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Uptake of Toxin K28 and Early Endocytic Site Formation in *S. cerevisiae*

By

Susheela Yogini Carroll

A dissertation submitted in partial satisfaction of the

requirement for the degree of

Doctor of Philosophy

in

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of the

University of California, Berkeley

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Professor David G. Drubin, Chair
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ABSTRACT

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K28 is an A/B toxin that targets yeast cells and depends on endocytosis and retrograde trafficking for toxicity. Knowledge of the specific proteins, lipids, and mechanisms required for trafficking and killing by K28 remains incomplete. In this work, over 5000 yeast mutants were screened and 365 were identified that affect K28 sensitivity. Hypersensitive mutants revealed cytoprotective pathways, including stress-activated signaling and protein degradation. Resistant mutants clustered to endocytic, lipid organization, and cell wall biogenesis pathways. Strikingly, the AP2 complex, which in metazoans links endocytic cargo to the clathrin coat, but had no assigned function in yeast, was critical for K28 toxicity. Yeast AP2 localizes to endocytic sites and has a cargo-specific function in K28 uptake. Furthermore, the AP2 complex arrives early during endocytic site formation, which is a step of endocytosis that is not well understood. My research in this area expanded our knowledge of the proteins present during the initial stages of endocytic site formation and defined their order of arrival. The roles that Ede1p (homolog of Eps15) and clathrin have in regulating early stages of endocytosis were also examined. My results show Ede1p functions early in endocytic site formation, whereas clathrin likely promotes site transition to the late coat stage. Since cargo also arrives during the early stages of endocytosis, its involvement in endocytic site regulation was investigated using a secretion mutant to deplete cargo from the cell surface. Our results are consistent with a role for cargo in regulating the transition of endocytic sites from the early to late stages. In total, this work comprehensively identified processes important for A/B toxin trafficking and killing, and analyzed the regulation of early endocytic sites.

This work is dedicated to the memories of Kiwi and Escarjoe.

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LIST OF ABBREVIATIONS

AP2:	Adaptor Protein complex 2
ANTH:	AP180 N-Terminal Homology
ENTH:	Epsin N-Terminal Homology
FCHo1/2:	Fer/Cip4 Homology domain-only proteins 1 and 2
TIRF:	Total Internal Reflection Fluorescence

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CHAPTER 1
GENERAL INTRODUCTION

Endocytosis is a process wherein cells internalize extracellular fluid, plasma membrane molecules, and particles from the surrounding environment. During receptor-mediated endocytosis, cargo molecules bind to cell surface receptors, the plasma membrane invaginates, and a vesicle containing the cargo is pinched off from the membrane and targeted to the endosome. Endocytosis has relevance to receptor internalization, cellular signaling, and viral/toxin entry.

There are several types of endocytosis in mammalian cells. Pinocytic pathways in mammalian cells include clathrin-mediated, caveolae-dependent, and clathrin- and caveolae-independent endocytosis (Doherty and McMahon, 2009). Phagocytosis and macropinocytosis are examples of endocytosis used to internalize larger particles, more extracellular fluid, and/or bigger areas of plasma membrane. Only one known endocytic pathway exists in yeast, which involves clathrin and actin (Engqvist-Goldstein and Drubin, 2003). An endocytic-type mechanism has also been discovered in bacteria (Lonhienne et al., 2010). In general, endocytosis is an essential eukaryotic process that is also found in some phyla of bacteria.

Endocytosis has been examined extensively in the budding yeast *Saccharomyces cerevisiae*. Using yeast as a model organism to study endocytosis has several advantages. Blocking one endocytic pathway in mammalian cells leads to complications because another endocytic pathway might become upregulated (Damke et al., 1995). This problem is eliminated when studying endocytosis in yeast because only one known endocytic pathway exists in this organism. Furthermore, many endocytic proteins in yeast are homologous to proteins involved in mammalian clathrin-mediated endocytosis (Engqvist-Goldstein and Drubin, 2003). Finally, yeast is a molecular-genetically tractable organism in which GFP tags can be integrated into the chromosome and entire genes can be deleted.

The events that occur during endocytic site formation, and the role that cargo plays during the early stages of endocytosis, are unclear. The proteins that arrive during the early stages of endocytosis are not well defined, but they can be examined using established fluorescent microscopy techniques (Kaksonen et al., 2003; Kaksonen et al., 2005). The uptake of cargo proteins on the plasma membrane is an important function of endocytosis and it is necessary to explore how cargo molecules influence this process. Some model cargos used to study endocytosis include toxins secreted by bacteria and plants, which are internalized by mammalian cells (Sandvig and van Deurs, 2005). K28, a protein toxin, and α-factor, a peptide pheromone, are examples of extracellular cargos that may be used to probe yeast endocytosis (Eisfeld et al., 2000; Toshima et al., 2006). Through using cargos to investigate yeast endocytosis, and imaging techniques to study early arriving proteins, several areas of endocytosis can be explored.

Endocytosis in Yeast

Live-cell fluorescence microscopy in *Saccharomyces cerevisiae* has revealed when individual proteins act at internalization sites and the timing of endocytic

machinery assembly and disassembly (Kaksonen et al., 2005). GFP-tagged endocytic proteins form structures on the cell surface called "patches". Endocytic proteins have been organized into five modules (early, coat, WASP/Myo, actin, and amphiphysin) based on their arrival to endocytic sites and on patch dynamics.

The early module consists of proteins that arrive early during endocytic site formation, which do not internalize with the vesicle, but instead disassemble before actin polymerization (Stimpson et al., 2009). Ede1p and Syp1p, which are homologs of mammalian Eps15 and FCHo1/2, are the only known members of this module. Ede1p is thought to have a role in endocytic site formation because *ede1*Δ yeast form fewer endocytic sites which contain later arriving endocytic machinery (Kaksonen et al., 2005; Stimpson et al., 2009). Syp1p is thought to be involved in forming endocytic sites at the bud neck, membrane tabulation, regulating the WASP/Arp2/3 complex, and serving as an adaptor for the endocytic cargo Mid2p (Stimpson et al., 2009; Reider et al., 2009; Boettner et al., 2009). How the early module proteins initiate endocytic site formation is a complete mystery.

The coat module proteins assemble on the plasma membrane and internalize with the vesicle before disassembly (Kaksonen et al., 2005). Clathrin is the earliest known coat module protein to arrive at endocytic sites (Kaksonen et al., 2005; Newpher et al., 2005). Clathrin is important for uptake of the cargo α-factor, and for proper dynamics of other endocytic proteins (Newpher and Lemmon, 2006; Chu et al., 1996). The next coat module proteins to arrive at endocytic sites are Sla2p, Ent1/2p, and the Pan1 complex (Pan1p, Sla1p, and End3p), which are homologs of mammalian Hip1R, epsins, and intersectin, respectively (Kaksonen et al., 2005; Toret et al., 2008).

Las17p, a homolog of WASP, which activates the Arp2/3 complex, is recruited to endocytic sites at about the same time as Sla1p (Sun et al., 2006). The Arp2/3 complex promotes actin assembly, which is thought to provide the force for membrane invagination (Kaksonen et al., 2003). Pan1p and Sla2p are proteins that are hypothesized to connect the endocytic coat to the actin meshwork (Kaksonen et al., 2006). Lastly, the amphiphysin module proteins localize to endocytic sites and are thought to be involved in vesicle scission (Kaksonen et al., 2003).

Cargo in Endocytosis

How cargo interacts with endocytic machinery is an important topic in endocytosis. There are two main sorting signals for receptor-mediated endocytosis in yeast: ubiquitin and $NPFX_{(1,2)}D$ motifs (Engqvist-Goldstein and Drubin, 2003). Cargos at the plasma membrane are mono-ubiquitinated on lysine residues by Rsp5p to mark them for internalization (Terrell et al., 1998; Dunn and Hicke, 2001). Several arrestin-like proteins may be involved in the uptake of specific cargos by acting as ubiquitin ligase adaptors for cargos (Nikko et al., 2008; Lin et al., 2008). Sla1p is thought to bind cargos with $NPFX_{(1,2)}D$ motifs and direct their internalization (Howard et al., 2002). Syp1p and Yap1801/2p are endocytic proteins that are important for uptake of only certain cargos, and are therefore likely to act as cargo adaptors (Reider et al., 2009; Burston et al., 2009).

The role cargo plays in endocytosis is an area of increasing interest. Studies in mammalian cells revealed that cargo may have a role in regulating clathrin-coated pits

(Loerke et al., 2009). Overexpressing the transferrin receptor led to a decrease in late abortive pits, suggesting that cargo may regulate a checkpoint that controls clathrin-coated pit maturation. In contrast, increasing LDL receptor levels increases clathrin-coated pit size and slows their maturation, meaning that individual cargos may regulate endocytosis differently (Mettlen et al., 2010). In yeast, experiments using a fluorescently-labeled α-factor derivative revealed that cargo arrive during early stages of endocytosis (Toshima et al., 2006). The early arrival of cargo at endocytic sites suggests that in yeast cargo may also regulate endocytosis, although the mechanism is unknown.

Using Toxins to Study Endocytosis and Trafficking

Protein toxins secreted by bacteria and plants have been used as tools to study mammalian cell biological processes (Sandvig et al., 2010). Cholera, Shiga, ricin and *Pseudomonas* exotoxin A are AB protein toxins that are internalized by and kill mammalian cells (Sandvig and van Deurs, 2005). The A moiety of AB toxins is the enzymatically active component and the B moiety is responsible for targeting the toxin to the correct intracellular compartment. Therefore B subunits of AB toxins have been used to study cell surfaces, endocytosis, and trafficking pathways.

Ricin is an example of an AB toxin that has been particularly useful when studying mammalian cell biology. Some of the first evidence for clathrin-independent endocytosis was generated from experiments using ricin toxin. In these studies clathrin-mediated endocytosis was blocked by hypotonic shock and potassium-depletion, but ricin toxin was still internalized (Moya et al., 1985). Ricin is now known to use clathrin-mediated endocytosis, clathrin- and caveolae-independent endocytosis, and macropinocytosis internalization pathways (Sandvig and van Deurs, 1996). Furthermore, ricin was used as a cargo in some of the first studies demonstrating protein recycling after endocytosis (Sandvig and Olsnes, 1979). Ricin has proved to be a useful tool when studying mammalian endocytosis and trafficking pathways.

Yeast Killer Toxin K28

The K28 toxin is secreted by yeast infected with the intracellular ScV-M28 virus and will kill yeast not infected with the virus. Since K28 is the only gene encoded by the ScV-M28 double-stranded RNA (dsRNA) virus, yeast infected with ScV-M28 must also be infected with an L-A helper virus (Schmitt and Tipper, 1990; Schmitt and Tipper, 1995). The L-A virus encodes the capsid protein (Gag) and the RNA-dependent RNA polymerase (Pol) used by both viruses (Wickner, 1993). K28 is made as a preprotoxin precursor in the cytosol and then post-translationally translocated into the ER (Breinig et al., 2006). The preprotoxin precursor is processed in the secretory pathway at the N-terminus, C-terminus, and internally to generate the mature K28 toxin (Riffer et al., 2002). The secreted K28 toxin consists of an α and β subunit connected by a disulfide bond.

In order to kill a target cell, K28 first attaches to the surface of another yeast cell by binding to a mannotriose side-chain of a cell wall mannoprotein (Schmitt and Radler, 1990; Schmitt and Radler, 1988). Studies suggest that K28 is internalized via endocytosis and enters early endocytic compartments (Eisfeld et al., 2000). K28 is

predicted to then traffic to the Golgi through an unknown mechanism. Erd2p is a receptor that normally transports resident ER proteins with a C-terminal H/KDEL sequence from the Golgi back to the ER (Semenza et al., 1990). The Erd2p receptor is thought to bind the HDEL sequence on the K28 β subunit and transport the toxin to the ER (Eisfeld 2000). The toxin then translocates into the cytosol, possibly through the Sec61 complex (Eisfeld et al., 2000; Heiligenstein et al., 2006). Once in the cytosol, the β subunit is ubiquitinated and degraded by the proteasome. The α subunit enters the nucleus and arrests cells in early S phase, with the single nucleus in the mother cell (Schmitt et al., 1996). Low doses of K28 elicit apoptosis-like death dependent on the presence of caspase homolog Yca1p (Reiter et al., 2005; Madeo et al., 2002). These yeast display DNA fragmentation, flipping of phosphatidylserine to the outer leaflet of the plasma membrane, and accumulation of reactive oxygen species. High doses of K28 cause necrotic cell death (Reiter et al., 2005).

Yeast that produce K28 are also resistant to the toxin. In these yeast, mature K28 enters the cell and traffics to the cytosol. In one model, the preprotoxin form of K28 translated from the ribosome forms a complex in the cytosol with the entering mature K28 toxin (Breinig et al., 2006). This complex is ubiquitinated and degraded by the proteasome before the K28 α subunit can kill the cell. According to this model, any yeast that is capable of making the preprotoxin precursor of the toxin in the cytosol will be resistant to the mature form of K28.

There are many questions that remain about the K28 pathway. Proof that K28 is internalized by receptor-mediated endocytosis is still lacking and only a few genes involved in the K28 trafficking and killing pathways have been identified. A genome-wide screen of yeast can reveal many more individual genes and complexes that mediate K28 action. From that information, we can gain a better understanding of how K28 functions in the cell and also gain new insights into yeast cell biology.

CHAPTER 2

A Yeast Killer Toxin Screen Provides Insights into A/B Toxin Entry, Trafficking, and Killing Mechanisms

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Background

As a member of the A/B protein toxin family, the yeast K28 "killer" toxin shows striking similarities to various clinically relevant toxins produced by plants and pathogenic bacteria. These A/B toxins usually consist of one or more β subunits that mediate cellular entry and intracellular targeting, and an α subunit that kills the invaded cell (Falnes and Sandvig, 2000). The K28 toxin is a virally encoded α/β heterodimer secreted by *Saccharomyces cerevisiae* infected with the M28 virus. After uptake by uninfected yeast, K28 follows a trafficking pathway that resembles those taken by numerous other A/B toxins, ultimately resulting in cell killing (Breinig et al., 2006). Studying A/B toxin pathways in a genetically tractable organism, such as yeast, promises to provide new insights into the interactions between A/B toxins and their molecular targets in mammalian cells.

Similar to cholera toxin, ricin, and Shiga toxin, K28 enters cells by endocytosis and traffics through endosomal and Golgi compartments to the ER, effectively bypassing degradation in the vacuole/lysosome (Eisfeld et al., 2000; Sandvig and van Deurs, 2002). The conserved 'KDEL' receptor, Erd2p in yeast, recognizes C-terminal K/HDEL sequences on K28 and cholera toxins and directs their retrograde trafficking from the Golgi to the ER of their target cells. Some of the cellular ER retrotranslocation machinery is then co-opted to allow export into the cytosol (Heiligenstein et al., 2006; Sandvig and van Deurs, 2002; Yu and Haslam, 2005). Once released from the ER a disulfide bond connecting the α and β subunits of K28 breaks and the β subunit is degraded by the proteasome (Heiligenstein et al., 2006). The K28 α subunit elicits a pre-S phase cell cycle arrest and kills the target cell by an unknown mechanism, possibly by activating a yeast apoptotic-like cascade (Reiter et al., 2005; Schmitt et al., 1996).

Although there have been systematic efforts to uncover yeast mutants with altered sensitivity to the ionophoric K1 killer toxin and the tRNase toxin zymocin (Huang et al., 2008; Page et al., 2003), no systematic screens for the A/B toxin K28 have been reported. Previously, a genetic approach was used to identify a limited number of K28 resistant mutants, such as the endocytic mutant *sla2*Δ and the Golgi-to-ER retention mutant *erd2*Δ, that have defects in toxin uptake and trafficking (Eisfeld et al., 2000). However, many questions remain spanning the entire proposed pathway of K28 uptake and killing. For example, the cell wall and plasma membrane factors important for K28 binding, the cellular components acted upon to induce cell death, and the cellular mechanisms used to mitigate the toxic effects of K28 are all unknown. Moreover, the extent to which common mechanisms are used by K28 and medically important toxins has not been fully investigated.

The 365 K28 resistant or hypersensitive mutants identified by our screen generate a detailed view of conserved A/B toxin trafficking and killing mechanisms. The mutants identified are statistically enriched in biological processes and components that are likely to be important for K28 action. Our screen results also provide insights into the biology of cells targeted by A/B toxins. For example the yeast AP2 complex, which has not previously been associated with any endocytic functions in yeast (Yeung et al., 1999), was identified by our screen as having a critical role in K28 action. Our

functional analysis of yeast AP2 suggests it has a previously unrecognized and conserved role in cargo-selective endocytosis. These results highlight the utility of K28 as a sensitive tool for analyzing cellular pathways and processes in intoxicated cells.

Results

A Genetic Screen for K28 Resistance or Hypersensitivity

To identify determinants of K28 sensitivity, we developed a 'killer' assay to systematically screen mutant yeast strains for K28 resistance or hypersensitivity. A dense spot of K28-secreting cells was plated onto a lawn of the strain to be tested, and the zone of clearance in the lawn around the spot was measured to give quantitative results (see Figure 2.1A). We screened 4806 strains in the *MAT*α deletion collection, which represents ~80% of all yeast genes, and an array of temperature sensitive (ts) alleles representing almost one-quarter of all essential yeast genes.

Our screen yielded 176 mutations causing K28 resistance (140 deletions, 36 ts alleles) and 189 mutations causing K28 hypersensitivity (116 deletions, 73 ts alleles). A manually annotated distribution of all K28 resistant or hypersensitive mutants identified is presented in Figures 2.1B and 2.1C. In general, K28 resistant hits support and expand upon the previously proposed pathway for K28 entry, trafficking, and killing (Schmitt and Breinig, 2006). The hypersensitive hits reveal the largely unexplored cellular pathways that resist toxin action. For example, over 25% of K28 hypersensitive mutations were found in genes related to protein translation. Although the mechanism of K28 killing is unknown, the ribosome is a common target for other toxins which follow a nearly identical trafficking pathway to K28 (Montanaro et al., 1973; Reisbig et al., 1981). Additionally, mutations in 21 genes of unknown function were found to alter K28 sensitivity, providing potential insights into the functions of these genes. The use of the ts strain collection allowed us to screen previously inaccessible aspects of yeast cell biology and identify determinants of K28 toxicity, such as the proteasome (hypersensitive) and glycophosphatidylinositol (GPI) -anchor biosynthetic machinery (resistant).

We next sought to gain an overview of K28 activity by identifying the cellular processes implicated repeatedly by different hypersensitive or resistant mutants. One unbiased approach to identify such processes is to calculate the enrichment of gene ontology (GO) terms associated with genes identified in the screen. GO annotations draw on many types of data from the literature to organize genes into cellular processes, components and functions (The Gene Ontology Consortium, 2000). We identified 250 GO terms that were statistically enriched (Bonferroni corrected $p < 0.05$) in our hit list. The fold enrichments for K28 resistant and hypersensitive GO terms representing the major groups of enriched cellular processes or components are shown in Figures 2.1D and 2.1E.

The GO terms enriched in our dataset reveal the aspects of cell physiology that determine how K28 interacts with and affects cells. The resistant hits are enriched for genes involved in cell wall organization, endocytosis, and trafficking. These analyses specifically identified several cellular components, such as the chromatin-silencing SIR complex and the clathrin adaptor AP2 complex, as determinants of K28 killing. The hypersensitive hits are enriched for genes involved in gene expression and translation, as well as genes that encode the protein sorting HOPS complex, and the chromatin remodeling SWR1 and ASTRA complexes (Shevchenko et al., 2008).

Figure 2.1

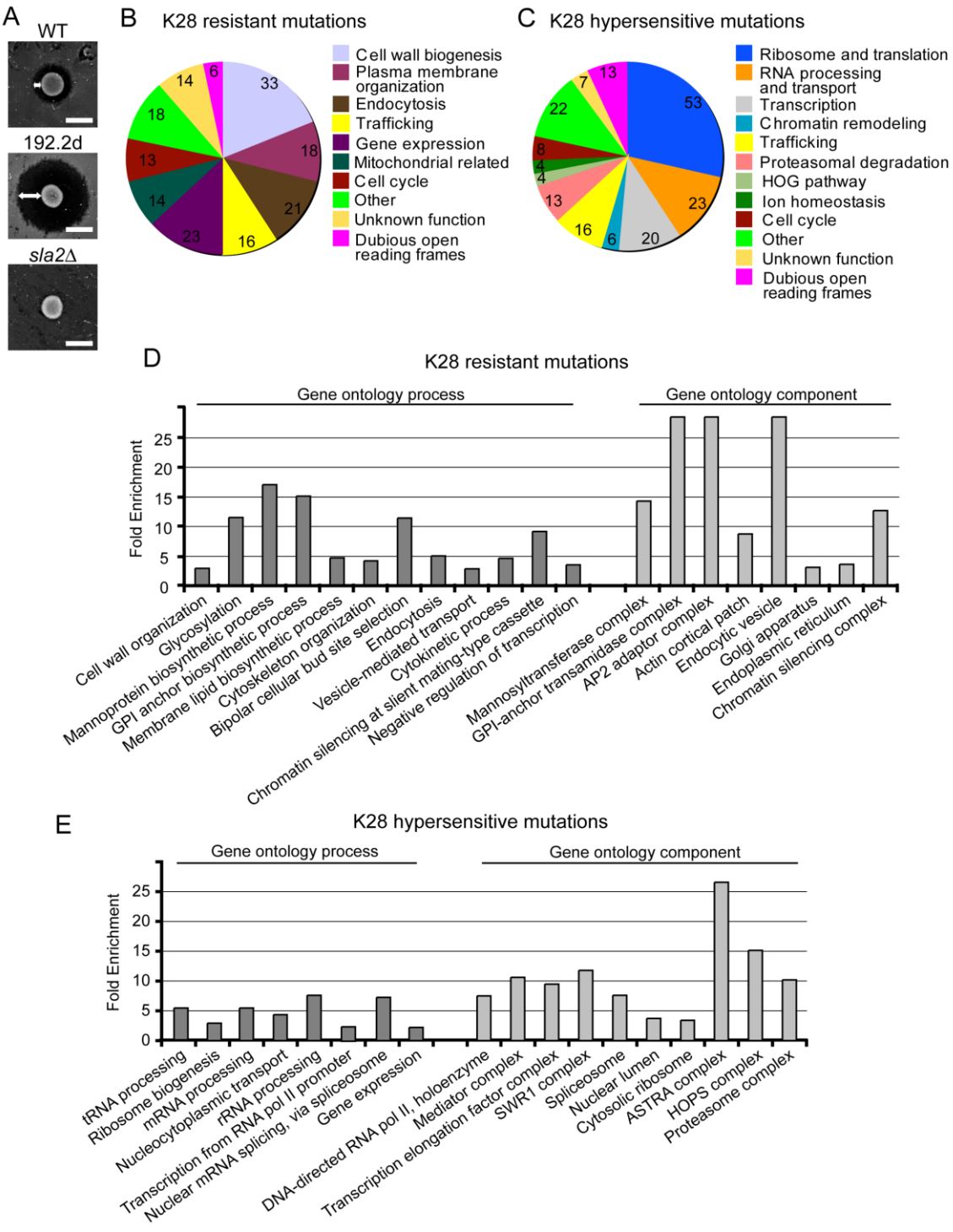

A
WT
192.2d
sla2Δ
B
K28 resistant mutations
33
18
21
16
23
14
13
18
14
6
Cell wall biogenesis
Plasma membrane organization
Endocytosis
Trafficking
Gene expression
Mitochondrial related
Cell cycle
Other
Unknown function
Dubious open reading frames
C
K28 hypersensitive mutations
53
23
20
6
16
13
4
4
8
22
7
13
Ribosome and translation
RNA processing and transport
Transcription
Chromatin remodeling
Trafficking
Proteasomal degradation
HOG pathway
Ion homeostasis
Cell cycle
Other
Unknown function
Dubious open reading frames
D
K28 resistant mutations
Gene ontology process
Gene ontology component
Fold Enrichment
0
5
10
15
20
25
Cell wall organization
Glycosylation
Mannoprotein biosynthetic process
GPI anchor biosynthetic process
Membrane lipid biosynthetic process
Cytoskeleton organization
Bipolar cellular bud site selection
Endocytosis
Vesicle-mediated transport
Cytokinetic process
Chromatin silencing at silent mating-type cassette
Negative regulation of transcription
Mannosyltransferase complex
GPI-anchor transamidase complex
AP2 adaptor complex
Actin cortical patch
Endocytic vesicle
Golgi apparatus
Endoplasmic reticulum
Chromatin silencing complex
E
K28 hypersensitive mutations
Gene ontology process
Gene ontology component
Fold Enrichment
0
5
10
15
20
25
tRNA processing
Ribosome biogenesis
mRNA processing
Nucleocytoplasmic transport
rRNA processing
Transcription from RNA pol II promoter
Nuclear mRNA splicing, via spliceosome
Gene expression
DNA-directed RNA pol II, holoenzyme
Mediator complex
Transcription elongation factor complex
SWR1 complex
Spliceosome
Nuclear lumen
Cytosolic ribosome
ASTRA complex
HOPS complex
Proteasome complex

Figure 2.1. A Genome-Wide Screen for K28 Resistance or Hypersensitivity. (A) Examples of killer assay results for BY4742 (WT), hypersensitive 192.2d and resistant *sla2*Δ strains. Arrows represent halo measurements. Scale bar, 5 mm. (B and C) Manually annotated groupings of K28 resistant (B) and hypersensitive (C) mutants. (D and E) Statistically enriched gene ontology (GO) terms among K28 resistant (D) and hypersensitive (E) mutations.

To assess the specificity of these enriched categories, we compared the GO terms enriched in our screen to those enriched in a screen for mutants resistant to the tRNase killer toxin zymocin (Huang et al., 2008). Other than GO terms relating to RNA processing and ribosome biogenesis, there was little overlap between GO terms enriched in the two screens (data not shown). This result suggests that the genes identified in our screen reflect the specific manner in which K28 interacts with cells.

In Figure 2.2 Osprey-generated networks (Breitkreutz et al., 2003) display examples of genes involved in processes and components important for K28 action based on enriched GO terms. These results are consistent with what is known about K28 action, but more comprehensively identify the cellular processes and components involved. For example, K28 is thought to initially interact with the yeast cell wall and possibly components of the plasma membrane (Heiligenstein et al., 2006; Schmitt and Radler, 1988; Schmitt and Radler, 1990), and we found a large number of mutations relating to cell wall and plasma membrane organization that were not previously linked to K28 resistance (Figure 2.2A). The importance of membrane lipids in toxin binding is exemplified by the toxin aerolysin, which binds to the GPI moiety of target cell surface proteins, and cholera and Shiga toxins, which recognize modified sphingolipids (Heyningen, 1974; Jacewicz et al., 1986; Nelson et al., 1997). It is important to note that in addition to binding the toxin, the correct lipid environment could be needed for K28 endocytosis or trafficking (Beh and Rine, 2004; deHart et al., 2002).

After endocytic uptake, K28 is proposed to traffic the secretory pathway in reverse and exit from the ER into the cytosol (Heiligenstein et al., 2006). Accordingly, we found both K28 resistant and hypersensitive mutants throughout the endomembrane system (Figures 2.1B and 2.1C). Resistant mutants associated with GO terms related to intracellular trafficking (Figure 2.2B) show that both secretion and retrograde traffic are important for K28 toxicity. We speculate that mutants causing defects in secretory traffic might affect the amount of K28 receptor available for toxin binding, whereas retrograde trafficking defects might affect K28 transport to the ER (Heiligenstein et al., 2006). Figure 2.2B also shows a group of hypersensitive mutants in the vacuolar HOPS complex. It is possible that vacuolar defects redistribute a factor that controls K28 killing. Alternatively a portion of internalized toxin might normally reach the vacuole, a lytic structure in yeast, where it is destroyed. When the vacuole is defective an excess of K28 might traffic to the ER, resulting in hypersensitivity. In support of the second hypothesis, the analogous organelle in metazoans, the lysosome, has been proposed to degrade a fraction of ricin and Shiga toxins after endocytic uptake (Sandvig and van Deurs, 1996). It is also noteworthy that mutations in the ubiquitin-proteasome pathway cause K28 hypersensitivity (Figures 2.1C and 2.1E). Thus, our screen suggests that vacuolar targeting, and proteasomal degradation in the cytosol, may act in tandem to control the cytosolic K28 load.

Figure 2.2

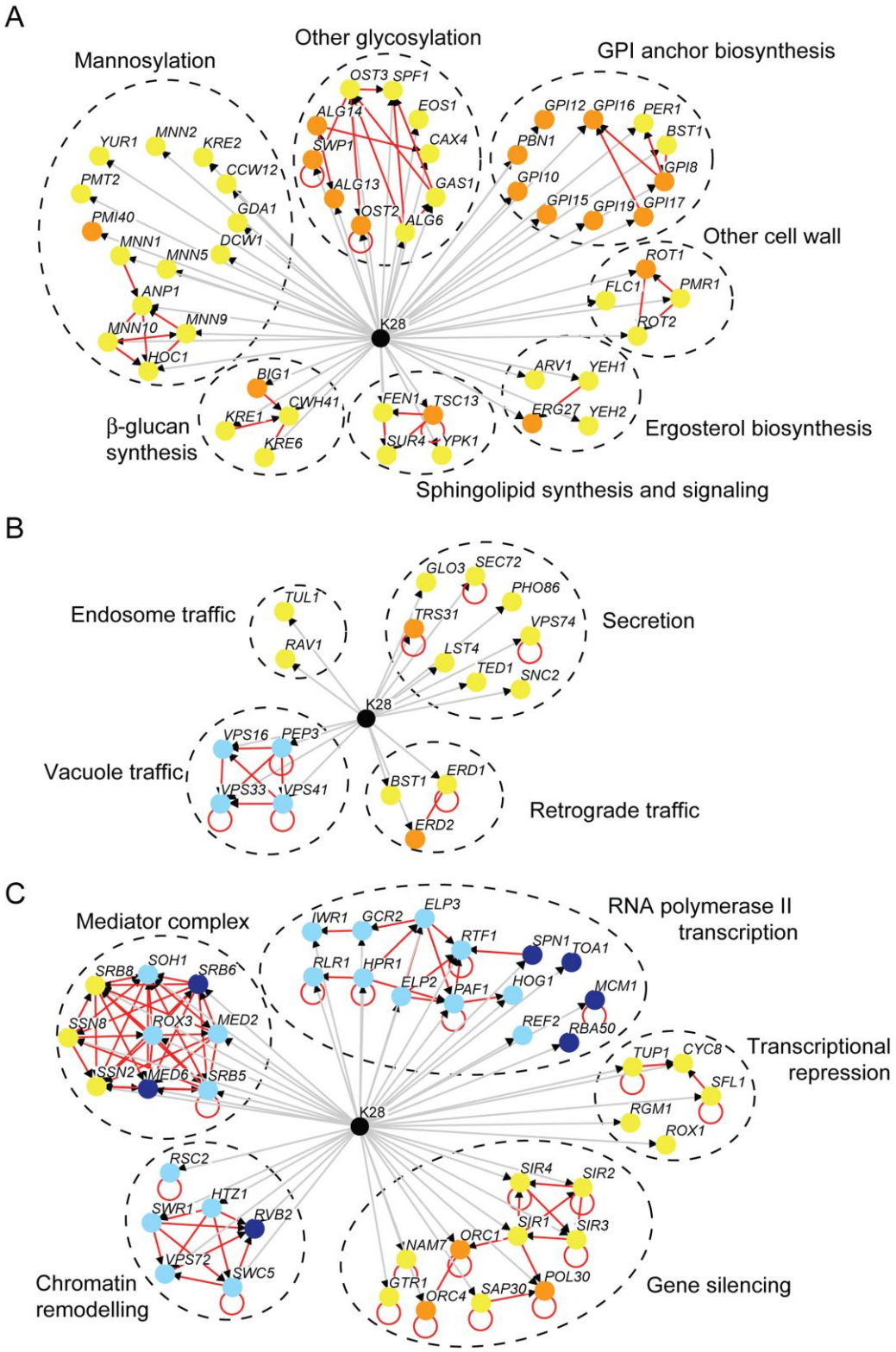

A
Mannosylation
Other glycosylation
GPI anchor biosynthesis
Other cell wall
β-glucan synthesis
Ergosterol biosynthesis
Sphingolipid synthesis and signaling
K28
B
Endosome traffic
Secretion
Vacuole traffic
Retrograde traffic
K28
C
Mediator complex
RNA polymerase II transcription
Transcriptional repression
Chromatin remodelling
Gene silencing
K28

Figure 2.2. Genes from Selected Complexes and Pathways Implicated in K28 Resistance or Hypersensitivity. Osprey generated network diagrams of genes related to (A) cell wall and lipid biogenesis, (B) vesicular trafficking, and (C) the regulation of gene expression. Yellow and orange circles represent K28 resistant deletions or ts alleles, respectively, and light blue and dark blue circles represent K28 hypersensitive deletions or ts alleles, respectively. Grey lines depict the K28 phenotype while red lines depict published physical, functional or genetic interactions within a subgroup of genes.

We identified a number of hypersensitive and resistant mutants associated with enriched GO terms related to transcriptional regulation (Figure 2.2C). The identification of multiple subunits of several complexes illustrates the specificity, coverage and sensitivity of our screen. For example in the mediator complex, which regulates transcription, loss of core subunits causes K28 hypersensitivity, while loss of the antagonistic CDK module subunits causes K28 resistance (Figure 2.2C) (Samuelsen et al., 2003). Similarly, mutation of multiple subunits of the chromatin silencing SIRcomplex causes K28 resistance while deletion of the antagonistic histone variant *HTZ1* or SWR1 complex components causes K28 hypersensitivity (Figure 2.2C) (Meneghini et al., 2003). Further cell biological analyses should help elucidate mechanistic roles for these complexes and others in K28 killing.

Finally, we generated double mutants to explore epistatic interactions between presumably early-acting resistant mutations and diverse hypersensitive mutations. To assess different parts of the toxin pathway we chose hypersensitive deletions of an osmolarity stress-activated kinase (*HOG1*), a vacuolar SNARE protein (*VAM7*) and a ribosomal subunit (*RPS8A*). Deletion of the cell wall biosynthetic enzyme *MNN2* was epistatic to all hypersensitive mutants presumably because it abrogates K28 binding (Table I and Figure 2.3) (Schmitt and Radler, 1990). Deletion of the endocytic gene *END3* or the gene *APM4*, which encodes a subunit of the AP2 complex, was epistatic to *vam7*Δ and *rps8a*Δ. Strikingly, deletion of *HOG1* was epistatic to both *end3*Δ and *apm4*Δ. These results show the importance of the HOG pathway to resist K28 killing, and suggest either that the reduced amounts of toxin entering the endocytic mutant cells are sufficient for killing when *HOG1* is absent, or that K28 might have a secondary killing mechanism, which is only revealed when the HOG pathway is absent. Strikingly, the metazoan *HOG1* homolog p38 is activated in mammalian cells treated with cholera, Shiga, and ricin toxins, highlighting the relevance of our screen to toxin biology in more complex eukaryotes (Schnitzler et al., 2007; Tamura et al., 2003; Walchli et al., 2008).

Table I. Epistatic Relationships Among Mutants with Altered K28 Sensitivity

*Double mutant phenotypes

	*hog1*Δ	*rps8a*Δ	*vam7*Δ
*mnn2*Δ	++	++	++
*end3*Δ	-	++	++
*apm4*Δ	-	+	+

*Single mutant phenotypes

*mnn2*Δ	++
*end3*Δ	++
*apm4*Δ	++
*hog1*Δ	- -
*rps8a*Δ	- -
*vam7*Δ	-

*Scoring is based on relative halo sizes compared to wild-type cells.
++ = very resistant, + = partially resistant
- = partially hypersensitive, - - = very hypersensitive

Some K28 Resistant Mutants Have Toxin-Binding Defects

Since many cell wall mutants are known to have defects in binding to the toxin (Schmitt and Radler, 1990), we wanted to determine which mutants were resistant because of weak K28 binding. Applying a toxin-cell binding assay to our 176 K28 resistant mutants biochemically defined requirements for K28-cell binding. We found 20 of 140 resistant gene deletions and 8 of 36 resistant ts alleles deplete K28 from culture supernatant significantly less well than wild-type cells ($p < 0.01$; Figure 2.3). The gene deletions with the most severe phenotypes relate to mannosylation. Less severe binding defects occur in deletion strains with aberrant lipid biogenesis or transcriptional mutations (Figure 2.3). Similarly, ts alleles with K28 binding defects are mutated in genes affecting protein mannosylation and lipid biogenesis (Figure 2.3). In particular, half of all binding-defective ts alleles affect attachment of GPI anchors to proteins. This assay sheds light on the mechanism of toxin resistance in >15% of the mutant strains and identified transcriptional networks likely to impact cell wall structure. For example, the importance of the Cyc8p/Tup1p transcriptional repressor and the CDK module of the mediator complex in K28-cell binding is consistent with established roles for *CYC8/TUP1* in manno-specific flocculation and the CDK module in regulation of other flocculation genes (Samuelsen et al., 2003; Stratford, 1992).

Figure 2.3

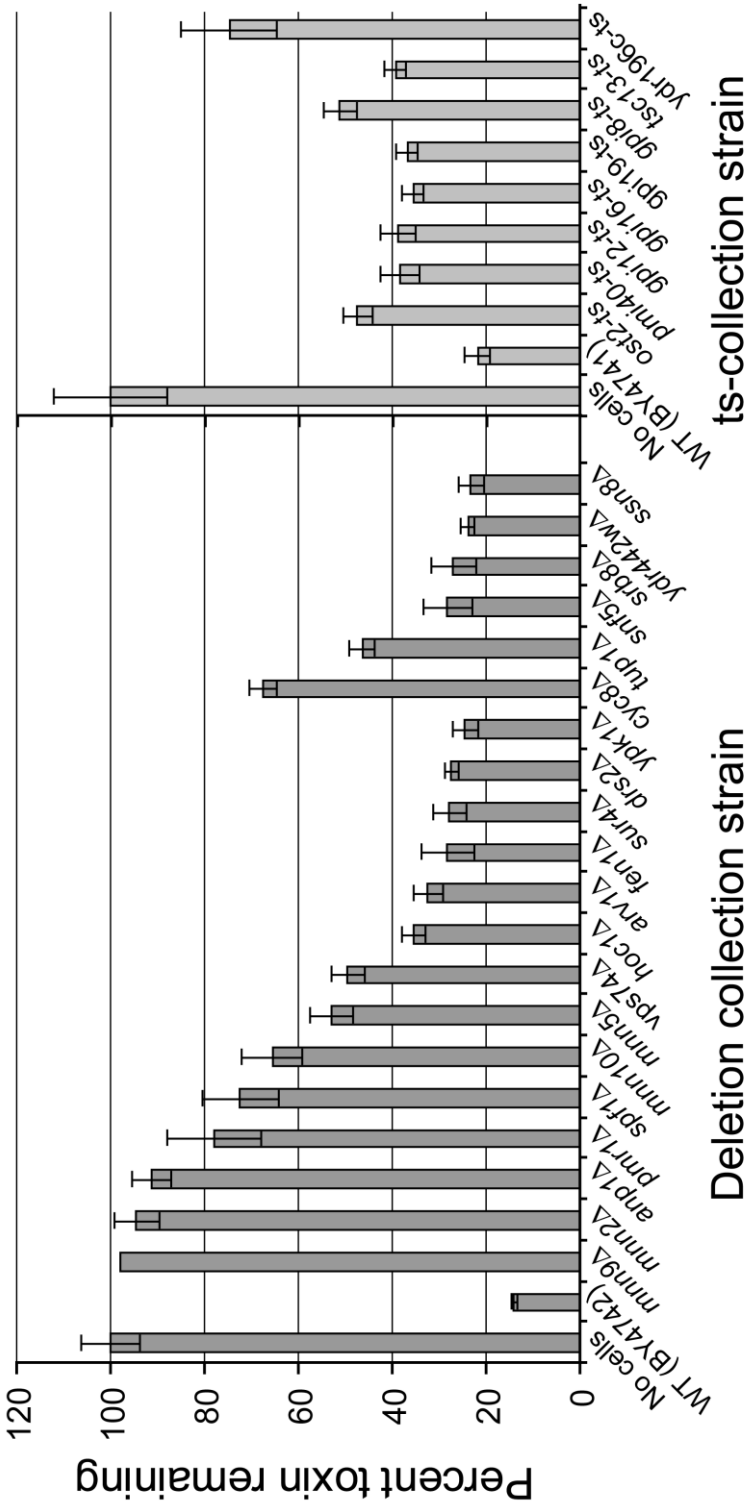

Percent toxin remaining
120
100
80
60
40
20
0
WT (BY4742)
No cells
mnn9Δ
mnn2Δ
anp1Δ
pmr1Δ
spf1Δ
mnn10Δ
mnn5Δ
vps74Δ
hoc1Δ
arv1Δ
ten1Δ
sur4Δ
drs2Δ
ypk1Δ
cyc8Δ
tup1Δ
snf5Δ
srb8Δ
ydr442wΔ
ssn8Δ
Deletion collection strain
WT (BY4741)
No cells
ost2-ts
pmi40-ts
gpi12-ts
gpi16-ts
gpi19-ts
gpi18-ts
tsc13-ts
ydr196c-ts
ts-collection strain

Figure 2.3. K28 Binding Defects Underlie a Proportion of Toxin Resistant Mutations. Relative toxin activity remaining after depletion of K28 containing culture supernatant with wild-type or mutant cells. Shown are the mean values of at least three experiments for mutants that deplete toxin activity significantly more poorly than wild-type ($p < 0.01$). Error bars indicate standard error of the mean.

AP2 Adaptins Are Endocytic Coat Proteins

One intriguing result of our screen was the identification of all four AP2 clathrin adaptor subunit deletions (*apl1*Δ, *aps2*Δ, *apl3*Δ and *apm4*Δ) as strongly K28 resistant (Figure 2.4A). The AP2 complex functions in metazoans to link endocytic cargo proteins to the clathrin coat. However, in *S. cerevisiae* AP2 homolog deletions previously had no detectable endocytic phenotype and were thought to be unimportant for endocytosis (Sorkin, 2004; Yeung et al., 1999). To our knowledge K28 resistance is the first specific phenotype in *S. cerevisiae* for AP2 loss-of-function; large chemical genetic screens have found altered sensitivities, the basis for which is obscure (Hillenmeyer et al., 2008; Parsons et al., 2006).

To better understand the cause of toxin resistance in the four AP2 subunit deletions, we analyzed dynamics of the yeast AP2 β (Apl1p) and μ (Apm4p) subunits. The Apl1-GFP and Apm4-GFP strains exhibited wild-type toxin sensitivity, suggesting that the GFP fusions are functional (data not shown). Both Apl1-GFP and Apm4-GFP localize to cortical patches that resemble sites of endocytosis (Figure 2.4B) (Kaksonen et al., 2003). The Apl1-GFP and Apm4-GFP patches are dynamic and have similar average lifetimes of 67 ± 32 seconds and 64 ± 29 seconds, respectively. The long and variable lifetimes of these proteins are similar to those of early arriving endocytic proteins clathrin (Kaksonen et al., 2005) and Ede1p (Toshima et al., 2006). The Apl1-GFP and Apm4-GFP patches internalize from the cell cortex before disappearing (95%, n=105 for both Apl1p and Apm4p) in a manner characteristic of endocytic coat proteins (Kaksonen et al., 2005). To confirm that this internalization corresponds to endocytic events, we recorded two-color movies of Apl1-GFP with the endocytic coat protein Sla1-mCherry or the late endocytic patch marker Abp1-mRFP. Apl1-GFP forms patches, which are joined subsequently by, and then internalized with, Abp1-mRFP and Sla1-mCherry (Figure 2.4C). Most endocytic patches contain AP2, as 91% (n=102) of Sla1-mCherry patches had detectable Apl1-GFP. These results indicate that AP2 is a part of the yeast endocytic machinery and that it internalizes with the endocytic coat upon assembly of actin as marked by Abp1p. Because of this dynamic behavior, yeast AP2 can be included in the previously defined endocytic coat module (Kaksonen et al., 2005).

Like mammalian AP2, yeast AP2 subunits physically associate, and a deletion of one subunit prevents the other subunits from interacting (Yeung et al., 1999). Consistent with this finding, Apm4-GFP failed to localize to cortical endocytic patches in *apl1*Δ cells (Figure 2.4D). This observation suggests that AP2 subunits likely form a complex at endocytic sites, and that they are interdependent on each other for proper localization.

Together, our data place the AP2 complex within the modular framework set out by Kaksonen et al. (2005). Figure 2.4E shows all K28 resistant endocytic hits arrayed, where possible, along the temporal endocytic pathway from coat to WASP/myosin to actin to scission modules, and includes the placement of AP2 in the coat module. Some of the other endocytic genes we identified cause only very mild endocytic phenotypes, such as *abp1*Δ and *myo5*Δ. In total, these results show that K28 can be used as an extremely sensitive tool to investigate cargo uptake by cells.

Figure 2.4

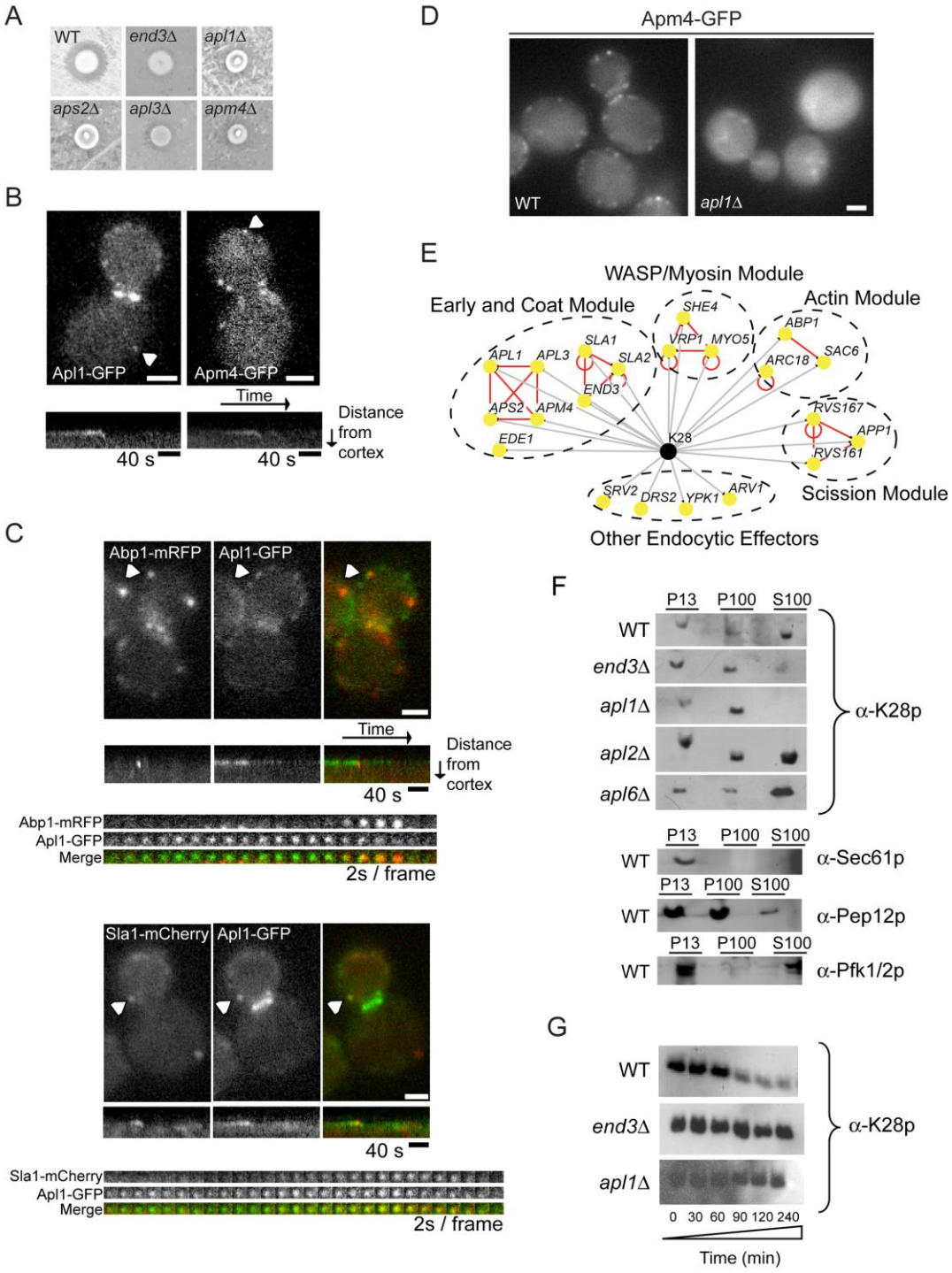

A
WT
end3Δ
apl1Δ
aps2Δ
apl3Δ
apm4Δ
B
Apl1-GFP
Apm4-GFP
Time
Distance from cortex
40 s
40 s
C
Abp1-mRFP
Apl1-GFP
Time
Distance from cortex
40 s
Abp1-mRFP
Apl1-GFP
Merge
2s / frame
Sla1-mCherry
Apl1-GFP
40 s
Sla1-mCherry
Apl1-GFP
Merge
2s / frame
D
Apm4-GFP
WT
apl1Δ
E
WASP/Myosin Module
Early and Coat Module
Actin Module
SHE4
ABP1
SLA1
VRP1
MYO5
APL1
APL3
SLA2
ARC18
SAC6
END3
APS2
APM4
RVS167
APP1
EDE1
K28
RVS161
SRV2
DRS2
YPK1
ARV1
Scission Module
Other Endocytic Effectors
F
P13
P100
S100
WT
end3Δ
apl1Δ
apl2Δ
apl6Δ
α-K28p
WT
α-Sec61p
WT
α-Pep12p
WT
α-Pfk1/2p
G
WT
end3Δ
apl1Δ
α-K28p
0 30 60 90 120 240
Time (min)

Figure 2.4. Yeast AP2 Subunits are Endocytic Coat Proteins. (A) Killer assays of BY4742 (WT), a control endocytic mutant, and AP2 subunit deletions. (B) Yeast expressing Apl1-GFP or Apm4-GFP. Kymographs of the patches indicated by the arrows from movies of cells expressing the GFP-tagged proteins. (C) Yeast expressing Apl1-GFP and Abp1-mRFP (above) or Sla1-mCherry (below). Kymographs and montages of single patches indicated by the arrows from two-color movies of cells expressing the indicated proteins. (D) Wild-type (WT) or *apl1*Δ yeast expressing Apm4-GFP. (E) Network diagram of the endocytic mutants identified in our screen. Grey lines depict the K28 phenotype and red lines depict published interactions within a subgroup of genes. (F) Subcellular fractionation for the indicated strains treated with K28. Fractions were probed with the indicated antibodies by Western blotting. P13 = 13,000 g pellet; P100 = 100,000 g pellet; S100 = 100,000 g supernatant. (G) Western blots of K28 remaining in cell-free culture supernatant after incubation of spheroplasts with K28 over time. Scale bars, 4 μm.

AP2 Appears to Perform a Cargo-Specific Function in Endocytosis

Since AP2 localizes to endocytic sites and is important for K28 toxicity, we tested its involvement in K28 internalization. Subcellular fractionation was used to follow K28 in cell lysates prepared from toxin-treated cells. The amount of K28 in each fraction varied between experiments, and therefore these data cannot be quantitatively analyzed. As shown in Figure 2.4F, wild-type cells and deletions of *APL2* or *APL6*, which are subunits of the AP2-related AP1 and AP3 complexes, accumulated K28 in the cytosol (S100 fraction). Conversely, deletion of AP2 subunits prevented K28 accumulation in the cytosol in each of three independent experiments (Figure 2.4F and data not shown). K28 was not detectable in the cytosolic fraction of *end3*Δ endocytic mutants in three out of four independent experiments, and appeared as a faint band in the fourth experiment, shown in Figure 2.4F. The presence of K28 in the P100 fraction (Golgi, endosomal and/or vesicle membranes) in *end3* and *apl1* mutants is likely caused by leakiness in the endocytic blocks of these mutants. To confirm that *END3* and *APL1* deletions had defects in K28 uptake, we measured K28 levels in cell-free culture supernatant of yeast spheroplasts incubated with K28. Figure 2.4G shows that wild-type but not *end3* or *apl1* mutant spheroplasts, deplete K28 from the supernatant in a time-dependent manner. Together these results suggest that AP2 mediates K28 uptake.

Mutating certain early-arriving endocytic proteins in yeast, such as clathrin or Ede1p, decreases both the lifetime of later patch components, such as Sla1p, and the number of endocytic sites in the cell (Kaksonen et al., 2005). Unlike *ede1*Δ yeast, there was no significant decrease in the Sla1-GFP lifetimes in *apl1*Δ yeast, nor were there decreases in Sla1-GFP patch number (Figure 2.5A). These data suggest that AP2 does not significantly impact the function of the core endocytic machinery. Finally, to test whether AP2 subunit deletions affect endocytosis of other cargos or the plasma membrane, we examined uptake of a fluorescent derivative of the peptide cargo α-factor and of the lipophilic dye FM4-64 (Toshima et al., 2006). Deletion of AP2 subunits did not affect the endocytosis of FM4-64 or α-factor (Figures 2.5B and 2.5C).

These results suggest yeast AP2 has a strong but cargo-specific effect on K28 endocytosis. It is well established that mammalian AP2 mediates endocytic cargo recognition (Sorkin, 2004), and we show here that the yeast AP2 complex has an endocytic role in K28 uptake. An alternative hypothesis, that AP2 is involved in a non-endocytic trafficking event, is unlikely because Apl1-GFP and Apm4-GFP localize only to the cell cortex. Our data lead us to suggest that yeast AP2 acts as a specific endocytic adaptor for the killer toxin receptor. This is one of the first reports of a cargo-selective component of the yeast endocytic machinery (Burston et al., 2009). Which cargos other than K28 and its yet-to-be identified receptor are selected by AP2 remains to be discovered, but is of considerable interest, as a growing number of factors, such as the arrestin-like proteins, seem to contribute selectivity in specification of the plasma membrane proteome composition (Lin et al., 2008).

Figure 2.5

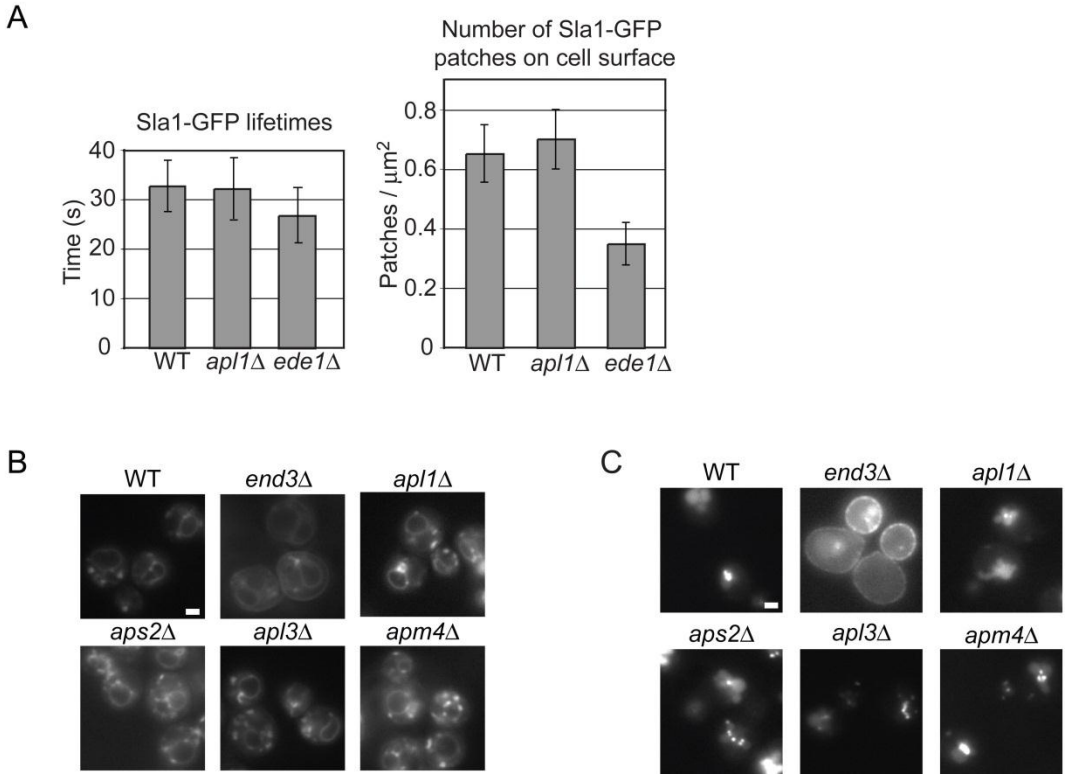


Figure 2.5. AP2 Loss Does Not Cause a General Endocytic Defect. (A) Lifetimes of individual Sla1-GFP patches ± standard deviation for wild-type (WT), *apl1*Δ and *ede1*Δ yeast. n = 50 patches for each strain. Sla1-GFP patch number/cell surface area (μm^2) ± standard deviation in wild-type, *apl1*Δ, and *ede1*Δ yeast. n = 20 patches for each strain. (B) FM4-64 labeling of the indicated strains. (C) Uptake of fluorescent α-factor in the indicated strains.

Conclusions

Our results define interconnected protein complexes and pathways that impact K28 function and suggest testable hypotheses about virtually every aspect of K28-target cell interaction. This study and the literature (Broeck et al., 2007; Sandvig and van Deurs, 2002; Schmitt and Breinig, 2006) suggest that K28 has striking mechanistic similarities to clinically relevant toxins. By exploiting the genomic tools available in yeast we identified 365 genes that mediate K28 sensitivity, thereby providing a resource that promises to be useful in understanding A/B toxin action. This type of screen could not be done with toxins that target mammalian cells, but still identified conserved pathways. Therefore, the results are expected to be relevant to understanding how cholera, Shiga and other toxins affect mammalian cells.

Materials and Methods

Strains

Yeast strains used in Chapter 2 are listed in Table II. The K28 toxin sensitivity screen was carried out using yeast deletion (Research Genetics) (Winzeler et al., 1999) and ts allele collections (Ben-Aroya et al., 2008). GFP tagged AP2 subunits were constructed as described (Longtine et al., 1998). Yeast were grown either in rich (YPD) or synthetic media essentially as described (Amberg et al., 2005).

Table II. Yeast Strains Used in Chapter 2

Strain Name	Background	Relevant Genotype	Source
DDY3779		MATα *ura3-52, leu2, ski2-2, {M28 virus infected, K28 secreting}*	Schmitt and Tipper, 1990
DDY3780		*ura3, leu2, {killer toxin hypersensitive}*	Schmitt et al., 1996
DDY3781	BY4741	MATa *ura*Δ*0, leu2*Δ*0, his3*Δ*1, met15*Δ*0*	Research Genetics
DDY3782	BY4742	MATα *ura*Δ*0, leu2*Δ*0, his3*Δ*1, lys2*Δ*0*	Research Genetics
deletion collection	BY4742	MATα *ura*Δ*0, leu2*Δ*0, his3*Δ*1, lys2*Δ*0, yfg1*Δ::KanMX4*	Research Genetics
ts collection	BY4741	MATa *ura*Δ*0, leu2*Δ*0, his3*Δ*1,yfeg1-ts*::KanMX4**	Ben-Aroya et al., 2008
DDY3783	BY4741	*bar1*Δ*::cgLEU2*	This study
DDY3784	BY4741	*apl1*Δ*::KanMX4*, *bar1*Δ*::cgLEU2*	This study
DDY3785	BY4741	*aps2*Δ*::KanMX4*, *bar1*Δ*::cgLEU2*	This study
DDY3786	BY4741	*apl3*Δ*::KanMX4, bar1*Δ*::cgLEU2*	This study
DDY3787	BY4741	*apm4*Δ*::KanMX4, bar1*Δ*::cgLEU2*	This study
DDY3788	BY4741	*end3*Δ*::KanMX4, bar1*Δ*::cgLEU2*	This study
DDY3789	DDY1102	MATα *ura3-52, leu2,3-112, his3*Δ*200, APL1-GFP::HIS3*	This study
DDY3790	DDY1102	MATα *ura3-52, leu2,3-112, his3*Δ*200, lys2-801, APM4-GFP::HIS3*	This study

DDY3791	DDY1102	MATa *ura3-52, leu2,3-112, his3Δ200, lys2-801, APL1-GFP::HIS3, ABP1-mRFP::HIS3*	This study
DDY3792	DDY1102	MATa *ura3-52, leu2,3-112, his3Δ200, lys2-801, APM4-GFP::HIS3, ABP1-mRFP::HIS3*	This study
DDY3793	DDY1102	MATα *ura3-52, leu2,3-112, his3Δ200, lys2-801, APL1-GFP::HIS3, SLA1-mCherry::HIS3*	This study
DDY3794	DDY1102	MATα *ura3-52, leu2,3-112, his3Δ200, lys2-801, APM4-GFP::HIS3, SLA1-mCherry::HIS3*	This study
DDY3795	DDY1102	MATα *ura3-52, leu2,3-112, his3Δ200, apl1Δ::cgURA3*	This study
DDY3796	DDY1102	MATα *ura3-52, leu2,3-112, his3Δ200, apl1Δ::cgURA3, APM4-GFP::HIS3*	This study
DDY3700	DDY1102	MATα *ura3-52, leu2,3-112, his3Δ200, SLA1-GFP::KANMX4*	Kaksonen et al., 2005
DDY3797	DDY1102	MATa *ura3-52, leu2,3-112, his3Δ200, apl1Δ::cgURA3, SLA1-GFP::HIS3*	This study
DDY3798	DDY1102	MATα *ura3-52, leu2,3-112, his3Δ200, ede1Δ::cgLEU2, SLA1-GFP::KANMX4*	Kaksonen et al., 2005
DDY3799	DDY1102	MATα *ura3-52, leu2,3-112, his3Δ200, lys2-801, ede1Δ::cgLEU2, apl1Δ::cgURA3, SLA1-GFP::KANMX4*	This study
DDY3800	BY4742	MATα *uraΔ0, leu2Δ0, his3Δ1, hog1Δ::KANMX4, end3Δ::KANMX4*	This study
DDY3801	BY4742	MATα *uraΔ0, leu2Δ0, his3Δ1, hog1Δ::KANMX4, apm4Δ::KANMX4*	This study

DDY3802	BY4742	MATα *ura∆0, leu2∆0, his3∆1, hog1∆::KANMX4, mnn2∆::KANMX4*	This study
DDY3803	BY4742	MATα *ura∆0, leu2∆0, his3∆1, met15∆0, vam7∆::KANMX4, end3∆::KANMX4*	This study
DDY3804	BY4742	MATα *ura∆0, leu2∆0, his3∆1, lys2∆0, met15∆0, vam7∆::KANMX4, apm4∆::KANMX4*	This study
DDY3805	BY4742	MATα *ura∆0, leu2∆0, his3∆1, lys2∆0, vam7∆::KANMX4, mnn2∆::KANMX4*	This study
DDY3806	BY4742	MATα *ura∆0, leu2∆0, his3∆1,rps8a∆::KANMX4, end3∆::KANMX4*	This study
DDY3807	BY4742	MATα *ura∆0, leu2∆0, his3∆1, rps8a∆::KANMX4, apm4∆::KANMX4*	This study
DDY3808	BY4742	MATα *ura∆0, leu2∆0, his3∆1, lys2∆0, rps8a∆::KANMX4, mnn2∆::KANMX4*	This study
**YFG1* = your favorite gene, representing any non-essential ORF in the collection ** *YFEG1* = your favorite essential gene, representing any of the ts-alleles in the collection. The *LYS2/lys2∆0* and *MET15/met15∆0* genotypes vary.			

Screen for Toxin Resistance or Hypersensitivity

MS300c yeast secreting K28 were spotted onto methylene blue agar (MBA) plates spread with a lawn of the mutant strain being tested. Deletion mutants were grown at room temperature, while replicates of the ts alleles were grown at 25, 30 and 34°C. The distance from the spot of MS300c cells to the lawn of sensitive cells was measured at 48 and 72 hours. *MAT*α (BY4742) deletion mutants that tested as resistant or hypersensitive were retested, and reproducible hits were confirmed with further testing in the *MAT*a or homozygous diploid deletion collections, or by PCR.

Statistical Validation of Cellular Pathways Related to K28 Function

Gene ontology processes, functions and components annotated to the 176 K28 resistant or 189 K28 hypersensitive genes were compared to a background set of all 5015 mutations for which we generated K28 sensitivity scores according to the method described in Boyle et al., (2004) (Generic GO Term Finder, http://go.princeton.edu/cgi-bin/GOTermFinder).

Toxin-Cell Binding Assay

10 OD/ml of wild-type or mutant cells were incubated in K28-containing supernatant for 15 minutes at 4°C, as previously described (Breinig et al., 2002). The cells were removed and the remaining supernatant was tested for activity. The zone of clearance in the lawn was converted into a percentage of the undepleted toxin activity.

Microscopy

Imaging of GFP-labeled proteins and two-color imaging of GFP and mRFP or mCherry labeled proteins was done using an Olympus IX-71 or IX-81 microscope essentially as described (Kaksonen et al., 2005). For single channel imaging, neutral density filters were used to reduce the intensity of excitation light and time-lapse movies were typically acquired at rate of 1 frame every 2 seconds.

Cell Fractionation and Toxin Uptake

For cell fractionations yeast were grown to early log phase, spheroplasted, washed in 0.8 M sorbitol buffer, and incubated for 1.5 h at 20°C in the presence of $2x10^5$ U/ml K28 toxin (Eisfeld et al., 2000; Schmitt and Tipper, 1990; Seaman et al., 1998). Thereafter, cells were washed, lysed, and subjected to differential centrifugation: P13, plasma, endosomal, Golgi and ER membrane fraction (13,000 g); P100, endosomal and Golgi membrane and vesicle fraction (100,000 g pellet); S100, cytosolic fraction (100,000 g supernatant). Protein samples were analyzed by Western blot using antibodies directed against K28 (Heiligenstein et al., 2006), Pfk1/2p, actin-β (mAbcam 8224), Pep12p (2C3-G4, Molecular Probes), and/or Sec61p.

Toxin uptake by yeast spheroplasts was determined by Western blotting as previously described (Heiligenstein et al., 2006). Briefly, anti-K28 antibodies detected the amount of toxin remaining in the cell-free culture supernatant after incubating $2.5X10^8$ spheroplasts at 30ºC with purified K28 over 4 hours.

Data Analysis and Software

Images were collected using Metamorph (Molecular Devices) and processed using Image J (http://rsbweb.nih.gov/ij/index.html) to subtract background fluorescence and to normalize for the effects of photo-bleaching. Network diagrams were generated using Osprey 1.2.0 (Breitkreutz et al., 2003).

CHAPTER 3

Analysis of Yeast Endocytic Site Formation and Maturation Through a Transition Point

Background

Endocytosis is the process by which cells internalize proteins and lipids from the plasma membrane and surrounding environment. Live-cell fluorescence microscopy in *Saccharomyces cerevisiae* and mammalian cells has revealed dynamics of the machinery that drives endocytosis (Kaksonen et al., 2003; Henne et al., 2010; reviewed in Perrais and Merrifield, 2005). Protein recruitment to endocytic sites occurs in a highly predictable order. Knowledge of endocytic protein spatiotemporal dynamics has provided valuable insight into the mechanisms underlying endocytic vesicle formation in yeast and mammalian cells (Liu et al., 2009; Stimpson et al., 2009; Toret et al., 2008; Barker et al., 2007; Sun et al., 2006; Smaczynska-de et al., 2010; Newpher et al., 2005; Merrifield et al., 2005; Ferguson et al., 2009). In budding yeast, about 40 endocytic proteins have been shown to assemble at endocytic 'patches', which have been classified into the early, coat, WASP/Myo, amphiphysin, or actin modules based on their dynamics at endocytic sites (Stimpson et al., 2009; Kaksonen et al., 2005; Carroll et al., 2009; summarized in Tonikian et al., 2009).

There has recently been much interest in the early stages of endocytic site formation in both yeast and mammalian cells, specifically focusing on proteins that are likely to be involved in site establishment (Henne et al., 2010; Stimpson et al., 2009; Boettner et al., 2009; Reider et al., 2009). In mammalian cells, the proteins FCHo1/2, Eps15, and intersectin are present during the earliest known stages of clathrin coat assembly and are thought to be important in the nucleation of endocytic sites (Henne et al., 2010). In comparison, Ede1p (an Eps15 homolog), Syp1p (an FCHo1/2 homolog), clathrin, and the AP2 complex are among the first proteins to appear at nascent endocytic sites in budding yeast (Stimpson et al., 2009; Newpher et al., 2005; Kaksonen et al., 2005; Carroll et al., 2009; Boettner et al., 2009; Reider et al., 2009; Toshima et al., 2006). However, the order that these proteins arrive to endocytic sites with respect to each other, and their roles in early site formation, have not been thoroughly studied. An exploration of the early endocytic stages in the genetically tractable organism, budding yeast, will elucidate mechanisms that regulate endocytic site establishment.

Proteins that arrive early during endocytic site formation have long and variable lifetimes, whereas later arriving proteins have highly regular lifetimes (Stimpson et al., 2009; Newpher et al., 2005; Kaksonen et al., 2005; Carroll et al., 2009). This observation suggests that a transition point exists wherein endocytic vesicle formation cannot progress until some event triggers recruitment and/or activation of later-acting components of the endocytic machinery. The mechanisms that govern this transition are unclear, although it is known that the early components are important for proper dynamics of later arriving components of the endocytic machinery. For example, Ede1p has a role in the recruitment of the later arriving proteins Sla2p, Ent1p, Ent2p, Sla1p, and Las17p to endocytic sites (Stimpson et al., 2009; Kaksonen et al., 2005). Additionally, in the absence of clathrin, some later arriving proteins have shorter lifetimes and others have extended lifetimes, suggesting that clathrin has a complex role in endocytic site maturation (Kaksonen et al., 2005; Newpher and Lemmon, 2006; Newpher et al., 2006). A more comprehensive study of the roles of Ede1p and clathrin in regulating early endocytic stages promises to provide insight into the initiation stage of endocytosis and how endocytic sites progress through the transition point.

Endocytic cargos are thought to arrive during the early, variable phase of endocytosis in yeast, and therefore may have a role in regulating early endocytic site progression (Toshima et al., 2006). Furthermore, there is evidence that some endocytic cargo molecules may be able to regulate clathrin-coated pit maturation in mammalian cells (Loerke et al., 2009; Mettlen et al., 2010). One hypothesis is that cargo recruitment may play a role in progression of the endocytic site through the transition point in yeast endocytosis; however this remains to be established.

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

Results

Arrival of proteins at the yeast early endocytic site

Ede1p, Syp1p, clathrin, and the AP2 complex are known to arrive at endocytic sites early in the pathway (Stimpson et al., 2009; Newpher et al., 2005; Kaksonen et al., 2005; Carroll et al., 2009; Boettner et al., 2009; Reider et al., 2009; Toshima et al., 2006). We predicted that the uncharacterized protein Ydr348cp might also be an endocytic protein because high-throughput studies have found that it has physical interactions with Ede1p, and Ydr348c-GFP localizes to the cell periphery and bud neck (Huh et al., 2003; Gavin et al., 2002; Gavin et al., 2006). Ydr348cp also has homology to the *Schizosaccharomyces pombe* protein Pal1p, which physically interacts with the endocytic protein Sla2p (Ge et al., 2005). We here name *S. cerevisiae* Ydr348cp as Pal1p, and demonstrate that Pal1-GFP forms dynamic patches at the cell surface (Figure 3.1A, left) that have an average lifetime 65 ± 35 seconds (n=50). Pal1-GFP patches internalize from the cortex before disassembly (94%, n=50), which is a characteristic of endocytic coat proteins (Kaksonen et al., 2005). 94% of Sla1-mCherry patches contained Pal1-GFP (n=50), indicating that Pal1-GFP is present at essentially all endocytic sites (Figure 3.1B). Based on these analyses, Pal1p can be considered an early arriving endocytic coat protein.

Yap1801p and Yap1802p, homologs of mammalian AP180, are thought to recruit clathrin to the plasma membrane (Newpher et al., 2005), and therefore may act as early arriving endocytic proteins. The dynamics of Yap1802p were analyzed to determine if the yeast AP180s arrive early to endocytic sites. Yap1802-GFP patches have an average lifetime of 63 ± 26 seconds (n=50), and like Pal1p, move off the cortex before disassembly (98%, n=50) (Figure 3.1A, right). In a two-color analysis, Yap1802-GFP arrives at endocytic sites before Sla1-mCherry (96%, n=25) (Figure 3.1C). Together these data place Yap1802p early in the coat module.

Figure 3.1

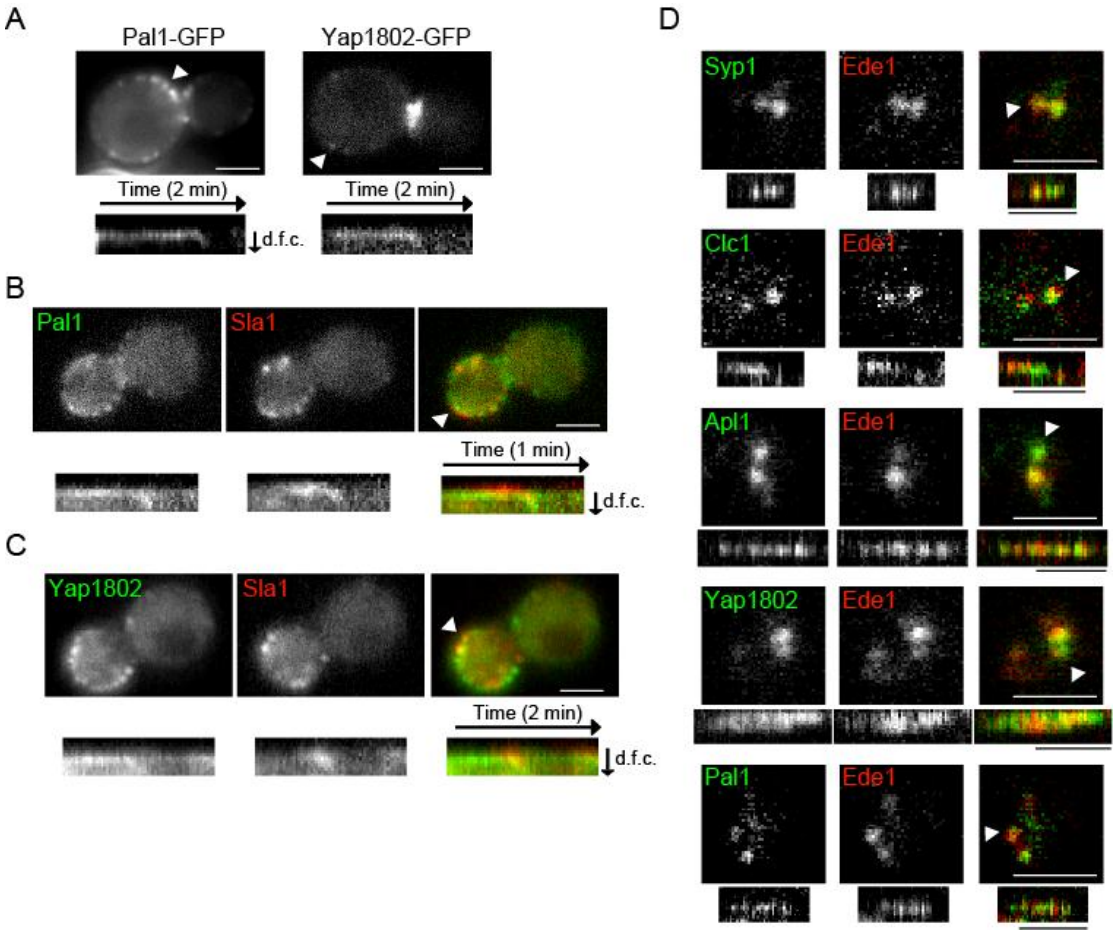


Figure 3.1. Analysis of the Yeast Early Endocytic Site. (A) Epifluorescence images of yeast expressing Pal1-GFP or Yap1802-GFP. Kymographs are of the patches indicated by the white arrowheads. (B) Epifluorescence images of a yeast cell expressing Sla1-mCherry and Pal1-GFP. Kymographs of the patch indicated by the white arrowheads are from two-color movies acquired at a rate of one frame per second. (C) Epifluorescence images of a yeast cell expressing Sla1-mCherry and Yap1802-GFP. Kymographs of the patch indicated by the white arrowheads are from two-color movies acquired at a rate of two frames per second. (D) Two-color TIRF microscopy images of yeast expressing Ede1-RFP and Syp1-GFP, Clc1-GFP, Apl1-3XGFP, Yap1802-GFP, or Pal1-GFP. GFP and RFP tagged proteins arrived within four seconds of each other ≥85% of the time (n=20 patches for each). Kymographs of the patch indicated by the white arrowheads are from two-color movies. Black time bars, 30 s. All white scale bars, 2 μm.

Dynamics and recruitment of many later arriving endocytic proteins have been previously described (Kaksonen et al., 2003; Kaksonen et al., 2005), which has been pivotal in our understanding of endocytic mechanisms. However, a detailed analysis of the order of arrival of early endocytic proteins is lacking, and knowing this sequence is central to understanding the mechanism of endocytic site establishment and how proteins that drive vesicle formation are subsequently recruited. Two-color TIRF microscopy was used to assess the order of arrival of early proteins at endocytic sites relative to Ede1-RFP recruitment. Clathrin light chain (Clc1p), Apl1p, and Yap1802p were used as markers for clathrin, the AP2 complex, and the yeast AP180s, respectively. We found that Ede1-RFP arrives at a similar time to Syp1-GFP, Clc1-GFP, Apl1-3XGFP, Yap1802-GFP, and Pal1-GFP in instances when both patches were clearly visible. None of the GFP-tagged proteins consistently arrived before or after Ede1-RFP; rather it seems the early endocytic proteins arrive at the nascent endocytic site at approximately the same time (Figure 3.1D). The fluorescent signal from these proteins is quite dim and the patches tend to fluctuate in intensity until their disassembly. Based on these observations, we define a new early coat module comprised of clathrin, the AP2 complex, Yap1802p, and Pal1p (Figure 3.5). The early coat module proteins internalize with the vesicle before disassembly, which is distinct from the early module proteins which disassemble before coat internalization (Stimpson et al., 2009).

Analysis of the roles of early arriving proteins in endocytosis

A more comprehensive analysis of the function of the early arriving proteins is needed to better understand endocytic site formation and progression to later stages. Since the function of Pal1p in endocytosis has not been characterized, the role of Pal1p in fluid-phase and receptor-mediated endocytosis was examined. Similar to what was observed in wild-type cells, *pal1*Δ yeast were able to internalize FM 4-64 and to clear fluorescently-labeled α-factor from the plasma membrane, showing that Pal1p is not required for bulk or receptor-mediated endocytosis (Figure 3.2A). The dynamics of Sla1-GFP were examined in *pal1*Δ yeast to determine how Pal1p affects other components of the endocytic machinery. Unlike *ede1*Δ yeast wherein Sla1-GFP lifetimes and patch number are known to decrease (Stimpson et al., 2009; Kaksonen et al., 2005), the lifetime and number of Sla1-GFP patches remain unchanged in *pal1*Δ yeast (Figure 3.2B).

Deleting *SYP1*, AP2 complex subunits, and *YAP1801/2* does not cause readily detectable defects in endocytosis, and these proteins are important for the internalization of only certain cargos (Stimpson et al., 2009; Kaksonen et al., 2005; Carroll et al., 2009; Reider et al., 2009; Huang et al., 1999; Burston et al., 2009). Ede1p and clathrin, however, are important for proper dynamics of later arriving endocytic proteins and for efficient cargo uptake (Stimpson et al., 2009; Kaksonen et al., 2005; Newpher and Lemmon, 2006; Chu et al., 1996; Gagny et al., 2000). Therefore, we decided to more thoroughly investigate the roles of Ede1p and clathrin in the initiation and maturation of early endocytic sites by assessing the localization and dynamics of early arriving proteins in *ede1*Δ and *clc1*Δ yeast.

Figure 3.2

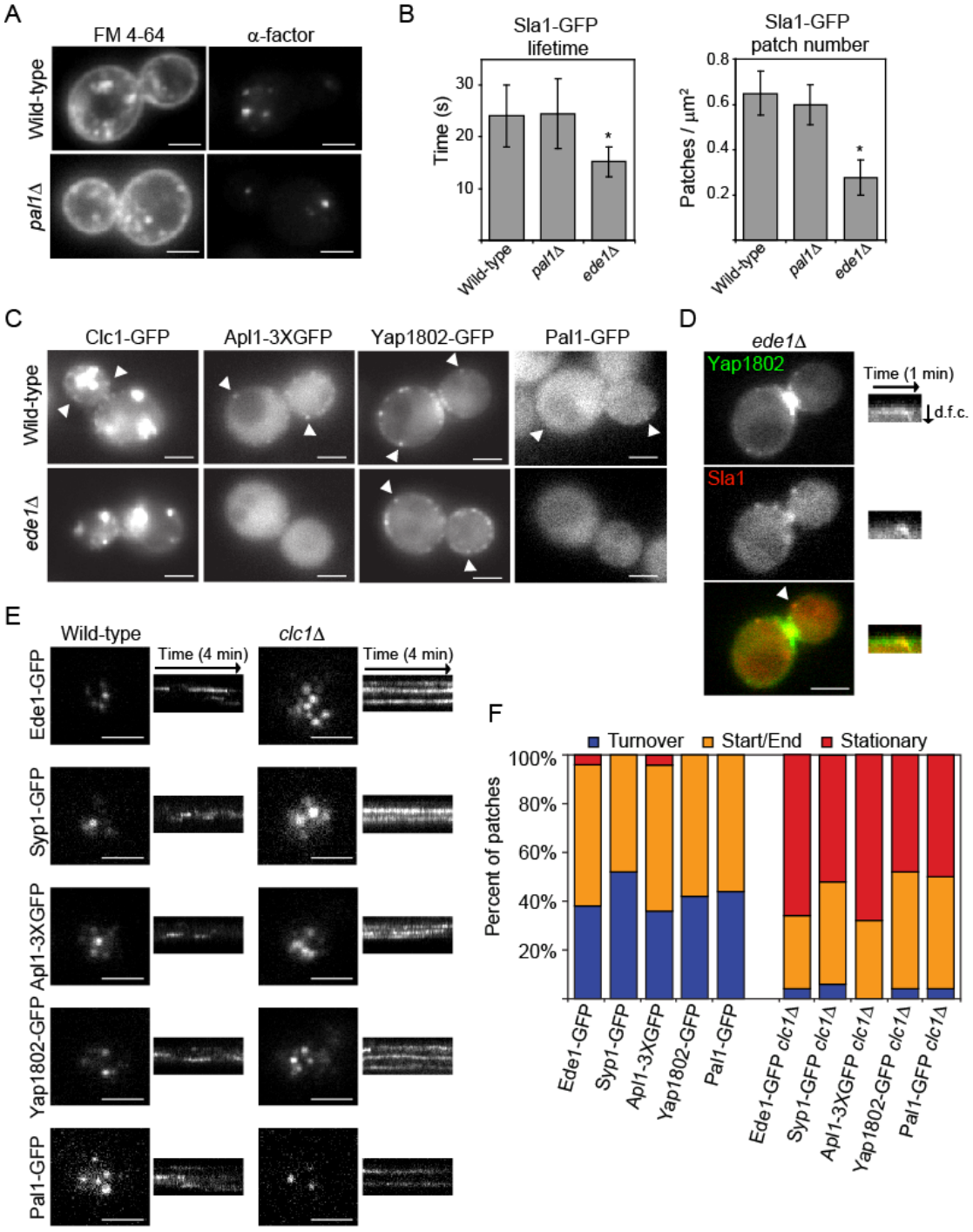

A
FM 4-64
α-factor
Wild-type
pal1Δ
B
Sla1-GFP lifetime
Time (s)
30
20
10
Wild-type
pal1Δ
ede1Δ
Sla1-GFP patch number
Patches / µm²
0.8
0.6
0.4
0.2
C
Clc1-GFP
Apl1-3XGFP
Yap1802-GFP
Pal1-GFP
Wild-type
ede1Δ
D
ede1Δ
Yap1802
Sla1
Time (1 min)
d.f.c.
E
Wild-type
clc1Δ
Time (4 min)
Ede1-GFP
Syp1-GFP
Apl1-3XGFP
Yap1802-GFP
Pal1-GFP
F
Turnover
Start/End
Stationary
Percent of patches
100%
80%
60%
40%
20%
Ede1-GFP
Syp1-GFP
Apl1-3XGFP
Yap1802-GFP
Pal1-GFP
Ede1-GFP clc1Δ
Syp1-GFP clc1Δ
Apl1-3XGFP clc1Δ
Yap1802-GFP clc1Δ
Pal1-GFP clc1Δ

Figure 3.2. Analysis of the Roles of Pal1p, Ede1p, and Clc1p in Endocytosis. (A) FM 4-64 uptake (left) and fluorescent α-factor uptake (right) in the indicated strains after 10 min. (B) Lifetimes of Sla1-GFP patches ± standard deviation (n = 50 patches) (left) and Sla1-GFP patch number per cell surface area (μm^2) ± standard deviation (n = 20 cells) (right) for the indicated yeast. Patch number was counted from maximum intensity Z-projections of unbudded or large-budded cells. Z-stacks were acquired through the entire cell at 0.15 μm intervals. Movies used to generate lifetime data were acquired at a rate of one frame per second. * indicates a statistically significant decrease compared to wild-type ($p < 0.0001$). (C) Images of wild-type and *ede1*Δ yeast expressing Clc1-GFP, Apl1-3XGFP, Yap1802-GFP, or Pal1-GFP. White arrowheads indicate examples of cortical patches. (D) Images of an *ede1*Δ yeast cell expressing Yap1802-GFP and Sla1-mCherry. Kymographs of the patch indicated by the white arrowhead are from two-color movies. (E) TIRF microscopy images of wild-type and *clc1*Δ yeast cell expressing Ede1-GFP, Syp1-GFP, Apl1-3XGFP, Yap1802-GFP, or Pal1-GFP. Kymographs are taken from four minute movies. (F) Percent of patches in the indicated strains that assemble and disassemble within a four minute interval (turnover), are present throughout the TIRF movie (stationary), or are present in either the first or last frames of the movie (Start/End) (n=50 patches). All white scale bars, 2 μm.

Clc1-GFP, Apl1-3XGFP, and Pal1-GFP all failed to form stable cortical patches in *ede1*Δ yeast (Figure 3.2C), which is similar to the behavior observed for Syp1-GFP in the absence of Ede1p (Stimpson et al., 2009). Surprisingly, Yap1802-GFP formed patches in *ede1*Δ yeast that colocalize with the endocytic site marker Sla1-mCherry (Figure 3.2, C and D). Thus, Ede1p is important for recruitment of most early arriving proteins to endocytic sites, although Yap1802p can localize correctly in the absence of Ede1p.

The role of clathrin in early endocytic site establishment and maturation was studied using a clathrin light chain deletion mutant. Ede1-GFP, Apl1-3XGFP, Yap1802-GFP, and Pal1-GFP were able to form cortical patches in *clc1*Δ yeast, indicating that clathrin light chain is dispensable for endocytic site establishment (Figure 3.2E). Contrary to previous reports (Boettner et al., 2009), we observed that Syp1-GFP was also able to form cortical patches in *clc1*Δ yeast (Figure 3.2E). However, the dynamics of Ede1-GFP, Syp1-GFP, Apl1-3XGFP, Yap1802-GFP, and Pal1-GFP patches were perturbed in clathrin light chain mutants: very few patches assembled and disassembled (turnover patches) within a four minute period when compared to wild-type cells (Figure 3.2, E and F), and there was an increase in the number of stationary patches (patches that persist for more than four minutes) in *clc1*Δ yeast for each of the early arriving endocytic proteins.

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

The extension in lifetimes of early arriving endocytic proteins in *clc1*Δ yeast is similar to the phenotype previously observed for Sla2p, a Hip1R homolog, in clathrin knockouts (Newpher and Lemmon, 2006; Newpher et al., 2006). Therefore, the recruitment and dynamics of later arriving endocytic machinery were also analyzed in *clc1*Δ yeast. As previously reported, we found that Sla2-GFP formed patches with extended lifetimes in *clc1*Δ yeast (Figure 3.3A). Interestingly, Ent1-GFP and Ent2-GFP also formed patches with extended lifetimes in *clc1*Δ yeast (Figure 3.3A). Similar to what was observed for Sla1-GFP and Las17-GFP (Kaksonen et al., 2005; Newpher and Lemmon, 2006), Pan1-GFP and End3-GFP patches had shorter lifetimes in clathrin light chain mutants (Figure 3.3B). It should be noted, however, that Pan1-GFP and End3-GFP did form a few stationary patches in *clc1*Δ yeast that were present throughout 90 second movies. Together these results show that clathrin light chain is important for proper dynamics of the endocytic early proteins, Sla2p, Ent1p, and Ent2p.

Because of their similar phenotypes in *clc1*Δ yeast, we examined Sla2p, Ent1p, and Ent2p more carefully. Consistent with previous observations, Sla2-GFP arrived at endocytic sites before Sla1-mCherry (Newpher and Lemmon, 2006), and we now find that Ent1-GFP and Ent2-GFP also arrive at endocytic sites before Sla1-mCherry (Figure 3.3C). However, Sla1-mCherry and Las17-GFP arrive at endocytic sites at a similar time point (Figure 3.3C). Since Sla2p, Ent1p, and Ent2p have a unique localization dynamics, we speculated that these proteins may have similar functions in endocytosis. In our strain background *ent1*Δ *ent2*Δ yeast are viable, and we found that they form abnormal actin structures, marked by Sac6-RFP, that are similar to those previously reported in *ent1*Δ *ent2*Δ yeast expressing an $ENTH^{Y100R}$ domain from Ent1p (Figure

3.3D) (Aguilar et al., 2006). The actin structures in *ent1*Δ *ent2*Δ yeast are also very similar to the elongated actin tails formed in *sla2*Δ yeast (Kaksonen et al., 2003), however, this phenotype is not caused by a lack of recruitment of Sla2p to endocytic sites in *ent1*Δ *ent2*Δ yeast (Figure 3.3D). These data suggest that Sla2p, Ent1p, and Ent2p act at a similar stage of endocytosis and should be considered components of a new, intermediate coat module (Figure 3.5).

Figure 3.3

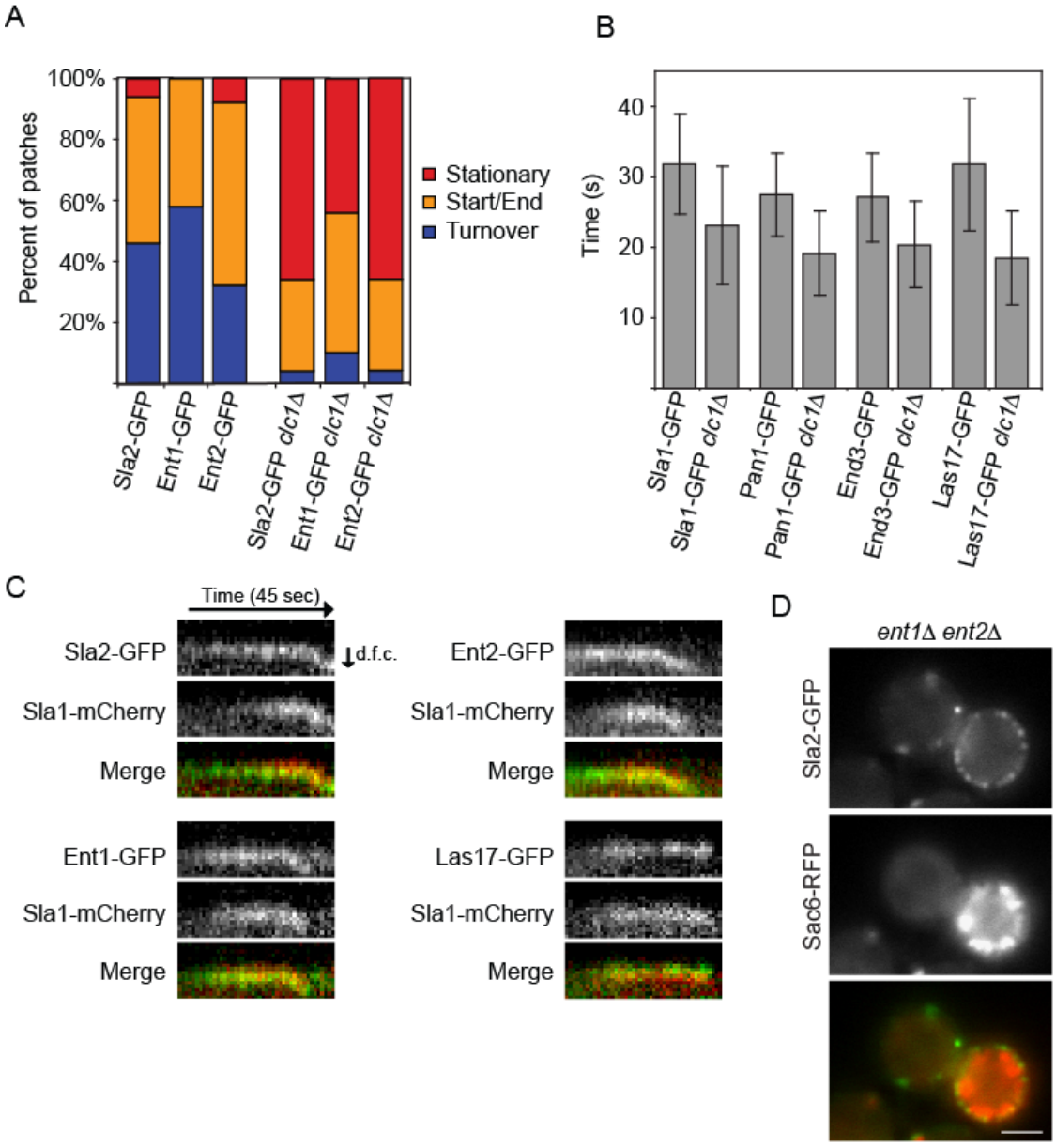

A
100%
80%
60%
40%
20%
Percent of patches
Stationary
Start/End
Turnover
Sla2-GFP
Ent1-GFP
Ent2-GFP
Sla2-GFP clc1Δ
Ent1-GFP clc1Δ
Ent2-GFP clc1Δ
B
40
30
20
10
Time (s)
Sla1-GFP
Sla1-GFP clc1Δ
Pan1-GFP
Pan1-GFP clc1Δ
End3-GFP
End3-GFP clc1Δ
Las17-GFP
Las17-GFP clc1Δ
C
Time (45 sec)
Sla2-GFP
d.f.c.
Sla1-mCherry
Merge
Ent2-GFP
Sla1-mCherry
Merge
Ent1-GFP
Sla1-mCherry
Merge
Las17-GFP
Sla1-mCherry
Merge
D
ent1Δ ent2Δ
Sla2-GFP
Sac6-RFP

Figure 3.3. Sla2p, Ent1p, and Ent2p Behave Similarly in *clc1*Δ Yeast, Have Similar Localization Dynamics, and Display Similar Knockout Phenotypes. (A) Percent of patches in the indicated strains that assemble and disassemble within a four minute interval (turnover), are present throughout the TIRF movie (stationary), or are present in either the first or last frames of the movie (Start/End) (n=50 patches). (B) Lifetime of Sla1-GFP, Pan1-GFP, End3-GFP, and Las17-GFP patches ± standard deviation in wild-type or *clc1*Δ yeast (n = 50 patches). Movies used to generate lifetime data were acquired at a rate of one frame per second. (C) Kymographs from epifluorescent two-color movies of yeast expressing Sla1-mCherry and Sla2-GFP, Ent1-GFP, Ent2-GFP, or Las17-GFP. Movies were acquired at a rate of one frame per second. (D) Fluorescent images of an *ent1*Δ *ent2*Δ yeast cell expressing Sla2-GFP and Sac6-RFP. White scale bars, 2 μm.

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

Having established how early arriving proteins affect endocytic site formation and maturation, we next sought to investigate the role of cargo in this process, since cargo also arrive early at endocytic sites (Toshima et al., 2006). The dynamics of the endocytic machinery were monitored in a secretion mutant, *sec18-1*^{ts}, wherein cell surface levels of endocytic cargo can be reduced. The endocytic cargo protein GFP-Snc1, which recycles continuously through the plasma membrane and endosomal compartments (Lewis et al., 2000), disappeared from the plasma membrane of *sec18-1*^{ts} yeast within 20 minutes of shifting to 37ºC (Figure 3.4A). This indicates that secretion to the plasma membrane is blocked in *sec18-1*^{ts} yeast under these conditions. We found that the lifetimes of Ede1-GFP, Clc1-GFP, and Sla2-GFP patches were extended in *sec18-1*^{ts} mutants when compared to wild-type cells after yeast were incubated for 30 minutes at 37ºC (Figure 3.4B and data not shown). The defect in Ede1-GFP patch dynamics could be rescued by returning the yeast to 25ºC for 20 minutes, indicating that the cells remained viable (Figure 3.4B). The increase in Ede1-GFP and Sla2-GFP patch lifetimes in *sec18-1*^{ts} mutants at the restrictive temperature was accompanied by a defect in FM 4-64 uptake (Figure 3.4C). The defect in endocytic patch dynamics in the *sec18-1*^{ts} mutant was likely not caused by a depletion of plasma membrane PtdIns(4,5)P_2 because there was no detectable change in the distribution of GFP-2XPH(PLCδ), which is a marker for this lipid (Figure 3.4F) (Stefan et al., 2002).

Figure 3.4

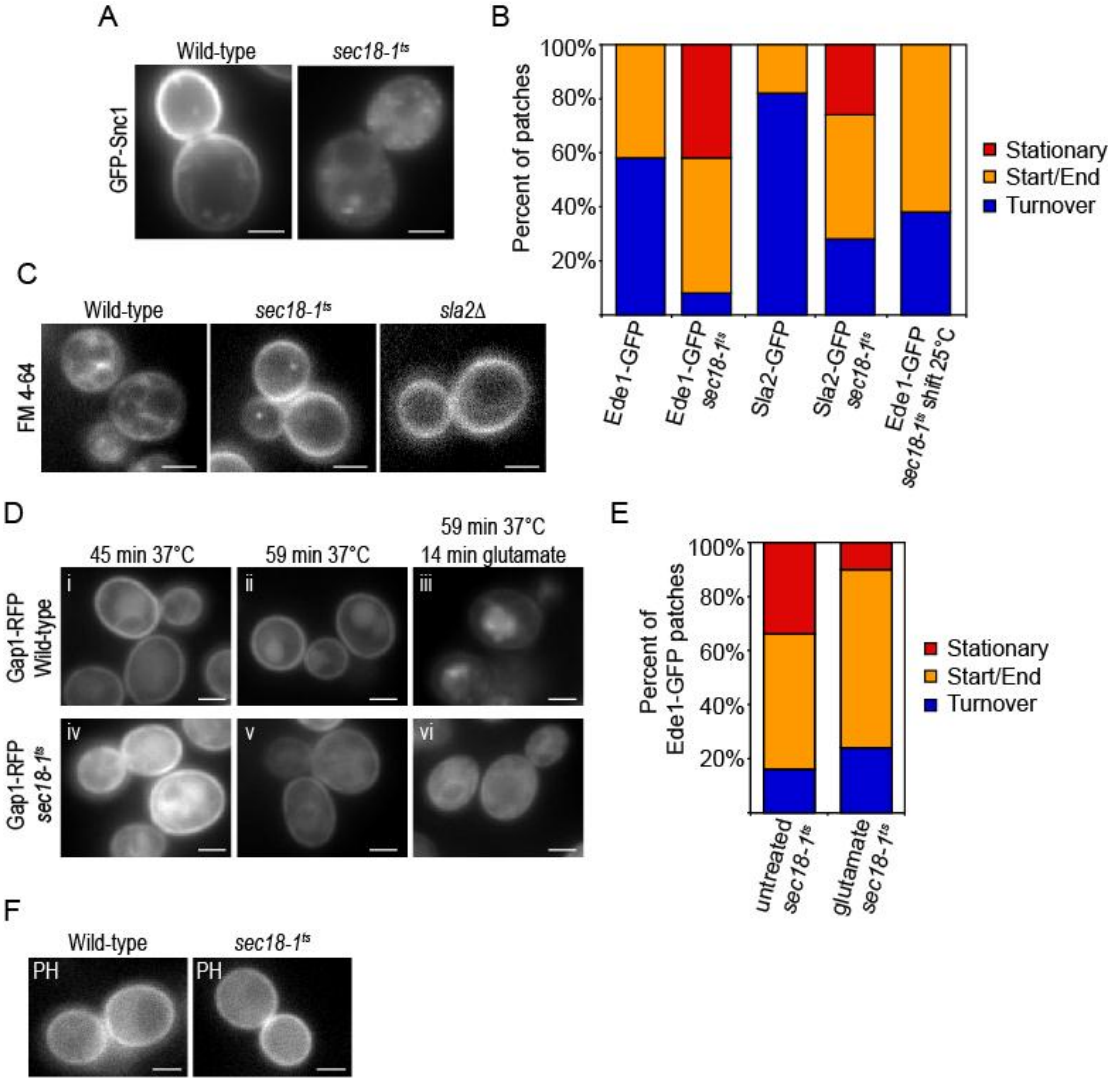

A
Wild-type
sec18-1ts
GFP-Snc1
B
Percent of patches
100%
80%
60%
40%
20%
Stationary
Start/End
Turnover
Ede1-GFP
Ede1-GFP sec18-1ts
Sla2-GFP
Sla2-GFP sec18-1ts
Ede1-GFP sec18-1ts shift 25°C
C
Wild-type
sec18-1ts
sla2Δ
FM 4-64
D
45 min 37°C
59 min 37°C
59 min 37°C 14 min glutamate
Gap1-RFP Wild-type
Gap1-RFP sec18-1ts
i
ii
iii
iv
v
vi
E
Percent of Ede1-GFP patches
100%
80%
60%
40%
20%
Stationary
Start/End
Turnover
untreated sec18-1ts
glutamate sec18-1ts
F
Wild-type
sec18-1ts
PH
PH

Figure 3.4. Endocytic Defects in *sec18-1^{ts}* Yeast. (A) Images of wild-type or *sec18-1^{ts}* yeast expressing GFP-Snc1. Yeast were incubated at 37ºC for 20 min before imaging. (B) Percent of patches in the indicated strains that assemble and disassemble within a four minute interval (turnover), are present throughout the TIRF movie (stationary), or are present in either the first or last frames of the movie (Start/End) (n=50 patches). Wild-type and mutant strains were incubated at 37ºC for 30 min before imaging, except for yeast incubated at 37ºC for 30 min and then shifted to 25ºC for 20 min before imaging (Ede1-GFP *sec18-1^{ts}* shift 25ºC). (C) FM 4-64 uptake in strains incubated at 37ºC for 30 min, and then labeled with FM 4-64 for 20 min at 37ºC before imaging. (D) Images of Gap1-RFP overexpressed in wild-type or *sec18-1^{ts}* yeast. Cells were incubated at 37ºC for 45 min (i and iv) or 59 min (ii and v), or cells were incubated at 37ºC for 45 min and then treated with 0.1% glutamate (w/v) for 14 min at 37ºC (iii and vi). (E) Percent of Ede1-GFP patches in *sec18-1^{ts}* yeast overexpressing Gap1-RFP that turn over, remain stationary, or start/end during a four minute TIRF microscopy movie (n=50 patches). Yeast were incubated at 37ºC for 55 min (untreated) or incubated at 37ºC for 45 min and treated with 0.1% glutamate for 10 min at 37ºC (glutamate) before imaging. (F) Images of wild-type and *sec18-1^{ts}* yeast expressing GFP-2XPH(PLCδ), which is a maker for PtdIns(4,5)P_2. Yeast were incubated at 37ºC for 30 min before imaging. All white scale bars, 2 μm.

In *sec18-1*^{ts} yeast at the restrictive temperature, constitutively recycled cargos are endocytosed from the plasma membrane and are not replaced. The decrease in endocytic cargo concentration at the plasma membrane might influence endocytic site progression through a regulatory transition point. To test this hypothesis, the amino acid permease, Gap1-RFP, was overexpressed in *sec18-1*^{ts} yeast. Gap1p is expressed at the cell surface when wild-type or *sec18-1*^{ts} yeast are grown on a poor nitrogen source (Figure 3.4D, i, ii, iv, and v). However, Gap1p is cleared from the plasma membrane and trafficked to the vacuole in the presence of a preferred nitrogen source, such as glutamate (Roberg et al., 1997; Soetens et al., 2001). Within 14 minutes of adding 0.1% glutamate, Gap1-RFP was cleared off the plasma membrane of wild-type cells and of the majority of *sec18-1*^{ts} cells incubated at the restrictive temperature (Figure 3.4D, iii and vi). Gap-RFP did not accumulate in the vacuole when glutamate was added to *sec18-1*^{ts} yeast, likely due to the mutant's trafficking defects (Hicke et al., 1997). The defects in Ede1-GFP patch dynamics at 37ºC in *sec18-1*^{ts} cells were partially rescued (more turnover and fewer stationary patches) when Gap1-RFP was targeted for endocytosis by the addition of glutamate (Figure 3.4E). These results suggest the endocytic defect of *sec18-1*^{ts} yeast can be partially rescued by inducing internalization of an endocytic cargo. In further support of this cargo-dependent effect, another method that can be used to induce internalization of multispan plasma membrane proteins is the addition of cycloheximide to yeast (Lin et al., 2008; Galan and Haguenauer-Tsapis, 1997). Cells incubated for two hours in 50 μg/ml cycloheximide cleared RFP-Snc1, Fur4-GFP, and Gap-RFP from the plasma membrane (data not shown). A cycloheximide induced depletion of membrane cargo also resulted in a decrease in the number of dynamic Ede1-GFP patches (data not shown). Admittedly, cycloheximide's effects on cell physiology are rather global, but this observation nonetheless is consistent with a role for cargo in timing of the endocytic pathway.

Discussion

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

Assembly of proteins at the early endocytic site

This work has expanded the number of proteins known to arrive early during yeast endocytic site formation. We find that Pal1p, a previously uncharacterized protein conserved in fungi (Ge et al., 2005), is a component of the *S. cerevisiae* endocytic machinery. The patch lifetimes and dynamics of Pal1-GFP and Yap1802-GFP are similar to those of clathrin and the AP2 complex subunits. Therefore, Pal1p, Yap1802p, clathrin, and the AP2 complex have been placed into the early coat module, which modifies a previously proposed model (Figure 3.5) (Stimpson et al., 2009).

Insight into the earliest stages of endocytosis was gained by precisely defining which proteins arrive earliest at endocytic sites. In one model of endocytic site formation, Ede1p and clathrin arrive first at endocytic sites because they are more important for efficient endocytic uptake (Stimpson et al., 2009; Kaksonen et al., 2005; Newpher and Lemmon, 2006; Chu et al., 1996; Gagny et al., 2000). However, we find that this model seems unlikely because Ede1p and clathrin arrive at endocytic sites at about the same time as the less important components Syp1p, the AP2 complex, Yap1802p, and Pal1p. Another possible model is that a signal at the plasma membrane simultaneously recruits the early arriving endocytic proteins and cooperative binding aids their stabilization at endocytic sites. It is also possible that some early arriving endocytic proteins physically interact in the cytosol prior to associating with the plasma membrane, which has been observed for other endocytic proteins (Lundmark and Carlsson, 2004). Further investigation is required to understand the mechanisms acting during the initial steps of endocytosis.

The order of recruitment of proteins to early endocytic sites in yeast is slightly different to the order reported in mammalian systems. In mammalian cells, FCHo1/2 (homolog of Syp1p), Eps15 (homolog of Ede1p), and intersectin (homolog of the Pan1 complex formed by Pan1p, Sla1p, and End3p) were reported to arrive earliest at sites on the plasma membrane, followed by the arrival of clathrin and the AP2 complex (Henne et al., 2010). It is unclear why clathrin and the AP2 complex appear to arrive earlier during yeast endocytosis. The AP2 complex plays a minor role in yeast endocytosis (Carroll et al., 2009), but a major role in mammalian endocytosis, and therefore clathrin and AP2 complex recruitment to endocytic sites may be regulated differently. Furthermore, in yeast, the Pan1 complex localizes to endocytic sites well after the early arriving proteins (Figure 3.5). Intersectin may act as a protein scaffold during early endocytic stages in mammalian systems (Henne et al., 2010; McPherson, 2002), but in yeast this function may be performed by other early arriving proteins.

Figure 3.5

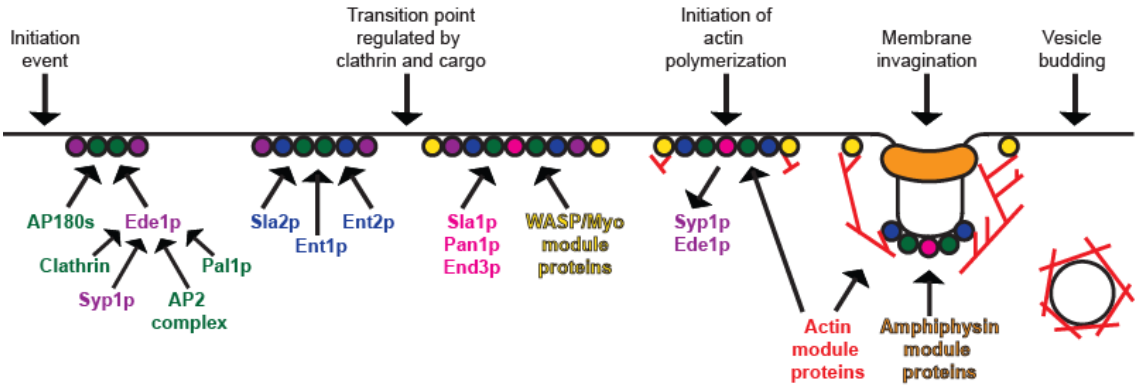


Figure 3.5. Model for the Temporal Recruitment of Endocytic Proteins. The early module proteins (Ede1p and Syp1p; purple) and the early coat module proteins (clathrin, the AP2 complex, Yap1802p, and Pal1p; green) arrive earliest during endocytic site formation. Ede1p is important for recruitment of Syp1p, clathrin, the AP2 complex, and Pal1p to endocytic sites. Next, the intermediate coat module proteins (Sla2p, Ent1p, and Ent2p; blue) arrive at endocytic sites. Endocytic sites seem to mature through a transition point regulated by clathrin and cargo before the late coat module proteins (Sla1p, Pan1p, and End3p; pink) and the WASP/Myo module proteins (yellow) are recruited to sites. Ede1p and Syp1p disassemble from endocytic sites at the start of actin polymerization. During membrane invagination, Sla2p, Ent1p, and Ent2p may be involved in connecting the actin network to the vesicle coat and/or plasma membrane.

Role of the early arriving proteins in endocytosis

This work has provided insight into how the early arriving proteins contribute to endocytic site formation. *PAL1* deletion mutants did not have detectable endocytic defects, which is consistent with the observation that deletions of only a few early endocytic components have strong defects in endocytosis. Other early arriving proteins function in a cargo-specific manner (Stimpson et al., 2009; Carroll et al., 2009; Reider et al., 2009; Huang et al., 1999; Burston et al., 2009). Pal1p may also be a cargo-specific adaptor, or it could act redundantly with other endocytic proteins.

Ede1p was hypothesized to be involved in endocytic site establishment because it affects the formation of productive endocytic sites (Stimpson et al., 2009; Kaksonen et al., 2005). However, previous studies only examined the dynamics of later arriving proteins, and therefore did not reveal the role of Ede1p in early stages of site formation. We reported here that clathrin, the AP2 complex, and Pal1p do not stably associate with endocytic sites in *ede1*Δ yeast. In contrast, Yap1802p was recruited in the absence of Ede1p. Conflicting reports exist over whether Syp1p is recruited to cortical patches in *ede1*Δ yeast (Stimpson et al., 2009; Boettner et al., 2009). It is unclear why some early arriving endocytic proteins are able to form patches in *ede1*Δ yeast, but our results confirm that Ede1p is an important factor in normal endocytic site formation and reveal that it is involved at the earliest known stages of site initiation. Yap1802p might localize to endocytic sites in the absence of Ede1p because it has an ANTH (AP180 N-terminal homology) domain (Legendre-Guillemin et al., 2004; Maldonado-Baez et al., 2008), which may bind PtdIns(4,5)P_2 on the plasma membrane. Our results also suggest Yap1802p alone is unable to recruit and stabilize the other early arriving proteins at endocytic sites.

Since the lifetimes of Sla1p and Las17p are shortened in *ede1*Δ and *clc1*Δ yeast (Stimpson et al., 2009; Kaksonen et al., 2005; Newpher and Lemmon, 2006), one might assume that Ede1p and clathrin have similar roles in endocytosis. However, these proteins have very different effects on the dynamics of early arriving endocytic machinery. Unlike what was observed in *ede1*Δ yeast, we find that the early arriving endocytic proteins are able to form cortical patches in *clc1*Δ yeast. Clathrin has been proposed to be important for endocytic site progression (Newpher and Lemmon, 2006; Newpher et al., 2006), however the exact stage of endocytosis that clathrin regulates was unclear. This study finds that clathrin is not necessary for recruitment of early arriving proteins to endocytic sites, but that it is important for site maturation and disassembly. Specifically, clathrin likely regulates the transition from the intermediate to late coat stages (Figure 3.5). Comparing the phenotypes of *clc1*Δ and *ede1*Δ yeast reveal that the lack of clathrin at endocytic sites in *ede1*Δ yeast does not result in the same phenotypes observed in *clc1*Δ yeast. For example, we find the patch lifetimes of Sla2p and Ent2p are extended in *clc1*Δ yeast, but the lifetimes of these proteins are shortened in *ede1*Δ yeast (Stimpson et al., 2009). It is possible that undetectable levels of clathrin might localize to and promote maturation of endocytic sites in *ede1*Δ yeast. Alternatively, endocytic proteins that normally inhibit endocytic site maturation may not be recruited to endocytic sites, or they might be regulated differently, in *ede1*Δ yeast.

Formation of an intermediate coat module

Sla2p, Ent1p, and Ent2p arrive at endocytic sites before Sla1p, and *ent1*Δ *ent2*Δ yeast have similar phenotypes to *sla2*Δ yeast. These results have now led us to propose existence of an intermediate coat module separate from the late coat module containing the Pan1 complex (Pan1p, Sla1p, and End3p) (Figure 3.5) (Kaksonen et al., 2003). The intermediate coat module may serve to link the early proteins involved in endocytic site formation to the later arriving proteins which drive membrane invagination. The similar phenotypes of *ent1*Δ *ent2*Δ yeast and *sla2*Δ yeast imply that Sla2p and Ent1/2p act at similar stages of the endocytic pathway. These proteins likely contribute independently to the same step of endocytosis, and are not simply involved in recruiting one another (Newpher et al., 2005). Sla2p has been hypothesized to connect the force of actin polymerization to membrane invagination (Kaksonen et al., 2006), so Ent1p and Ent2p may also function in this step of endocytosis.

Secretion and cargo may regulate progression of endocytic events

It is perhaps not surprising that blocking secretion leads to defects in endocytosis, but how the two cellular processes are connected is not understood. Previous studies have found that several *sec* mutants have defects in endocytosis (Hicke et al., 1997; Riezman, 1985). We are now able to identify the precise stage of endocytosis that is affected by the process of secretion in yeast. In *sec18-1*^{ts} yeast, endocytic sites are formed, which contain the early arriving and intermediate coat module proteins, but the sites do not progress to completion. Studies have suggested that cargo regulates clathrin-coated pit maturation in mammalian cells, but the mechanism is unclear (Loerke et al., 2009; Mettlen et al., 2010).When cargos are depleted from the yeast cell surface in a secretion mutant or by the addition of cycloheximide, the lack of cargo may activate a checkpoint preventing the endocytic site from maturing past the intermediate coat stage. In further support of this model, Ede1p lifetimes are shorter in yeast buds which presumably contain higher concentrations of cargo than in mother cells which contain less cargo (data not shown).This model is also consistent with the observation that cargo normally arrives during the early stages of endocytosis (Toshima et al., 2006).

Depletion of endocytic cargo may not be the only reason why endocytosis is defective in *sec18-1*^{ts} yeast. Adding glutamate to induce internalization of the endocytosed cargo Gap1p in *sec18-1*^{ts} yeast did not fully rescue the endocytic defects, suggesting that other factors may also be involved in creating this phenotype. Although the distribution of PtdIns(4,5)P_2 did not seem to change in *sec18-1*^{ts} yeast, the lipid environment at the plasma membrane may be altered in *sec* mutants (Yakir-Tamang and Gerst, 2009). Trafficking is perturbed in *sec* mutants, and important machinery needed for endocytosis might not be recycled or delivered to the plasma membrane. It is also possible that in yeast exocytic events are linked to endocytic events directly. In fact, in mammalian cells compensatory endocytosis is thought to follow an exocytic event in some cells, and mechanisms linking exocytosis and endocytosis have also been proposed (Yao et al., 2009; Gundelfinger et al., 2003; Pechstein et al., 2010; Bai et al., 2010). How secretion regulates endocytosis, and the role of cargo in endocytosis, are

areas that require further investigation. This work has provided an important framework for future studies about how endocytic sites form and factors that control site maturation.

Materials and Methods

Strains

Yeast strains used in this study are listed in Table III. Single C-terminal GFP tags were integrated into the chromosome as previously described (Longtine et al., 1998). C-terminal 3XGFP tags were created as previously described (Sun et al., 2007). Gene deletions were generated by replacing the gene open reading frame with *Candida glabrata LEU2*, *URA3*, or *HIS3* cassettes. Yeast expressing GFP and RFP tagged Snc1p were transformed with the plasmids pRS416-*GFP-SNC1* and pRS416-*RFP-SNC1*, respectively. Yeast were transformed with the plasmid pRS426-GFP-2XPH(PLCδ) to monitor PtdIns(4,5)P_2 levels (Stefan et al., 2002).

Table III. Yeast Strains Used in Chapter 3

Strain Name	Relevant Genotype
DDY904	*MATα ura3-52 leu2-3,112 his3Δ200 lys2-801*
SCY766	*MATα ura3-52 leu2-3,112 his3Δ200 PAL1-GFP::HIS3*
SCY691	*MATα ura3-52 leu2-3,112 his3Δ200 YAP1802-GFP::HIS3*
SCY832	*MATα ura3-52 leu2-3,112 his3Δ200 PAL1-GFP::HIS3 SLA1-mCherry::HIS3*
SCY874	*MATa ura3-52 leu2-3,112 his3Δ200 YAP1802-GFP::HIS3 SLA1-mCherry::HIS3*
DDY3700	*MATα ura3-52 leu2-3,112 his3Δ200 SLA1-GFP::KanMX6*
SCY458	*MATα ura3-52 leu2-3,112 his3Δ200 SLA1-GFP::KanMX6 pal1Δ::cgURA3*
DCT612	*MATα ura3-52 leu2-3,112 his3Δ200 SLA1-GFP::KanMX6 ede1Δ::cgLEU2*
DDY2808	*MATα ura3-52 leu2-3,112 his3Δ200 lys2-801 sla2Δ::cgLEU2*
SCY431	*MATα ura3-52 leu2-3,112 his3Δ200 pal1Δ::cgURA3*
DDY2873	*MATa ura3-52 leu2-3,112 his3Δ200 bar1Δ::cgLEU2*
SCY788	*MATa ura3-52 leu2-3,112 his3Δ200 bar1Δ::KanMX6 pal1Δ::cgURA3*
DDY3871	*MATα ura3-52 leu2-3,112 his3Δ200 lys2-801 SYP1-GFP::KanMX6 EDE1-RFP::HIS3*
SCY799	*MATα ura3-52 leu2-3,112 his3Δ200 lys2-801 CLC1-GFP::HIS3 EDE1-RFP::KanMX6*
SCY779	*MATα ura3-52 leu2-3,112 his3Δ200 lys2-801 APL1-3GFP::HIS3 EDE1-RFP::KanMX6*
SCY790	*MATα ura3-52 leu2-3,112 his3Δ200 YAP1802-GFP::HIS3 EDE1-RFP::KanMX6*
SCY829	*MATα ura3-52 leu2-3,112 his3Δ200 PAL1-GFP::HIS3 EDE1-RFP::KanMX6*

DDY3060	*MATα ura3-52 leu2-3,112 his3Δ200 lys2-801 CLC1-GFP::HIS3*
SCY519	*MATα ura3-52 leu2-3,112 his3Δ200 APL1-3GFP::HIS3*
SCY718	*MATα ura3-52 leu2-3,112 his3Δ200 CLC1-GFP::HIS3 ede1Δ::cgLEU2*
SCY724	*MATα ura3-52 leu2-3,112 his3Δ200 APL1-3GFP::HIS3 ede1Δ::cgLEU2*
SCY875	*MATα ura3-52 leu2-3,112 his3Δ200 YAP1802-GFP::HIS3 SLA1-mCherry::HIS3 ede1Δ::cgLEU2*
SCY856	*MATα ura3-52 leu2-3,112 his3Δ200 PAL1-GFP::HIS3 SLA1-mCherry::HIS3 ede1Δ::cgLEU2*
DCT520	*MATα ura3-52 leu2-3,112 his3Δ200 EDE1-GFP::HIS3*
DCT559	*MATα ura3-52 leu2-3,112 his3Δ200 SYP1-GFP::KanMX6*
SCY631	*MATa/MATα ura3-52/ura3-52 leu2-3,112/leu2-3,112 his3Δ200/his3Δ200 lys2-801/LYS2 EDE1-GFP::HIS3/EDE1 clc1Δ::cgLEU2/CLC1*
SYC756	*MATa/MATα ura3-52/ura3-52 leu2-3,112/leu2-3,112 his3Δ200/his3Δ200 lys2-801/LYS2 ade2-1/ADE2 SYP1-GFP::KanMX6/SYP1 clc1Δ::cgLEU2/CLC1*
SCY690	*MATa/MATα ura3-52/ura3-52 leu2-3,112/leu2-3,112 his3Δ200/his3Δ200 lys2-801/LYS2 ade2-1/ADE2 APL1-3GFP::HIS3/APL1 clc1Δ::cgLEU2/CLC1*
SCY693	*MATa/MATα ura3-52/ura3-52 leu2-3,112/leu2-3,112 his3Δ200/his3Δ200 lys2-801/LYS2 ade2-1/ADE2 YAP1802-GFP::HIS3/YAP1802 clc1Δ::cgLEU2/CLC1*
SCY853	*MATa/MATα ura3-52/ura3-52 leu2-3,112/leu2-3,112 his3Δ200/his3Δ200 lys2-801/LYS2 ade2-1/ADE2 PAL1-GFP::HIS3/PAL1 clc1Δ::cgURA3/CLC1*
DDY2796	*MATα ura3-52 leu2-3,112 his3Δ200 SLA2-GFP::HIS3*
SCY636	*MATa/MATα ura3-52/ura3-52 leu2-3,112/leu2-3,112 his3Δ200/his3Δ200 ade2-1/ADE2 SLA2-GFP::HIS3/SLA2 SLA1-GFP::KanMX6/SLA1 clc1Δ::cgLEU2/CLC1*
DDY3696	*MATα ura3-52 leu2-3,112 his3Δ200 ENT1-GFP::HIS3*
SCY632	*MATa/MATα ura3-52/ura3-52 leu2-3,112/leu2-3,112 his3Δ200/his3Δ200 lys2-801/LYS2 ENT1-GFP::HIS3/ENT1 clc1Δ::cgLEU2/CLC1*
DDY3697	*MATα ura3-52 leu2-3,112 his3Δ200 ENT2-GFP::HIS3*
SCY633	*MATa/MATα ura3-52/ura3-52 leu2-3,112/leu2-3,112 his3Δ200/his3Δ200 lys2-801/LYS2 ENT2-GFP::HIS3/ENT2 clc1Δ::cgLEU2/CLC1*
DDY2735	*MATα ura3-52 leu2-3,112 his3Δ200 PAN1-GFP::HIS3*

SCY689	*MATa/MATα ura3-52/ura3-52 leu2-3,112/leu2-3,112 his3Δ200/his3Δ200 ade2-1/ADE2 PAN1-GFP::HIS3/PAN1 clc1Δ::cgLEU2/CLC1*
DCT270	*MATa ura3-52 leu2-3,112 his3Δ200 END3-GFP::HIS3*
SCY737	*MATa/MATα ura3-52/ura3-52 leu2-3,112/leu2-3,112 his3Δ200/his3Δ200 lys2-801/LYS2 END3-GFP::HIS3/END3 clc1Δ::cgURA3/CLC1*
DDY2736	*MATa ura3-52 leu2-3,112 his3Δ200 LAS17-GFP::HIS3*
SCY634	*MATa/MATα ura3-52/ura3-52 leu2-3,112/leu2-3,112 his3Δ200/his3Δ200 lys2-801/LYS2 LAS17-GFP::HIS3/LAS17 clc1Δ::cgLEU2/CLC1*
SCY676	*MATα ura3-52 leu2-3,112 his3Δ200 SLA2-GFP::HIS3 SLA1-mCherry::HIS3*
SCY612	*MATα ura3-52 leu2-3,112 his3Δ200 ENT1-GFP::HIS3 SLA1-mCherry::HIS3*
SCY679	*MATα ura3-52 leu2-3,112 his3Δ200 ENT2-GFP::HIS3 SLA1-mCherry::HIS3*
SCY825	*MATα ura3-52 leu2-3,112 his3Δ200 lys2-801 LAS17-GFP::HIS3 SLA1-mCherry::HIS3*
SCY514	*MAT? ura3-52 leu2-3,112 his3Δ200 SLA2-GFP::HIS3 SAC6-RFP::KanMX6 ent1Δ::cgLEU2 ent2Δ::cgHIS3*
SCY385	*MATα ura3-52 leu2-3,112 his3Δ200 lys2-801* [*pRS416-GFP-SNC1*]
SCY684	*MATα ura3-52 leu2-3,112 his3Δ200 lys2-801 sec18-1::LEU2* [*pRS416-GFP-SNC1*]
SCY686	*MATα ura3-52 leu2-3,112 his3Δ200 lys2-801 EDE1-GFP::HIS3 sec18-1::LEU2*
SCY728	*MATα ura3-52 leu2-3,112 his3Δ200 lys2-801 SLA2-GFP::HIS3 sec18-1::LEU2*
SCY682	*MATα ura3-52 leu2-3,112 his3Δ200 lys2-801 sec18-1::LEU2*
HSY300	*MATa ura3-52 leu2-3,112::P_{TPI}-GAP1-RFP::LEU2 his3Δ200 EDE1-GFP::HIS3*
SCY823	*MATa ura3-52 leu2-3,112::P_{TPI}-GAP1-RFP::LEU2 his3Δ200 EDE1-GFP::HIS3 sec18-1::LEU2*
SCY759	*MATα ura3-52 leu2-3,112 his3Δ200 SAC6-RFP::KanMX6* [*pRS426-GFP-2XPH(PLCδ)*]
SCY760	*MATα ura3-52 leu2-3,112 his3Δ200 lys2-801 SAC6-RFP::KanMX6 sec18-1::LEU2* [*pRS426-GFP-2XPH(PLCδ)*]
SCY710	*MATα ura3-52 leu2-3,112 his3Δ200 EDE1-GFP::HIS3* [*pRS416-RFP-SNC1*]
SCY421	*MATα ura3-52 leu2-3,112 his3Δ200 lys2-801 FUR4-GFP::HIS3* [*pRS426*]

Microscopy

Yeast strains used for imaging were grown to log phase at 25ºC in synthetic media lacking tryptophan (imaging media) and immobilized on concanavalin A-coated coverslips. To avoid suppressor mutations in *clc1*Δ yeast, strains were maintained as heterozygous diploids, which were sporulated and dissected before use. The spores were grown overnight in YPD and were then grown for four hours in imaging media prior to imaging. Since *ent1*Δ *ent2*Δ yeast and *sla2*Δ yeast have growth defects, these strains were also grown overnight in YPD, and then grown for four hours in imaging media. The *sec18-1*^{ts} yeast were grown overnight at 25ºC, and then incubated at 37ºC for the time indicated and imaged in a 37ºC temperature controlled chamber.

Olympus IX71 and IX81 microscopes with 100X/NA 1.4 objectives, Orca cameras (Hamamatsu), appropriate filter sets, and neutral density filters were used to image live yeast cells. Simultaneous two-color imaging was performed as described previously using a 488-nm argon-ion laser (Melles Griot) to excite GFP and either a mercury lamp filtered through a 575/20 nm filter (see Figures 1B, 1C, 2D, 3C, and 3D) or a 561-nm argon-ion laser (Melles Griot) (see Figure 3.1F) to excite RFP or mCherry (Stimpson et al., 2009). TIRF microscopy was performed using an IX81 microscope with a 100X/1.65 NA objective and an adjustable angle laser beam, which was lowered to reduce background signal. Two-color TIRF microscopy in Figure 3.1D was performed by lowering the angle of both the 488-nm and 651-nm laser beams separately to reduce background signal. Movies were acquired at a rate of one frame every two seconds, unless indicated otherwise. Images were collected using Metamorph software and processed using Image J (http://rsbweb.nih.gov/ij/index.html).

FM 4-64 and fluorescent α-factor uptake assays

For FM 4-64 staining, cells were incubated with 8 μM FM 4-64 in imaging media for the time indicated and then imaged. Fluorescent 488-α-factor uptake experiments were performed essentially as described previously (Toshima et al., 2006). Cells were grown to log phase in imaging media. The cells were pelleted and then resuspended to ~40 OD/ml in imaging media and incubated with 200 ng of fluorescent α-factor on ice for one hour. The cells were washed three times in ice-cold imaging media, warmed to room temperature, and imaged after the time indicated.

CHAPTER 4
CONCLUSIONS AND FUTURE DIRECTIONS

My dissertation research focused on the full uptake, trafficking, and killing pathway of the cargo K28, the formation and maturation of early endocytic sites, and the role cargo plays in controlling the timing of endocytosis. The genes identified in the killer toxin screen implicate new pathways in K28 toxicity. A subset of resistant mutants was found to have defects in K28 binding to the cell surface, thereby illuminating the mechanism of resistance in these strains. The killer toxin screen also provided insight into the function of several uncharacterized proteins, especially the AP2 complex. These results exemplify how toxins can be used as tools to study cell biological processes. Furthermore, investigating the cellular pathways implicated in K28 action may elucidate how A/B toxins target and kill mammalian cells.

This work has also expanded our understanding of events occurring during the earliest known stages of yeast endocytosis. I discovered that the early arriving proteins are recruited to endocytic sites at about the same time. Whereas Ede1p is essential for proper endocytic site formation, clathrin is important for the transition from the intermediate to late coat stages. I also found evidence for a role for endocytic cargo in progression through a transition point in the yeast endocytosis pathway.

Investigating K28 Pathways and its Intracellular Target

Large scale approaches to identifying mediators of K28 action

Prior to this work, only a few genes that are involved in K28 trafficking and killing were known (Heiligenstein et al., 2006; Reiter et al., 2005; Eisfeld et al., 2000; Schmitt et al., 1996; Schmitt and Radler, 1988). Our screen of the collections of mutants of nonessential and essential yeast genes identified a total of 365 genes that alter K28 sensitivity, which provides a more complete view of the entire K28 pathway. This genetic approach to investigating K28 action proved to be successful. Additional screening of double knockout yeast may further elucidate the relationships between genes that mediate K28 sensitivity. For example, deletion of endocytic genes was epistatic to the deletion of a ribosomal subunit, but surprisingly the endocytic mutants were not epistatic to the deletion of the kinase *HOG1*. Screening of a double knockout collection would also uncover genes that act redundantly within the same pathway.

The assay used in the killer toxin screen was reproducible and semi-quantitative, but only measured how single genes affect the viability of cells treated with K28. Exploiting other assays may reveal more subtle intricacies of the K28 pathway. A cellular microarray could be used to detect genes that are upregulated when yeast are exposed to K28, thereby identifying possible signaling pathways activated by the toxin. Another approach would be to analyze how the yeast proteome changes when cells are exposed to K28. In a method termed SILAC, mass spectrometry is used to compare proteins from yeast labeled with heavy amino acids to proteins from untreated yeast (Ong et al., 2002). Proteins that are present in a different abundance, or that have altered phosphorylation states, when K28 was added could indicate which pathways are involved in K28 action. This technique has been successful in exploring host-pathogen interactions in other systems (Shui et al., 2009). Furthermore, co-immunoprecipitation

could be used to determine which cellular proteins physically interact with K28 in different intracellular compartments. Any complexes formed between K28 and host proteins would be further investigated to assess if the interaction was important for K28 toxicity. Lastly, structural information about K28 could be derived from protein crystallography experiments. The structure of K28 could be compared to that of other protein toxins, which may be useful for predicting common entry, trafficking, or killing mechanisms.

Identifying the K28 plasma membrane receptor

A targeted approach could also be used to investigate unanswered questions about K28 action. The identify of K28's plasma membrane receptor is currently not known. It may be a lipid since many lipid biosynthesis mutants were resistant to K28 due to cell surface binding defects. In a liposome flotation assay, K28 was not able to bind liposomes comprised of phosphatidylcholine, phosphatidylethanolamine, and PtdIns(4,5)P_2 (data not shown). K28 may not have interacted with the liposomes if the necessary lipids were not present or if the relative concentrations of the lipids were not optimal for toxin binding. This assay could be modified, however, to use liposomes containing lipids derived from the yeast plasma membrane, which is more likely to contain any possible lipid receptors. Lipid binding arrays can also be used to determine if K28 binds to lipids.

The K28 cell surface receptor could be a plasma membrane protein. The AP2 complex, which is important for K28 uptake, is an adaptor of protein cargos in mammalian endocytosis, suggesting that the K28 receptor may also be a protein. Some plasma membrane protein mutants were K28 resistant, but the phenotypes were not strong enough to suggest that any of these proteins were the K28 receptor. Other studies have proposed that Erd2p, a receptor that retrieves proteins from the Golgi to the ER, is the plasma membrane receptor for K28 (Schmitt and Breinig, 2006). To test this hypothesis, TIRF microscopy and cellular fractionation could be used to determine if Erd2p localizes to the cell surface and if *erd2*Δ yeast have K28 uptake defects. Another possibility is that K28 may utilize many different cell surface receptors for internalization. Ricin is an example of a toxin that can use multiple cell surface receptors to enter cells (Sandvig et al., 2010).

It is important to note that some endocytosed toxins do not seem to have a cell surface receptor. Gelonin is an example of a protein plant toxin that is internalized only by fluid-phase endocytosis (Sandvig and van Deurs, 2005). After gelonin enters the endosome and the membrane is disrupted, the toxin can enter the cytosol and kill the cell. K28 may also lack a plasma membrane receptor and enter cells by fluid-phase endocytosis. In this model, once K28 reaches the endosome, Erd2p may bind to the toxin and mediate its trafficking to the ER.

Elucidating the K28 trafficking pathway

The K28 trafficking pathway could be examined more thoroughly if a fluorescently-labeled toxin was available. In such studies, trafficking mutants identified from the toxin screen could be tested to identify the intracellular compartment in which K28 accumulates. For example, I proposed that vacuolar mutants were hypersensitive

because a portion of K28 might normally traffic to the vacuole where it is degraded, but when trafficking to the vacuole is defective, an excess of K28 is diverted to the ER. This hypothesis could be tested by comparing the amount of fluorescently-labeled K28 in vacuoles of wild-type and mutant yeast. In general, these types of experiments could determine where along the entry pathway transport of K28 is blocked in each of the trafficking mutants.

Identifying the intracellular target of K28

Another major question in the killer toxin field focuses on the mechanism by which K28 kills cells. Attaching a nuclear location signal to the α subunit of K28 has been reported to increase its toxicity (Schmitt and Breinig, 2006). Studies have also found that K28 treated cells arrest in early S phase with pre-replicated DNA (Schmitt et al., 1996). These results suggest that K28 might kill cells by targeting the DNA replication machinery. The K28 killer toxin screen identified resistant and hypersensitive mutants related to the cell cycle and DNA replication. Additional experiments could be performed to test if K28 directly binds to DNA or interferes with the DNA replication machinery using an *in vitro* assay.

The largest category of hypersensitive mutants from the toxin screen affected translation, especially rRNA biogenesis and ribosome assembly. The very large number of hits in this pathway could indicate that translation is a target of K28. If K28 did reduce the activity of a component of the translational machinery, then additional mutations affecting other steps in translation could further weaken the strain and cause a hypersensitivity phenotype. Moreover, the ribosome is a common target for many toxins, including Shiga toxin and ricin (Montanaro et al., 1973; Reisbig et al., 1981). Future experiments could determine if K28 directly affects ribosome assembly or protein translation. It is important to note, however, that the K28 hypersensitive mutants of the translation machinery may be non-specific because mutants involved in ribosome function also sensitized cells to K1 toxin, which is predicted to have a different killing mechanism (Page et al., 2003; Martinac et al., 1990).

Using K28 as a Tool to Study Cell Biology

Protein toxins have been used as tools to study several cell biological processes, including endocytosis (Sandvig et al., 2010). For example, Shiga toxin has been used to study how cargo can initiate internalization because Shiga toxin has the ability to tubulate membranes and may activate signaling pathways important for endocytosis (Sandvig et al., 2010; Romer et al., 2007; Cao et al., 2010; Walchli et al., 2009). K28 may also prove to be a useful tool for studying yeast endocytosis in future studies. The extent to which K28 can tubulate membranes, or can initiate internalization upon binding to its plasma membrane receptor, needs to be investigated further. An alternative possibility is that K28 may act as a hitchhiker on proteins or lipids that are constitutively recycled, thereby eliminating the need for K28 to induce its own endocytosis.

How endocytic cargos are sorted in yeast is another area of endocytosis that could be explored using K28 as a tool. Using a tagged receptor that binds extracellular cargo as a marker for cargo sorting is problematic because receptor biosynthetic

pathways are also labeled using this method. The extracellular yeast mating pheromone α-factor has been used to study the endocytic pathway, however, α-factor traffics through only one possible endocytic pathway, which leads to the vacuole (Dulic et al., 1991; Toshima et al., 2006). K28, which likely traffics to the Golgi (Eisfeld et al., 2000), could be used to address questions such as whether different cargo localize to the same endocytic sites, and when different cargos are separated for trafficking to the vacuole versus the Golgi. Previous studies have suggested that cargos are sorted to different intracellular compartments from a common endosome in yeast, but these studies focused on only one cargo with multiple intracellular destinations (Strochlic et al., 2008). In future experiments, fluorescently-labeled K28 and α-factor could be added simultaneously to cells and differences in their internalization and trafficking pathways could be analyzed visually.

K28 can also be used to investigate cell stress response pathways. Mutations in the high osmolarity glycerol (HOG) pathway caused hypersensitivity to K28 and also sensitized cells to the K1 and *Pichia membranifaciens* killer toxins (Page et al., 2003; Santos et al., 2005). Both K1 and *Pichia membranifaciens* killer toxins are suggested to form pores in the plasma membrane (Martinac et al., 1990; Santos and Marquina, 2004), thereby explaining the need for a HOG pathway response in the presence of these toxins. However, K28 is not thought to disrupt the cellular membrane, but is thought to enter cells by endocytosis (Eisfeld et al., 2000). Moreover, Shiga toxin, which appears to follow a similar cellular entry pathway to K28, activates the mitogen-activated protein kinase p38 in mammalian cells, which is a homolog of Hog1p in yeast (Walchli et al., 2008; Foster and Tesh, 2002). This result illustrates that the K28 toxin can be used to investigate cell stress response and other cellular protective pathways in yeast that are similar to the pathways activated by toxins targeting higher eukaryotes.

The K28 killer assay can be used to test the effect of mutations or tags on the functionality of proteins identified in the screen. For example, the AP2 complex subunit Apl3-GFP was found to have different dynamics than the AP2 subunits Apl1-GFP and Apm4-GFP (data not shown). It was discovered, however, that Apl3-GFP yeast were partially resistant to K28, indicating that the GFP tag resulted in a nonfunctional protein. This could explain why Apl3-GFP had different dynamics than other AP2 complex subunits. In general, the killer assay could be used for structure-function studies of the AP2 complex, or any other protein found in the K28 screen.

It is important to note that K28 can be a challenging protein to handle. Generating a fluorescently-labeled K28 derivative has proved to be very difficult. K28 is processed at the N-terminus, C-terminus, and internally (Riffer et al., 2002), which makes GFP-tagging K28 problematic. K28 can be chemically conjugated to fluorescent dyes, although optimal labeling reactions usually require incubations in basic pH buffer conditions that can inactivate the toxin. Subcellular fractionation can be used as an alternative method for tracking K28 localization, however these experiments are also technically challenging. Furthermore, I had difficulty in purifying K28 for many of these experiments. Advances in K28 purification, fluorescent-labeling, and subcellular fractionation techniques are necessary for pursuing future projects involving K28.

Characterization of the Early Endocytic Site

Further analysis of the yeast AP2 complex revealed that it arrives early during endocytic site formation, which is a stage of endocytosis that is poorly understood. Other proteins that arrive early at endocytic sites include Ede1p (an Eps15 homolog), Syp1p (an FCHo1/2 homolog), and clathrin (Toshima et al., 2006; Stimpson et al., 2009; Kaksonen et al., 2005; Newpher et al., 2005; Boettner et al., 2009; Reider et al., 2009). Proteins that localize to endocytic sites before the coat protein Sla1p are considered to be early arriving proteins (Stimpson et al., 2009; Carroll et al., 2009). Prior to this study, however, the order in which these proteins arrive at endocytic sites relative to each other was not known.

After establishing that Pal1p and Yap1802p (an AP180 homolog) are also early arriving proteins, the order of protein recruitment to early endocytic sites was examined more closely. Ede1p, Syp1p, clathrin, the AP2 complex, Yap1802p, and Pal1p localize to endocytic sites at about the same time. This result may provide insight into how sites are established in mammalian systems. FCHo1/2, Eps15, and Intersectin are reported to arrive first to sites of clathrin-coated pit formation in mammalian cells, and then clathrin and the AP2 complex are recruited (Henne et al., 2010). Since Yap1802p localized early during yeast endocytic site formation, the recruitment of AP180 in mammalian cells should be examined more carefully to determine if it also arrives early at endocytic sites. It is important to note that the order of protein arrival in mammalian cells was determined in studies were the proteins were overexpressed (Henne et al., 2010), which may alter the dynamics of endocytic proteins and cargo uptake.

The role of several early arriving proteins in endocytic site formation had not been fully characterized prior to this work. I found that Ydr348cp/Pal1p localizes early at endocytic sites, but appears to be dispensable for proper Sla1-GFP dynamics or uptake of FM 4-64 and α-factor cargos. Pal1p seems to be conserved only in fungi, and its *S. pombe* homolog is involved in cell morphogenesis (Ge et al., 2005). Future studies may provide insight into whether Pal1p in *S. cerevisiae* has a function in linking endocytosis to cell shape.

In addition to Pal1p, Yap1802p was also identified as an early arriving protein. The yeast AP180s are not essential for normal endocytic machinery dynamics or uptake of most cargos, but seem to function primarily in the uptake of Snc1p (results not shown) (Kaksonen et al., 2005; Huang et al., 1999; Burston et al., 2009; Wendland and Emr, 1998). My work has shown Yap1802p does not require Ede1p to localize to endocytic sites, thereby implying that the presence of Yap1802p at endocytic sites is not sufficient to compensate for the endocytic defects in *ede1*Δ yeast. Interestingly, *ede1*Δ *ent1*Δ *ent2*Δ yeast expressing Ent2p lacking functional ubiquitin-interacting motifs have greater defects in α-factor uptake when *YAP1801* and *YAP1802* are also deleted (Dores et al., 2010). Endocytic machinery and cargo uptake should be examined more carefully in *ede1*Δ *yap1801*Δ *yap1802*Δ yeast to determine if the presence of Yap1802p at endocytic sites in *ede1*Δ yeast contributes to productive endocytosis.

Clathrin is an early arriving protein that has been suggested to be important for maturation of endocytic sites (Newpher and Lemmon, 2006). This work has helped elucidate the exact stage of endocytosis that is delayed in clathrin mutants. More specifically, clathrin mediates efficient exit from the intermediate coat stage, in which only the early arriving proteins, Sla2p, Ent1p and Ent2p have been recruited to

endocytic sites. Studies have proposed that clathrin light chain promotes site maturation by relieving an endocytic inhibition caused by Sla2p (Newpher et al., 2006). Since Ent1p and Ent2p also form stable cortical patches in *clc1*Δ yeast, the roles these proteins may have in inhibiting endocytic site progression should be investigated. Furthermore, Ent1p and Ent2p have clathrin binding domains (Aguilar et al., 2003; Wendland et al., 1999), which clathrin may use to interact with and regulate these proteins.

Role of Cargo in Endocytic Progression

The role that cargo plays in endocytic site maturation is a topic of recent interest (Loerke et al., 2009; Mettlen et al., 2010), and this study has provided valuable insight into the regulatory function of cargo during yeast endocytosis. Inducing cargo uptake by adding α-factor to cells did not seem to shorten the variable phase in yeast endocytosis (data not shown), but experiments using a secretion mutant suggests that a lack of cargo delays endocytic site maturation. Furthermore, the possibility also exists that the endocytic defects observed in *clc1*Δ yeast are caused by a lack of cargo accumulation at endocytic sites. One hypothesis is that inducing uptake of a single cargo is not sufficient to shorten the variable phase of endocytosis; however, a checkpoint is activated at this stage of endocytosis that prevents yeast endocytic sites from maturing if cargo has not been recruited to sites.

One question that arises from these experiments centers on how cargo is able to regulate endocytosis. Inducing Gap1p internalization partially rescued endocytic defects in *sec18-1*^{ts} mutants. Further research could determine if any cargo can rescue endocytosis in secretion mutants, or if different cargos and their associated adaptors have distinct regulatory capabilities similar to that observed in mammalian systems (Mettlen et al., 2010; Mettlen et al., 2009). These studies will reveal if cargos that promote endocytic maturation have common characteristics, sequences, or motifs that are necessary for the cargos to perform regulatory functions.

The Intermediate Coat Module

Results from several experiments have led to the proposal that an intermediate coat module consisting of Sla2p, Ent1p, and Ent2p exists. Previous studies showed that Sla2p, Ent1p, and Ent2p have similar vesicle uncoating dynamics (Toret et al., 2008). This work finds that Ent1p and Ent2p have longer lifetimes in *clc1*Δ yeast, which is similar to the phenotype observed for Sla2p in clathrin mutants (Newpher and Lemmon, 2006). I also observed that as previously reported for Sla2p, Ent1p and Ent2p arrive at endocytic sites before Sla1p (Newpher and Lemmon, 2006). The unique dynamics of Sla2p, Ent1p, and Ent2p has led to the modification of our previous model to include a separate module for these proteins (Kaksonen et al., 2005).

In general, Sla2p, Ent1p, and Ent2p seem to have similar functions in yeast endocytosis. This study finds that the abnormal actin structures formed in *sla2*Δ yeast are similar to those observed in *ent1*Δ *ent2*Δ yeast, and that the rate of actin flux through the filament networks is also similar (results not shown). An Sla2p truncation that expresses the ANTH domain was shown to be sufficient for normal growth and the protein localized properly to endocytic sites (Sun et al., 2007). However when the

ANTH domain lipid binding residues were mutated, the strain had growth defects and the protein was mislocalized (Sun et al., 2007). Interestingly, Ent1p and Ent2p have homologous ENTH domains that bind PtdIns(4,5)P_2, and expressing only the ENTH domain from Ent1p (ENTH1) can rescue growth defects of *ent1*Δ *ent2*Δ yeast (Aguilar et al., 2003; Aguilar et al., 2006). Furthermore, my unpublished results show that a truncation of the ENTH1 domain that excludes the putative α0 lipid binding helix was not able to rescue growth defects of *ent1*Δ *ent2*Δ yeast and the mutant ENTH1 domain no longer localizes to endocytic sites. In general, Sla2p, Ent1p, and Ent2p seem to have a role in connecting the force of actin polymerization to membrane invagination, and the lipid binding activities of these proteins are important for this function.

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