal UBA domain (Hoeller et al., 2006). Given that Ede1p is also extensively phosphorylated (Chi et al., 2007; Li et al., 2007; Smolka et al., 2007), the possibility exists that these modifications may function in a coordinated manner.

While it had previously been shown that Rsp5p is responsible for the Ede1p ubiquitination (Dores et al., 2010), our data demonstrate that Ubp2p and Ubp7p are responsible for deubiquitination of Ede1p and likely other endocytic proteins. Moreover, the data presented here implicate dynamic ubiquitination and deubiquitination of the endocytic machinery in regulation of endocytic coat assembly, maintenance and disassembly.

MATERIALS AND METHODS

Strains
Yeast strains used in this study are listed in Table III. Gene deletions were generated by replacing the gene open reading frame with *Candida glabrata LEU2, URA3*, *hphNT1* or the *KanMX4* cassettes. C-terminal GFP tags were integrated as previously described (Longtine et al., 1998). The N-terminal GFP tag for Rsp5 was integrated as previously described (Janke et al., 2004). N-terminal tagging of Ubp7 was performed as previously described (Prein et al., 2000).

Microscopy
Yeast strains for imaging were grown to log phase at 30ºC (25ºC for *sla2Δ* and *ark1Δ prk1Δ*) in synthetic media lacking tryptophan and immobilized on concanavalin A-coated coverslips.

Microscopy was performed on Olympus IX71 and IX81 microscopes with 100x/numerical aperture (NA) 1.4 objectives and Orca cameras (Hamamatsu, Hamamatsu, Japan) at 25ºC. Simultaneous two-color imaging was performed using a argon-ion laser (CVI Melles Griot, Albuquerque, NM) to excite GFP and a 561-nm diode-pumped solid-state laser (CVI Melles Griot) to excite RFP. TIRFM was performed using an IX81 microscope equipped with a 100x/NA 1.65 objective with independently adjustable 488- and 561-nm lasers. Images were acquired with a frame rate of 1 frame per second using MetaMorph software (Molecular Devices, Sunnyvale, CA) and patch lifetimes were calculated using ImageJ (National Institutes of Health, Bethesda, MD).

His6-Ubiquitin Purification
Yeast were grown to log phase in 500mL of YPD at 30ºC and collected via centrifugation at 3500 RPM in a Sorvall SLA-3000 rotor. Cell pellets were resuspended in two volumes of lysis buffer (15mM Tris pH 8.0, 0.1M NaH2PO4, 8M Urea, 1mM beta-mercaptoethanol) and frozen in liquid nitrogen. Cells were lysed by bead beating (Biospec Mini Beadbeater) and clarified by centrifugation at 14,000 RPM for 15 minutes. Supernatants were incubated with nickel-NTA beads (Qiagen) for 30 min and collected by centrifugation. Beads were washed with 40 volumes of lysis buffer followed by 3 volumes of wash buffer (15mM Tris pH 6.3, 0.1M NaH2PO4, 8M Urea) and eluted in 3 volumes of elution buffer (15mM Tris pH 4.5, 0.1M NaH2PO4, 8M Urea, 100mM imidazole) and analyzed by SDS-PAGE. Rabbit anti-Ede1 and anti-Rvs167 were generous gifts from Linda Hicke. Anti-rabbit HRP was purchased from GE Healthcare and blots were read using a BioRad ChemiDoc XRS+ system.

Table III *S. cerevisiae* strains used in this study

Strain	Genotype	Source
DDY1102	*MATa/MATα his3Δ200/his3Δ200 leu2-3, 112/leu2-3, 112 ura3-52/ura3-52 ade2-1/ADE2 lys2-801/LYS2*	Drubin Lab
DDY4315	*MATα his3Δ200, leu2-3,112, ura3-52, lys2-801, ubp7 ΔUCH - GFP::cgHIS3*	This study
DDY4316	*MATα his3Δ200, leu2-3,112, ura3-52, lys2-801, ubp7 ΔUCH - GFP::cgHIS3, Abp1-RFP::His3*	This study
DDY4317	*MATα his3Δ200, leu2-3,112, ura3-52, lys2-801, sac6-RFP::KanMX, ubp7ΔUCH-GFP::His3, sla2Δ::LEU2*	This study
DDY4318	*MATα his3Δ200, leu2-3,112, ura3-52, NatNT2::pGPD GFP-Rsp5*	This study
DDY4319	*MATα his3Δ200, leu2-3,112, ura3-52, NatNT2::pGPD GFP-Rsp5, sla2Δ::LEU2*	This study
DDY4320	*MATα his3Δ200, leu2-3,112, ura3-52, NatNT2::pGPD GFP-Rsp5, ark1Δ::cgHIS3, prk1Δ::cgLEU2*	This study
DDY4321	*MATα his3Δ200, leu2-3,112, ura3-52, ubp7Δuch-GFP::His3, Sac6-RFP::KanMX4, rvs167Δ::Leu2*	This study
DDY4322	*MATα his3Δ200, leu2-3,112, ura3-52, ubp7Δuch-GFP::His3, Abp1-RFP::His3, sla1Δ::Leu2*	This study
DDY4323	*MATα his3Δ200, leu2-3,112, ura3-52, lys2-801, ubp2Δ::cgURA3, ubp7Δ::cgLEU2, Ede1-GFP::HIS3*	This study
DDY3866	*MATα his3Δ200, leu2-3, 112, ura3-52, Ede1-GFP::HIS3*	(Stimpson et al., 2009)
DDY4324	*MATα his3Δ200, leu2-3,112, ura3-52, lys2-801, ubp2Δ::cgURA3, ubp7Δ::cgLEU2, Yap1802-GFP::HIS3*	This study
DDY4054	*MATα his3Δ200, leu2-3, 112, ura3-52, Yap1802-GFP::HIS3*	(Carroll et al., 2012)
DDY4325	*MATα his3Δ200, leu2-3,112, ura3-52, lys2-801, ubp2Δ::cgURA3, ubp7Δ::cgLEU2, Sla2-GFP::HIS3*	This study

DDY2796	*MATα his3Δ200, leu2-3, 112, ura3-52, lys2-801, Sla2-GFP::HIS3*	(Carroll et al., 2012)
DDY4326	*MATa his3Δ200, leu2-3,112, ura3-52, lys2-801, ubp2Δ::cgURA3, ubp7Δ::cgLEU2, Ent2-GFP::HIS3*	This study
DDY3697	*MATα his3Δ200, leu2-3, 112, ura3-52, Ent2-GFP::HIS3*	(Toret et al., 2008)
DDY4327	*MATα his3Δ200, leu2-3,112, ura3-52, lys2-801, Pan1-GFP::KanMX, Δubp2::cgURA3, Δubp7::cgLEU2*	This study
DDY2341	*MATa his3Δ200, leu2-3, 112, ura3-52, lys2-801, Pan1-GFP::KanMX*	Drubin Lab
DDY4328	*MATα his3Δ200, leu2-3,112, ura3-52, lys2-801, Sla1-GFP::HIS3, Abp1-RFP::HIS3, Δubp2::cgURA3 Δubp7::cgLEU2*	This study
DDY3062	*MATα his3Δ200, leu2-3, 112, ura3-52, lys2-801, Sla1-GFP::HIS3, Abp1-RFP::HIS3*	(Kaksonen et al., 2005)
DDY4329	*MATa his3Δ200, leu2-3,112, ura3-52, lys2-801, ubp2Δ::cgURA3, ubp7Δ::cgLEU2, Lsb3-GFP::HIS3*	This study
DDY3957	*MATa his3Δ200, leu2-3, 112, ura3-52, Lsb3-GFP::HIS3*	Drubin Lab
DDY4330	*MATα his3Δ200, leu2-3,112, ura3-52, lys2-801, ubp2Δ::cgURA3, ubp7Δ::cgLEU2, Las17-GFP::HIS3*	This study
DDY2736	*MATa his3Δ200, leu2-3, 112, ura3-52, lys2-801, Las17-GFP::HIS3*	(Kaksonen et al., 2003)
DDY4331	*MATα his3Δ200, leu2-3,112, ura3-52, lys2-801, Myo5-GFP::KanMX, ABP1-RFP::HIS3, Δubp2::cgURA3 Δubp7::cgLEU2*	This study
DDY2813	*MATα his3Δ200, leu2-3, 112, ura3-52, lys2-801, Myo5-GFP::KanMX*	Drubin Lab
DDY4332	*MATα his3Δ200, leu2-3,112, ura3-52, lys2-801, Bbc1-GFP::HIS3, Δubp2::cgURA3 Δubp7::cgLEU2*	This study
DDY3095	*MATα his3Δ200, leu2-3,112, ura3-52, Bbc1-GFP::HIS3*	(Kaksonen et al., 2005)
DDY4333	*MATa his3Δ200, leu2-3,112, ura3-52, lys2-801, ubp2Δ::cgURA3, ubp7Δ::cgLEU2, Sac6-GFP::HIS3*	This study

DDY3264	*MATα his3Δ200, leu2-3, 112, ura3-52, lys2-801, Sac6-GFP::HIS3*	Drubin Lab
DDY4334	*MATa his3Δ200, leu2-3,112, ura3-52, lys2-801, ubp2Δ::cgURA3, ubp7Δ::cgLEU2, Abp1-GFP::HIS3*	This study
DDY2931	*MATa his3Δ200, leu2-3, 112, ura3-52, lys2-801, Abp1-GFP::HIS3*	(Martin et al., 2005)
DDY4335	*MATα his3Δ200, leu2-3,112, ura3-52, lys2-801, Rvs167-GFP::HIS3, Δubp2::cgURA3 Δubp7::cgLEU2*	This study
DDY3938	*MATα his3Δ200, leu2-3, 112, ura3-52, Rvs167-GFP::HIS3, Bzz1-RFP::HIS3*	(Kishimoto et al., 2011)
SUB280	*MATa lys2-801 leu2-3,112 ura3-52 his3-Δ200 trpl-1 ubi1::TRP1 ubi2-Δ2:ura3 ubi3-Δub2 ubi4-Δ2::LEU2 [pUB39][pUB100]*	(Finley et al., 1994)
SUB592	*MATa lys2-801 leu2-3,112 ura3-52 his3-Δ200 trpl-1 ubi1::TRP1 ubi2-Δ2:ura3 ubi3-Δub2 ubi4-Δ2::LEU2 [pUB221][pUB100]*	(Spence et al., 2000)
DDY4336	*MATa lys2-801 leu2-3,112 ura3-52 his3-Δ200 trpl-1 ubi1::TRP1 ubi2-Δ2:ura3 ubi3-Δub2 ubi4-Δ2::LEU2 [pUB39][pUB100] ubp2Δ::KanMX4, ubp7Δ::hphNTI*	This study
DDY4337	*MATa lys2-801 leu2-3,112 ura3-52 his3-Δ200 trpl-1 ubi1::TRP1 ubi2-Δ2:ura3 ubi3-Δub2 ubi4-Δ2::LEU2 [pUB221][pUB100] ubp2Δ::KanMX4, ubp7Δ::hphNT1*	This study
DDY3878	*MATa his3Δ200, leu2-3,112, ura3-52, ede1Δ::cgLEU2, Sla2-GFP::HIS3*	(Stimpson et al., 2009)
DDY4338	*MATα his3Δ200, leu2-3,112, ura3-52, ubp2Δ::HIS3, ubp7Δ::LEU2, ede1Δ::LEU2, Sla2-GFP::HIS3*	This study
DDY4339	*MATa his3Δ200, leu2-3,112, ura3-52, ede1Δ::cgLEU2, Sla2-GFP::HIS3, LHP2798*	This study
DDY4340	*MATα his3Δ200, leu2-3,112, ura3-52, ubp2Δ::HIS3, ubp7Δ::LEU2, ede1Δ::LEU2, Sla2-GFP::HIS3, LHP2798*	This study
DDY4341	*MATα his3Δ200, leu2-3,112, ura3-52, lys2-801, GFP-Ubp7*	This study
DDY4342	*MATα his3Δ200, leu2-3,112, ura3-52, lys2-801, GFP-Ubp7, Abp1-RFP::His3*	This study

DDY4343	*MATa his3Δ200, leu2-3,112, ura3-52, ubp7Δuch-GFP::His3, Sac6-RFP::KanMX4, bzz1Δ::hphNT1*	This study
DDY4344	*MATα his3Δ200, leu2-3,112, ura3-52, ubp7Δuch-GFP::His3, Sac6-RFP::KanMX4, rvs167Δ::Leu2, bzz1Δ::hphNT1*	This study
DDY4345	*MATa his3Δ200, leu2-3,112, ura3-52, ubp7Δuch-GFP::His3, Sac6-RFP::KanMX4, abp1Δ::Ura3*	This study
DDY4346	*MATα his3Δ200, leu2-3,112, ura3-52, ubp7Δuch-GFP::His3, Sac6-RFP::KanMX4, bbc1Δ::Leu2*	This study
DDY4347	*MATα his3Δ200, leu2-3,112, ura3-52, ubp7Δuch-GFP::His3, lsb3Δ:: hphNT1*	This study
DDY4348	*MATα his3Δ200, leu2-3,112, ura3-52, ubp7Δuch-GFP::His3, lsb4Δ::KanMX4*	This study
DDY4349	*MATα his3Δ200, leu2-3,112, ura3-52, ubp7Δuch-GFP::His3, lsb3Δ::hphNT1, lsb4Δ::KanMX4*	This study

Table IV Plasmids used in this study

Plasmid	Description	Source
LHP561	PAN1-UBI	Linda Hicke
LHP2798	EDE1-UB	Linda Hicke

CHAPTER 3

CONCLUSIONS AND FUTURE DIRECTIONS

CONCLUSIONS

Clathrin-mediated endocytosis is a very complex, regular and highly regulated process (Kaksonen et al., 2003; Kaksonen et al., 2005). In order to more fully understand the signals governing the recruitment, disassembly and activity of the ~60 proteins that act at endocytic sites (Figure 1.2) my dissertation focused on the role of the post-translational modification ubiquitin in regulating the endocytic machinery. Multiple endocytic proteins are known to be ubiquitinated (Dores et al., 2010; Gupta et al., 2007; Stamenova et al., 2004), but phenotypes or specific functional consequences were not known (Stamenova et al., 2004). Deletion of ubiquitin-binding domains of endocytic adaptor proteins reduces endocytosis (Dores et al., 2010), but since many endocytic cargos are ubiquitinated prior to internalization (Egner and Kuchler, 1996; Kolling and Hollenberg, 1994; Volland et al., 1994), what role ubiquitination of the endocytic machinery might have remained unclear.

Recruitment of Ubp7p to endocytic patches (Figure 2.1A-D) at around the time of endocytic vesicle formation suggested a role for deubiquitination in vesicle uncoating. While it is possible that Ubp7p acts on the endocytic cargos, later trafficking of cargo depends on ubiquitin modifications being present in sorting vesicles (Lam et al., 2009). Purification of ubiquitinated proteins revealed that deletion of the genes encoding the DUBs Ubp2/7p increases the ubiquitination of Ede1p and Rvs167p (Figure 2.6), revealing that the DUBs are acting on the endocytic machinery itself. While this does not exclude the possibility that the DUBs are also acting on the cargo, it means that the phenotypes observed in *ubp2Δ ubp7Δ* cells may be the result of excessive ubiquitination of the endocytic machinery.

While there is no direct evidence that Ubp2p localizes to endocytic sites, this protein is known to be in a complex with the E3 ubiquitin ligase Rsp5p, which modifies both Ede1p and Rvs167p. A tagged version of the ligase localizes to the plasma membrane in mutants where endocytosis is blocked (Figure 2.1E), suggesting that it reaches the plasma membrane and that endocytosis is required for its clearance.

Deletion of the genes encoding the DUBs Ubp2/7p results in two interesting endocytic phenotypes: extension of the cortical coat lifetime and recruitment of endocytic proteins to cytoplasmic puncta likely to be early endosomes. The extended lifetime phenotype is observed for coat proteins, but not proteins of the WASP/myo, actin or scission modules (Figure 2.4), implying that actin recruitment and polymerization occur normally and that coat recruitment is specifically impaired.

We hypothesized that the second phenotype, ectopic protein recruitment to early endosomes, was the result of a ubiquitin not being removed at the end of endocytosis, and therefore continuing to signal to initiate endocytosis on the wrong membrane. As to the identity of the DUB targets, they could be cargo molecules or proteins of the endocytic machinery.

In order to demonstrate that ubiquitin signals for ectopic coat recruitment, I expressed Ede1, a candidate ubiquitinated protein, as a ubiquitin fusion, mimicking absence of the DUBs. This mutant replicated both the extended coat lifetime and cytoplasmic puncta phenotypes (Figure 2.7). These effects were specific for Ede1p as expression of another endocytic protein, Pan1p, as a ubiquitin fusion did not produce either phenotype. However, deletion of *EDE1* in a *ubp2Δ ubp7Δ* background only rescued the coat protein lifetime phenotype and not the cytoplasmic puncta phenotype. This implies that Ubp2/7 are acting on multiple endocytic targets including Ede1p.

The next logical step in this project is to identify the other endocytic targets of Ubp2/7p and the upstream E3 ligase that ubiquitinates them. I propose both a candidate gene approach and an unbiased approach.

The His6-myc-ubiquitin purification demonstrated that Ede1p and Rvs167p are more highly ubiquitinated in the *ubp2Δ ubp7Δ* background. Given either an antibody or a tagged version of a protein, this approach could be applied to any endocytic protein. Using a *natNT2* marked 6HA tag, candidate genes can be tagged in the His6-Ub purification strains and the ubiquitination status evaluated.

High-priority candidate proteins include Ent1/2p, which have ubiquitin binding domains and are ubiquitinated *in vivo* (Dores et al., 2010; Gupta et al., 2007), and Bbc1p, which is ubiquitinated *in vivo* and has PY motifs which may mediate binding to Rsp5p. As with Ede1p, candidate proteins that are more highly ubiquitinated when the DUBs are deleted will be expressed as ubiquitin fusion proteins and assayed for the phenotypes displayed by *ubp2Δ ubp7Δ* cells: extended cortical coat lifetimes and endocytic protein recruitment to cytoplasmic puncta. As with *EDE1*, deletion of candidate genes in *ubp2Δ ubp7Δ* cells will allow us to determine if the gene product is necessary for the phenotypes while the ubiquitin fusion expression allows us to test for sufficiency.

In order to identify endocytic proteins that are ubiquitinated, and especially those that are ubiquitinated excessively in *ubp2Δ ubp7Δ* cells, I propose performing mass spectrometry on purified clathrin coated vesicles (CCVs). In *ubp2Δ ubp7Δ* cells, endocytic targets retain their modifications and this leads to recruitment of endocytic proteins to cytoplasmic membrane structures believed to be early endosomes. These will likely retain many, if not all, of the ubiquitinations that are removed by the DUBs. CCVs purified by classical approaches do not normally contain large amounts of endocytic proteins because internalizing vesicles uncoat within a few hundred nanometers of the plasma membrane (Kaksonen et al., 2005). Addition of the protein-protein crosslinking agent DSP at 1.5 mM for 45 minutes at 30º during the spheroplasting step slightly increases the amount of the endocytic protein Ede1p purified (Figure 3.1). Purification of CCVs from *ubp2Δ ubp7Δ* cells reveals an increase in Ede1p, and purification of CCVs from *ubp2Δ ubp7Δ* cells after addition of DSP reveals increased levels of copurifying endocytic proteins including Ede1p, Sla1p, Sla2p, Sac6p, and Abp1p (data not shown). Additionally, there are a large number of ubiquitinated proteins purified as assayed by immuno-blot against a 3HA-tagged ubiquitin. However, immuno-blots against Golgi proteins Sec22p and Pep12p reveal a significant number of Golgi-derived vesicles, implying that additional purification steps are required.

Figure 3.1

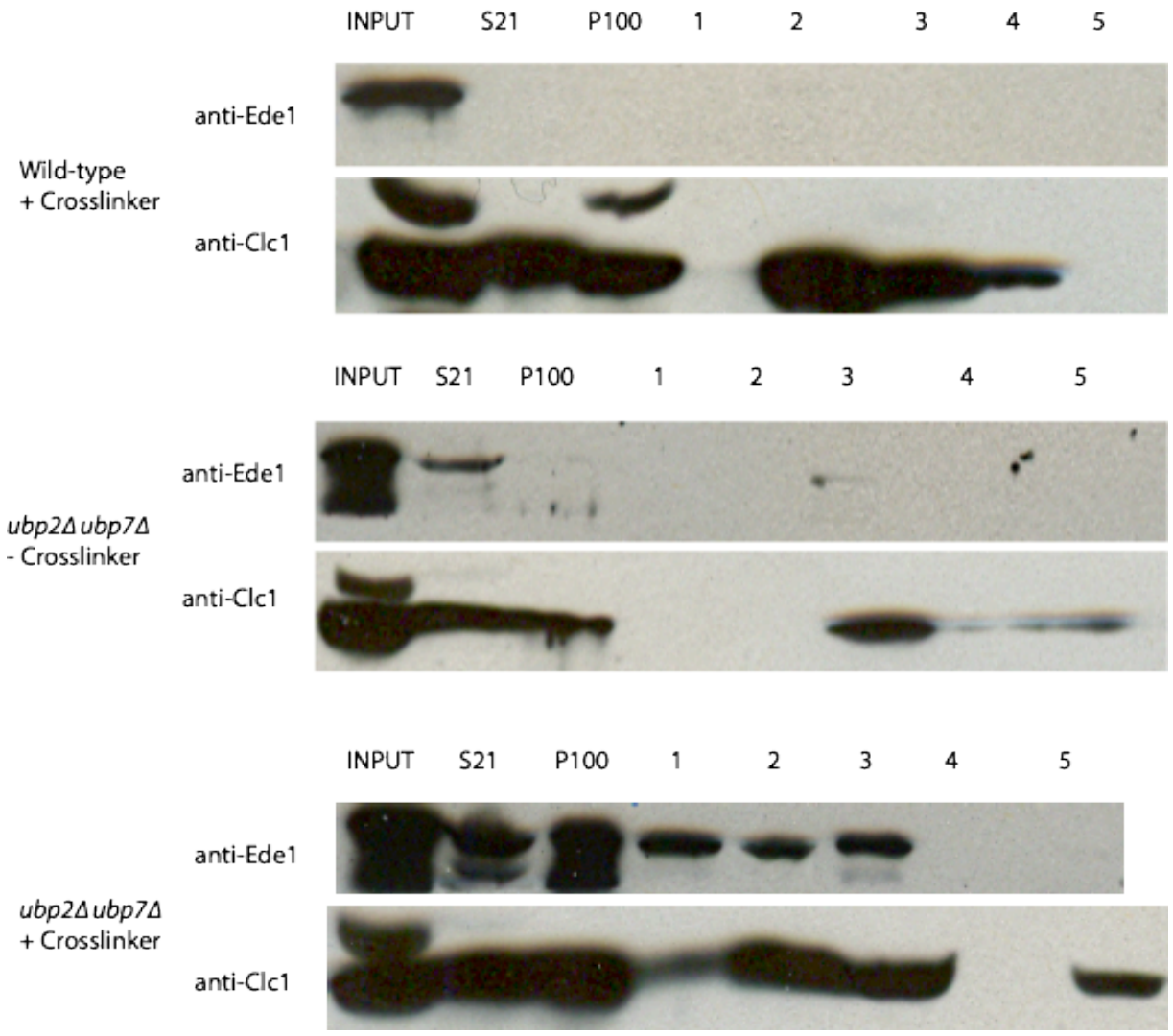

INPUT S21 P100 1 2 3 4 5
anti-Ede1
Wild-type
+ Crosslinker
anti-Clc1
INPUT S21 P100 1 2 3 4 5
anti-Ede1
ubp2Δ ubp7Δ
- Crosslinker
anti-Clc1
INPUT S21 P100 1 2 3 4 5
anti-Ede1
ubp2Δ ubp7Δ
+ Crosslinker
anti-Clc1

Figure 3.1 Clathrin-coated vesicle purification
Input, low-speed supernatant, high-speed pellet and pooled fractions from the S-1000 column were assayed by immuno-blot for the presence of clathrin light-chain and Ede1p. CCVs from wild-type cells, with 1.5 mM DSP added for 45 minutes, had clathrin but no detectable Ede1p. CCVs from *ubp2Δ ubp7Δ* cells without crosslinker had low levels of co-purifying Ede1p. CCVs from *ubp2Δ ubp7Δ* cells with crosslinker had high levels of Ede1.

Therefore I propose a secondary purification to enrich for endocytic CCVs over Golgi-derived CCVs prior to mass spectrometry. The mass spec would enable both identification of the proteins present on the crosslinked CCVs as well as identification of the ubiquitinated and phosphorylated proteins and the sites of the modifications.

Proteins identified by the mass spectrometry could then be assayed individually as described above, using the His6-Ub purification to verify their presence, constructing Ub-fusion proteins to test function, and performing genetic interaction tests with *ubp2Δ ubp7Δ* mutations to confirm that the modifications are removed by the endocytic DUBs and have functional consequences relating to the regulation of the endocytic machinery.

Deletion of the DUBs or expression of a permanently ubiquitinated Ede1p are capable of driving formation of cytoplasmic puncta marked by endocytic proteins. Whether these internal structures are the result of failure to uncoat, or if uncoating proceeds and then proteins are recruited to early endosomes *de novo* is not clear. In order to make this determination, rapid 4D imaging is required. Imaging a stack through the entire cell with a speed of ~1Hz will allow determination of whether a cytoplasmic puncta labeled by an early endocytic protein arose from an endocytic site that failed to uncoat. Alternatively, by using fast 4D imaging and FM4-64 or a membrane-bound GFP/RFP construct, one could follow the internalization of a vesicle, note if it uncoats, and then determine if endocytic proteins are later recruited to a fully uncoated endosome.

Ede1p regulation by ubiquitination is the focus of much of this dissertation. However, Ede1p is also extensively phosphorylated. Whether there is an interaction between the two kinds of post-translational modifications is not known. One of the kinases capable of phosphorylating Ede1p is Hrr25p (Connie Peng, personal communication), which localizes to endocytic patches very early in the pathway. By combining *in vitro* ubiquitination and *in vitro* phosphorylation assays, we can determine if phosphorylation affects the ability of the ligase to ubiquitinate Ede1p or visa versa.

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