Mechanisms of Clathrin-Mediated Endocytosis in Budding Yeast

by

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Mechanisms of Clathrin-Mediated Endocytosis in Budding Yeast

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Abstract

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Clathrin-mediated endocytosis (CME) is a major endocytic pathway that is highly conserved among eukaryotes. CME is critical to maintain cell homeostasis, attenuate receptor signaling, generate turnover of proteins at the plasma membrane (PM), and modulate lipid composition of the PM. In the budding yeast *S. cerevisiae* CME, cargo on the PM is captured, then invaginated into a tubule that pinches into a vesicle to travel into the cell interior. This process relies on the progressive recruitment and coordination of the activities for approximately 60 different proteins. Here, I describe three studies to investigate how the recruitment and activities of endocytic proteins are coordinated during various steps of endocytosis to ensure successful endocytic events.

One way to understand how a complex network of proteins functions is to simplify the process to its most basic components. The Pan1/End3 coat protein complex plays a crucial role in coupling cargo capture to actin assembly, which provides force for PM invagination. I aimed to simplify CME by coupling the upstream coat protein Sla2 to the downstream coat protein Sla1 in order to eliminate the need for the intermediate Pan1/End3 complex. I successfully generated an Sla1-Sla2 fusion protein that was functional for endocytosis. The Sla1-Sla2 fusion protein was recruited to endocytic sites even in the absence of End3 and able to partially rescue some End3 function. However, the Sla1-Sla2 fusion protein was not able to rescue any Pan1 function. These results suggest that Pan1 and End3 have distinct and separable functions in CME. Importantly, generation of this fusion protein is an important initial step in simplifying CME to its minimal functional components.

In budding yeast, the N-BAR protein heterodimer, Rvs161/167, plays an important role in scission of the vesicle from the PM. This step is dependent on several additional scission factors. How these factors coordinate with Rvs161/167 to ensure proper scission is poorly understood. Rvs167 contains an N-BAR domain, which contributes to membrane binding and bending, in addition to an SH3 domain. Las17/WASp is a nucleation promoting factor (NPF) that activates Arp2/3-mediated branched actin polymerization, which contributes to vesicle scission. Since Rvs167 and Las17 are predicted to interact through the Rvs167 SH3 domain. I investigated a possible role for Rvs167 in regulation of actin assembly at endocytic
sites. I found that Rvs167 can enhance or inhibit Las17 activated Arp2/3-mediated actin assembly in vitro, but found no evidence that this regulation occurs in vivo. To identify a potential role for SH3-mediated protein interactions in vivo, I generated truncation mutants of Rvs167 that lacked the SH3 domain to analyze their recruitment to endocytic sites and function. I found that the N-BAR domain alone is sufficient for Rvs167 recruitment to endocytic sites. However, I found that the N-BAR domain did not rescue negative genetic interactions between rvs167Δ and other endocytic mutations. These results reveal that while not important for localization to endocytic sites, SH3-mediated protein interactions might be important for Rvs167 function in endocytosis.

To better understand vesicle scission, it is important to understand how lipid composition changes. I investigated a potential role for the phosphatidate phosphatase (PAP), App1 in regulating endocytosis. PAPs are enzymes that catalyze the conversion of phosphatidic acid (PA) to diacylglycerol (DAG). The relative concentration of these lipids is important for regulation of lipid composition as well as regulation of membrane fission/fusion events. Since App1 has numerous genetic and physical interactions with endocytic factors, I investigated its localization and effects on endocytic dynamics. I found that App1-3GFP signal is predominately cytoplasmic with enrichment on mitochondria. In cells lacking App1, I determined there were subtle defects in endocytic vesicle scission. Overall, these results do not support a role for specific lipid regulation by App1 at endocytic sites.

These studies in S. cerevisiae provide insights into the mechanistic nuances of how protein recruitment and activity are coordinated in CME and provide a foundation for future work.
For Colin
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Chapter 1

General introduction

1.1 Overview of clathrin-mediated endocytosis

Endocytosis is a process that is critical for cell survival since it is responsible for transport external particles such as nutrients and fluids from the exterior of the cell to the interior. This process is also crucial to maintain cell homeostasis, attenuate receptor signaling, generate turnover of proteins at the plasma membrane (PM), and modulate lipid composition of the PM. In mammalian cells, there are a number of different endocytic pathways, but clathrin-mediated endocytosis (CME) is a major receptor-mediated pathway and is a highly conserved process amongst eukaryotes. In CME, the triskelion protein, clathrin, assembles into a cage-like structure around a small portion of the membrane, which invaginates toward the interior of the cell until it pinches off into a vesicle that travels into cell [1, 2]. The budding yeast *S. cerevisiae* is an ideal model organism in which study this process since yeast are easy to manipulate genetically and since CME is a highly conserved process.

1.2 Timeline of protein recruitment to endocytic sites

In budding yeast CME, about 60 different proteins are progressively recruited to endocytic sites [2–4]. In the mid-1980’s, fluorescently labeled actin and associated proteins were observed as diffraction limited spots on the cortex called ”actin patches” [5, 6]. These ”actin patches” represent the late stages of endocytosis and these proteins are just a few of many endocytic proteins which have now been identified. Using two-color live-cell microscopy, pairs of proteins can be simultaneous tracked over time. By tracking, the relative timing of protein recruitment to endocytic sites can determined, thus establishing a time line endocytic events [3, 7]. One of the most striking features of CME is the precise spatio-temporal regulation of so many proteins. Based on when proteins arrive at endocytic sites and their known or predicted function, these proteins can be grouped into the following modules: early, coat, WASP/myosin, actin, and amphipathins [3]. The arrival of the early proteins occurs approximately 75 - 135 s before scission and marks the initial stage of CME. The coat module
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Figure 1.1: **Modules of endocytic proteins in yeast.** Cartoon representation of the progressive recruitment of different proteins during of budding yeast CME. Several proteins from each module are listed.

proteins arrive progressively. They can be further sub-categorized into early, intermediate, and late [8, 9]. This module is generally responsible for the association of the invaginating pit with the actin machinery and for capturing cargo [7, 10–12]. The WASP/myosin module arrives approximately 30 s before scission and contains actin nucleation-promoting-factors (NPFs) that trigger actin assembly. Upon assembly of an actin network, the membrane undergoes dramatic remodeling as it invaginates into a tubule. The amphiphysin module is recruited late in the pathway and aids in vesicle scission (Figure 1.1) [3, 13].

**CME site initiation**

In budding yeast, site formation is indicated by the arrival of the early module of proteins including Ede1 and Syp1 and the early coat module including clathrin and the AP2 complex. These proteins have a highly variable lifetimes at endocytic sites, ranging from 30 s to 240 s [3, 9–12]. These proteins appear to arrive at endocytic sites simultaneously, making it difficult to identify any upstream signals that might mark a specific location on the PM for endocytic site initiation [9]. While it is unclear what initiates the arrival of these proteins to a specific location, we know that endocytic sites are not completely selected at random. During the early phases of the cell cycle, yeast becomes highly polarized and endocytic sites accumulate toward the bud tip. Ede1 has been identified as a key regulator for site formation since loss of this protein results in patch destabilization. In *ede1Δ* cells, there is a decrease in the number of patches, and localization of several early proteins is disrupted. While Ede1 plays a key role, it is not a essential for endocytic site initiation since some proteins are
still able to localize to properly to endocytic sites [14]. The inability to identify a master regulator complicates our understanding of endocytic site initiation. However, this feature could be beneficial for cells by making the process more robust and flexible.

CME site maturation

The initial phase of protein accumulation and recruitment during site initiation can last for a variable amount of time. As more proteins accumulate and the endocytic site matures, there is a transition to a "regular phase" of endocytosis, wherein proteins exhibit regular lifetimes at the PM. This transition is possibly dependent on cargo. It is possible that where sites must accumulate sufficient cargo to trigger a transition to the regular phase. Once this transition occurs, endocytic sites mature with regular timing as they proceed to vesicle scission. At this transition point, the intermediate coat proteins including Sla2 and Ent1/2 will accumulate approximately 40 s before vesicle scission [3]. The coat module is responsible for the association of the invaginating membrane tip with the actin network. Sla2 contains an ANTH domain that binds to PI(4,5)P$_2$ at the PM and a THATCH domain that binds to actin filaments [15, 16]. As a result of uncoupling the actin network from the invaginating membrane tip, actin in a sla2Δ mutant will polymerize at the PM and continually flux into the cell interior [7]. Approximately 5 s after the intermediate coat module proteins arrive, the late module including the Pan1/End3/Sla1 complex arrive at endocytic sites [9]. This complex is important for physically coupling the initiation machinery from the early, early coat, and intermediate coat modules to the later phase proteins, such as WASP/Myo, actin, and amphiphysin [17, 18]. Pan1 and End3 arrive as a complex slightly before the Sla1/Las17 complex, and are critical for its recruitment [18, 19].

As the site matures, there are some common features among proteins that assemble. These overlapping features can be seen when looking at the domain structures of the proteins. Since the coat module is important for coupling the PM of invaginating tubule to the actin machinery, membrane binding domains such as the ANTH and ENTH domains are important for these proteins. The ANTH membrane binding domain, which is present in Sla2, is also present in AP1801 and AP1802. ENTH domains also bind PI(4,5)P$_2$ and are found in Ent1/2. Endocytic proteins associate via an extensive protein-protein interaction network. Many of the proteins such as Ede1, Sla2, Pan1, Ent1/2 and End3 contain coil-coil domains, which can associate and may help these proteins serve as scaffolds. Additionally, Ede1, Pan1, and End3 contain EH domains that can bind to the tri-peptide asparagine-proline-phenylalanine (NPF) motif. There are several proteins that contain SH3 domains and poly-proline PXXP motifs that are recruited to endocytic sites. The early protein, Syp1, contains a proline rich region, as does Pan1. Sla1 has three SH3 domains. As endocytosis progresses further, the protein interaction network mediated by SH3-PXXP motifs expands, as described further below [20].
Actin nucleation and polymerization

The most dramatic membrane deformation during yeast CME is dependent on actin polymerization [7]. Actin nucleation at endocytic sites is mediated by the Arp2/3 complex, which must be activated by nucleation promoting factors (NPFs) to promote actin polymerization [4, 21–23]. NPFs are recruited approximately 30 s before vesicle scission and are part of the WASP/myosin module [24]. There are several endocytic proteins that exhibit NPF activity, but Las17 (homolog of mammalian WASP), and the Myo3/5 (type I myosins) with Vrp1, have the strongest NPF activity and are the most crucial for CME [25–27]. Other proteins that arrive with the module are Bzz1 and Bbc1 [3]. Even after the WASP/myosin module proteins are recruited to endocytic sites, actin polymerization is delayed for approximately 15 s. The burst of actin polymerization that is required to invaginate the membrane is thought to be precisely timed. To ensure accurate timing of actin polymerization, NPF activity must be regulated. Las17 is constitutively active, therefore inhibitory regulation is imperative to prevent aberrant actin polymerization. Las17 is recruited to endocytic sites as a complex with Sla1, which is a potent inhibitor of Las17 NPF activity [19, 28]. SH3 domain interactions with the Las17 proline-rich domain is thought to be the basis for this inhibitory interaction. As Bzz1 arrives, competitive binding may relieve this inhibition. Bbc1 is an additional inhibitory factor, which can inhibit both Las17 and Myo3/5 [27]. Syp1, which is one of the earliest proteins to arrive also has inhibitory activity toward th Las17 NPF activity [29].

Actin begins to polymerize at endocytic sites approximately 15 s before vesicle scission. During this step, actin is spatially organized so that plus ends of growing filaments are at base of invaginating tubules. As monomers are added to the base, the coat protein couples actin to the tip of the invaginating tubule. In addition to the Arp2/3 complex actin nucleator, there is an entire network of actin associated proteins that are coincidently recruited to endocytic sites to ensure proper regulation of actin assembly and function. Capping proteins cap the barbed ends of actin filaments and restrict their growth to only regions where polymerization is needed. There are also proteins that directly bind and decorate filaments, such as Abp1 and the actin bundling protein, Sac6. Actin filament turnover is mediated by severed by proteins such as Cof1 (cofilin) and Aip1 [30].

Vesicle scission and protein dissociation

Just before the scission step, the amphiphysin module, comprised of the obligate heterodimeric BAR protein Rvs161/Rvs167, is recruited to endocytic sites. Scission of the vesicle from the PM is primarily dependent on Rvs161/167 since loss of this dimer causes the invaginating membrane to retract back to the cortex [3]. This invagination-retraction phenotype is unique, but only occurs in approximately 20% of endocytic events [13]. Using the rvs167Δ mutant as a sensitized background allowed for the identification of other scission factors, whose loss increased the penetrance of this phenotype. These additional factors include the F-BAR protein Bzz1, the lipid phosphatase synaptojanin Sjl2, and actin.
Scission in yeast varies from that of mammalian cells. The scission step in mammalian cells relies heavily on the mechanical pinching force generated by the GTPase. In yeast, the dynamin homolog Vps1, has been implicated in the scission event, but it is not required [31].

Like the invagination step of CME, this scission step is critically dependent on tight coordination between the membrane lipids and the proteins that act to remodel the membrane. There are several ways that proteins interact with the membrane to change organization and dynamics of lipid molecules within the membrane and therefore, the membrane’s physical properties. First, the Rvs161/167 heterodimer forms a rigid curved structure from its N-BAR domain, which is capable of molding the membrane [32–34]. Additionally, there are factors that influence lipid organization within a two dimensional space. By binding to the membrane, BAR-domain proteins have been shown to restrict lipid flow and Sjl2 catalyzes the conversion of PI(4,5)P$_2$ to PI(4)P and PI [35, 36]. Both of these mechanisms that can segregate lipids to generate a line tension, which is proposed to promote scission [37]. Temporal and spatial changes in lipid composition may happen rapidly as lipids are modified by enzymes such as Sjl2. In addition to two dimensional segregation, changes in lipid geometry on only one side of the lipid bilayer can affect the spontaneous curvature of the membrane bilayer, allowing the membrane to adopt a curved structure.

Once vesicle scission has occurred, the endocytic site proteins rapidly dissociate. This uncoating process relies on the the Ark1/Prk1 kinases to phosphorylate endocytic proteins, causing their dissociation. In an ark1Δ prk1Δ mutant, failure to uncoat causes to aggregates of endocytic proteins to accumulate in the cell interior [38, 39].

### 1.3 Current outlook on CME

Through work of many labs over the years, been enormous strides have been made in understanding CME in both yeast and mammalian cells. In budding yeast, we have a thorough view of which proteins are present at endocytic sites and when they arrive. We are also gaining a more comprehensive view of the function of these proteins and how they are able to coordinate with each other in a complex network during endocytosis. There are still many things we do not fully understand. To start, the initiation mechanism remains poorly understood. The process for selecting a region of the PM for endocytic site initiation is remains elusive. In the early stages of endocytosis, we also do not understand the regulation of the transition from the variable timing to regular timing phase. During the regular phase of endocytosis, there are a large number of proteins, many of which have overlapping functions. It remains both unclear which of these functions and proteins are the most important for CME and also under what conditions the complexity of such a myriad of proteins becomes important. In Chapter 2, I have used a synthetic biology approach to generate fusion proteins to simplify the intermediate and late coat modules and thus gain insight into the most crucial functions of this module. In the later phases of endocytosis it is unclear how cytoskeleton assembly, membrane binding proteins and membrane itself are controlled to allow for successful scission. In Chapter 3, I explored various ideas to determine
how protein-protein interactions involving Rvs167 might contribute to these later phases. In Chapter 4, I investigated the role of a putative endocytic protein and its potential role in CME.
References


CHAPTER 1. GENERAL INTRODUCTION


Chapter 2

An Sla1-Sla2 fusion protein provides insight into how the Pan1/End3/Sla1 complex functions in endocytosis

2.1 Introduction

Endocytosis is the process by which the plasma membrane (PM) first invaginates to form a tubule or a sphere and then pinches into a vesicle that travels into the cell interior. This process is critical to maintain cell homeostasis, attenuate receptor signaling, generate turnover of proteins at the plasma membrane (PM), and modulate lipid composition of the PM. Clathrin mediated endocytosis (CME) is a major endocytic pathway and is highly conserved amongst eukaryotes [1].

In the budding yeast *S. cerevisiae*, CME depends on precise spatio-temporal recruitment of approximately 60 proteins to endocytic sites and their regulation [2]. Based on their timing of arrival and their known or predicted function, these proteins have been grouped into the following modules: early, coat, WASP/myosin, actin, and amphiphysin [3]. The arrival of the early proteins marks the initial stage of CME. The coat module proteins arrive progressively and is further sub-categorized into early, intermediate, and late [4, 5]. This module is generally responsible for the association of the invaginating pit with the actin machinery [6–9]. The WASP/myosin module proteins contains actin nucleation-promoting-factors (NPFs) that trigger actin and proteins associated with the actin module proteins. Upon actin assembly, the membrane undergoes dramatic remodeling, resulting in PM tubule. The amphiphysin module is recruited last and aids in vesicle scission [3, 10].

Early proteins of the coat module arrive at the earliest detection of endocytic sites and are important for cargo selection. As endocytosis, the intermediate coat module proteins including the Sla2/Ent1/2 protein complex are assembled at endocytic sites. This complex provides a linkage that is crucial to transmit actin forces to the PM, which results in the formation of a tubule [3, 11, 12]. A Pan1/End3 complex arrives next, followed by the Sla1/Las17 complex proteins.
CHAPTER 2. AN SLA1-SLA2 FUSION PROTEIN PROVIDES INSIGHT INTO HOW THE PAN1/END3/SLA1 COMPLEX FUNCTIONS IN ENDOCYTOSIS

[13, 14]. The Pan1/End3/Sla1 complex is required for proper endocytosis and cell viability. A critical role of the Pan1/End3/Sla1 complex was recently revealed in work by Sun et al., where simultaneous loss of Pan1 and End3 caused early proteins, including Sla2, to become spatially uncoupled from Sla1 and downstream proteins [14]. While current research provides us with a general view of how this module functions, we still do not fully understand how all of the proteins and complexes within this module act together to ensure proper endocytosis.

There are a couple features of the intermediate coat module that have limited our ability to fully understand it. First, proteins in this module assemble through a network of protein-protein interactions, so disrupting one protein can cause disruptions in others. Secondly, many of the proteins in this module work together, so elimination of one protein may not reveal which general functions are the most critical for proper endocytosis. These features are not unique to this module. From a molecular standpoint, the CME process is extraordinarily complex and relies on a large number of proteins, many of which have seemingly redundant functions. Additionally the process requires rapid assembly and disassembly, which requires extensive protein-protein and protein-lipid interactions. Thus, proper CME depends on rapid, cooperative action between numerous proteins and lipids. These features allow for the CME to be robust and dynamic, but make it difficult to pinpoint which exact protein functions are most crucial for productive endocytic events.

One approach to understanding this intricate process is to simplify a module into its most basic components. This approach has been applied to the WASP/Myosin module and has provided key insights into how this module functions [15]. I employed a similar synthetic biology approach in an attempt to simplify the intermediate coat module. Because the Pan1/End3/Sla1 complex links endocytic site initiation proteins to the actin assembly machinery, I reasoned that artificially coupling the endocytic site initiation machinery with the actin assembly machinery might bypass the need for the entire complex. Since Sla2 is one of the last proteins to assemble with the early machinery, and Sla1 is critical for recruiting the NPF, Las17, I tethered these proteins together as a first step toward the simplification this module.

2.2 Results

An Sla1-Sla2 fusion protein can replace Sla1 and Sla2 function in endocytosis

Since budding yeast clathrin mediated endocytosis (CME) is a robust process that relies on many proteins with seemingly redundant functions, it can be difficult to parse out which of these functions is the most crucial for proper CME. To pinpoint the most important factors for CME, I focused on simplifying the intermediate coat module. Sun et al. showed that simultaneous loss of Pan1 an End3 spatially uncouples Sla2 and endocytic site initiation proteins from Sla1 and actin assembly proteins. I hypothesized that artificially recruiting Sla1 to upstream proteins by linking it to Sla2 might bypass the need for the Pan1/End3/Sla1
CHAPTER 2. AN SLA1-SLA2 FUSION PROTEIN PROVIDES INSIGHT INTO HOW THE PAN1/END3/SLA1 COMPLEX FUNCTIONS IN ENDOCYTOSIS

Figure 2.1: An Sla1-Sla2 and Sla2-Sla1 fusion protein can replace Sla1 and Sla2 function for proper yeast growth and morphology. Continued on next page.
CHAPTER 2. AN SLA1-SLA2 FUSION PROTEIN PROVIDES INSIGHT INTO HOW THE PAN1/END3/SLA1 COMPLEX FUNCTIONS IN ENDOCYTOSIS

Figure 2.1: An Sla1-Sla2 and Sla2-Sla1 fusion protein can replace Sla1 and Sla2 function for proper yeast growth and morphology. (A) Protein extracts from two independently derived replicates for each genotype were separated by SDS-PAGE and blotted using anti-Sla2 or anti-Sla1. (B) Growth of various SLA1-SLA2 and SLA2-SLA1 yeast after 48 h at 30°C, 37°C, and 39°C. (C) Representative brightfield images of wild-type, sla1Δ sla2Δ, SLA1-SLA2, and SLA2-SLA1 yeast at 23°C.

complex. To test this hypothesis, I generated yeast strains expressing a fusion protein comprised of Sla1 and Sla2. Since it can be difficult to predict if a fusion protein will function properly, I generated an SLA1-SLA2 strain as well as an SLA2-SLA1 strain. First, I tested SLA1-SLA2 and SLA2-SLA1 cells for expression of the fusion protein by western blotting. I found a band immuno-reactive for anti-Sla2 and anti-Sla1 close to 245 kDa predicted molecular weight of fusion protein (Figure 2.1A). To determine whether the Sla1-Sla2 and Sla2-Sla1 fusion proteins functioned properly, I tested SLA1-SLA2 and SLA2-SLA1 cells for their ability to rescue the growth phenotypes of sla1Δ or sla2Δ mutants at 30°C, 37°C, and 39°C. I found that SLA1-SLA2 yeast nearly phenocopied the growth of wild-type yeast at all temperatures tested (Figure 2.1B). Using brightfield microscopy, I observed that SLA1-SLA2 cells could also rescue morphological defects of sla1Δ sla2Δ yeast (Figure 2.1C). These data suggest that the Sla1-Sla2 fusion is capable of replacing Sla1 and Sla2 in basic cellular functions. SLA2-SLA1 cells were also able to rescue morphological defects of sla1Δ sla2Δ yeast, but were not able to fully rescue the growth defects of sla1Δ yeast at higher temperatures of 37°C or 39°C (Figure 2.1B and C). Since SLA1-SLA2 yeast appeared more normal, I used this strain for all future experiments.

Once I had established that Sla1-Sla2 was able to replace the basic cellular functions normally provided by Sla1 and Sla2, I wanted to investigate Sla1-Sla2 function at endocytic sites. I started by tagging Sla1-Sla2 with GFP and mCherry to compare localization and characteristics of Sla1-Sla2 with Sla1 and Sla2. I tested the tagged version of the protein for functionality using a growth assay. I found that SLA1-SLA2-GFP and SLA1-SLA2-mCherry strains grow as well as SLA1-SLA2 and do not exhibit the growth phenotypes of sla1Δ or sla2Δ mutants (Figure 2.1A). This result suggests that fluorescently tagged Sla1-Sla2 is as functional as the untagged version (Figure 2.2A). In endocytosis, there is a tight temporal regulation of each protein. The proteins appear as dynamic puncta on the cortex and exhibit a characteristic lifetime at endocytic sites. I used live-cell fluorescence microscopy to observe the Sla1-Sla2-GFP and Sla1-Sla2-mCherry signal in cells. I found that Sla1-Sla2-GFP and Sla1-Sla2-mCherry form cortical puncta similar to Sla1-GFP or Sla2-GFP (Figure 2.2B). The lifetimes of fluorescently tagged proteins are easily visualized in kymographs, where a line drawn through the cortex is projected over time (Figure 2.2B). I observed an Sla1-Sla2-GFP lifetime of 38.4 ± 9.4 s which is not statistically different from the Sla1-Sla2-mCherry lifetime of 40.4 ± 10.9 s. The lifetime of Sla1-Sla2 is longer than the Sla1-GFP lifetime (24.1 ± 4.5 s) or the Sla2-GFP lifetime (29.6 ± 6.9 s) (Figure 2.2C). While the fluorescent signals from Sla1
Figure 2.2: **Comparison of Sla1-Sla2, Sla1, and Sla2 endocytic lifetimes.** Continued on next page.
and Sla2 disappear as an endocytic site disassembles and the vesicle travels to the interior of the cell, occasionally Sla1-Sla2 puncta were observed inside of the cell (data not shown). Sometimes I could see these puncta originate on the cortex and move inward, and other times they would appear in the interior of the cell. Since these movies were collected within a single z-plane, it is possible that these puncta also originated at the cortex and subsequently appeared in the cell interior by moving in the plane of focus. These data demonstrate that Sla1-Sla2 exhibits similar, but not identical, endocytic dynamics to Sla1 or Sla2.

After I established that Sla1-Sla2 endocytic dynamics are similar to those of Sla1 and Sla2, I asked if Sla1-Sla2 could function in place of Sla1 and Sla2 to promote proper endocytosis. Sla2 is crucial in endocytosis, during which it functions to transfer the force produced by actin assembly to the plasma membrane, resulting in tubule formation. A fluorescently labeled actin-associated protein, such as Abp1 or Sac6, may be used to track the actin network at endocytic sites. These proteins normally exhibit a lifetime of 15 s before internalizing ([9], Figure 2.3A). In sla2Δ cells, live-cell microscopy of Sac6-RFP reveals an actin network that continuously polymerizes from cortex into the cell ([9], Figure 2.3A). This phenotype is exacerbated in sla1Δ sla2Δ mutants (Figure 2.3A). In SLA1-SLA2 sla2Δ or SLA1-SLA2 sla1Δ sla2Δ I observed that Sac6-RFP patches form at the at the cell cortex and internalize in a similar manner to wild-type cells (data not shown, Figure 2.3A). Sla1 interacts with the actin nucleation promoting factor (NPF), Las17, to help recruit it to endocytic sites and inhibit its activity [13, 16]. As a result, fewer actin patches form in a sla1Δ mutant. However, these patches are larger and persist longer than in wild-type cells ([17], Figure 2.3 and 2.4). The Sac6-RFP lifetime in sla1Δ mutant (15.9 ± 2.9 s) was longer and more variable than in wild-type cells (11.9 ± 1.6 s) (Figure 2.3B). This phenotype is largely rescued in SLA1-SLA2 sla1Δ or SLA1-SLA2 sla2Δ sla1Δ sla2Δ strains, which have a slightly shorter and more variable Sac6-RFP lifetime (10.8±2.1 s) when compared to wild-type cells. Using a circumferential kymograph that depicts all the Sac6-RFP patches of a cell, I found that SLA1-SLA2 cells do not have enlarged Sac6-RFP patches like those found an sla1Δ mutant (Figure 2.4A). Also, the decreased number of Sac6-RFP patches in a sla1Δ mutant compared to wild-type is rescued in SLA1-SLA2 cells (Figure 2.4B). Although SLA1-SLA2 cells do not exhibit actin dynamics identical to wild-type cells, lack the majority of defects exhibited by sla1Δ, sla2Δ, or sla1Δ sla2Δ mutants. These data indicate that Sla1-Sla2 can compensate for the majority of Sla1 and Sla2 functions in endocytosis.
Figure 2.3: *SLA1-SLA2* cells exhibit similar actin patch dynamics to wild-type cells. (A) Single frame from live-cell fluorescent imaging movie of the actin marker Sac6-RFP in the indicated strains at 23°C. Representative kymograph over 90 s from a single patch, below. (B) Lifetimes of fluorescent patches quantified from live-cell imaging movies represented in panel A. (* = statistically significant difference of $p<0.05$, **** = statistically significant difference of $p<0.0001$).
CHAPTER 2. AN SLA1-SLA2 FUSION PROTEIN PROVIDES INSIGHT INTO HOW THE PAN1/END3/SLA1 COMPLEX FUNCTIONS IN ENDOCYTOSIS

Figure 2.4: SLA1-SLA2 cells have similar actin patch numbers to wild-type cells. (A) Single frame from live-cell fluorescent imaging movie of actin marker, Sac6-RFP, in the indicated strains at 23°C. Circumferential kymographs over 90 s, right. (B) Number of Sac6-RFP patches per cell per 90 s quantified from live-cell imaging movies represented in panel A. (*** = statistically significant difference of $p<0.001$, **** = statistically significant difference of $p<0.0001$, n.s. = no statistical significance).
Figure 2.5: Sla1-Sla2 is recruited to the cortex in the absence of End3 and partially rescues growth defects of end3Δ cells. (A) Single frame from live-cell imaging of mixed populations of cells. Representative line scans drawn through cortical puncta, below. Blue lines: cells expressing Sla1-GFP, Purple lines: cells expressing Sla1-Sla2-GFP. (B) Growth assay comparison of SLA1-SLA2, end3Δ, and end3Δ SLA1-SLA2 yeast after 48 h at 25°C, 30°C, and 37°C.
CHAPTER 2. AN SLA1-SLA2 FUSION PROTEIN PROVIDES INSIGHT INTO HOW THE PAN1/END3/SLA1 COMPLEX FUNCTIONS IN ENDOCYTOSIS

An Sla1-Sla2 fusion protein can partially compensate for loss of End3

I generated the Sla1-Sla2 fusion protein to determine if artificially localizing Sla1 to endocytic sites could bypass the need for the Pan1/End3/Sla1 complex. Since Sla1 is normally recruited to endocytic sites by Pan1 and End3, it was important to determine if Sla1-Sla2 was capable of localizing to endocytic sites in the absence of Pan1 or End3. First, I compared Sla1-Sla2 cortical recruitment to that of Sla1. To ensure that cells were examined under identical conditions, I mixed SLA1-SLA2-GFP and SLA1-GFP cells on the same coverslip. Although the characteristics of Sla1-Sla2-GFP and Sla1-GFP patch dynamics differ (Figure 2.2), cortical localization intensity of Sla1-Sla2-GFP is similar to that of Sla1-GFP. Next, I compared the localization of Sla1-Sla2-GFP and Sla1-GFP in end3Δ cells. In end3Δ cells, Sla1-GFP cortical puncta are barely distinguishable from the cytoplasmic GFP signal, indicating failed recruitment of Sla1-GFP to endocytic sites (Figure 2.5). By comparison, Sla1-Sla2-GFP still forms clear cortical puncta, which indicates that Sla1-Sla2 is capable of proper localization even in the absence of End3 (Figure 2.5 B).

Sla1-Sla2 is capable of providing most Sla1’s function (Figures 2.1, 2.2, 2.3, and 2.4) and is recruited to the cortex in the absence of End3. From these results, I recognized an opportunity to use Sla1-Sla2 to investigate the role End3 without disrupting Sla1. Both end3Δ and sla1Δ mutants exhibit growth defects at high temperatures (Figure 2.1 and 2.5B). If growth defects exhibited by an end3Δ mutant is caused in part by lack of Sla1 recruitment to endocytic sites, then this defects could be rescued by Sla1-Sla2. I found that end3Δ SLA1-SLA2 sla1Δ sla2Δ yeast grew more robustly at 37°C than the end3Δ mutant, but not as well as wild-type yeast. This result demonstrates the ability of the Sla1-Sla2 fusion protein to partially rescue functions of End3.

Cells expressing an Sla1-Sla2 fusion protein still require Pan1 for cell viability

My results up to this point demonstrated that Sla1-Sla2 could function at endocytic sites and could partially bypass the need for End3. Since Pan1 is also responsible for recruitment of Sla1 to endocytic sites, I hypothesized that Sla1-Sla2 would be recruited to endocytic sites in the absence of Pan1 and thus could rescue some endocytic defects in cells lacking Pan1. It has previously been reported that pan1Δ mutants are not viable. However, in our strain background, pan1Δ growth is severely impaired at 25°C and lethal at 30°C or higher (Figure 2.6). There is no noticeable difference in the growth of pan1Δ spores compared to pan1Δ SLA1-SLA2 sla1Δ sla2Δ spores (Figure 2.6A). Since Pan1 has multiple functions, I used pan1 mutants which are viable but lack the coil-coil domain and proline-rich region to see if the Sla1-Sla2 fusion could rescue growth defects of these mutants [18]. The pan1 a.a. 1-98,386-855 mutant is additionally lacking an EH domain that interacts with Sla1. In the case of both pan1 mutants, combining them with SLA1-SLA2 caused a slight negative synthetic growth interaction rather than rescuing growth defects (Figure 2.6B). I attempted
Fig. 2.6: Synthetic growth interactions between SLA1-SLA2 and pan1 mutants. (A) Individual spores from tetrad dissections of PAN1/pan1Δ or PAN1/pan1Δ SLA1-SLA2/SLA1-SLA2 sla1Δ/sla1Δ sla2Δ/sla2Δ diploids. (B) Growth of SLA1-SLA2 with pan1 a.a.1-855 and pan1 a.a.1-98, 386-855 mutants after 48 h at 30°C, 37°C, and 39°C.

I found that pan1Δ cells exhibit morphological defects and that Sla1-GFP that is slightly enhanced on the cortex but remains static (Fig. 2.7). Since the pan1Δ mutant strain was so ill, I was unable to generate a strain with Sla1-Sla2-GFP for comparison. Together, these data demonstrate that Sla1-Sla2 is not capable of rescuing functions of Pan1, and in some cases seems to exacerbate defects caused by loss of Pan1 function.
CHAPTER 2. AN SLA1-SLA2 FUSION PROTEIN PROVIDES INSIGHT INTO HOW THE PAN1/END3/SLA1 COMPLEX FUNCTIONS IN ENDOCYTOSIS

2.3 Discussion

Understanding the role of the Pan1/End3/Slal complex in endocytosis

There have been challenges in understanding the intermediate coat module and its role in endocytosis. One challenge arises from studying a large number of proteins that act cooperatively. In such a system, elimination of one protein may not reveal which general functions are the most critical for proper endocytosis. It was only by eliminating Pan1 and End3 together that Sun et al. were able to identify the important role that the Pan1/End3/Slal complex plays in spatially coupling cargo capture with the actin machinery [14]. Another challenge arises from studying a module that depends on a network of protein-protein interactions, where disruption of one protein may cause failed recruitment of downstream proteins. One approach to overcome these challenges is to reduce a module into its minimal functional components. Pan1/End3 plays a clear role in coupling Sla2 and endocytic initiation proteins to Sla1 and actin assembly proteins. I therefore aimed to simplify the module by artificially coupling the upstream and downstream components to eliminate the need for Pan/End3. To accomplish this, I generated a strain expressing an Sla1-Sla2 fusion protein. I characterized the dynamics of this protein and assessed its ability to function in endocytosis. Once I determined that Sla1-Sla2 protein is functional in endocytosis, I was able to use it as a tool to further study the role of the Pan1/End3/Slal complex in endocytosis.

The initial goal of generating the Sla1-Sla2 protein was to tether Sla1 to the upstream
factors involved in site initiation. As shown in Figure 2.5, I succeeded in achieving this, since Sla1-Sla2 localized to cortical patches in the absence of End3. Sla1 recruitment to endocytic sites is primarily dependent on End3, which can make it difficult to separate the functions of End3 and Sla1. Artificially recruiting Sla1 function to endocytic sites allowed me to study the effects of End3 loss without disrupting Sla1. I hypothesized that some of the growth defects of end3Δ were due in part to failed recruitment of Sla1. I found Sla1-Sla2 was able to partially rescue these defects, which supports this hypothesis. There was, however, not a full rescue. This result was expected since the growth defect of the end3Δ mutant is more severe than that of the sla1Δ mutant.

Pan1 can also recruit Sla1 to endocytic sites. I therefore hypothesized that using the Sla1-Sla2 fusion protein to artificially recruit Sla1 to endocytic sites might rescue some of the defects that result from loss of Pan1. In contrast to my expectations, data in Figure 2.6 demonstrate that Sla1-Sla2 caused more severe defects when expressed in pan1 mutants. Although Pan1 and End3 form a stable complex, this study supports the idea that End3 and Pan1 have distinct and separable functions. It is likely that the primary role of End3 is to recruit Sla1 to endocytic sites, whereas Pan1 plays additional roles in ensuring the proper progression of endocytosis.

A synthetic biology approach to simplifying the intermediate coat module

Generating the Sla1-Sla2 fusion protein was a first step in simplifying the intermediate coat module down to the most basic components. This approach enabled elimination of End3 without disrupting Sla1-Sla2 localization. This study also illuminated a crucial role for Pan1. I established that the Sla1-Sla2 fusion protein is functional in endocytosis, and therefore can be the foundation for generating a simplified intermediate coat module.

Future directions

Extensive protein-protein and protein-lipid interactions allow CME to be robust and dynamic, but can make difficult to pinpoint which protein functions are most crucial for productive endocytic events. Using a synthetic biology approach to strip a module of proteins down to its most essential components allow us to identify a minimal functional system. Future steps toward generating a minimal functional coat protein module will include eliminating domains from the Sla1-Sla2 fusion. Since Sla1-Sla2 can localize to endocytic sites on without End3, the End3 binding domain of Sla1 is a good candidate domain for elimination. Future experiments should also look at localization and dynamics of Sla1-Sla2 in cells where Pan1 and End3 have been simultaneous eliminated. This could provide information about the nature of Pan1’s additional roles in endocytosis. Understanding Pan1’s function in endocytosis might aid in future designs of a minimal fusion protein which can include essential domains of Pan1.
Identifying the minimal components required for a functional coat protein model will provide valuable information about how this module functions and will also be a useful tool for future studies. Lewellyn et al. have generated a minimally functional protein to replace the WASP/MYO module proteins [15]. If a minimal coat protein could also be generated, it might be possible to combine these minimal proteins modules \textit{in vivo} to better understand which components are required for a successful endocytic event. Using \textit{in vitro} systems has provided a bottom up approach to studying many biological processes. Since CME requires many proteins, recapitulating this process \textit{in vitro} can be difficult. Michelot et al. have made strides in this direction by using an \textit{ex vivo} cell extract system [19]. Future studies aimed at reconstructing an \textit{in vitro} system to study endocytosis should consider using the minimal protein modules as a tool to construct a more simple system. Additional future studies should also consider applying this approach to other protein modules such as the early module or the actin module.

\section*{2.4 Materials and methods}

\subsection*{Yeast strains and plasmids}

Yeast strains used in this study were derived from S288C and are listed in Table 2.1. \textit{SLA1-SLA2} and \textit{SLA2-SLA1} were generated by cloning the first gene plus 500 bp of the 5’ UTR with the second gene plus 500bp of the 3’ UTR, connected with a (GlySerSer)_4 linker, into a BamHI digested pRS306 plasmid using Gibson Assembly. The plasmid was linearized and then transformed into wild-type yeast for integration into the URA3 locus. \textit{SLA1-SLA2-GFP} and \textit{SLA1-SLA2-mCherry} strains were generated using homologous recombination [20]. Yeast strains were grown in rich media (YPD) or synthetic media with appropriate amino acid supplements at 25°C unless otherwise stated.

\subsection*{Yeast growth analysis}

Yeast spot growth assays were performed by diluting an overnight liquid YPD culture to 1 OD$_{600}$, performing 1/10 serial dilutions, and spotting 4 µl of each dilution onto a YPD plate. Yeast were grown at the indicated temperatures for 48 hours before imaging. Yeast tetrad analysis was performed by separating spores from a single ascus onto a YPD plate, then grown for 5 days at 25°C before imaging.

\subsection*{Microscopy}

Images were acquired using a Nikon Ti with a 100X NA1.4 objective and an Andor Neo 5.5 SCMOS camera (Andor Technology USA, South Windsor, CT). Single-color movies were collected at 1 s intervals with 500 ms exposure for the indicated length of the movie. Cells containing MTW1-mCherry for identification purposes were imaged as a single-color GFP.
movie followed by an mCherry Z-stack containing 13 planes 0.3 \( \mu \)m apart represented as a maximum intensity projection. Images were analyzed using ImageJ and a plugin (courtesy of Jasper Weinberg) that generates radial kymographs in 2-degree increments. Fluorescent lifetimes of proteins were calculated by measuring the time a patch persisted on the cortex before moving into the cell or disappearing. All imaging was performed at \( 23^\circ \)C in synthetic media. Cells were imaged in a live-cell imaging chamber made by coating a coverslip with Concavalin A (1\( \mu \)g/ml), then subsequently inverting the coverslip onto a slide and sealed with vacuum grease.

**Western Blotting**

Yeast were cultured in YPD starting 0.2 \( \text{OD}_{600} \) and harvested at \( \text{OD}_{600} \) 0.6 - 0.8. Lysis of yeast and extraction of protein was performed by standard trichloroacetic acid protocol. Equal amounts of protein extracts were separated on SDS-PAGE gels, transferred to a nitrocellulose membrane, then probed with rabbit anti-Sla1 ([21]), or anti-Sla2 ([22]). Primary antibodies were detected using LI-COR IRDye and VRDye secondary antibodies and imaged on an Odyssey infrared scanner (LI-COR Biosciences).

**Statistical Analysis**

The p-value between two data sets was calculated using the Student’s t-test. The p-values for three or more data sets were calculated using one-way ANOVA with a Tukey’s post hoc test.
TABLE 2.1: YEAST STRAINS USED THIS STUDY

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Yeast strains used this study (continued)

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AKY112  MATa  his3-Δ200  ura3-52  leu2-3,112  ura3::SLA1-SLA2-mCherry::KanMX6::URA3 SAC6-RFP::KanMX sla2Δ::cgLEU2

AKY492  MATa  his3-Δ200  ura3-52  leu2-3,112  ura3::SLA1-SLA2-mCherry::KanMX6::URA3 SAC6-RFP::KanMX sla1Δ::NatNT2

AKY488  MATa  his3-Δ200  ura3-52  leu2-3,112  ura3::SLA1-SLA2-mCherry::KanMX6::URA3 SAC6-RFP::KanMX sla1Δ::NatNT2 sla2Δ::cgLEU2

AKY583  MATa  his3-Δ200  ura3-52  leu2-3,112  end3Δ  SLA1-GFP::KanMX6 MTW1-3mCherry::LEU2

AKY585  MATa  his3-Δ200  ura3-52  leu2-3,112  end3Δ  SLA1-GFP::KanMX6 MTW1-3mCherry::Leu2  end3Δ::HIS3

AKY561  MATa  his3-Δ200  ura3-52  leu2-3,112  end3Δ::HIS3  ura3::SLA1-SLA2-GFP::KanMX6::URA3 sla1Δ::NatNT2 sla2Δ::cgLEU2

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References


Chapter 3

Investigating the role of the SH3 protein interaction domain of Rvs167 in clathrin-mediated endocytosis

3.1 Introduction

Clathrin-mediated endocytosis (CME) is a vital cellular process that is critical to maintain cell homeostasis, attenuate receptor signaling, generate turnover of proteins at the plasma membrane (PM), and modulate lipid composition of the PM. In the budding yeast *S. cerevisiae*, there are approximately 60 proteins that coordinate to remodel the PM into a tubule that then pinches into a vesicle that travels to the cell interior. Endocytic sites progressively assemble with precise spatio-temporal regulation over approximately 75 - 135 s [1]. First, early endocytic scaffold proteins and cargo adapters assemble at endocytic sites [2–5]. These proteins are followed by assembly of coat proteins which are responsible for the association of the invaginating pit with the actin machinery [6–9]. Next, nucleation-promoting-factors (NPFs) are assembled approximately 30 s before vesicle scission. Although NPFs are present at the PM, there is approximately a 15 s delay before these NPFs activate Arp2/3-mediated actin assembly. The majority of membrane remodeling occur in these last 15 s, as the actin network associates with the coat and the polymerization provides force to remodel the PM into a tubule. In the last step, the amphiphysin module is recruited and acts as a key factor in scission [1, 2, 6].

CME is a highly conserved process amongst eukaryotes; however, budding yeast and mammalian cells exhibit some differences in the membrane remodeling steps of endocytosis [10]. Gaining a thorough understanding of differences and similarities between yeast and mammals may provide evolutionary insight into how the cytoskeleton can interact with and shape the PM. In yeast, the cell wall allows cells to grow under high pressure, necessitating an increased force to invaginate the PM [11, 12]. Thus, budding yeast CME relies heavily more on actin polymerization for membrane invagination than in mammalian cells, making it an
ideal model organism to study the role of actin in CME [2]. Another difference arises in the scission step. Dynamin is required for scission in mammalian cells, while the heterodimeric BAR proteins Rvs161/167 play a more prominent role in yeast [10]. The Rvs161/167 heterodimer was identified as the key factor for proper scission since loss of this dimer results in a unique phenotype, where 20% of endocytic sites begin to invaginate but then retracts back to the cortex [2]. The rvs167Δ mutant was used as a sensitized background to identify additional scission factors, whose loss increased the internalization-retraction phenotype up to 80% of events. Additional scission factors included the F-BAR protein Bzz1, the lipid phosphatase synaptojanin Sjl2, and NPF CA domains that are required to promote actin polymerization [13].

Although we know which factors contribute to scission, we still do not understand how these factors coordinate with one another to ensure faithful scission. Rvs161 and Rvs167 dimerize via their N-BAR domains to bind and tubulates membranes [14–16]. Rvs167 also contains a Gly-Pro-Ala rich region that is predicted to be unstructured, as well as an SH3 domain that can bind poly-proline (PXXP) motifs ([14–17], Figure 3.1A). During yeast CME, a network of SH3-PXXP interactions is important for protein recruitment and regulation. The role of Rvs167 SH3-mediated protein interactions has not been well studied. There have been several examples of BAR proteins that coordinate membrane binding/bending activity with regulation of actin assembly at the membrane [18–20]. From a large-scale SH3-PXXP interaction study, the Rvs167 SH3 domain was found to have the highest predicted interaction score with a PXXP motif found in the yeast nucleation promoting factor (NPF), Las17 [21]. Additionally, the Rvs161/167 heterodimer cooperates with actin during scission [13]. Based on these data, I hypothesized that Rvs167 SH3 interactions with Las17 PXXP regulate actin assembly during the scission step of endocytosis. To investigate this possibility, I tested Rvs167 for its ability to influence Las17 activated Arp2/3-mediated actin dynamics in vitro and in vivo.

3.2 Results

Rvs161/167 affects actin polymerization in vitro

Vesicle scission facilitated by Rvs161/167 since loss of this dimer causes the invaginating membrane to retract back to the cortex [2]. Rvs161/167 N-BAR domains form a heterodimer that binds and bends membranes. In addition to the N-BAR domain, Rvs167 also contains an SH3 domain that can bind poly-proline (PXXP) motifs ([14–17], Figure 3.1A). During yeast CME, a network of SH3-PXXP interactions is important for protein recruitment and regulation. The role of Rvs167 SH3-mediated protein interactions has not been well studied. There have been several examples of BAR proteins that coordinate membrane binding/bending activity with regulation of actin assembly at the membrane [18–20]. From a large-scale SH3-PXXP interaction study, the Rvs167 SH3 domain was found to have the highest predicted interaction score with a PXXP motif found in the yeast nucleation promoting factor (NPF), Las17 [21]. Additionally, the Rvs161/167 heterodimer cooperates with actin during scission [13]. Based on these data, I hypothesized that Rvs167 SH3 interactions with Las17 PXXP regulate actin assembly during the scission step of endocytosis. To investigate this possibility, I tested Rvs167 for its ability to influence Las17 activated Arp2/3-mediated actin dynamics in vitro and in vivo.
Figure 3.1: **Rvs167 protein domains.** (A) Cartoon representation of Rvs161 and Rvs167 dimerization via N-BAR domains. (B) Domain structure of full-length Rvs167 and truncations used in this study.

I first tested whether Rvs161/167 affects on Las17-activated, Arp2/3-mediated actin polymerization *in vitro* using pyrene actin assembly assay. In this assay, fluorescence intensity of pyrene-labeled G-actin monomers increases over time as actin polymerizes to yield F-actin. Arp2/3 requires activation by an NPF such as Las17. I purified Las17, the Arp2/3 complex, actin, and Rvs161/167 to test the effects of Rvs161/167 on actin polymerization (Figure 3.2A). As expected, Las17 increased actin polymerization compared to Arp2/3 and actin alone (Figure 3.2B and C). Addition Rvs161/167 at low concentrations enhances actin polymerization while higher concentrations inhibit actin polymerization (Figure 3.2B). I had hypothesized that Rvs167 regulation of actin dynamics would occur by directly binding to Las17. I therefore tested a version of the Rvs161/167 lacking the SH3 domain. I found that addition of Rvs16/167∆SH3 to Las17, Arp2/3, and actin resulted in a similar effect as Rvs161/167 (Figure 3.2B and C). At low concentrations, Rvs161/167∆SH3 enhanced actin polymerization. At high concentrations polymerization was inhibited. These results were not consistent with the idea that the Rvs167 SH3 domain mediates specific interactions with Las17 to influence actin polymerization. It is possible that these results reflect an *in vitro* artifact and therefore do that not accurately represent the biological behavior of these proteins. This could mean that the importance of specific protein interactions was not tested in this assay. It could also mean that in the complex environment of an endocytic site, Rvs161/167 does not influence actin polymerization.

Results from the *in vitro* polymerization assays are most informative when coupled with *in vivo* data. Actin polymerization can be tracked *in vivo* using an actin marker such as Sac6 or Abp1. Normally, fluorescently tagged Sac6 appears as cortical puncta on the PM that persist for 15 s before moving into the cell and disappearing (Figure 3.3A). One way to
Figure 3.2: Rvs161/167 can enhance or inhibit Las17 activated Arp2/3-mediated actin polymerization in vitro. (A) Purified Las17, Arp2/3 complex, Rvs161/167, and Rvs161/167Δ were separated by SDS-PAGE and stained with SYPRO Ruby Protein Gel Stain. (B) Actin was polymerized in the presence of purified Arp2/3 complex, Las17, and indicated concentrations of Rvs161/167 or (C) Rvs161/167ΔSH3.
Figure 3.3: **Rvs167 does not affect Sac6-GFP fluorescence intensity endocytic sites.** (A) Single frame from live-cell fluorescence microscopy of cells expressing Sac6-GFP. (B) Average Sac6-GFP fluorescence intensity of single patches tracked over time for mixed populations of indicated cells (error bars represent SEM).
CHAPTER 3. INVESTIGATING THE ROLE OF THE SH3 PROTEIN INTERACTION DOMAINS OF RVS167 IN CLATHRIN-MEDIATED ENDOCYTOSIS

compare actin levels at endocytic sites is to record Sac6-GFP fluorescence intensity over the lifetime of a cortical patch. To ensure an accurate comparison between two cell types, I mixed the cell types of interest on the same coverslip and used expression of kinetochore marker, MTW1-3mCherry, to distinguish between the two cell types. After comparing Sac6-GFP of rvs167Δ and MTW1-3mCherry cells, I found no noticeable change (Figure 3.3B). There are several endocytic proteins that regulate actin polymerization. For example, Bbc1 is a known inhibitor of the NPFs Las17 and Myo5. In a bbc1Δ mutant, the Sac6-GFP intensity is increased and projects farther into the cell than in wild-type cells ([2, 22], Figure 3.3B). However, mutants of some of the genes involved in actin regulation do not have as pronounced effects. For example, Syp1 negatively regulates Las17, but actin phenotypes marked by Sac6 fluorescence are nearly undetectable (Figure 3.3B). Syp1 was only identified as a negative regulator of Las17 in a screen in which SYP1 overexpression was able to suppress endocytic phenotypes exhibited by the arp2-7, which causes unregulated actin assembly [23]. Taken together, these data do not support the idea that Rvs167 regulates actin assembly. However, it is possible that Rvs167 regulation of actin assembly was not detectable under the conditions I tested.

The BAR-domain of Rvs167 is sufficient for its localization to endocytic sites but cannot fully rescue Rvs167 function

Since there were no noticeable changes to Sac6-GFP in rvs167Δ cells compared to wild-type cells, where the Rvs167 played a role in directly regulating the actin network has unclear. I had initially hypothesized that Rvs167 interacts with Las17 directly through an SH3-PXXP interaction. While Rvs167 had the highest predicted interaction score with Las17, there were several other potential binding partners and potential roles for protein-protein interactions. In CME, there are many SH3 and PXXP motifs that interact to promote rapid assembly, cooperation, and regulation of proteins at endocytic sites. I considered several possible roles for Rvs167 SH3-PXXP interaction: 1. an SH3-PXXP interaction might be responsible for the recruitment of Rvs167 to endocytic sites, 2. an SH3-PXXP interaction might recruit downstream proteins to endocytic sites, 3. an SH3-PXXP interaction might regulate Rvs167 function, and 4. an SH3-PXXP interaction could regulate other proteins at endocytic sites. To investigate these possibilities, I generated two Rvs167 truncation mutants, one removing the SH3 domain and the other leaving just the BAR domain (Figure 3.1B).

I first checked if the SH3 domain contributes to proper Rvs167 localization. I generated GFP tagged versions of the truncated strains, then confirmed that they were expressed at their expected sizes by western blotting (Figure 3.4). I found that Rvs167ΔSH3-GFP and Rvs167BAR-GFP exhibited similar phenotypes to the full length, Rvs167-GFP. Using Slal-mCherry as a marker for endocytic sites, I found that the Rvs167 truncations were recruited to the Slal-mCherry marked sites at approximately the same time as Rvs167-GFP. Additionally, Rvs167ΔSH3-GFP and Rvs167BAR-GFP exhibited similar fluorescence intensity levels to Rvs167-GFP, indicating that the truncated versions of Rvs167 were as
Figure 3.4: **Rvs167 truncations localize to endocytic sites.** Continued on next page.
Figure 3.4: Rvs167 truncations localize to endocytic sites. (A) Protein extracts from indicated strains were separated by SDS-PAGE and blotted using anti-GFP and anti-Pgk1. (B) Single frame from dual-color live-cell microscopy of various Rvs167-GFP, Rvs167ΔSH3-GFP, and Rvs167BAR-GFP with Sla1-mCherry. Montage of a single puncta, right. (C) Single frame from live-cell microscopy of indicated mixed cell populations. Fluorescence intensity plots of patch over time, right.

abundant as Rvs167 at endocytic sites (Figure 3.4). Previous work has shown that disruption of the Rvs167 N-BAR domain disrupts localization of the protein [15]. Together, these data support the idea that the SH3 domain is dispensable for Rvs167 localization to endocytic sites, while the N-BAR domain is required and sufficient for its localization.

I determined that the SH3 domain did not contribute to Rvs167 localization, which indicated that the SH3 domain might recruit other proteins to endocytic sites, or might be required for regulation of Rvs167, or might regulate the function of other proteins. Since there were many possible interaction partners and potential consequences of SH3-PXXP interactions, I chose a more general approach to investigate functional role of SH3 mediated protein interactions. There are many genetic interactions between rvs167 mutants and other endocytic mutants. Some are negative interactions that result in increased growth defects, while others result in increased internalization-retraction phenotype at endocytic sites. I generated several double mutants, combining endocytic mutants with rvs167, rvs167Δ, and rvs167bar to better understand the functional relationships between the protein products of these mutant genes. If negative interactions that normally occur with rvs167Δ are preserved in the double mutant with rvs167ΔSH3 and rvs167bar, this would suggest that this synthetic interaction was due to loss of the SH3 domain. If in the double mutant with rvs167ΔSH3 and rvs167bar exhibited a phenotypic rescue, this would suggest that the Rvs167 N-BAR domain is sufficient to replace Rvs167 function in this double mutant. It was important that the Rvs167 truncations were recruited to endocytic sites with a similar efficiency to Rvs167. Additionally, previous work found that the SH3 domain of Rvs167 is dispensable for membrane tubulation, suggesting that the truncated versions of Rvs167 would still maintain their membrane binding and bending activity. Thus, it would be unlikely that interactions between the rvs167 truncation mutants would result in failed mutant recruitment or non-functionality. Since there are a number of known genetic interactions, I started by testing a few: myo5ΔSH3, sac6Δ, bzz1Δ and las17Δ (Figure 3.5A). I found negative growth interactions with myo5ΔSH3, sac6Δ, bzz1Δ. In all cases for which there was a negative interaction with rvs167Δ, there was also a negative interaction with both the Rvs167 truncations. These data indicate that N-BAR domain may not be enough for proper Rvs167 functionality and point to the idea that the SH3 domain is important for at least some aspects of Rvs167 function.
Figure 3.5: Synthetic growth interactions between *rvs* mutants and other endocytic mutants. (A) Summary of mutants tested for growth interactions. (B) Negative synthetic growth interaction between *rvs* mutants and *bzz1Δ* or *myo5ΔSH3* mutants after 48 h at 25°C, 30°C, and 37°C.
3.3 Discussion

A potential role for Rvs161/167 in actin regulation at endocytic sites

Endocytosis is dependent on a network of protein-protein interactions that aid in rapid assembly of an endocytic site and ensure proper regulation of protein function. Rvs161/167 has been identified as a key component of the scission module, but how it works to ensure faithful scission is not fully understood. The Rvs167 N-BAR domain is required for Rvs167 recruitment to endocytic sites and is capable of tubulating membranes. Rvs167 also contains an SH3 protein interaction domain that could mediate protein-protein interactions during the scission step. Rvs167 was predicted to interact with the nucleation promoting factor (NPF) Las17. Since actin was identified as an additional scission factor, I initially hypothesized that Rvs167 might regulate Las17 NPF activity. There is precedent for regulation of actin networks at a membrane by BAR proteins. I tested this hypothesis in vitro and in vivo.

I determined the Rvs161/167 affects on Las17 activated Arp2/3-mediated actin polymerization using an in vitro actin pyrene assay. I determined that low concentrations of Rvs167 enhanced actin polymerization, while high concentrations inhibited actin polymerization (Figure 3.2B). If Rvs161/67 regulation of actin polymerization was dependent on SH3-PXXP interactions between Rvs167 and Las17, I would expect a decreased effect in the absence of the SH3 domain. I found that Rvs167 lacking the SH3 domain, showed similar effects on Las17-mediated Arp2/3 activation to full-length Rvs167 (Figure 3.2C). Overall, these results did not support the idea that the biochemical are mediated by the Rvs167 SH3 domain. I tested whether Rvs167 for in vivo regulates actin assembly by quantitation of the actin marker, Sac6-GFP using live-cell microscopy. I found that there were no noticeable changes in the extent of actin assembly at endocytic sites (Figure 3.3). Taken together, these data do not support the idea that Rvs167 regulates Las17 NPF activity. It is possible that the in vitro effects of Rvs161/167 on actin polymerization were due to an artifact and do not occur at endocytic sites.

While my data currently point to the idea that the in vitro results are due to an artifact, it is still possible that Rvs161/167 regulates Las17 NPF activity at endocytic sites. In the case of Bbc1, which inhibits Las17 and Myo5, there is a clear actin phenotype in the bbclΔ mutant. However, Syp1 inhibits Las17 in vitro and yet in vivo evidence from tracking fluorescently labeled actin markers to support such a role in cells. Endocytosis is a robust process that relies on many proteins. While Las17 has the strongest NPF activity, there are several NPFs present at endocytic sites. In a complex system such as this, it is possible that redundancy in protein function can mask in vivo effects of specific mutations. Therefore, we cannot rule out the possibility that Rvs161/167 plays a role in NPF regulation.
Investigating the importance of Rvs167 protein interactions in endocytosis

Rvs167 plays a central role in the scission step of endocytosis, but how it functions in this step is not entirely understood. The contributions of Rvs167 membrane bending activity and SH3-mediated proteins interaction have not been fully investigated. To understand better how these protein functions contribute to endocytosis, I generated truncations of Rvs167. Previous work found the Rvs167 N-BAR domain is required for proper localization to endocytic sites, and I found that it is also sufficient for proper localization (Figure 3.4). Since the SH3 domain did not contribute to localization, I wanted to investigate its functional contribution. There are several known negative genetic interactions between \textit{rvs167}\textsuperscript{∆} and other endocytic mutants. Rvs167 mutants were recruited to endocytic sites and the N-BAR domain still functioned to tubulate membranes. This provided an opportunity to look for genetic interactions between \textit{rvs167}\textsuperscript{∆}SH3 or \textit{rvs167}bar and endocytic mutants. I found that negative genetic interactions with \textit{rvs167}\textsuperscript{∆} resulting in growth defects were also present for \textit{rvs167}\textsuperscript{∆}SH3 and \textit{rvs167}bar (Figure 3.5). These data indicate that resultant gene products from \textit{rvs167}\textsuperscript{∆}SH3 or \textit{rvs167}bar are unable to rescue function of Rvs167, and suggest that the SH3 domain plays an important role in cells.

Future directions

I initially hypothesized that Rvs167 might play a role in actin assembly regulation. Comparing fluorescence intensity of actin markers was informative for some NPF regulators, but not others. We know which NPFs have the strongest \textit{in vitro} activity and also have insight to how these NPFs initiate actin polymerization \textit{in vivo} [22]. It would still be interesting to investigate these NPFs more thoroughly \textit{in vivo} to compare the relative amounts of actin assembly at endocytic sites. A simple set of follow up experiments would be to compare fluorescence intensity of actin markers in various mutants that lack essential NPF domains such as \textit{las17}CA and \textit{myo5}CA. It is possible that NPF activity of Las17 or Myo5 can compensate for NPF activity of the other when one is lost. Additionally, these experiments might hint at the relative contribution of each NPF. If one NPF can partially compensate for the other, using \textit{las17}CA or \textit{myo5}CA mutants could serve as a sensitized background to study regulation of remaining NPFs by other genes. Quantitative molecule counting at endocytic sites has provided information about site composition and regulation of actin in \textit{S. pombe} [24]. It would be even more informative if data from fluorescence intensity comparisons could be coupled with molecule numbers at endocytic sites.

Combining \textit{rvs167} truncations with other endocytic mutants revealed that the N-BAR domain of Rvs167 is not sufficient to rescue all of Rvs167 function. These results indicate that there is a potentially important role for the SH3 domain of Rvs167. In addition to the four endocytic mutants I tested, there are several other endocytic mutants that exhibit negative interactions with \textit{rvs167}\textsuperscript{∆}. Extending the study to include these genes could provide even more information. I determined that \textit{las17}\textsuperscript{∆} \textit{rvs167}\textsuperscript{∆} double mutant does not have
a growth phenotype. From tracking Sla1-GFP in live-cell microscopy, Kishimoto et. al. determined that increased rate of the internalization-retraction phenotype which is quantified using live-cell microscopy of Sla1-GFP [13]. It will be important to investigate if the SH3 domain contributes to the scission step of endocytosis. Using live-cell microscopy to observe Sla1-GFP in las17Δ with rvs167 truncation mutants would indicate if the BAR domain is sufficient to rescue the increase in internalization-retraction phenotype normally exhibited by the las17Δ rvs167Δ double mutant.

The growth interaction data is promising, but does not provide any mechanistic information. To fully understand the importance of any potential interactions, future studies should focus on understanding the molecular basis of these genetic interactions. I determined that the Rvs167BAR is sufficient for Rvs167 localization to endocytic sites in wild-type cells. First, it should be confirmed that the localization of Rvs167 truncations are not disrupted in the double mutants. Tracking endocytic dynamics can provide important information about how endocytosis is disrupted. Using two-color live-cell microscopy to track a coat marker such as Sla1-GFP and an actin marker such as Sac6-RFP can allow us to see what is happening to endocytic dynamic in these double mutants. The double mutant rvs167Δ sac6Δ was not viable. The auxin-inducible degron tag allows for induced degradation of a protein of interest. This system is ideal for studying loss of a protein where a full gene deletion would normally result in be an ideal system to use to better understand the molecular basis for understanding what happens when both Rvs167 and Sac6 are lost in cells. Additionally, in these studies I used truncation mutants to broadly probe at the function of Rvs167 domains. Since my data indicate that the SH3 domain could play an important role in endocytosis, future studies should use a loss of function point mutation in the SH3 domain as a more specific test of this hypothesis.

3.4 Materials and methods

Yeast strains and plasmids

Yeast strains used in this study were derived from S288C and are listed in Table 2.1. The rvs167 truncation mutants were generated by integrating a natNT2 selection cassette or GFP tag with HIS3 selection cassette at +1281 for the rvs167ΔSH3 mutant and at +755 for the rvs167bar mutant. Correct integration and sequences were verified by PCR and DNA sequencing.

Protein purification

Las17 and the Arp2/3 complex were purified essentially as reported [25]. DDY1810 yeast was transformed with Pgal-Las17-TEV-13Myc or Pgal-Arc18-TEV-13Myc plasmids. Overnight cultures were used to inoculate a 1 L culture SM/2% raffinose/DO-URA at 0.2 OD600 and grown for 24 hours at 30°C. Cultures were induced using 2% galactose for 10
hours. Cells were harvested by centrifugation, drop frozen in liquid nitrogen and lysed using a blender (Waring) cooled with liquid nitrogen. Cell powder was thawed in 1/4 v/w ratio of 100 mM HEPES (pH 7.5), 5 mM EDTA with complete Protease Inhibitor Cocktail (Roche). Crude lysate was centrifuged for 20 min at 80,000 rpm in a Beckman TLA100.3 rotor (Beckman Coulter). Clarified lysate was incubated with 800 µl of protein A-CL4B (Amersham Pharmacia Biotech) beads that had been saturated with anti-myc 9E10 monoclonal antibody (Pierce) for 1.5 h. Beads were washed 5X with 10X volume of 20 mM Hepes (pH7.5), 1 mM EDTA, 500 mM KCl. Las17 beads were washed once extra with the same buffer containing 1 M KCl. Beads were resuspended in 200 µl of 20 mM Hepes (pH 7.5), 1 mM EDTA, 50 mM KCl. 6HIS-TEV protease was incubated with beads for 2 h at room temperature, then removed using Ni-beads (Roche). Supernatant was collected and combined with eluate from 2X bead washes with 1X volume of 20 mM Hepes (pH 7.5), 1 mM EDTA, 50 mM KCl (1 M KCl for Las17). The protein was concentrated using a centricon, dialyzed overnight at 4°C into 20 mM Hepes (pH 7.5), 1 mM EDTA, 50 mM KCl, then flash frozen in liquid N2.

Rosetta E. coli cells were transformed with dual expression cassettes with Rvs161/Rvs167-6HIS. E. coli were grown under antibiotic selection. A 1 L culture was grown to 0.6 OD600, then induced for expression using 0.5 mM IPTG. After 2.5 hours, were harvested by centrifugation, washed in 500 ml 20 mM HEPES (pH 7.5). Cells were resuspended in 20 mM HEPES (pH7.4), 250 mM KCl, with one tablet of Roche complete EDTA free protease inhibitor cocktail, then lysed using sonication (Newbrunswick). Crude lysate was centrifuged at 40,000 rpm in a Ti60 rotor (Beckman). Clarified lysate was injected over a 1 ml HIS-trap column (GE Healthcare) connected to an AKTA FPLC (GE Healthcare). The HIS-trap column was washed with 20 ml 20 mM imidazole, then eluted over 10 ml with a 20 mM - 500 mM imidazole gradient. Fractions containing the protein were pooled, dialyzed overnight into 20 mM HEPES (pH7.5), 500 mM KCl, then snap frozen into liquid N2.

Actin was purified from rabbit skeletal muscle acetone powder as previously described [26]. Briefly, rabbit skeletal muscle acetone powder was re-hydrated in G-buffer (5 mM Tris-HCl, pH 8.0, 0.2 mM ATP, 0.1 mM CaCl2, and 0.5 mM DTT), polymerized, and washed with buffer containing 1 M KCl to remove associated proteins. Polymerized actin was resuspended in G-buffer, dounced, sonicated (New Brunswick), then dialyzed into G-buffer for 3 days, exchanging buffer every 12 h. Monomeric actin was centrifuged at 90,000 rpm in a TLA100 rotor (Beckman) for 3 hr to remove oligomers/nuclei. Actin was further separated from oligomers/nuclei by gel filtration chromatography using on a Sephacryl S-300 (GE Healthcare). Pyrene actin was generated by labeling Cys-374 to a stoichiometry of 0.8:1.0 with pyrene iodoacetamide.

Purified protein and BSA standards were separated on SDS-PAGE gels then stained with SYPRO Ruby Protein Gel Stain (Molecular Probes). Protein concentrations were calculated using a BSA standard curve generated by calculating band intensity from ImageJ.
Pyrene actin assembly assay

Pyrene actin assembly assays were performed with 2 µM rabbit muscle actin containing 3% pyrene actin in the presence of 5 nM Arp2/3 complex, 5 nM Las17 and the indicated concentrations of Rvs161/167 or Rvs161/167∆SH3. Mg-ATP-actin was first prepared by incubating Ca-ATP-actin on ice with 0.2 mM EGTA and 11-fold molar excess of MgCl₂ for 10 min. Actin and relevant proteins were added to a 96-well plate containing KMEI to a final concentration of 100 mM KCl, 2 mM MgCl₂, 1 mM EGTA, and 10 mM imidazole (pH7.0). Fluorescence intensity was measured using a Tecan Infinite M1000 plate reader (Tecan Trading).

Western blotting

Yeast were cultured in YPD starting at a density of 0.2 OD₆₀₀ and harvested at OD₆₀₀ = 0.6 - 0.8. Yeast lysis and protein extraction were performed by standard trichloroacetic acid protocol. Equal amounts of protein extracts were separated on SDS-PAGE gels, transferred to a nitrocellulose membrane, then probed with rabbit anti-GFP antibody (Molecular Probes, Eugene, OR), or rabbit anti-Pgk1 (Invitrogen). Primary antibodies were detected with secondary LI-COR IRDye and VRDye and imaged on an Odyssey infrared scanner (LI-COR Biosciences, Lincoln, NE).

Microscopy

Images were acquired using a Nikon Ti with a 100X NA1.4 objective and an Andor Neo 5.5 SCMOS camera (Andor Technology USA, South Windsor, CT). Two-color movies were acquired using an FF505/626-Di01 dual-pass dichroic mirror and FF01-524/628-25 dual-pass emission filters (Semrock, Rochester, NY) with sequential 750 ms exposure times with a 500 ms lag between each exposure. Cells expressing MTW1-mCherry for identification were imaged as a single-color GFP movie followed by an mCherry Z-stack containing 13 planes 0.3 µm apart represented as a maximum intensity projection. Images were analyzed using ImageJ and a plugin (courtesy of Jasper Weinberg) that generates radial kymographs in 2-degree increments. Fluorescent lifetimes of proteins were calculated by measuring the time a patch persisted on the cortex before moving into the cell or disappearing. Fluorescence intensity of a patch over time was collected using Matlab to select a 232.5 nm x 232.5 nm box around a well-isolated patch. The average intensity of the box was plotted over time. Background fluorescence was subtracted using an exponential decay fit to at least 5 frames before and after the patch. Multiple patch intensities were aligned using the maximum intensity. All imaging was performed at 23°C in synthetic media. Cells were imaged in a live-cell imaging chamber made by coating a coverslip with Conaavalin A (1µg/ml). The coverslip was subsequently inverted onto a slide and sealed with vacuum grease.
Yeast growth analysis

Yeast spot growth assays were performed by diluting an overnight liquid YPD culture to 1 OD_{600}, performing 1/10 serial dilutions, and spotting 4µl of each dilution onto a YPD plate. Yeast was grown at the indicated temperatures for 48 hours before imaging.
### Table 3.1: Yeast strains used this study

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\text{rvs167}\Delta\text{SH3::NatNT2}
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\text{rvs167bar::natNT2}
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AKY331  
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AKY361  
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\text{rvs167}\Delta::\text{NatNT2}
\]

AKY357  
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\text{rvs167}\Delta\text{SH3::NatNT2}
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AKY359  
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\text{rvs167bar::NatNT2}
\]

AKY456  
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\]
CHAPTER 3. INVESTIGATING THE ROLE OF THE SH3 PROTEIN INTERACTION DOMAIN OF RVS167 IN CLATHRIN-MEDIATED ENDOCYTOSIS

References


CHAPTER 3. INVESTIGATING THE ROLE OF THE SH3 PROTEIN INTERACTION
DOMAIN OF RVS167 IN CLATHRIN-MEDIATED ENDOCYTOSIS

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Chapter 4

Investigating a role for App1 in clathrin-mediated endocytosis

4.1 Introduction

Clathrin-mediated endocytosis (CME) is an essential cellular process that is responsible for the uptake of nutrients and for proper lipid distribution within a cell. In this process, the plasma membrane (PM) invaginates into a tubule which is pinched into a vesicle that travels into the cell. In budding yeast, over 60 different proteins localize to endocytic sites. They can be be grouped into modules based on their time of arrival at endocytic sites and their predicted function [1–3]. By understanding how these proteins coordinate the dramatic membrane remodeling steps we not only gain insights into the mechanisms that govern endocytosis, but also increase our basic understanding of the membrane as a dynamic structure.

Endocytic sites develop over the course of 60 - 120 s. However, the most dramatic membrane remodeling steps occur just 15 s before vesicle scission. During this time, actin-associated proteins aid in the polymerization of actin at the cortex, which is thought to provide the majority of the force required for membrane invagination [4]. Scission of the vesicle is primarily dependent on the heterodimeric BAR protein, Rvs161/Rvs167 since loss of this dimer causes the invaginating membrane to retract back to the cortex [1]. Additional factors such as the F-BAR protein Bzz1, the lipid phosphatase synaptojanin Sjl2, and nucleation promoting factors (NPFs), which promote actin polymerization, also contribute to scission [5]. Since BAR-domain proteins have a rigid curved structure, they are capable of molding the membrane [6–8]. Additionally, these factors can change the organization and dynamics of lipid molecules within the membrane and therefore, the membrane’s physical properties. BAR-domain proteins have been shown to restrict lipid flow [9] and Sjl2 catalyzes the conversion of PI(4,5)P$_2$ to PI(4)P and PI [10]. Both of these mechanisms can segregate lipids to generate a line tension which is thought to promote scission [11]. In order to gain a more comprehensive picture of this process, it is important to investigate other potential
CHAPTER 4. INVESTIGATING A ROLE FOR APP1 IN CLATHRIN-MEDIATED ENDOCYTOSIS

membrane modifying proteins that may be contribute to membrane remodeling.

One enzyme that may contribute to membrane remodeling during endocytosis is Actin Patch Protein App1. Samanta et al. identified App1 as a protein that associates with actin patches, which are sites of endocytosis, after using network-based statistical algorithm applied to large-scale interaction data to assign unannotated proteins to functions [12]. Later, Chae et al. revealed App1 to be phosphatidate phosphatase (PAP), which catalyzes de-phosphorylation of phosphatidic acid (PA) to yield diacylglycerol (DAG) [13]. A careful biochemical analysis involving the four known PAPs in budding yeast found that there are no global changes to the lipid composition in app1Δ cells, suggesting that App1 may not be involved in lipid synthesis, but rather in regulation of local PA and DAG concentration [14].

Studies show that regulation of PA and DAG concentration facilitates many fusion/fission events in both yeast and mammalian cells [15]. It is possible that the same mechanisms that facilitate these events also act in endocytosis. First, there are many examples of proteins that bind preferentially to PA or DAG, causing specific recruitment of various proteins [16–18]. Alternatively, conversion of PA to DAG may alter the lipid geometry to facilitate membrane deformation. Although both PA and DAG favor negative membrane curvature, absence of the phosphate moiety in DAG results in a reduced headgroup size compared to PA [15]. Four PAPs have been identified in yeast: Pah1, Lpp1, Dpp1 and App1. Pah1 is responsible for de novo lipid synthesis at the endoplasmic reticulum (ER), producing DAG as an intermediate step in the formation of triacylglycerol (TAG) [19], whereas Lpp1 and Dpp1 act locally at the Golgi and vacuole, respectively [20–24]. App1 has been recently identified as a PAP but its cellular function has not been identified. Due to the large number of App1 associations with endocytic proteins and the importance of PA to DAG conversion in fission/fusion events, I investigated a potential role for App1 in endocytosis.

4.2 Results

App1 is enriched on mitochondria and occasionally in puncta on the plasma membrane

App1 was been identified as a potential endocytic protein due to its interactions with known endocytic proteins [12]. App1 is a phosphatidate phosphatase (PAP), which catalyzes the conversion of phosphatidic acid (PA) to diacylglycerol (DAG) [13]. Since PA to DAG conversion can aid in membrane fission, I investigated a potential role for App1 in endocytosis. First, I aimed to track localization of App1 to identify any subcellular structures at which it might function. Visualizing App1-GFP was difficult due to its low expression level, so I tagged endogenous APP1 with 3GFP and tested for expression by western blotting. I found that App1-3GFP ran close to the expected size of 99kDa (Figure 4.1A). Unfortunately, I could not test the functionality of the tagged protein since the null has no detectable phenotype and there are no known synthetic growth interactions with app1Δ in our strain background. App1-3GFP signal was dim and in all cells it was predominately distributed
throughout the cytoplasm. GFP signal was enriched on structures that were fairly stable over a 60 s movie (Figure 4.1B). These structures were identified as mitochondria by their colocalization with MitoTracker Red (Figure 4.1C). In addition, 3% of cells, there were additional puncta that formed on the cortex of cell and persisted for approximately 10 s before disappearing (Figure 4.1D). Previously, App1 enzymatic activity as reported to be enriched on mitochondria, in the cytoplasm, and on the plasma membrane, which fits well with the App1-3GFP localization I observed.

To identify a potential role for App1 in endocytosis, I first checked for synthetic growth interactions between \textit{app1}\textsuperscript{\textDelta} and endocytic mutants. However, I did not identify any interactions (Figure 4.2). In budding yeast, endocytic sites progress with a stereotyped spatiotemporal accumulation of proteins, which is often disrupted in endocytic mutants [1]. I observed the endocytic coat protein Sla1-GFP dynamics in both wild-type and \textit{app1}\textsuperscript{\textDelta} cells. There were no noticeable defects Sla1-GFP in \textit{app1}\textsuperscript{\textDelta} (Figure 4.3A). The lifetime of Sla1-GFP at endocytic sites in wild-type cells was 29.9 ± 5.5 s, which was not significantly different from the lifetime of 29.4 ± 5.4 s in \textit{app1}\textsuperscript{\textDelta} cells (Figure 4.3B). Since App1 is a PAP, I reasoned that it could be acting in the later stages of endocytosis where the membrane undergoes major deformation. Many of the endocytic factors involved with the scission step of endocytosis were only identified by their strong synthetic genetic interaction with \textit{rvs167}\textsuperscript{\textDelta}, which resulted in an increased internalization-retraction phenotype, that can be identified by tracking the Sla1-GFP signal in mutants [5]. In \textit{app1}\textsuperscript{\textDelta} \textit{rvs167}\textsuperscript{\textDelta} cells, I found a slight increase in the number of Sla1-GFP patches that do not internalize or that exhibit the internalization-retraction phenotype (Figure 4.3C).

### 4.3 Discussion

**Potential biological roles for App1**

App1 is a phosphatidate phosphatase (PAP), an enzyme that catalyzes the conversion of phosphatidic acid (PA) to diacylglycerol (DAG) [13]. The relative concentration of these lipids is important for regulation of lipid composition, differential recruitment and binding of proteins to a specific membrane, as well as aiding in fission and fusion events [15–18]. Although App1 has been identified as a PAP, its biological function has yet to be identified. I observed that App1-3GFP has a cytoplasmic localization with enrichment on mitochondria. Additionally, in very small subpopulation of cells, I found that small puncta appear on the plasma membrane (Figure 4.1). This localization corresponds well with where the enzymatic activity was found, which was primarily cytoplasm, on the mitochondrial membrane, and on the plasma membrane (PM), suggesting that the fluorescence localization faithfully reflects the biological location of App1. In mammalian cells, mitochondrial fusion is promoted by the conversion of cardiolipin to PA, which is catalyzed by MitoPLD, and fission is promoted by the conversion of PA to DAG, which is catalyzed by Lipin1. Additionally, PA concentrations in mammalian cells are important for kinesin-mediated association of mitochondria with
Figure 4.1: Localization of App1-3GFP. Continued on next page.
Figure 4.1: (A) Protein extracts from \textit{APP1-3GFP} yeast strains were separated by SDS-PAGE and blotted for using anti-GFP. (B) Single frame from live-cell fluorescent imaging movie of \textit{APP1-3GFP} cells on the left, montage of 1 frame per s for 30 s on the right. (C) Single frame from dual-color live-cell fluorescent imaging movie of \textit{App1-3GFP} cells stained with MitoTracker Red. (D) Single frame from live-cell fluorescent imaging movie of subpopulation of \textit{APP1-3GFP} cells on the left. Montage of 1 frame per s for 30 s on the right. Kympgraph of single puncta, below.

Figure 4.2: \textbf{Synthetic growth test of \textit{app1Δ} with endocytic mutants.} Growth of \textit{app1Δ}, endocytic mutants, or double mutants at after 48 h at 30°C, 37°C

mitrotubules [15]. Since App1 was predominately enriched on mitochondria, it is possible that App1 could act as a functional homolog to Lipin1. In the future it is important to investigate a potential role for App1 in regulation of PA/DAG levels at mitochondria.

\textbf{App1 in endocytosis}

App1 was hypothesized to be an endocytic protein because it hares an extensive interaction network with other endocytic components. However, it has never been shown to play a role in endocytosis. It is clear that App1 localizes to mitochondria, but that does not exclude it from also functioning at endocytic sites. In a small subpopulation of cells, I found that App1-3GFP localizes to bright puncta at the PM. It is possible that App1 localizes to the
Figure 4.3: **Endocytic dynamics marked by Sla1-GFP in wild-type and app1Δ cells.** (A) Single frame from live-cell fluorescent imaging movie of SLA1-GFP in wild-type or app1Δ cells. (B) Sla1-GFP lifetimes calculated from movies collected in A (n.s. = not statistically significant, Student’s t-test). (C) Kymographs from movies in A were scored as Sla1-GFP signal disappearing without movement away from the cortex (no movement), movement away from the cortex (internalization), or movement away from the cortex followed by retraction back to the cortex (internalization-retraction).
plasma membrane more robustly under specific conditions that I was not able to identify. App1 might be required at endocytic sites when the PM is resistant to deformation due to factors such as high membrane tension, or when the cell is under stress. Therefore, I tested a few conditions such as osmotic shock by adding high salt or sorbitol, as well as heat shock, and found no change in App1 localization. Since endocytosis is a highly dynamic process that is confined to a diffraction limited point on the plasma membrane, it can be difficult to visualize enzymes that may be present at low concentrations and for short periods of time. It is possible that App1 acts at endocytic sites, but it is too dim to visualize using live-cell microscopy. In app1Δ cells, I found subtle defects in scission exacerbated by rvs167Δ (Figure 4.2 and 4.3). I checked for colocalization between App1-GFP and the endocytic actin marker Sac6-RFP, but in these cells I did not observe the puncta at the membrane. Based on this observation, it is possible that there was a subpopulation of App1-3GFP cells with stable localization to puncta and that these cells were not selected when I generated the App1-GFP Sac6-RFP strain. Since I did not observe App1 localization at endocytic sites or a strong endocytic phenotype in app1Δ cells, I was unable to demonstrate a direct role for App1 in endocytosis.

**Future directions**

Since App1-3GFP colocalizes with mitochondria, future experiments should investigate a potential role for App1 at mitochondria. The S228c yeast strain background is a widely used laboratory strain, especially for studies of CME, but contains a mutated allele of HAP1. The HAP1 gene encodes for a transcriptional regulator of electron transport, which is important for mitochondrial function [25]. Thus, S288c is not suitable for mitochondrial studies and the role of App1 should be investigated in another strain background, such as W303. Live-cell microscopy to visualize App1-3GFP should therefore be repeated in the appropriate strain background. Mitochondrial studies could start by looking for a role for App1 in regulating mitochondrial fusion/fission events.

Lipid bilayers are modified and regulated in many ways. Future studies should aim to understand how these modifications allow the PM to behave properly. In these future studies it will be interesting to understand how membranes behave under standard as well as various stress conditions. A key to performing these experiments will be to develop a robust *in vitro* system that includes many of the endocytic components.

### 4.4 Materials and methods

**Yeast strains**

Yeast strains used in this study were derived from S288C and are listed in Table 4.1. APP1-3GFP and app1Δ strains were made as previously described [26, 27]. Yeast strains
were grown in rich media (YPD) or synthetic media with appropriate amino acid supplements at 25°C unless otherwise stated.

**Yeast growth test**

Yeast spot growth assays were performed by diluting an overnight liquid YPD culture to 1 OD$_{600}$, performing 1/10 serial dilutions, and spotting 4 µl of each dilution onto a YPD plate. Yeast were grown at indicated temperatures for 48 hours before imaging.

**Microscopy**

Images of App1-3GFP were acquired using a Nikon Ti with a 100X NA1.4 objective and an Andor Neo 5.5 SCMOS camera (Andor Technology USA, South Windsor, CT). Single-color movies were collected at 1 s intervals with 1 s exposure at 25 power for GFP. Two-color movies were acquired using a FF505/626-Di01 dual-pass dichroic mirror and FF01-524/628-25 dual-pass emission filters (Semrock, Rochester, NY) with sequential 1 s exposure times with a 400 ms lag between each exposure. Single-color movies of SLA1-GFP were acquired on an IX71 Olympus epifluorescence microscope using a 100X NA 1.4 objective and an Orca-ER camera (Hamamatsu, Hamamatsu City, Japan) with a 1 s exposure time collected during a 90 s movie. Images were analyzed using ImageJ and a plugin (courtesy of Jasper Weinberg) that generates radial kymographs in 2-degree increments. Fluorescent lifetime of Sla1-GFP was calculated by measuring the time a patch persisted on the cortex before moving into the cell or disappearing. Sla1-GFP internalization, internalization-retraction, and no movement phenotypes were scored from kymographs as described previously [5]. All imaging was performed at 23°C in synthetic media. Cells were imaged in a live-cell imaging chamber made by coating a coverslip with Concanavalin A (1µg/ml). The coverslip was subsequently inverted onto a slide sealed with vacuum grease.
### Table 4.1: Yeast strains used this study

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<th>Genotype</th>
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CHAPTER 4. INVESTIGATING A ROLE FOR APP1 IN CLATHRIN-MEDIATED ENDOCYTOSIS

References


