DISSERTATION

CARGO INDUCED RECRUITMENT OF THE ENDOCYTIC ADAPTOR SLA1 AND THE ROLE OF SLA1-CLATHRIN BINDING IN ENDOCYTOSIS

Submitted by

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ABSTRACT

CARGO INDUCED RECRUITMENT OF THE ENDOCYTIC ADAPTOR SLA1 AND THE ROLE OF SLA1-CLATHRIN BINDING IN ENDOCYTOSIS

Clathrin-mediated endocytosis is a highly dynamic process that is essential in all eukaryotes. This process is utilized for a number of functions including the uptake of extracellular nutrients, manipulation of the plasma membrane content, downregulation of cell signaling pathways, and viral entry. While differences in protein composition, sequence, and structure do exist between species for this process, many core protein functions and the mechanistic steps involved in endocytic vesicle formation and internalization are highly conserved. This has allowed findings from one species to be applicable to another. For this reason *Saccharomyces cerevisiae* has been characterized as a highly useful model organism for studying and identifying key proteins and conserved mechanisms in clathrin-mediated endocytosis that are found in all eukaryotes. In yeast, roughly 60 proteins have been identified as being part of the endocytic machinery. Clathrin-mediated endocytosis begins with the recruitment of early endocytic proteins that establish the site of endocytosis. This includes scaffolding and coat proteins, such as clathrin, that aggregate at the plasma membrane through interactions with lipids, protein cargo, and other components of the endocytic machinery. This is followed by recruitment of other late coat proteins that further prepare the site for internalization. Following coat formation the mobile phase of membrane invagination is initiated by the recruitment of the actin polymerization machinery. Actin polymerization then generates an inward force at the site of endocytosis that causes invagination of the plasma membrane. The

invagination is then separated from the plasma membrane through the recruitment of scission proteins that pinch off the endocytic vesicle. Lastly the internalized vesicle undergoes a process of coat protein disassembly before being targeted to its proper destination in the cell. While much of this process has been well characterized, significant gaps in our understanding of how different steps in endocytic progression are coordinated and how endocytic proteins function still exist. Using a combination of yeast genetics, fluorescent microscopy, electron microscopy, and biochemistry we have furthered our understanding of clathrin-mediated endocytosis, focusing on the role adaptor-clathrin and adaptor-cargo binding plays in formation and progression of the endocytic process.

Our work began by focusing on the role of the adaptor protein Sla1, a clathrin and cargo binding protein that serves essential roles in endocytosis. It was previously established that Sla1 binds clathrin through a variable clathrin box of sequence LLDLQ. Loss of clathrin binding by mutation of this clathrin box has a dramatic effect on endocytosis such as an increased patch lifetime of Sla1 at endocytic sites, and dramatic defects in internalization of endocytic protein cargo. While these experiments demonstrated the importance of Sla1-clathrin binding in endocytosis, they did not explain why Sla1-clathrin binding was important and how this interaction contributes mechanistically to endocytic progression. By imaging Sla1 and clathrin, our work demonstrates that Sla1 contributes to proper clathrin recruitment to endocytic sites. A loss of proper recruitment of clathrin to endocytic sites by mutation of the Sla1 variable clathrin box also resulted in significant accumulation of other endocytic proteins, including those involved in actin polymerization. The lifetime of these additional endocytic components lasted for significantly longer at endocytic sites, some having a disruption in normal recruitment dynamics. Despite this accumulation of the actin polymerization machinery, there is a significant

delay in actin polymerization and an increase in actin polymerization time and levels at endocytic sites. Our results also demonstrate defects in the formation of the endocytic invagination and delays in scission. Thus, the Sla1-clathrin interaction is needed for normal progression through different stages of the endocytic process.

A second question in the endocytic field that has received little attention is the role cargo plays in the recruitment of the endocytic machinery. The conventional view is that first the endocytic machinery forms an endocytic site and then cargo is concentrated by binding adaptor proteins. Sla1 has previously been shown to bind to endocytic protein cargo that contains the amino acid sequence NPFxD through its SHD1 domain. It has also been shown through biochemical experiments that Sla1 binds Ubiquitin via its third SH3 domain. Both NPFxD and Ubiquitin have been shown to be important signals of cargo for entry into the endocytic pathway. The question, however, remained as to whether cargo binding via these signals contributes to recruitment of the adaptor Sla1 to endocytic sites. The work described in this dissertation will present evidence that this is indeed the case.

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CHAPTER 1

INTRODUCTION

1.1 Overview

In order for eukaryotic cells to maintain homeostasis, proper protein trafficking between intracellular organelles is crucial. One of these important trafficking pathways is clathrin-mediated endocytosis (CME). This process serves numerous functions including nutrient uptake, regulation of intracellular signaling pathways, controlling membrane lipid composition, cell wall synthesis, and can even be hijacked as a mechanism for viral entry. The process itself takes place at the plasma membrane, where endocytic proteins aggregate in a highly dynamic and coordinated fashion eventually resulting in the internalization of an endocytic vesicle. These endocytic proteins are recruited through interactions with both proteins and lipids of the plasma membrane, as well as other components of the endocytic machinery. This highly choreographed process functions to induce membrane receptor protein clustering, remodeling of the plasma membrane composition and structure, and finally the internalization of an endocytic vesicle that will be targeted to its proper destination in the cell, usually endosomes.

Clathrin-mediated endocytosis begins with the initial recruitment of scaffold and adaptor coat proteins that establish the site of endocytosis. These proteins are necessary for the recognition and aggregation of endocytic cargo, as well as the recruitment of a number of endocytic proteins including clathrin, the defining unit of the process. Following coat formation, actin polymerization generates a force perpendicular to the plasma membrane thereby driving invagination of the endocytic site. A combination of changes in lipid composition and membrane

curvature then induces recruitment of scission proteins that sever the neck of the membrane invagination, resulting in the internalization of the coated vesicle. Following internalization the protein coat is disassembled and the vesicle is targeted to an intracellular compartment, usually endosomes. While numerous studies have brought significant clarity to how clathrin-mediated endocytosis occurs and why this process is so important to cell homeostasis and viability, science has yet to fully define how this process takes place, but also what proteins are involved and how they interact with each other and components of the plasma membrane. Different parts of the endocytic machinery are still being identified and new roles and functions for endocytic proteins are currently being identified. In our studies, we have brought insight into the role endocytic adaptors play in clathrin recruitment, as well as the role cargo plays in recruitment of the endocytic machinery and progression of endocytosis.

Sla1 (synthetic lethal with actin binding protein 1) is an endocytic clathrin adaptor protein that has been demonstrated to be necessary for proper endocytic site formation and progression in *Saccharomyces cerevisiae*. Work from our lab and others have demonstrated that among the numerous roles Sla1 plays in endocytosis, its role in clathrin binding and protein cargo binding are of significant importance. It was demonstrated that Sla1 directly binds clathrin and that this interaction is important for proper endocytosis and receptor internalization. It has also been shown that Sla1 directly binds to a membrane receptor sorting signal necessary for the proper internalization of specific membrane proteins. While these revelations were very informative as to the role of Sla1 and adaptors in endocytosis, they also left a number of questions unanswered. For instance, what is the role of Sla1-clathrin binding in endocytosis and how is this interaction important for normal endocytic progression? In the endocytic field it is also unclear as to whether binding of membrane protein cargo is important for adaptor

recruitment, versus serving a more passive role, and not being necessary for endocytosis to occur.

In the studies presented here it will be demonstrate through the use of fluorescence microscopy, biochemistry, and electron microscopy techniques in yeast that Sla1 contributes to clathrin recruitment to endocytic sites and that this interaction is important for normal recruitment of the endocytic machinery. It will also be shown that proper Sla1-clathrin binding is necessary for proper transition from the late coat formation phase of endocytosis to actin polymerization, and that reduced levels of clathrin at endocytic sites disrupts normal membrane shaping during invagination of the endocytic site. Our results also demonstrate that Sla1-cargo binding plays an important role in adaptor Sla1 recruitment, that ubiquitinated cargo may be targeted by Sla1 while also contributing to Sla1 recruitment, and that improper recruitment of Sla1 to endocytic sites results in its nuclear localization.

1.2 Intracellular trafficking and clathrin-independent endocytosis

1.2.1 Membrane protein trafficking and endocytosis

All life as we know it can be divided into the three domains: Archaea, Bacteria, and Eukarya. Organisms under the taxon Eukarya are referred to as eukaryotes and are commonly defined as cells containing both a nucleus and membrane-enclosed organelles. Both the membrane-enclosed nucleus and organelles are distinctly defined from one another based upon properties including lipid, protein, and DNA composition, as well as a specific pH and ionic concentrations. In order for the cell to maintain homeostasis of these structures a proper trafficking pathway for lipids and proteins is necessary. These pathways can be broadly simplified into a secretory pathway, an endocytic pathway, and a recycling pathway.

The secretory pathway begins with the production of both soluble and integral membrane proteins and lipids in the endoplasmid reticulum (ER), and trafficking of properly folded proteins and lipids, in the form of vesicles to the golgi apparatus. Some of these proteins and lipids remain components of the golgi network; however, if necessary, further vesicular budding and transport from the golgi network to downstream endomembrane compartment or the extracellular environment then occurs. The forward trafficking between the ER and the golgi apparatus is referred to as anterograde transport and serves as the starting point for the development of the endomembrane system. Trafficking between the ER and golgi apparatus is bidirectional, with the reverse flow of material (retrograde transport) from the golgi to the ER also occurring in order to capture unfolded or escaped ER proteins. This cyclical flow of vesicles between the two organelles is facilitated by distinct cytoplasmic machinery that are selective for the cargo and lipids being transported. Two crucial components of the transport machinery in retrograde and anterograde trafficking between the ER and golgi network are the coat proteins COPI and COPII. The golgi associated COPI protein facilitates retrograde transport to the ER from the golgi, while COPII facilitates anterograde transport from the ER to the cis-golgi network. After trafficking to the golgi network, proteins then traffic through the golgi cisternae through vesicle budding by COPI-coated vesicles, and exit through the trans-golgi network, again via vesicle bud formation. The trans-golgi budding events are sites for vesicle formation and further downstream trafficking to other membrane bound organelles such as endosomes, lysosomes, and the plasma membrane. This trans-golgi network trafficking is facilitated by recruitment of the coat protein clathrin which, just as is the case with COPI and COPII, generates a membrane coat that assists in vesicle formation from the targeted organelle [1, 2].

Many of the proteins and lipids that are trafficked to the plasma membrane will inevitably be internalized through endocytosis. It is estimated that cells will internalize 1 to 5 times the equivalent of their total membrane every hour, and thus a well-functioning recycling pathway is necessary for maintaining the composition of the plasma membrane [3, 4]. Endocytic cargo that is initially targeted to endosomes can further be trafficked to late endosomes, lysosomes, the trans-golgi network, or to recycling endosomes that will bring the cargo back to the plasma membrane. Different markers can be used to identify early endosomes including the PI(3) kinase Vps34, the very important GTPase Rab5, and its effector EEA1 [5, 6]. The lumen of early endosomes is mildly acidic, and facilitates the release of ligands from receptors, at which point proteins and endosome lumen contents are then sorted. The generation of endosomal membrane tubules can result in entry into the fast endosomal recycling pathways, or entry into a later endocytic recycling compartment of which recycling endosomes will emerge [4]. Some receptors such as the transferrin and LDL receptors appear to be recycled back to the plasma membrane in the absence of any specific recognition or sorting sequence. This however may not always be the case as is the argument for protein receptors internalized by the cell in clathrin independent endocytic pathways. Proteins such as Rab and Arf GTPases, their effectors, scaffolding proteins, and motor proteins that help transport recycling endosomal carriers, all contribute to maintaining the composition of the plasma membrane through recycling of lipids and proteins internalized via endocytosis [4].

The endocytic pathway can be defined as the process by which cells actively take up molecules from the extracellular space, as well as plasma membrane proteins and lipids. These molecules are then targeted to the proper destination in the cell, in most cases beginning with early endosomes. Endocytosis serves a number of functions including nutrient uptake, regulation

of membrane protein signaling, pathogen entry, cell polarity and cell migration. Different types of endocytosis exist and can be distinguished by the protein machinery involved, the size of the internalizing vesicle, and the cargo being targeted. The process of clathrin-mediate endocytosis is the best characterized and most heavily studied; however other endocytic pathways exist including phagocytosis, macropinocytosis, and Caveolin-mediated endocytosis, all of which will be discussed here briefly.

1.2.2 Phagocytosis

The process by which a cell engulfs microorganisms, other cells, and foreign particles, usually of size greater than 0.5 μm, is referred to as phagocytosis. This mechanism can be performed by simple unicellular organisms for nutrient acquisition, as well as by the immune cells of more complex metazoans for the removal of pathogens and debris. Although the process of phagocytosis can vary from organism to organism, and between the types of molecules involved, all forms of phagocytosis require the finely regulated rearrangement of the actin cytoskeleton [7].

In mammals numerous forms of phagocytosis and mechanisms of activation exist. In the case of Fc-receptor-mediated phagocytosis foreign particles are first targeted for removal by immunoglobulin (Igs) binding. Igs act as opsonins that target the foreign particle or cell for recognition by phagocytes such as macrophages and neutrophils. Fc receptors found on professional phagocytes target the conserved Fc region on Igs for phagocytosis. The most common Ig is that of IgG which binds to the receptor FcγR. Binding of the IgG ligand by FcγR results in phosphorylation of specific tyrosine residues on motifs termed ITAM's [7, 8]. The phosphorylation of these ITAM motifs then act as a binding site for the tyrosine kinase SYK

leading to its phosphorylation and activation [9-11]. This results in downstream signaling events that end in the recruitment of the actin machinery, which causes actin polymerization and the formation of membrane protrusion that will encapsulate the foreign entity.

In the case of complement-receptor mediated phagocytosis, complement-opsonized particles are often targeted non-specifically and are internalized in more of a "sinking" fashion, requiring minimal disturbance to the membrane, versus the encapsulation by membrane protrusions. In one example of this process C3b or its modified version C3bi binds to the microbial surface and acts as an opsonin for recognition by complement receptors 1, 3, and/or 4 (CR1, CR3, CR4). All three of the receptors are expressed in macrophages and neutrophils and function in combination to initiate phagocytosis of the opsonized microbe [7]. Such as is the case with Fc-receptor-mediated phagocytosis, CR3 and CR4 are also phosphorylated following phagocyte activation [7, 12, 13]. This activation again leads to downstream signaling events that results in the recruitment of the actin machinery and actin polymerization, resulting in engulfment of the foreign particle.

While the examples given here have been well studied and researched, other receptors and means of phagocytosis have been characterized. Although distinctly diverse mechanisms exists, all forms of phagocytosis require the robust reorganization of the actin network and machinery. The major actin nucleator in cells is the Arp2/3 complex, and can be found to localize to, and is involved in, both FcγR and CR3 phagocytic events [14, 15]. In mammalian professional phagocytes it has been shown that Rho GTPases, such as Rac1 and Cdc42, are extremely important for membrane ruffling and phagocytosis, and both proteins have been well established as playing significant roles in generation of actin stress fibers as well as lamellipodia and filopodia membrane formation [16-20]. Key regulators of actin polymerization like WASP

have been shown to be important for efficient IgG-mediated phagocytosis [21]. WASP has been well characterized to function through interactions with Arp2/3 to nucleate actin filaments and initiate actin polymerization [22, 23]. Activation of WASP by Cdc42 and PIP2 for Arp2/3 triggered actin polymerization has also been demonstrated *in vitro* [24]. These and other results have established the significant role that actin and the actin machinery play in phagocytosis.

1.2.3 Macropinocytosis

Macropinocytosis is another regulated process of endocytosis by which cells uptake sizeable amounts of non-selective molecules in a bulk fashion of large "gulps". Macropinocytosis often occurs as a signal-dependent process initiated in response to growth factor stimulation by molecules such as epidermal growth factor, platelet-derived growth factor, and the tumour-promoting factor phorbol myristate acetate [25, 26]. This, however, is not always true as is the case with dendritic cells that perform constitutive macropinocytosis [27, 28].

Actin-mediated ruffling of the plasma membrane to form lamellipodia mechanistically initiates macropinocytosis. These membrane protrusion extend out into the extracellular space and fold back inward to fuse with the plasma membrane and form a large irregular shaped vesicle, of size >0.2 μm, termed a macropinosome [25]. Notably, just as is the case with phagocytosis, these membrane protrusions are formed as a result of recruitment and activation of the actin polymerizing machinery. Down regulation of Rho GTPases Rac1 and Cdc42 activity in A431 epidermis cells by the addition of amiloride, which causes a reduction in pH, also obliterated macropinocytosis that had previously been induced by the addition of EGF [29]. Furthermore, deletion of the WASP-like protein Scar results in a significant reduction in macropinocytosis and reduced actin polymerization [30].

1.2.4 Caveolae-mediated endocytosis

It was in 1953 that small bulb-shaped plasma membrane invaginations of size 50-80 nm were first visualized through electron microscopy (EM) imaging of blood capillaries [31]. It wouldn't be until 1992 that caveolin-1 was identified as a main component of what was described as caveolae membrane coats. Further investigation would later identify caveolin-2 and muscle-specific caveolin-3, thus establishing a caveolin gene family [32-35]. Since its initial discovery, caveolae formation has been shown to be involved in various cellular functions including lipid homeostasis, signal transduction regulation, transcytosis, and endocytosis [36, 37].

Caveolin-1 is a small integral membrane protein with a cytosolic N-terminal domain that interacts with cholesterol and also binds to and regulates important signaling molecules. Caveolin-mediated endocytosis begins at the Golgi apparatus. Here caveolin oligomerization is initiated before being trafficked to the plasma membrane. Oligomerization is dependent upon lipids and cholesterol, as is also the case at sites of plasma membrane invagination [38-40].

Formation of caveolae membrane invaginations is also dependent upon the recruitment of another protein family called cavins [41, 42]. Cavins form an electrostatic interaction between the negatively charged head groups of phosphatidylserine (PS) and PI(4,5)P_2 lipids, and the cavin domains helical region 1 (HR1) and HR2 . When purified cavins were added to PS enriched liposomes they were capable of inducing membrane tubulation, suggesting that they play a role in initiating membrane invagination [43]. It is believed that the clustering of these specific lipids by caveolin are responsible for inducing recruitment of the cavin family of proteins. The proper organization of the caveolae coat is not fully understood, however EM

imaging has demonstrated that cavins and caveolin form characteristic stripes surrounded by globular protein crescents on membrane invaginations [31].

Live cell imaging has demonstrated that caveolae are static structures and that their endocytosis is regulated by ligand binding to cargo receptors concentrated in caveolae. While all of the steps involved in progression of caveolae-mediated endocytosis are not fully understood, it is known that budding of caveolae from the plasma membrane is regulated by kinases and phosphatase [31]. Lastly, just as is the case in clathrin-mediated endocytosis, release of caveolin-coated vesicles from the plasma membrane requires the active pinching off from the plasma membrane by the large GTPase dynamin [44].

1.2.5 Clathrin-mediated endocytosis

Through electron microscopy imaging clathrin-mediated endocytosis can easily be distinguished from other forms of endocytosis due to the presence of a characteristically defined coat of proteins that forms on the plasma membrane. The process itself is highly dynamic, and requires the well-orchestrated and properly timed recruitment of numerous factors. These include adaptor proteins, components of the actin polymerizing machinery, and membrane binding proteins that help to not only shape the internalizing vesicle but also to sever it from the plasma membrane. The functions of this endocytic pathway range from nutrient uptake and receptor internalization to regulation of intracellular signaling pathways, cell wall synthesis, and a mechanism for viral entry into the cell. It is the process of clathrin-mediated endocytosis in *Saccharomyces cerevisiae*, and the role of the clathrin binding adaptor protein Sla1, that will be the focus of this dissertation. A model of clathrin-mediated endocytosis is represented in Figure1.1, which describes basic components and steps involved in the process.

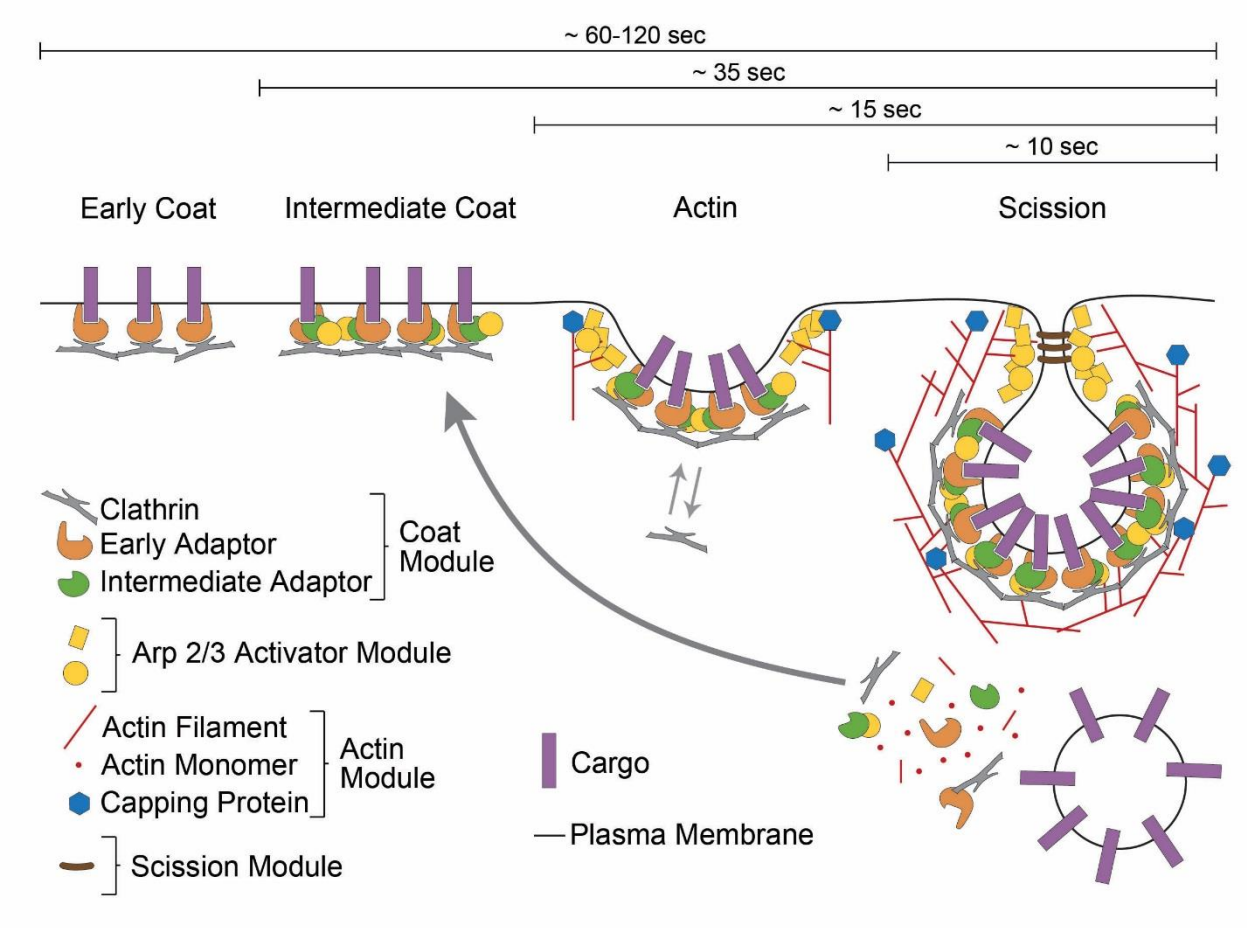

Figure 1.1: Stages and basic components of clathrin-mediated endocytosis. Early proteins of the endocytic machinery such as adaptors are recruited to the plasma membrane where they form the early coat and link both cargo and clathrin to the endocytic site. Later components of the endocytic machinery are then further recruited to enhance coat formation and growth of the endocytic site. This is followed by recruitment of the Arp2/3 activator module that then triggers actin polymerization, resulting in enhanced membrane invagination. Scission proteins are then recruited to the invagination neck where they pinch-off the endocytic vesicle from the plasma membrane. Uncoating of the endocytic vesicle then occurs, and the machinery is recycled to newly forming endocytic sites.

1.3 Clathrin-mediated endocytosis

1.3.1 A history of clathrin-mediated endocytosis

In 1964 Tom Roth and Keith Porter imaged coated membrane invaginations on the surface of mosquito oocytes taking up yolk proteins in electron micrograph sections [45]. In their publication Roth and Porter coined the term ‘coated pit’ and ‘coated vesicle’ as they speculated that these internalizing structures played a role in the uptake of yolk material, and that the coat forming on these internalizing structures might have a mechanical function and specificity for the material being absorbed. While this work arguably established the beginning of the endocytosis field, it would be another 10 years before a scientist by the name of Barbara Pearse would serendipitously view structures described as ‘vesicles in a basket’ via electron microscopy images of pig brain sections while in the search of formed microtubules. It was at this point that Pearse decided to change the direction of her project to purify these coated vesicles. SDS-PAGE analysis of her purest fraction would result in the identification of a highly abundant protein of molecular weight ~180 KDa. Pearse would go on to name this protein clathrin and proposed a role for it in the pinching off of membranes to form vesicles [46].

Pearse would continue her studies of clathrin-coated vesicles through collaborations with Tony Crowther and John Finch. Through high resolution electron micrograph images of purified clathrin-coated vesicles they were able to demonstrate that the vesicle coat was a closed cage-like structure composed of a repeating variable number of hexagons and pentagons [47]. This beautifully organized coat structure would further be appreciated through images captured by John Heuser. Heuser’s technique of unroofing fibroblasts allowed for the capture of high resolution images of the coat that appeared under the plasma membrane during clathrin-mediated endocytosis [48]. Heuser’s images showed with impressive resolution how the coat appears to

grow from its outer edge on the plasma membrane, and that as the coated membrane begins to pucker from its planar geometry the organization of the coat is converted from one consisting primarily of hexagons with few pentagons and heptagons, to one consisting of a higher population of pentagons.[49]

While Pearse hypothesized that the repeating pattern of pentagons and hexagons found on coated vesicles were likely that of organized clathrin molecules, it was the work of James Keen and associates that would confirm this. Keen purified coated vesicles from isolated bovine brain tissue using a modified version of the method used by Pearse. Keens purification revealed vesicles composed primarily of clathrin at ~175,000 KDa and two smaller fractions of size 110 KDa and 55 KDa molecular weight. The coat was dissociated with high concentrations of a Tris-HCl buffer and then fractionated by gel filtration into a clathrin-containing fraction and one containing the 110 KDa polypeptide. When the clathrin fraction was dialyzed against a low ionic strength solution containing calcium, basket formation could be observed via electron microscopy, demonstrating clathrin was the molecule responsible for the pentagon and hexagon structures observed in coated vesicles. Interestingly, the clathrin basket was not able to form under dialysis against a solution of moderate ionic strength, but upon addition of the 110 KDa polypeptide fraction basket formation could again be readily observed through electron microscopy. This lead Keens to conclude that the 110 KDa polypeptide fraction acted as a clathrin assembly factor [50].

Around the same time that clathrin was being identified as an important coat protein in vesicle formation, work performed by Joseph L. Goldstein, Michael S. Brown, and coworkers from 1972-1982 was identifying cellular defects that resulted in familial hypercholesterolemia (FH). Patients with FH suffer from elevated levels of cholesterol in the blood that predispose

them to heart attacks early in life. In 1972 their work began by focusing on HMG-CoA reductase, an enzyme which had been shown to catalyze a rate-limiting step in cholesterol production, the activity of which is reduced when rats from which the cells were obtained ingested cholesterol [51]. Goldstein and Brown demonstrated that HMG-CoA activity in cultured human fibroblasts was high in the absence of the cholesterol carrying low density lipoprotein (LDL); however this activity was quickly reduced upon the addition of LDL [52]. Further work showed that cells obtained from patients with homozygous FH, with high HMG-CoA activity, showed no reduction in this activity upon the addition of LDL. However, addition of cholesterol in a form capable of absorption into the plasma membrane caused a reduction in HMG-CoA activity in normal and FH cells [53, 54]. These results strongly suggested that a membrane receptor specific for the LDL particle was responsible for cholesterol uptake and that patients with FH had a defect in receptor activity. Goldstein, Brown, and colleagues eventually proved the existence of the LDL receptor (LDLR), purified LDLR from bovine adrenal glands, and cloned the human cDNA [55, 56]. This work laid the foundation for the identification of more than 1100 mutations in the LDL receptor gene found in FH patients [51].

The LDL receptor and bound LDL were demonstrated to be internalized via endocytosis through experiments involving the internalization of LDL coupled to electron dense ferritin [57, 58]. Furthermore, the efficiency of LDL particle internalization was dependent upon its clustering into coated pits that were quickly identified as clathrin endocytic sites. This work demonstrated that clathrin-mediated endocytic sites could serve as gathering points for specific cell surface receptor internalization [58]. The function of coated pits as gathering points for receptor internalization was additionally shown to occur in fibroblasts that were capable of binding the LDL particle by the determined receptor, but could not internalize LDL properly [59,

60]. Receptors in these cells had a mutation that caused them to be excluded from the coated pits, further establishing the role of clathrin-mediated endocytosis in uptake of receptors and their bound ligands [61]. These results were significant in establishing the role clathrin-coated pits play in uptake of receptor bounds molecules [51]. Later work with these cells would demonstrate a key point mutation of a Tyrosine to a Cysteine in the cytoplasmic domain of the LDL receptor that was responsible for preventing receptor clustering into coated pits [62]. It would then be discovered that this tyrosine was part of a protein sequence NPVY that was responsible for targeting the receptor to clathrin-coated pits [63]. This established a role for endocytic sorting signals in receptor-mediated endocytosis.

Following these early findings, further investigations would identify a plethora of endocytic adaptors, scaffolding and accessory proteins, as well as membrane proteins, receptors, and extracellular cargo to be involved in the process of clathrin-mediated endocytosis. Many of these discoveries began with studies performed in mammalian cells; however further investigation would establish the significance of this process in all eukaryotes. The model organism *Saccharomyces cerevisiae* (budding yeast) has proven to be a useful tool for studying specific aspects of CME. To understand the significance yeast has played in our understanding of clathrin-mediated endocytosis, it is important to first obtain an understanding of what is known about the process in mammalian cells.

1.3.2 Mammalian clathrin-mediated endocytosis

Even though several clathrin independent endocytic pathways exist, the clathrin dependent one is the major internalization route in the vast majority of mammalian and other eukaryotic cells. Many of the original discoveries leading to our understanding of clathrin-

mediated endocytosis were first developed from experiments using mammalian cells. This groundwork paved the way for studies in other eukaryotes such as drosophila and yeast that have resulted in CME as being the most well-characterized endocytic mechanism to date. The process occurs through variously defined stages, beginning with initiation of site formation, cargo selection, growth of the endocytic coat, actin polymerization, and ending in vesicular scission and internalization of an endocytic vesicle. While numerous factors are involved in this process, three essential components for mammalian clathrin-mediated endocytosis are the clathrin heavy chain (CHC), the clathrin light chain (CLC), and the four subunits of the heterotetrameric complex adaptor protein 2 (AP2).

The earliest stages in mammals clathrin-mediated endocytosis involves the recruitment of AP2 to the plasma membrane where it binds to, recruits, and links clathrin to the plasma membrane. AP2 is recruited, in part, due to its ability to bind membrane regions enriched with the phosphatidylinositol lipid $PI(4,5)P_2$, which is important for endocytic site initiation [64-66]. The role of negatively charged phospholipids in bud formation has been well established in numerous studies, with depletion of $PI(4,5)P_2$ from the plasma membrane resulting in a significant depletion of clathrin-coated pit formation on the plasma membrane, as measured using fluorescently tagged AP2 and clathrin light chain [67]. A paper describing the first 5 seconds of a clathrin-coated pit demonstrates that in the majority of forming endocytic patches an average of two molecules of AP2 and one molecule of clathrin are recruited at the early onset of clathrin-mediated endocytosis site initiation [68]. Following this event, additional AP2 and clathrin molecules are rapidly recruited to the plasma membrane through interactions of the CHC n-terminal β-propeller domain and the β2 subunit of AP2. Upon binding to the plasma membrane, AP2 functions as a primary cargo recognition molecule interacting with multiple

receptors such as CD4, CTLA-4, and the well characterized transferrin receptor (Tfr) [69-71]. The interaction between AP2 and PI(4,5)P_2 at the plasma membrane results in a conformational change in AP2 that then exposes clathrin, cargo, and additional PI(4,5)P_2 binding motifs on AP2 that further enhances membrane binding and formation of clathrin-coated pits [72, 73].

The clathrin triskelion, from the Greek for a three legged structure, is a hexameric protein composed of three clathrin heavy chains, and three clathrin light chains [74, 75]. The clathrin heavy chain has an important amino-terminal β propeller domain that is required for interacting with the clathrin binding domain of various adaptor proteins. This is followed by 42 α-helical zig zags, a single longer α-helix that ends at the contact point between the three heavy chain subunits, and a structurally ill-defined carboxy-terminal segment. The 42 α-helical zig zags that make up most of the polypeptide chain is referred to as a leg of the clathrin triskelion, and ends with the β-propeller terminal domain at the tip of the amino terminal leg. At the center of the clathrin triskelion heavy chain contact point three long α-helices from each heavy chain projects outward, each of which is followed by a less ordered, carboxy-terminal segment. The three α-helices interact in a way to form a set of close contacts that result in the formation of a tripod-like structure, the base of which holds the heavy chain trimer together [76]. Mammalian cells express two different forms of the clathrin light chain, LCa and LCb, of which different isophorms exist [77]. Clathrin light chains associate with the leg of the clathrin heavy chain, proximally to the C-terminus, through a long central α-helix [76, 78, 79]. Work seems to indicate that the light chain is not necessary for clathrin triskelion formation since no contact points between individual light chains exist, triskelion formation has been generated in the presence of only the heavy chain, and the C-terminal third of the clathrin heavy chain alone is capable of trimerization [74, 80]. Work in yeast however, did demonstrate that deletion of the light chain

results in a dramatic reduction in heavy chain triskelion formation [81]. Furthermore, heavy chain trimerization experiments using recombinantly expressed bovine CHC, demonstrated that binding of the CHC by the CLC enhanced trimer formation [82].

Clathrin triskelions are capable of assembling into a lattice like structure at a mildly acidic pH of 6.2 [74]. However, at a more neutral pH, clathrin cage formation requires the presence of clathrin adaptor proteins such as mammalian AP2, one of many that have been shown to have this affect [50, 83-85]. This assistance in lattice cage formation is facilitated through interactions with the N-terminal β-propeller domain of clathrin. Most clathrin adaptors bind to the clathrin heavy chain between blades 1 and 2 of the β-propeller terminal domain through the use of a protein motif of sequence L[L/I][D/E/N][L/F][D/E], referred to as a clathrin box [86, 87]. Additionally, a second clathrin binding motif, the W-box, of sequence PWxxW, has been shown to bind to the top face of the β-propeller [87]. A fully closed clathrin cage creates a structure, reminiscent of a soccer ball, with 12 open spaces being made of pentagons with the rest being hexagons [79]. EM imaging of the clathrin coat, and sophisticated modeling programs, have demonstrated that as the plasma membrane bends the clathrin coat goes from a flat sheet to a curved one. This requires the rearrangement of the clathrin coat from pits with open spaces of pentagons, hexagons, and heptagons, to a structure containing fewer heptagons and hexagons and more pentagons [49]. The size and shape of the clathrin coat is thus dependent upon the number of heptagonal and pentagonal openings [88].

Early in the formation of clathrin-coated pits (CCP), scaffolding proteins are also recruited to the plasma membrane, which include FCHo1/2, Eps15, and intersectin. FCHo1/2 is an amino-terminal F-BAR domain containing protein. BAR domains are elongated bundles of three curved α-helices [89]. BAR domains can be divided into subgroups termed F-BAR, N-

BAR, and I-BAR [76, 90]. In the case of F-BAR proteins, such as FCHo1/2, the homodimerization of the bar domain creates a symmetrical arc like structure that binds to the negatively charged lipid head groups of curved membranes [76]. In vitro F-BAR domains have been shown to preferentially bind curved membranes, but have also been shown to cause membrane bending and tubulation [91, 92]. The FCHo proteins are important nucleators of endocytosis as they appear early at endocytic sites, just as their yeast homologue Syp1, before AP2 and clathrin. Furthermore, a complete loss of clathrin-coated pit formation has been observed upon double RNAi knockdown of FCHo1/2 [92]. FCHo1/2 has been shown to localize to the outer rim of a growing clathrin pits along with Eps15 in EM images [92, 93]. Further experimentation demonstrated that Eps15 and Intersectin1 interacted directly with the FCHo2 μ-homology domain and that there localization to the plasma membrane was dependent upon FCHo2 [92].

Eps15 (EGFR Protein Tyrosine kinase Substrate #15) arrives early to endocytic sites and was first identified as a substrate for epidermal growth factor receptor (EGFR) signaling [94]. Eps15 interacts directly with α-adaptin, a component of the AP2 complex, and has been shown through fluorescence microscopy to localize with both clathrin and AP2 at endocytic sites [95, 96]. The Eps15 protein has multiple EH domains (Eps15 homology) that have been shown to bind Ca^{2+} with high affinity, as well as the protein sequence motif NPF [97]. One of the very first Eps15 binding partners discovered was Epsin1, which binds the EH domains of Eps15 with its NPF motif [98]. Eps15 has also been shown to bind other components of the endocytic machinery including intersectin, stonin, and synaptojanin [99]. Eps15 also contains two ubiquitin interacting motifs (UIM) that were shown to interact with the two E3-ligases Nedd4 and Parkin, that are also responsible for monoubiquitination of Eps15 upon EGFR activation [100-102].

While it is still unknown exactly how ubiquitination of Eps15 affects the function of the protein, evidence does suggest that ubiquitination of the protein inhibits internalization of EGFR, and that this inhibition may be regulated by its second UIM [103]. In addition Eps15 was shown to be a tyrosine kinase substrate of the EGFR. Stimulation of cells with EGF results in phosphorylation of Eps15, and overexpression of an Eps15 mutant lacking the phosphorylation site effectively blocked endocytosis of EGFR. This work seems to suggest that phosphorylation of Eps15 effectively works to recruit the protein to the receptor or the endocytic machinery [104]. Eps15 function is thus regulated through posttranslational phosphorylation and ubiquitination. This and other work indicates that Eps15 plays an important role in endocytic site formation as well as receptor cargo internalization and regulation via endocytosis.

Another early arriving component of the endocytic machinery is that of Intersectin 1 (ITSN1) [92]. ITSN1 is a multifunctional adaptor and scaffolding protein and is the first of two Intersectin proteins, the latter being ITSN2, which share high sequence and structural similarities [105]. The gene of ITSN1 encodes for two main isoforms, one being a short form ITSN1-s, and a longer form ITSN1-L [106]. The short form is made up of two EH domains followed by a coiled-coil region and five Src Homology 3 domains (SH3), while the longer form has three additional domains, the Dbl homology (DH) domain, a Pleckstrin homology (PH) domain, and a C2 domain [106, 107]. Just as is the case with Eps15, the EH domains of ITSN1 have been shown to bind the peptide sequence NPF, such as those found in Epsin, stonin2, and a family of secretary carrier membrane proteins (SCAMPS) [108-110]. Evidence suggests that the coiled-coil domain of ITSN1 is involved in the formation of homodimers or heterodimerization with Eps15 for complex formation [111]. The intersectin SH3 domains have been shown to interact with a variety of other endocytic proteins containing traditionally recognized polyproline motifs, PxxP

[112]. ITSN1 forms a complex with CIN85/Ruk through binding of its first SH3 domain and the P3 and P4 proline rich domains of CIN85. This complex is involved in downregulation of receptor tyrosine kinases (RTK), but its formation is not dependent upon RTK stimulation, suggesting constitutive association between the two proteins [113]. It has been well established that among other interactions, protein SH3 domains will frequently be found to bind polyproline motifs found in cytoskeletal proteins [114-116]. With that point being made, ITSN1 has also been shown to be involved in regulation of actin polymerization during endocytosis [117]. Specifically ITSN1 and ITSN2 have both been shown to interact with WASP, the primary actin nucleation promoting factor involved in actin polymerization at the end of endocytosis [118, 119]. Furthermore, the Dbl homology (DH) domain of ITSN1 has been shown to act as a guanine nucleotide exchange factor (GEF) for Cdc42 and stimulates its activity, an interaction that is involved in activation of WASP and actin polymerization [118]. Lastly, the SH3 domains of ITSN1 have been shown to interact with the proline rich sequences of the scission GTPase protein dynamin, and evidence suggests a role for Intersectin in dynamin recruitment [111].

The Epsin family of proteins act as accessory molecules, essentially all of which contribute to membrane bending [120]. Named for its interaction with Eps15, Epsin1 is one of the best characterized members of the protein family, and is actively involved in clathrin-mediated endocytosis [120]. Epsin1, like all epsins, contains an Epsin-N-terminal Homology (ENTH) domain [121]. ENTH domains bind $PI(4,5)P_2$ lipids and are involved in membrane binding and bending [122-124]. Just downstream of the ENTH domain are several UIM's that have been shown to be involved in ubiquitin binding and internalization of ubiquitinated cargo [120, 125, 126]. Following this region are multiple DPW amino acid motifs that have been shown to bind to the α-appendage domain of AP2 and are flanked on each side by clathrin

binding domains [127-129]. The final carboxy-terminal region contains three NPF amino acid repeats that are important for interactions with EH domain containing proteins Eps15 and Intersectin [98, 108]. Epsin1 and its various isoforms all participate in clathrin-mediated endocytosis [120]. In particular Epsin plays an important role in deforming the plasma membrane via its ENTH domain. The ENTH domain is a compact superhelix composed of seven α-helices [130]. This domain binds to $PI(4,5)P_2$ lipids enriched on the plasma membrane allowing for further insertion of the N-terminal alpha-helix into the inner leaflet of the plasma membrane thus inducing membrane curvature. It is this interaction that is believed to contribute to membrane bending during endocytosis [123].

Similar to the ENTH domain is the highly related ANTH domain that can be found in adaptor protein AP180 and its homologue CALM [131]. Just as is the case with the ENTH domain, the ANTH domain is capable of binding $PI(4,5)P_2$, although the regions of lipid binding are distinct and unique between the two, despite structural similarities [132]. While ENTH and ANTH domain containing proteins Epsin and AP180 share similar properties including domains involved in clathrin binding and direct interactions with the α-appendage domain of AP2, their means of action in endocytosis are distinct [132]. For example the ENTH domain has been shown to cause tubulation of liposomes while the ANTH domain was not capable of inducing such deformation [123]. Interestingly, in vitro experiments demonstrated that while both Epsin1 and AP180 recruit clathrin to $PI(4,5)P_2$ monolayers and stimulate clathrin lattice formation, only Epsin1 was capable of inducing membrane curvature in these lattices. These results also suggest that clathrin assembly by itself may not be able to induce membrane curvature. The ANTH domain was, however, capable of generating more uniform clathrin lattice structures than the ENTH domain, potentially indicating a role in lattice size [123].

Following the early and intermediate stages of clathrin-mediated endocytosis such as site establishment, cargo loading, and coat formation, actin polymerization is activated to generate a driving force that assists in membrane invagination. While actin polymerization is not essential for CME in cultured mammalian cells, an increase in membrane tension does generate a requirement for the process, as coated pit assembly stalls in the absence of actin polymerization under conditions increasing membrane tension [133, 134]. In actin dependent cases of clathrin-mediated endocytosis the two major players Arp2/3 and N-WASP play a significant role in triggering actin polymerization. The Arp2/3 complex serves as a major regulator of actin nucleation and polymerization in the cell, however its activation requires the interaction with actin nucleation promoting factors (actin-NPF's) such as the N-WASP protein [135, 136]. In cultured fibroblasts it was well demonstrated through the use of total internal reflection fluorescence (TIRF) microscopy, that in the majority of endocytic sites plumes of actin could be seen as clathrin began to retreat into the cell [137]. Following this work it was further demonstrated that both Arp2/3 and N-WASP colocalized with clathrin during internalization, and that the percent of clathrin patches that colocalized with Arp2/3 was similar to that seen with actin [138]. Furthermore, fluorescent imaging of N-WASP knockout cells resulted in an approximate 50% reduction in the accumulation of both actin and Arp2/3 at endocytic sites, a phenotype also associated with a reduction in internalization of EGF, a classic endocytic cargo [139]. It should also be noted that wild type and N-WASP knockout cells had a significant population of 40-70% clathrin-coated structures that internalized without any detectable actin assembly [139]. This further supports previous observations that actin polymerization is not necessary for all clathrin-coated vesicle internalization events in cultured mammalian cells.

In order for actin polymerization to be translated into a force generating mechanism for the invagination of an endocytic vesicle the actin network must be linked to components of the endocytic site. It appears that this link is accomplished by the Huntington interacting protein 1 (HIP1) and HIP1-related (HIP1R). HIP1 was first identified for its interaction with the N-terminal domain of huntington protein, a protein with a mutant version in the brain associated with Huntington's disease [140, 141]. HIP1R does not bind the huntington protein, distinguishing a functional role for the two proteins. HIP1 is expressed in neurons and has been shown to bind to the clathrin heavy chain and AP2, while both HIP1 and HIP1R have been shown to bind liposomes containing either PI(3)P, PI(3,4)P_2, or PI(3,5)P_2, but not PI(4,5)P_2, through their ANTH domains [142, 143]. HIP1R does not bind AP2, and binds more weakly to the clathrin heavy chain, further distinguishing unique functions between HIP1 and HIP1R [144]. Interestingly, both proteins contain a talin-like THATCH domain capable of binding filamentous actin, however the talin-like domain of HIP1 has a much lower affinity for actin binding than that of HIP1R, and the HIP1 THATCH domain alone does not localize with filamentous cellular actin, while the talin-like THATCH domain of HIP1R does [144, 145]. HIP1 and HIP1R are also capable of heterodimerization through their helical domains, and colocalize with each other at endocytic sites [144, 146, 147]. Additionally, the helical domains of HIP1 and HIP1R were both shown to bind the clathrin light chain, and both full length proteins were capable of inducing clathrin assembly *in vitro* [144, 147, 148]. HIP1R has also been shown to functionally link F-actin to clathrin coats *in vitro*, and cellular unroofing EM experiments demonstrated labeling of filamentous structures by an anti-HIP1R antibody that were connected to clathrin-coated pits [147]. This body of work thus suggests dimerization of HIP1 and HIP1R links actin polymerization to the endocytic machinery and the lipid membrane. It should be noted, however,

that this model may need to be refined as more current work demonstrated that HIP1 and HIP1R coiled-coil domains preferentially formed homodimers over heterodimers, and the binding of the clathrin light chain by both proteins caused a conformational change that actually reduced actin binding [149]. Nonetheless, experiments involving the yeast HIP1/HIP1R homologue Sla2 further suggest that these proteins serve to link actin to the endocytic machinery, as will be discussed in detail later [140, 145].

The final stage of coated vesicle internalization requires the recruitment of lipid binding proteins that deform the plasma membrane in such a way as to induce membrane fission and internalization of the vesicle. Two important components that facilitate this function are the amphiphysins and dynamin. Amphiphysins are N-BAR domain containing proteins that bind clathrin, the AP2 complex, and dynamin, a combination of interactions suggesting a role for amphiphysin in linking dynamin to the endocytic machinery [89, 150-153]. This is supported by the fact that amphiphysin contains an SH3 domain that binds the proline rich domains of dynamin, as well as synaptojanin-1, a $PI(4,5)P_2$ phosphatase concentrated at nerve terminals [154-156]. Dynamin recruitment to the plasma membrane was further supported by work indicating that it was dependent upon binding both of the BAR-domain containing proteins endophilin and amphiphysin. This recruitment was in line with other results showing that the proline rich domain of dynamin, which interacts with the SH3 domains of both endophilin and amphiphysin, where responsible for this recruitment. This interaction is cooperative in that dynamin binding also contributes to release of endophylin and amphiphysin's auto inhibition of their membrane binding BAR domain [157]. Like other BAR domain containing proteins, the amphiphysin N-BAR domain is capable of binding and tubulating membranes, and also has the ability to sense and preferentially bind more highly curved membranes [89].

Dynamin plays a key role in the scission and release of clathrin-coated vesicles. Dynamin is a GTPase that was originally co-purified with brain microtubules [158]. Although multiple isoforms of this protein have been identified in mammalian cells, and their exact roles remain incompletely understood, their role in endocytosis has been firmly established [159]. An endocytic function for dynamin was first determined following studies of a temperature-sensitive paralytic phenotype in Drosophila melanogaster resulting from mutations in the *shibire* gene, which would later be identified as a dynamin gene [160, 161]. These mutations caused a depletion of neuronal synaptic vesicles, and a build-up of stalled 'collared' endocytic invaginations at defective temperature [162]. These long membrane invaginations were also observed in mammalian presynaptic plasma membranes after being treated with GTP-γS, a slowly hydrolysable form of GTP. Immunogold labeling and electron micrograph imaging revealed that these long invaginations were heavily decorated with dynamin along the tubule length, many of which were positive for clathrin at the bud tip [163]. Future work would go on to establish that dynamin is a component of the clathrin endocytic machinery and that its GTPase activity is important for vesicle fission and endocytosis of transferrin and the EGF receptor [164, 165]. The means by which dynamin contributes to membrane fission was demonstrated when dynamin was shown to polymerize into rings and ring stacks under conditions of low ionic strength [166]. These stacks resembled the collars that formed at membrane invaginations in *shibire* mutant flies. Further experiments have suggested that following polymerization of dynamin around the neck of an endocytic bud, GTP hydrolysis causes a structural rearrangement that induces constriction of the membrane neck inducing scission [167, 168]. In terms of its recruitment time to endocytic sites, TIRF imaging of endogenously expressed dynamin-GFP showed that dynamin appeared at endocytic sites at the end of endocytosis. Furthermore,

dynamin was shown to appear at endocytic sites following the appearance of actin, thus supporting its role in vesicle scission [169]. Lastly, these experiments have also demonstrated that actin polymerization plays an important role in dynamin recruitment, and is additionally a contributing factor in vesicle scission [169].

While the endocytic protein machinery plays well established roles in shaping the plasma membrane, it is important to acknowledge the role membrane lipids play in endocytic site maturation. The recruitment of early, intermediate, and late components of the endocytic machinery and cargo internalization has clearly been demonstrated to be dependent upon high $PI(4,5)P_2$ levels at endocytic sites [170]. Work using phospholipid specific fluorescent sensors nicely demonstrated a conversion of lipid composition from high $PI(4,5)P_2$ and low PI(4)P levels early in endocytic site formation, to high PI(4)P, $PI(3,4)P_2$, PI(3)P and low $PI(4,5)P_2$ levels in fully internalized endocytic vesicles, more similar in composition to early endosomes [171]. While nicely demonstrating the existence of lipid compositional changes occurring during endocytosis, this work suggested little conversion occurred until after vesicle scission. This concept is brought into question by others who have demonstrated lipid conversion and generation of $PI(3,4)P_2$ as being important in the maturation of late-stage clathrin-coated-pits before vesicle fission [172]. Additionally it was demonstrated that the generation of $PI(3,4)P_2$ by the PI(3) kinase C2α, and sequential formation of PI(3)P, appear to contribute to recruitment of the actin polymerization machinery [172, 173]. It should additionally be noted that the HIP1 and HIP1R proteins, that are believed to link actin polymerization to the endocytic vesicle during membrane invagination, preferentially bind $PI(3,4)P_2$ membranes over $PI(4,5)P_2$, possibly indicating an enhanced interaction that occurs at the end stages of endocytosis before vesicle scission [143].

1.3.3 Yeast clathrin-mediated endocytosis

In the field of clathrin-mediated endocytosis the model organism *Saccharomyces cerevisiae*, or budding yeast, has proven to be an invaluable tool for gaining insight into the role of various components of the endocytic machinery and the cargo it transports. One of the key benefits to studying clathrin-mediated endocytosis in yeast is that it serves as the primary source of endocytosis for the organism. In fact, it was not until recently that a second form of endocytosis was even discovered to exist in yeast, and is believed to only minimally contribute to the total amount of plasma membrane material internalized during the process [174]. Easy genetic manipulation and availability of tools, such as the knock out collection, have been instrumental in the establishment of S. cerevisiae as a prime model organism to study endocytosis [175, 176]. Important as well, is the ability to easily fluorescently tag and visualize endogenous proteins in yeast, a challenging and fairly recent process performed in mammalian cells through the tedious use of the CRISPER/cas9 system [177]. This allows clathrin-mediated endocytosis to be studied more easily with little interference and noise from other endocytic events. CME in yeast requires the properly timed recruitment and activity of ~60 proteins [178]. It has been through the studies of many of these proteins that key aspects of mammalian CME were then identified, validated, and shown to be conserved across eukaryotes. Studies of CME in yeast have also identified novel proteins that were later shown to have functionally similar mammalian homologues [178, 179]. Table 1.1 indicates the known protein machinery involved in yeast clathrin mediated endocytosis, the related mammalian homologs, and established domains in the yeast proteins. Here the process of CME will be discussed as it occurs in *Saccharomyces cerevisiae* or budding yeast. Particular attention will be given to the proteins,

Table 1.1: Proteins characterized to be involved in clathrin-mediated endocytosis. Proteins with no mammalian homolog listed have no currently identified mammalian homolog. Adapted from Weinberg and Drubin and Goode et al. [178, 180].

Stage in Yeast (Lifetime)	Yeast Protein	Mammalian Homolog	Domains/Subunits/Motifs
Early site initiation and coat formation (~60-120 sec)	Syp1	FCHo1/2	F-BAR, μ-homology domain, PRD
	Ede1	Eps15	EH, CC, UBA
	CHC	Clathrin Heavy Chain	β-propeller
	CLC	Clathrin Light Chain	
	AP2	AP2	Apl1, Apl3, Apm4, Aps2
	Pal1		NPF
	Yap1801/2	AP180/CALM	ANTH, NPF motif, CBM
Intermediate Coat (~40-60 sec)	Sla2	HIP1/HIP1R	ANTH, CC, THATCH
	Ent1/2	Epsin1/2	ENTH, UIM, NPF motif, CBM
Late Coat (~30-40 sec)	Pan1	Intersectin	EH, CC, PRD, WH2, Acidic motif
	End3	Eps15	EH, CC
	Sla1	Intersectin/CIN85	SH3, SHD1, SHD2, SR repeats, CBM
	Lsb3	SH3YL1a	SH3
	Lsb4/Ysc84	SH3YL1a	SH3
	Lsb5	GGA	VHS, GAT
	Gts1	SMAP2	UBA
	Ubx3		UBX, UAS, W-box
WASP/MYO (~15-35 sec)	Las17	WASP/N-WASP	WH1, WH2, PRD, LGM, Acidic Motif
	Vrp1	WIP/WIRE	WH2, PRD
	Bzz1	Syndapin	F-BAR, SH3
	Scd5		
	Myo3/5	Myosin-1E	Motor domain, TH1, TH2, SH3, Acidic motif
	Bbc1		SH3, PRD
	Ldb17	DIP/WISH/SPIN90	PRD
Actin Polymerization (~15 sec)	Act1	Actin	
	Arc15,18,19,35,40 and Arp2/3	Arp2/3	ARP, β-propeller
	Abp1	ABP1	ADFH, SH3, PRD, Acidic motif
	Cap1	Capping protein alpha	

	Cap2	Capping protein beta	
	Sac6	Fimbrin	CH
	Scp1	Transgelin	CH, PRD, CLR
	Twf1	Twinfilin	ADFH
	Crn1	Coronin	β-propeller, CC
	Cof1	Cofilin	ADFH
	Aip1	Aip1	β-propeller
	Srv2	Cyclase-associated protein (CAP)	HFD, β-sheet, PRD, WH2
	Gmf1	GMF	ADFH
	Bsp1		
	Pfy1	Profilin	
	Aim3		PRD, NPF motif
	Tda2	TcTex1 dynein light chain	TcTex1 Fold
	Aim21		
	Ark1, Prk1, Akl1	AAK1, GAK	Ser/Thr kinase, PRD
	Abp140		
Scission (~10 sec)	Rvs161	Amphiphysin	N-BAR
	Rvs167	Amphiphysin/endophilin	N-BAR, SH3
	App1		
	Sjl1/2	Synaptojanin-1	PRD
	Vps1	Dynamin	GTPase
Uncoating	Ark1, Prk1, Akl1	AAK1, GAK	Ser/Thr kinase, PRD
	Swa2	Auxilin	CBD
Ubiquitin regulation	Rsp5	Nedd4	WW domain, C2, HECT
	Ubp2		UCH
	Ubp15		UCH
	Ubp7/11	Usp2	UCH
	Art1/5	Alpha-arrestin	PY motif
	Art2/Csr2	Alpha-arrestin	PY motif
	Rog3/Rod1	Alpha-arrestin	PY motif
	Aly1/2	Alpha-arrestin	PY motif
Other	Arf3	Arf6	
	Mss4		PIP5K
	Glc7		
	Lsb1		SH3
	Lsb2		SH3
	Swf1	Palmitoyl transferase 1	

lipids, and posttranslational modifications involved, as well as the various biochemical, microscopy, and yeast genetic manipulation techniques used to characterize how this mechanism occurs.

The development of live-cell fluorescence microscopy has drastically altered our ability to study clathrin-mediated endocytosis in yeast and mammals. By tagging proteins with fluorescently labeled markers, scientists have been able to characterize the dynamic recruitment of the endocytic machinery. In yeast, the early arriving ubiquitin binding protein Ede1, the yeast Eps15 homologue, and the membrane binding F-BAR protein Syp1, the FCHo1/2 homologue, have been established as being involved in the formation and placement of endocytic sites [181]. While not essential for clathrin-mediated endocytosis, as is the case with many endocytic proteins, deletion of Ede1 has been shown to reduce the frequency of endocytic events [181]. Through various analyses the two proteins have been shown to interact directly, and the use of live cell fluorescent imaging was able to demonstrate that both endocytic proteins share similar dynamics [182]. In these experiments Syp1 was shown to also serve a role in the polarized distribution of endocytic sites to the neck and daughter cell of budding yeast, while Ede1 served a role in modulating endocytic site formation. Both proteins, however, contribute to the other's proper localization on the plasma membrane and endocytic site formation [181]. Interestingly, Syp1 is also a cargo adaptor protein, as its overexpression enhances internalization of the cell-wall stress sensor, Mid2, and this enhanced internalization is dependent on its carboxy-terminal μ-homology domain that has been shown to bind DxY motifs found in both endocytic cargos Mid2 and Snc1 [183, 184]. Like the FCHo homologues, Syp1 is an F-BAR domain containing protein with the ability to bind and cause tubulation of liposomes [183]. Syp1 also seems to have a role in preventing Arp2/3-mediated actin polymerization via inhibition of Las17 nucleation

promoting activity [182]. This ability of large multi-domain endocytic proteins to have multiple functions at different time points in endocytosis is a common feature as has been previously discussed.

While Ede1 and Syp1 are two of the first components of the endocytic machinery to be recruited to the plasma membrane, and seem to be involved in endocytic site formation, how endocytic sites are initiated remains a mystery. Ede1 has been shown to bind to ubiquitin and phosphoinositides, and Syp1 has been shown to bind endocytic cargo and membranes through its F-BAR domain, both of which could be responsible for site initiation [184, 185]. While CME in mammalian cells seems to be heavily dependent upon recruitment of many of the early arriving endocytic proteins, the process in yeast seems to be highly flexible with regard to recruitment of the early components of the endocytic machinery. This was demonstrated in yeast cells where co-deletion of seven early arriving endocytic proteins including Syp1, Ede1, and the AP180 homologues Yap1801/2, resulted in a number of interesting findings [186]. First, the long early phase of endocytosis was not necessary for site initiation at the plasma membrane. Second, the arrival of other "early" proteins, such as clathrin, Sla2, and Ent1, was dramatically delayed until just before the recruitment of the late arriving coat protein Sla1, which also had a shorter patch lifetime. Lastly, the early phase of protein recruitment was not required for membrane uptake, but was required for the internalization of specific membrane cargo [186].

In mammalian cells AP2 functionally acts as the early arriving clathrin adaptor protein that recruits the first few molecules of clathrin to endocytic sites [68]. However, in the case of yeast, even though AP2 does arrive early to endocytic sites around the time of Ede1 and Syp1, experiments have demonstrated no sign of clathrin binding to AP2 [187, 188]. In addition, the deletion of AP2 subunits has no reported effect on clathrin-mediated endocytosis, except in a

single circumstance in which AP2 deletion enable yeast to become resistant to exposure of killer toxin K28 [189]. This indicates that AP2 is involved in targeting the toxin for internalization but does not play an essential role, such as clathrin recruitment, in the process of clathrin-mediated endocytosis.

While cargo internalization is the main purpose of clathrin-mediated endocytosis, it is not clear as to the precise role cargo plays in progression of endocytosis, nor has the exact timing of cargo accumulation into endocytic sites been determined. There is, however, one example of cargo accumulation occurring shortly after Ede1 recruitment to endocytic sites. In these experiments fluorescently labeled alpha factor that binds to the yeast mating receptor Ste2, was shown to accumulate at endocytic sites subsequent to Ede1 recruitment but prior to recruitment of the late coat protein Sla1 [190]. These results, in combination with the fact that early arriving endocytic proteins seem to have more variable patch lifetimes compared to the highly consistent patch lifetimes of later arriving endocytic proteins, suggests that cargo accumulation may serve as a checkpoint for endocytic progression [178]. In this hypothesis endocytosis progression is stalled until proper cargo accumulation has occurred, at which point the rapid and more regular progression of endocytosis continues through the recruitment of later components of the endocytic machinery. Our work, demonstrated in chapter 3, investigates the role that cargo plays in recruitment of later components of the endocytic machinery.

Since yeast AP2 seems to have no known clathrin binding ability, the means by which clathrin recruitment occurs is not fully understood. Both components of the clathrin triskelion, the clathrin heavy chain (CHC) and the clathrin light chain (CLC), are recruited at the early onset of endocytic site formation [191, 192]. The early arriving clathrin adaptor proteins, and AP180 homologues, Yap1801/2, and later arriving, functionally redundant, Epsin proteins Ent1/2, have

been shown to play an important role in clathrin recruitment to endocytic sites through interactions of their C-terminal clathrin box motifs [192-194]. In these experiments fluorescently tagged clathrin light chain was shown to concentrate at the cell cortex after cells were treated with latrunculin A, an actin sequestering drug used for preventing actin polymerization. Deletion of Yap1801, Yap1802, Ent2, and all but the ENTH domain of Ent1, dramatically reduced the levels of cortical clathrin, which could partially be recovered by the expression of any one of the full length adaptors. It should be noted that these experiments did not completely eliminate clathrin from the cell cortex, and the Yap1801/2 and Ent1/2 proteins have patch lifetimes that suggest their arrival occurs after clathrin, indicating other means of clathrin recruitment exist [195, 196]. For these reasons a role for Sla1 in clathrin recruitment, and its role in endocytic progression, will be a significant focus of this thesis and detailed in chapter 2.

Other than a role in clathrin binding, the Yap1801/2 (AP180/CALM) proteins have been shown to contain a PtdIns(4,5)P_2 membrane binding ANTH domain, five NPF protein motifs, and play a cargo selective role for the recycling of the v-SNARE protein Snc1 [197-199]. Yeast two hybrid experiments have demonstrated that the NPF motifs in Yap1801/2 and Ent1 proteins interact with the scaffolding proteins Pan1 and Ede1, and these interactions are important for endocytic cargo internalization and normal protein dynamics [185, 193, 197]. This interaction is logical since both Ede1 and Pan1 contain EH domains that have a conserved role in binding NPF motifs, an interaction that has been seen between the mammalian homologues Eps15 and Intersectin respectively, as described previously. Further fluorescent microscopy experiments also demonstrated that Ent1/2 and Yap1801/2 were important for maintaining normal Pan1 temporal dynamics at endocytic sites [197]. In addition, mutation of the three EH domains in Ede1 that inhibit NPF binding resulted in higher levels of Ede1-RFP at endocytic sites, a similar

effect seen in cells absent of Ent1/2 and Yap1801/2 with the essential Ent1 ENTH domain present [197]. It was thus concluded that these later arriving adaptor proteins are necessary for the normal distribution of early arriving Ede1.

Following endocytic site specification, the recruitment of early endocytic factors such as scaffolding and adaptor proteins, and the transition from a hypothetical cargo checkpoint, the process of coat maturation continues to proceed. Proteins with a more regular temporal lifetime such as Sla2, Ent1/2, and the complex forming proteins Sla1/Pan1/End3 are recruited. Sla2 and Ent1 both contain $PI(4,5)P_2$ binding ANTH and ENTH domains respectively [185, 200]. In the case of Ent1/2 the lipid binding ENTH domain is proposed to contribute to its recruitment [185]. Ent1/2 also binds to ubiquitin through its UIM, an interaction that is important for its recruitment to the plasma membrane. Importantly, both protein cargo and endocytic machinery are subject to ubiquitination. This Ent1/2 UIM most likely interacts with ubiquitin on other components of the endocytic machinery since mutation of the UIM resulted in equally defective internalization of ubiquitinated and non-ubiquitinated version of the mating pheromone receptor Ste2 [201]. This interaction, along with binding of the Ent's multiple NPF motifs by the EH domains of other endocytic machinery, likely stabilize its localization to the plasma membrane [185].

Sla2, the Hip1R and Hip1 homologue, is another endocytic adaptor protein that arrives after endocytic site initiation, and can be considered to be part of the early/mid coat. Sla2 arrives after clathrin to endocytic sites and is one of the few endocytic proteins that bind directly to the clathrin light chain through a central coiled-coil domain that also mediates Sla2 dimerization [202-204]. It should be noted that the interaction of Sla2 with the clathrin light chain does not contribute to clathrin recruitment to endocytic sites, unlike its mammalian counterpart [192]. Sla2 has also been shown to interact with other components of the endocytic machinery, Pan1

and Sla1, through its coiled-coiled domain, which was also capable of inhibiting Pan1's actin nucleation promoting activity *in vitro* [205, 206]. As mentioned previously, Sla2 also has an amino-terminal ANTH domain. The interaction of Sla2 with PtdIns(4,5)P_2 through its ANTH domain is involved in coupling actin polymerization to membrane invaginations and vesicle internalization, and this mechanism occurs through cooperative binding of the ANTH domain with the Ent1 ENTH domain following membrane binding [200, 207]. Lastly, Sla2 contains a c-terminal THATCH domain that mediates interactions with F-actin [195, 208]. The combination of these interactions indicates that one of the main functions of Sla2 seems to be translating actin assembly at the end stages of endocytosis into membrane invagination. This is supported by the fact that in *sla2Δ* cells endocytic patches become stalled and do not invaginate, while also producing long F-actin assemblies at endocytic sites referred to as actin comet tails [209].

In the mid/late stages of coat formation endocytic scaffolding and adaptor proteins Pan1, End3, and Sla1 are recruited to endocytic sites. While these three proteins have been shown to form a complex, Pan1 and End3 appear to arrive at endocytic sites shortly before Sla1. Pan1 is a large scaffolding and coat protein that has four established domains. Pan1 has two N-terminal long repeat regions, LR1 and LR2, that contain two EH domains [210]. The EH domains of LR1 and LR2 have been shown to have a classical role in binding to NPF motifs found in the early arriving adaptor protein Yap1801, and likely Yap1802 [193]. LR1 has also been shown to associate with the C-terminal SR repeats of the endocytic adaptor protein Sla1, while the LR2 has been shown to interact with the C-terminal repeats of End3 [211, 212]. Pan1 also contains a central coiled-coil domain located more near the C-terminus that plays an important role in Pan1 dimerization as well as interactions with Sla2 that down regulates Arp2/3 activated actin polymerization by Pan1 [205, 213]. Lastly, Pan1 contains a WH2-like motif in its coiled-coil

domain that is conserved in various actin binding proteins, and a C-terminal proline rich region. These two regions bind to components of the actin machinery including Arp2/3, the type-I myosins Myo3/5, and F-actin [214-216]. The two roles of Pan1 thus seem to be to act as a actin nucleation promoting factor, and as a scaffolding protein that functions to recruit other components of the endocytic machinery. Sla1, Pan1, and End3 have all been shown to bind to one another for complex formation [211, 212]. These interactions are strongly believed to be disrupted through the phosphorylation of their interacting domains by the Ark1/Prk1 kinases after vesicle internalization [217]. It should also be noted that Pan1's role in endocytosis is essential, as deletion of the protein is lethal in budding yeast [210].

End3 has been described as a constitutive binding partner of Pan1 in clathrin-mediated endocytosis [212]. Through yeast two hybrid experiments the C-terminal domain of End3 was shown to interact with the Pan1 LR2 region as previously mentioned [218]. Other published work has demonstrated that End3 contains two binding tandem repeats (E3R) located within its C-terminal predicted coiled-coil domain, and it is these repeats that are involved in Pan1 binding [219]. Mutation of these regions prevented Pan1 binding *in vitro*, and just as is the case with *end3Δ* cells, causes hyper-phosphorylation of Pan1 and disruption of endocytosis [219]. Interestingly, fluorescence microscopy imaging revealed that the C-terminus of End3 is sufficient for its recruitment to endocytic sites through interactions with Pan1 [220]. Furthermore, End3 patch lifetime at endocytic sites is closely linked to Pan1, and gel filtration experiments demonstrate that Pan1 and End3 form a stable complex before recruitment to the plasma membrane [220].

Through fluorescence microscopy imaging, Sla1 has been shown to be recruited to endocytic sites shortly after Pan1 and End3, with an average lifetime of 30sec [220, 221]. Just as

is the case with Pan1, Sla1 is a large, multi-domain endocytic protein as shown in Figure 1.2. The N-terminal region of Sla1 contains three src-homology 3 (SH3) domains, two of which have been shown to be involved in generating a stable complex with the actin nucleation promoting factor (actin-NPF) Las17 [222, 223]. The interaction with the Las17 polyproline motifs 8-12 with Sla1 SH3 domains 1 and 2 was demonstrated to be important for inhibition of Las17 actin nucleation promoting ability by blocking a monomeric actin binding site in Las17 [223, 224]. While the function of the third SH3 domain in Sla1 has not been established, it has been shown to bind to both ubiquitin as well as a single polyproline motif found, interestingly enough, in mammalian dynamin [225]. In an interesting turn of events the Sla1 mammalian homologue CIN85 has also been shown to bind ubiquitin through its third SH3 domain [225]. Sla1 is also a clathrin and cargo binding adaptor protein [221, 226]. Following the third SH3 domain is the Sla1 homology domain 1 (SHD1) that binds to the protein-cargo sorting signal NPFxD located in the cytoplasmic portion of various membrane proteins and receptors [226-229]. Binding of this signal by SHD1 is either entirely necessary for membrane protein internalization, as is the case with Wsc1, or partially important for binding of endocytic cargo that is targeted for internalization through ubiquitination, as is the case with Ste2 and Ste3 membrane receptors [226, 229]. The ability of Sla1 to bind clathrin lies in its variable clathrin box (vCB), located C-terminally to its SHD1 and SHD2 domains [221]. The vCB sequence of LLDLQ makes it a variant due the presence of a Glutamine in the fifth position instead of an acidic amino acid. While the exact role of Sla1-clathrin binding is unknown, mutation of the vCB results in reduced endocytosis of the membrane cargo protein Wsc1, as well as an extended patch lifetime of Sla1 at endocytic sites.

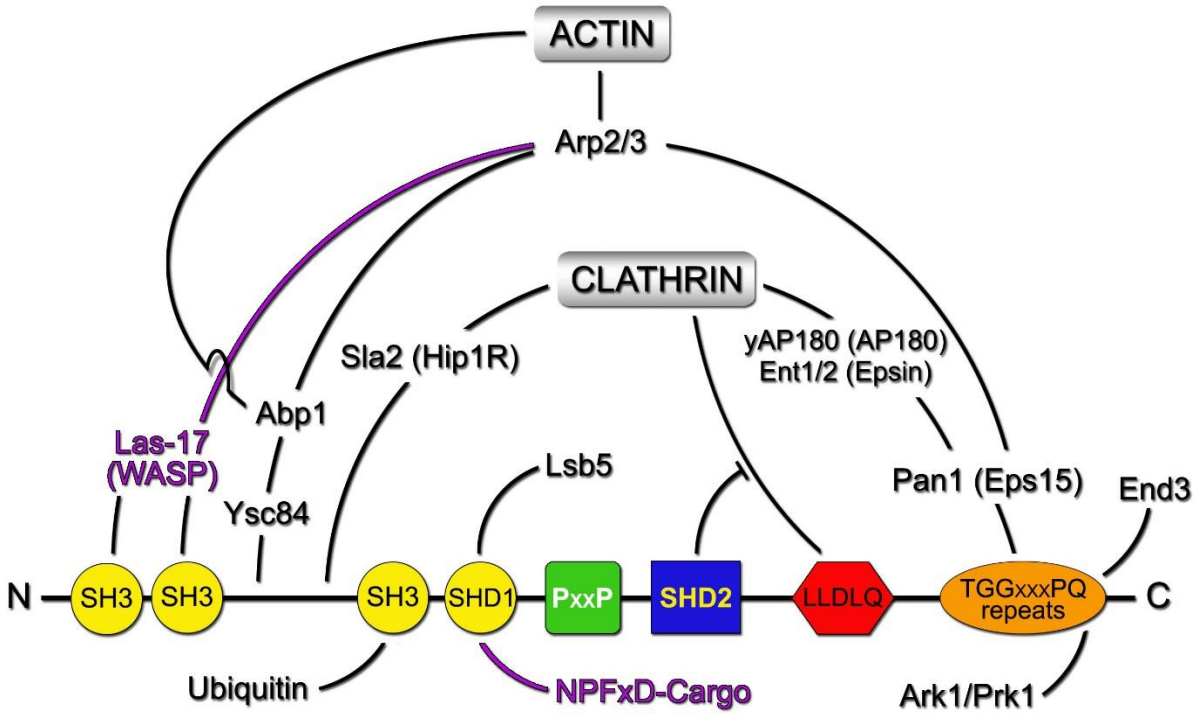

Figure 1.2: Diagram of a linearized model of Sla1, its established domains, and the proteins it interacts with. The first two SH3 domains of Sla1 have been shown to bind Las17 and regulate actin polymerization, while the third SH3 domain has been shown to bind monoubiquitin. The SHD1 domain binds to NPFxD containing cargo and targets it for endocytosis. No function is currently known for the PxxP motif found in Sla1. The SHD2 domain is capable of homodimerization and competing with clathrin for binding to the variable clathrin box (LLDLQ) shown in red. The C-terminal tail region of Sla1 is involved in binding with two other scaffolding and coat components of the endocytic machinery Pan1 and End3.

The function of the SHD2 domain still remains elusive; however, it has been demonstrate to have the ability to oligomerize, as well as compete for intramolecular binding of the vCB with clathrin [221]. It is possible that this interaction both regulates clathrin binding by the vCB and in tandem regulates oligomerization of the protein during coat formation. Lastly, the Sla1 C–terminus contains a series of Sla1 repeats, with an approximate consensus sequence of TGGxxxPQ termed SR repeats, shown to be responsible for the binding of Sla1 to End3 and Pan1 [211, 217]. Deletion of this region of Sla1 results in a mixed phenotype of Sla1-GFP localizing both diffusely at the cell cortex and in membrane patches that localize with and without other components of the endocytic machinery, such as Pan1 [220, 230]. Interestingly, Sla1 has also been shown to localize to the nucleus in protein truncations of the SR domain, a result that is possibly due to the presence of nuclear localization signals, and the absence of nuclear export signals that may reside in the C-terminal tail region [230, 231].

Around the end stages of coat formation recruitment of the actin polymerization module begins. Unlike the case with mammalian cells, yeast require a dynamic actin network for generating membrane invagination and completion of clathrin-mediated endocytosis [209, 232]. Actin nucleation is the rate limiting step in polymerization, and is facilitated by the function of the Arp2/3 complex and its activators, referred to as actin nucleation promoting factors (actin-NPFs) [233]. Las17, Myo3, Myo5, Abp1, and Pan1, have all been shown to contain actin nucleation promoting properties in which they enhance Arp2/3 activity and deliver actin monomers to the Arp2/3 active site [214, 234-236]. Additionally, endocytic proteins such as Syp1, Sla1, Sla2, Vrp1, Bzz1 and Bbc1 regulate the ability of the actin-NPFs to activate the Arp2/3 complex [182, 205, 223, 237-243]. In yeast, it appears that actin polymerization also marks the initiation of membrane bending. This was determined by direct correlated fluorescence

microcopy of protein pairs with electron tomography. These experiments utilized the ability to visualize two different fluorescently tagged proteins, one with a GFP and one with an RFP, in sections of yeast cells that had been exposed to high pressure freezing and resin embedding, while at the same time being able to perform EM imaging of the samples [244]. This allowed for an accurate measure of the shape of the plasma membrane at sites of endocytosis, as it correlates with the timed recruitment of endocytic adaptor proteins. Furthermore, in yeast, the formation of a dense branched actin network is essential for endocytosis to occur, a process found to be important in mammalian clathrin-mediated endocytosis in specific circumstances or cell types. This is due to the force generating nature of actin polymerization linked to the plasma membrane that is believed to overcome the internal turgor pressure of the yeast cells that would cause them to burst were it not for the presence of a cell wall [245].

In yeast the strongest of the endocytic actin nucleation promoting factors (actin-NPF's) is Las17, the mammalian WASP homologue [242]. Las17 contains a conserved N-terminal WASP homology (WH1) domain, a central stretch of multiple polyproline motifs, a C-terminal WH2 and acidic motif that are responsible for binding G-actin and stimulation of the Arp2/3 complex respectively, and a recently identified Las17 G-acting binding motif (LGB) that exists within its polyproline region [224, 234, 246-248]. In combination with Pan1, recruitment of Las17 can be thought of as the beginning of the recruitment phase for the actin machinery, arriving ~20 seconds before actin polymerization [209]. Just as was described above, Las17 forms a stable complex with Sla1, and is recruited to endocytic sites shortly after Pan1 [223]. The delay in Las17 activity of inducing Arp2/3 activated actin polymerization is attributed to its interaction with Sla1. Sla1 binds Las17 through its polyproline motifs to form a stable cytosolic "SLAC" complex, and it is this interaction that is necessary for delaying actin nucleation [223]. Sla1

binding is also important for proper Las17 recruitment to the plasma membrane [223]. Sla1 inhibits Las17 activity, in part, by competing with monomeric G-actin for the polyproline region that contains a second monomeric actin binding site [224]. Syp1 is also a negative regulator of Las17 activity by binding the Las17's unstructured central region to attenuate stimulation of the Arp2/3 complex [182]. In the case of Pan1, actin-NPF activity is believed to be inhibited by Sla2, however, how this regulation occurs is unknown [205]. The endocytic site thus waits at this stage, primed for actin nucleation and polymerization by Las17, Pan1, and other actin-NPFs.

Shortly after Las17 recruitment, and before the rapid actin assembly phase of endocytosis, the F-BAR domain containing protein Bzz1 and the WIP homologue Vrp1 are recruited to the clathrin-coated pit [242]. While the function of the F-BAR domain at the N-terminus of Bzz1 is currently unknown, the C-terminal region contains two SH3 domains that have been shown to interact directly with Las17 polyproline domains, and it is this interaction that is believed to be responsible for relieving inhibition of Las17 actin-NPF activity by Sla1 [240-242]. A second possibility is that Las17 inhibition is relieved by the proline rich region of Vrp1 that acts to compete with the Las17 polyproline region for Sla1 SH3 binding. This competition could also relieve inhibition by Sla1 [195]. Interestingly, it has also been shown that the Rab GTPase Sec4, which is involved in exocytosis, also contains the ability to relieve inhibition of Las17 actin-NPF activity by Sla1, as was demonstrated through *in vitro* pyrene actin polymerization assays. Additionally, Sec4 was shown to accumulate at endocytic sites shortly before actin polymerization, and was shown to bind to Las17 *in vivo* and *in vitro*, conclusively establishing its role in relieving inhibition of Las17 actin-NPF activity by Sla1 [249].

At about the same time as the initiation of actin polymerization, and after Bzz1 and Vrp1 recruitment, Type I myosins (Myo3/5) can be seen at cortical patches [242, 250]. Before their recruitment however, their activity and assembly is prevented through the binding of cytosolic calmodulin [251]. Myo3/5 are the second most potent actin-NPFs, after Las17, and their recruitment to endocytic sites and activation is dependent upon Vrp1 [242, 252]. Fluorescent imaging of yeast cells have shown that mutation of Myo3/5 actin-NPF domains results in a significant amount of fluorescently labeled endocytic patches that fail to internalize, and deletion of Myo3/5 and Vrp1 failed to internalize any patches labeled with Sla1-GFP [242]. This is a similar phenotype of cells treated with Latrunculin A, an actin sequestering drug that prevents actin polymerization [209]. When the plasma membrane begins to invaginate upon actin polymerization, fluorescent imaging demonstrated Myo5 remains immobile at the surface, however immuno-EM imaging has shown that a second smaller population can be seen at the tip of the internalizing vesicle [250, 253]. Fluorescence recovery after photobleaching (FRAP) experiments in *Δsla1Δbbc1* cells that generate long actin comet tails, have demonstrated actin polymerization likely begins at the plasma membrane and moves inward [191]. It has been suggested that while Las17 and the majority of Myo5 are functioning to drive actin polymerization at the base of the plasma membrane, the small population of Myo5 at the tip of the vesicle may be acting to bridge the actin network to the endocytic machinery. Two other possibilities are that Myo5 is acting to nucleate filaments for a thus far undetermined reason, or possibly it is responsible for a motor driven force that helps push the vesicle inward [178].

In order for the actin network to properly generate an inward force on the plasma membrane a number of additional actin binding proteins are required. The yeast fimbrin Sac6 is an actin bundling protein that crosslinks actin filaments [254]. Deletion of actin bundling

proteins Sac6 does not prevent actin polymerization at endocytic sites, but does result in non-productive endocytic patches that do not move off the plasma membrane [191]. The protein Scp1 functions with Sac6 to bundle actin filaments, and like Sac6 also localizes to cortical actin patches [191, 255, 256]. While the two proteins serve similar roles in actin bundling at endocytic sites, a difference in recruitment times to cortical patches and apparent differing roles in membrane invagination and scission gives distinct roles for the two proteins [257].

Actin filament disassembly is also crucial for controlling the actin filament architecture at endocytic sites. Three important proteins involved in this process are Cofilin, Coronin, and Aip1. All three of these proteins have been shown to localize to cortical actin patches with similar patch lifetimes and appear after the initiation of actin polymerization [258]. Cofilin is an actin severing protein that preferentially severs older ADP-containing filaments [259]. Aip1 and Coronin work with Cofilin to enhance the severing and turnover of actin filaments in vivo [260, 261]. Furthermore, Cofilin was shown to be required for the localization of Aip1 and Coronin to cortical actin patches, while Aip1 was necessary for preventing Cofilin and Coronin localization to actin cables vs membrane patches [258, 260].

The final step in clathrin-mediated endocytic vesicle internalization is recruitment of the membrane scission machinery and severing of the endocytic vesicle from the plasma membrane. In order for membrane fission to occur the two membrane bilayers must be brought into close proximity. In mammalian cells the energy driven GTPase dynamin is responsible for scission of clathrin-coated vesicle. Yeast have a dynamin-like protein Vps1 that plays a role in membrane fission. Vps1 colocalizes at the end stages of endocytosis with other components of the endocytic machinery, and deletion of Vps1 caused an increase in the patch lifetime of fluorescently tagged endocytic proteins and an increase in the number of retraction events [262]. While Vps1 does

play a role in membrane fission, it is the yeast amphiphysin proteins Rvs161 and Rvs167 that are believed to be primarily responsible for scission events at endocytic sites. Rvs161/167 are N-BAR domain containing proteins that arrive at the end stages of endocytosis, after actin polymerization [191]. These proteins have been shown to bind liposomes and promote tubule formation in vitro [263]. Furthermore, deletion of either Rvs167, Rvs161, or both, causes a phenotype in which patches labeled with Sla1-GFP failed to internalize in about 30% of the patches, indicating that the proteins function together for vesicle scission [191]. Interestingly, the SH3 domain of Rvs167 was shown to bind Vps1 and is important for recruitment of Vps1 to endocytic sites, and disruption of this interaction causes elongated membrane invaginations [264].

Over the last 50 years studies in the field of clathrin-mediated endocytosis have been highly rewarding. Key findings in this process have yielded Nobel Prize winners, better treatment of the sick, and a general greater understanding of how cells and their protein components function. While the successes of studying CME have been many, a plethora of unanswered questions still remain. Many components of the endocytic machinery have already been identified through various kinds of genetic, protein binding, and fluorescent microscopy screens, that interestingly enough have also indicated that unknown components may still exist. In addition, the proteins that we have already identified contain domains of unknown function, and the mechanisms by which they assist in endocytic progression, are incompletely understood. Even fundamental questions in the field of endocytosis still remain unanswered and debatable. Is cargo loading necessary for endocytosis to proceed? How endocytic sites are initiated? What is the organizational pattern of many of these proteins in both space and time as endocytosis progresses? Answering these questions are currently all active fields of research.

1.4 Clathrin adaptors, cargo binding, and endocytic sorting signals

1.4.1 Clathrin adaptors and recognition of cargo sorting signals

The set of proteins known as Clathrin Adaptors are an essential component of the clathrin-mediated endocytic machinery. The term "adaptor protein" was first coined to describe a series of molecules named AP1, AP2, AP3, AP4, and AP5, that all function in intracellular trafficking to link clathrin to membrane cargo and lipids of budding vesicles. While proteins AP1 and AP3 were demonstrated to be involved in trafficking from the golgi network and endosomes respectively, mammalian AP2 was demonstrated to be a component involved in trafficking from the plasma membrane [265-268]. AP4 is involved in trafficking from the golgi network, but interestingly does not associate with clathrin, nor contain a clathrin binding motif [269-272]. Although AP2 was the first to be identified as being involved in clathrin-mediated endocytosis, further studies of endocytosis in different organisms have determined the existence of a variety of clathrin adaptors that interact with different cargos, lipids, and components of the endocytic machinery, some of which have unique mechanism responsible for regulating these interactions.

While evidence suggests that AP2 in yeast does not bind to clathrin, nor play a crucial role in progression of endocytosis, its mammalian homologue serves as a unique model protein for studying adaptor function in clathrin and cargo binding and recruitment to endocytic sites [187]. Mammalian AP2 is a heterotetrameric adaptor protein composed of α, β2, μ2, and σ2 protein subunits [84, 273]. AP2 localization to the plasma membrane is driven by its binding to $PI(4,5)P_2$ by its α and μ2 subunits, the α subunit of which has also been shown to bind PIP_3 [64, 274-277]. The binding of $PI(4,5)P_2$ induces a conformational change in the protein from a "locked" or "inactive" state to an "open" or "active state" that is capable of binding membrane cargo, an alteration that further stabilizes AP2 in the open state [72, 277]. AP2 has been shown to

recognize two established cargo sorting signals. The first is the tyrosine hydrophobic signal Yxxφ, where φ is a bulky hydrophobic residue, recognized by the C-terminal domain of the μ2 subunit [278, 279]. The second is the acid dileucine motif [ED]xxxL[LI] recognized by combined regions of the α and σ2 subunits [69, 280, 281]. While both the α and β2 subunits interact with other components of the endocytic machinery including clathrin adaptors, it is the β2 subunit that is responsible for interactions with clathrin [128, 282-284]. Just as is the case with the α subunit, the β2 subunit contains a flexible hinge connected to an appendage domain. It is the flexible hinge of β2 that contains a canonical clathrin box motif of sequence LLNLD that is responsible for clathrin binding [283]. The C-terminal appendage domain also interacts with clathrin to a weaker extent, however both interactions are required for efficient clathrin binding [284]. Interestingly, just as is the case for a regulated mechanism of cargo binding, the ability of AP2 to bind clathrin is also regulated. In the "locked" conformation the β2 hinge is sequestered in the core of the heterotetrameric AP2 complex and is thus incapable of properly generating clathrin coat formation, however upon membrane recruitment, via lipid and cargo binding, the hinge is released from the core and proper clathrin binding can occur [73].

While AP2 has been demonstrated to be the most important of the clathrin binding adaptor proteins in mammals, other cargos use monomeric clathrin adaptor proteins referred to as clathrin-associated sorting proteins or CLASPs [285]. Disabled-2 (Dab2) is one example of these CLASP adaptor proteins. Dab2 contains a N-terminal phosphotyrosine-binding domain (PTB) that binds to $PI(4,5)P_2$ and an FxNPxY sorting signal found on many endocytic cargos such as the LDL receptor, the amyloid precursor protein (APP), APP-like protein 1 (APLP1), APLP2 and β1 integrin [286-289]. Fluorescent microscopy imaging revealed that Dab2 localizes with endocytic cargos such as the LDL receptor, as well as the adaptor protein AP2 and clathrin at

sites of endocytosis [286]. It was further demonstrated that a direct interaction between the Dab2 c-terminal region and the α-adaptin appendage domain of AP2 occurred in vivo, and that this interaction likely contributes to recruitment of Dab2 to endocytic sites [286]. In addition to these interactions, Dab2 has also been shown to bind clathrin and induce triskelion formation into a regular polyhedral coat [287]. Interestingly, Dab2 has also been shown to localize to the nucleus, and this nuclear localization appears to be cell cycle dependent [287].

The autosomal recessive with hypercholesterolemia protein ARH is another example of a CLASP that is a significantly important protein in the regulation of cholesterol internalization in mammals. The low density lipoprotein receptor (LDLR) binds to the LDL particle and plays a significant role in cholesterol uptake by the cell. It was the analysis of mutant LDL receptors that lead to the identification of the NPxY cytoplasmic tail sequence as the first endocytic sorting signal of a transmembrane protein that would later be shown to be present in other membrane receptors [62, 63]. Further research on patients with a rare autosomal-recessive form of hypercholesterolemia lead to the identification of ARH as an important PTB containing protein that has a significant role in uptake of the LDL receptor and its cholesterol containing ligand [290-292]. Given these results, further research demonstrated that the ARH PTB domain could bind directly to the NPxY motif found in the LDLR tail [293]. Furthermore, just as is the case with Dab2, ARH was shown to bind liposomes in a fashion favorable to those that contain $PI(4,5)P_2$ [294]. Similarly, ARH was also shown to interact with clathrin via a canonical clathrin box (LLDLE) that bound the clathrin heavy chain. Additionally, ARH was shown to bind the β2 subunit of the adaptor protein AP2, and colocalizes with the endocytic machinery [293, 294]. The body of work focusing on the LDL receptor, ARH, and related proteins such as Dab2 demonstrates a conserved role for endocytic adaptor proteins in the targeting of endocytic cargo

while simultaneously interacting with clathrin and other important components of the endocytic machinery. On a final note, Dab2 and ARH are simply two examples of a family of PTB containing proteins that recognize the NPxY motif found in various membrane receptors, many of which are part of a family of LDL receptor-related proteins (LRPs) [295].

In *Saccharomyces cerevisiae*, the currently identified clathrin adaptor proteins and clathrin binding proteins include Ent1/2, Yap1801/2, Ubx3, Sla2, and Sla1 [193, 194, 202, 221, 296]. While each of these proteins have been shown to directly interact with clathrin, the established ability to also simultaneously bind membrane cargo via a sorting signal appears to be a key property unique to Sla1 [221, 226, 227]. It is for these reasons, of which will be discussed in further detail, that Sla1 serves as a particularly useful molecule for studying mechanisms of clathrin-adaptor-cargo binding in a model organism such as yeast where proteins with specific mutations and modification can be expressed at endogenous levels.

Sla1 (synthetic lethal with actin binding protein 1) was first identified for its synthetic lethality in yeast upon its combined deletion with the actin binding protein 1 (Abp1) [222]. As mentioned above, Sla1 is a large multidomain protein that serves numerous functions in endocytosis (Figure 1.2). The Sla1 amino-terminus is involved in stable complex formation with Las17 and down-regulation of the actin nucleation promoting activity of Las17, while the carboxy-terminus has been shown to be involved in complex formation with End3 and Pan1 via its SR (Sla1 repeat) region, an interaction that is regulated through phosphorylation and appears to be involved in localization of Sla1 to endocytic sites [211, 217, 223, 230]. It is, however the ability of Sla1 to bind cargo through its SHD1 domain, ubiquitin through its SH3-3 domain, and clathrin through it vCB, that is the focus of this dissertation.

The Sla1 homology domain 1 (SHD1) and homology domain 2 (SHD2), were rightfully named for their unique structure found in Sla1 homologues of *Saccharomyces cerevisiae* (budding yeast) and *Schizosaccharomyces pombe* (fission yeast) [297]. While the SHD2 domain appears to have a role in regulating Sla1-clathrin binding and Sla1 oligomerization, the SHD1 domain has a role in cargo binding and active cargo transport into the endocytic pathway [221, 226]. The SHD1 domain was first demonstrated to have a role in cargo binding in studies involving expression of a plasma membrane Ste2p/Kex2p chimera protein containing the NPFSD amino acid sequence, a motif that targets plasma membrane proteins for endocytosis [226, 298]. In this work it was shown that the Sla1 SHD1 domain bound to the NPFSD sequence, and that this interaction was necessary for endocytosis of the chimera protein, and was also important for normal endocytosis of the pheromone receptor Ste3 [226]. Following this, the structure of the SHD1 domain in complex with an NPFxD containing peptide identified key residues of a hydrophobic pocket responsible for the interaction [227]. In addition to these findings a database search of plasma membrane proteins identified candidates that contain NPFxD sequences, one of which is the cell wall stress sensor Wsc1 [229]. Wsc1 has been shown to function as a cell wall stress sensor involved in maintenance of cell wall integrity and mostly localizes to the bud of mitotic yeast cells [299]. Using this knowledge, experimentation demonstrated Wsc1 contained a functional NPFxD endocytic sorting signal that was specifically recognized by the SHD1 domain of Sla1 for its internalization [229]. It is this recognition, internalization, and recycling of Wsc1 that maintains it at the outgrowth of budding yeast cells. In addition to these findings the yeast P4-ATPases Drs2p and Dnf1 were also shown to contain functional NPFxD sequences that are targeted by the Sla1 SHD1 domain for entry into the endocytic pathway [228]. While these findings demonstrate that the NPFxD sequence can act as

a targeted protein sorting signal for CME, it has not been established as to whether this sequence can function in the active recruitment of the adaptor protein Sla1 to endocytic sites, or whether any endocytic cargo signals directly contributes to the recruitment of any component of the endocytic machinery.

These clathrin adaptors and the linear motifs to which they bind are some of the best examples of endocytic machinery targeting endocytic cargo. These, however, are not the only examples of how protein cargo is targeted by the endocytic machinery. Other mechanisms such as post-translational modifications of ubiquitination, phosphorylation, and unique folded protein domains have been shown to contain information that targets cargo for endocytosis. The various forms of endocytic cargo sorting signals are listed in Table1.2 along with the components of the endocytic machinery that target them for endocytosis [300].

1.4.2 Ubiquitination targets proteins for clathrin-mediated endocytosis

In addition to cargo sorting signals acting as a means by which membrane bound proteins and receptors are targeted for clathrin-mediated endocytosis, ubiquitination of membrane protein cargo targets them for endocytosis. Ubiquitin is attached to its target substrate through the concerted action of three enzymes, the E1 ubiquitin activating enzymes, E2 ubiquitin conjugating enzymes and E3 ubiquitin ligases [301, 302]. The first evidence for ubiquitin as having a role in receptor endocytosis came from studies in yeast involving the G-protein-coupled receptor (GPCR) Ste2, and membrane transporters such as the uracil permease Fur4 and the ABC transporter Ste6 [303-306]. Additional work in *Saccharomyces cerevisiae* identified Rsp5 as the Nedd4-like E3 ubiquitin ligase responsible for the ubiquitination of numerous endocytic cargos,

Table 1.2: Endocytic Cargo Sorting Signals and Adaptors. Question marks indicate that speculation still exists as to whether the adaptor targets the sorting signal. Adapted from [300].

Mammalian Yeast Both

Amino Acid Signals, Domains, or Modifications	**Adaptor**	**Adaptor subunit(s) or Domain**
Yxxϕ	AP2	μ2
[DE]xxxL[LI]	AP2	α/σ2
Acidic clusters	AP2?	α?
[YF]xNPx[YF]	ARH; Dab2; Idol; SNX17, 27, and 31	PTB domain
$NPFx_{(1,2)}D$	Sla1	SHD1 domain
DxY	Syp1	μ-homology domain
Ubiquitin	Eps15, Ede1?, Epsin1/2?, Ent1/2?	UIM and UBA domains
GPCR phosphorylation	β-arrestin 1/2	N-terminal region
Synaptotagmin I C2A (C2B) domain	Stonin 2	μ-homology domain
Alk8 cytosolic domain	Fcho1	μ-homology domain
VAMP 7 longin domain	Hrb, AP-3	C-terminal unstructured region
VAMP 2, VAMP 3, and VAMP 8 SNARE motifs	CALM	ANTH domain

first identified by looking at the permeases Gap1 and Fur4, and sub sequentially a number of other plasma membrane channels, transporters, and receptors [307-311]. In mammalian cells, receptor tyrosine kinases (RTKs) have been highly studied for being targets of ubiquitination by the E3 ligase Cbl for downregulation and entry into the endocytic pathway [312, 313]. These and other studies have very clearly demonstrated that membrane protein cargo ubiquitination serves as an important sorting signal for entry of plasma membrane proteins into the endocytic pathway. While it has been established that ubiquitin acts as a signal for targeting membrane proteins for endocytosis, exactly how ubiquitin is recognized by the endocytic machinery is not as clear as its function as an endocytic sorting signal. The mammalian Eps15 and Epsin proteins, and their yeast homologues Ede1 and Ent1/2, are examples of candidate proteins that may link ubiquitinated cargo to the endocytic machinery. As previously described, Eps15 is an early arriving endocytic protein involved in the internalization of EGFR, which is ubiquitinated by c-Cbl, and contains two ubiquitin interacting motifs (UIM) capable of binding ubiquitinated proteins [94, 314, 315]. Furthermore, overexpression of c-Cbl enhances ubiquitination of EGFR, while also enhancing recruitment of Eps15 to the plasma membrane upon stimulation with EGF [315]. The Epsin family of proteins also contain UIMs, which in Epsin1 have been shown to bind to mono and polyubiquitin chains [120, 125, 126, 314].

In yeast, the ability of Ede1 and Ent1/2 to bind and target ubiquitinated cargo for internalization seems to be up for debate. In one example the UIMs of Ent1 and the UBA domain of Ede1 are capable of binding ubiquitin and yeast membranes in an ubiquitin dependent fashion [185, 316]. This work seems to suggest that ubiquitinated cargo may be targeted by Ede1 and Ent1, and that this could contribute to Ede1 and Ent1 recruitment. Further work, however, indicates that this may not be the case. In endocytic assays that measured the internalization of

radio labeled alpha factor by its ubiquitinated plasma membrane receptor Ste2, the alpha factor was internalized via endocytosis in a normal fashion regardless of the presence of the Ent1/2 UIM or the Ede1 UBA ubiquitin binding domains [201]. This indicates that possibly neither of the Ede1 or Ent1/2 proteins are involved in targeting ubiquitinated cargo for endocytosis, and another component of the endocytic machinery is capable of performing this function. Further endocytic assays revealed that the UIM of Ent1 was important for endocytosis of both ubiquitinated and non-ubiquitinated Ste2, suggesting a defect in endocytic machinery protein-protein interactions that are not specific to ubiquitinated cargo. In fact, this work seemed to suggest that ubiquitin binding by Ent1 merely serves an overlapping function of the Ent1 NPF motif with the Ede1 EH domains [201]. Hypothetically, this interaction is plausible as Ede1 has been shown to be itself ubiquitinated during progression of endocytosis [317].

A third, and most recently identified candidate for targeting ubiquitinated cargo for endocytosis is Sla1 and its mammalian homologue CIN85. A screen for endocytic proteins capable of binding monoubiquitin identified the third SH3 domain of Sla1 [225]. This interaction was confirmed through pulldown experiments and the location of key residues responsible for ubiquitin binding were identified by NMR. Furthermore, in a serendipitous turn of events the third SH3 domain of CIN85 was also shown to bind ubiquitin, suggesting a conserved role for these two proteins in the binding of monoubiquitin [225]. This work however does not indicate if Sla1 binds to either ubiquitinated cargo or other components of the endocytic machinery that are ubiquitinated. The question of which components of the endocytic machinery are responsible for targeting ubiquitinated cargo for endocytosis thus remains unclear.

Membrane protein cargos including receptors, permeases, and ion channels have thus been shown to be targeted for endocytosis through the cytosolic exposure of protein sorting

signals and the post translational modification of ubiquitination. While these insights bring great clarity to the process by which cargo is selected for clathrin-mediated endocytosis, a variety of questions related to the role endocytic cargo plays in progression of endocytosis remain. For instance, it is unclear as to whether membrane cargo plays an active role in the recruitment of the endocytic machinery or activation of that machineries function. Along those same lines it is unclear whether or not cargo loading is necessary for progression of endocytosis, or if cargo itself contains a functional role in aspects of endocytosis such as positioning of the endocytic machinery or shaping of the plasma membrane. Furthermore, it is additionally unclear as to the exact timing at which different cargos are recruited to the endocytic sites, and whether specific cargo sorting signals determine the timed incorporation of the machinery into an endocytic site.

With this being the case, certain publications do seem to hint that cargo plays an active function in endocytosis. One example was demonstrated through what seems to be the active recruitment of the endocytic adaptor proteins Dab1 and ARH through binding of its established endocytic cargo the LDL receptor (LDLR). In this work cells expressing low levels of the endocytic adaptor proteins Dab1 and ARH and the receptor LDLRs were used. Upon overexpression of a CD8/LDLR protein chimera containing the FxNPxY cargo sorting signal, fluorescently tagged Dab1 and ARH protein recruitment to the plasma membrane was enhanced. Furthermore, point mutations made to the PTB domain of ARH that prevent LDLR binding, also prevented membrane recruitment of ARH [318]. In addition to these findings, ARH was shown to localize to the nucleus when no CD8/LDLR was being overexpressed, a phenotype that was also seen upon mutation to the ARH PTB cargo binding domain [318]. While this result does suggest an active role in recruitment of the endocytic machinery by its target cargo, certain caveats still remain. For instance the ability of cargo to recruit Dab1/ARH in the defined system

could be a result of artificial overexpression of either the endocytic adaptor proteins or the endocytic cargo, a recruitment effect that has been shown for other endocytic proteins such as Dynamin when overexpressed [169, 319, 320]. Furthermore, these experiments were performed in cells that express low levels of the endocytic cargo of interest and their adaptors, bringing into question whether the endocytic processes performed in these cells is typical of what is seen in mammalian clathrin-mediated endocytosis. This is reflected in the appearance of larger levels of clathrin at endocytic sites upon low level expression of CD8/LDLR, and the appearance of giant clathrin-coated structures under high expression of CD8/LDLR. Lastly, these results do not seem to answer whether all endocytic cargo sorting signals are capable of recruiting their relative binding partners, and whether this mechanism of recruitment is conserved across species. Insight into these questions will be addressed in chapter 3.

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CHAPTER 2

THE SLA1 ADAPTOR-CLATHRIN INTERACTION REGULATES COAT FORMATION AND PROGRESSION OF ENDOCYTOSIS

2.1 Summary

The following chapter is reproduced with alterations from an article published by us in the scientific journal *Traffic* [1]. Clathrin-mediated endocytosis is a fundamental transport pathway that depends on numerous protein-protein interactions. Testing the importance of the adaptor protein-clathrin interaction for coat formation and progression of endocytosis *in vivo* has been difficult due to experimental constrains. Here we addressed this question using the yeast clathrin adaptor Sla1, which is unique in showing a cargo endocytosis defect upon substitution of three amino acids in its clathrin-binding motif (*sla1*^{AAA}) that disrupt clathrin binding. Live cell imaging showed an impaired Sla1-clathrin interaction causes reduced clathrin levels but increased Sla1 levels at endocytic sites. Moreover, the rate of Sla1 recruitment was reduced indicating proper dynamics of both clathrin and Sla1 depend on their interaction. *sla1*^{AAA} cells showed a delay in progression through the various stages of endocytosis. The Arp2/3-dependent actin polymerization machinery was present for significantly longer time before actin polymerization ensued, revealing a link between coat formation and activation of actin polymerization. Ultimately, in *sla1*^{AAA} cells a larger than normal actin network was formed, dramatically higher levels of various machinery proteins other than clathrin were recruited, and the membrane profile of endocytic invaginations was longer. Thus, the Sla1-clathrin interaction is important for coat formation, regulation of endocytic progression, and membrane bending.

2.2 Introduction

Endocytosis is necessary for a variety of fundamental cellular activities, including nutrient internalization, regulation of signal transduction, and cell surface remodeling. Clathrin-mediated endocytosis (CME) is a major endocytic pathway involving numerous proteins that collect cargo into a membrane patch, invaginate the membrane, and pinch off a vesicle [2-7]. Quickly after being released, the vesicle loses the coat of clathrin and other machinery components and then fuses with endosomes. This process is conserved throughout evolution and proceeds through a well-defined sequence of events [3, 4, 8-10].

Saccharomyces cerevisiae has been a very fruitful system to study CME using a combination of genetics, live-cell fluorescence microscopy, and biochemistry [8, 11-13]. Several studies have revealed CME takes place through discrete stages and a well-choreographed assembly and disassembly pathway of endocytic machinery components. First, clathrin and other proteins, such as Ede1 and Syp1, arrive to the plasma membrane and begin collecting transmembrane cargo (e.g., receptors) [11, 12, 14-17]. This step is immobile, relatively long and variable in time (~ 1 min). Second, other fundamental components of the immobile phase arrive, including Sla1, Pan1 and Las17, approximately 20 sec before actin polymerization [13]. Third, a fast, mobile stage of endocytosis occurs concomitant with Arp2/3-mediated actin polymerization [18]. Components of the coat, such as Sla1, move into the cell together with the bending membrane [11, 18, 19]. This mobile stage of endocytosis is brief (~15 sec) and culminates with vesicle scission, which is facilitated by the BAR-domain proteins Rvs161/167. Fourth, immediately after the scission step, most components of the coat disassemble and the released vesicle moves towards endosomes. While the various stages of endocytosis have been very well established, the regulation of the transition and progression through these stages is less well understood.

The clathrin 'triskelion', the soluble form of clathrin, is composed of three heavy chains and three light-chain subunits [5, 6, 20-22]. As clathrin is unable to bind directly to membrane components, coat assembly requires adaptors that link clathrin to membrane proteins and/or lipids [23, 24]. The clathrin box (CB) is a type of clathrin-binding motif present in adaptor proteins that binds the N-terminal domain of the clathrin heavy chain [20, 25-27]. In addition to assist in clathrin recruitment, adaptors also select and concentrate transmembrane protein cargo by binding to cytoplasmic sorting signals in cargo proteins [28]. Interestingly, binding to clathrin and cargo may also stabilize adaptors as part of the coat [25, 29]. Additionally, adaptors interact with a host of accessory proteins that function in different stages of endocytosis. Sla1 binds clathrin through a variant clathrin box (vCB) sequence (LLDLQ) and also binds and collects transmembrane protein cargo containing the NPFxD endocytic signal [30-34]. Thus, Sla1 is a good example of an endocytic clathrin adaptor. Moreover, it was previously shown that mutation of the Sla1 vCB motif from LLDLQ to AAALQ in the *SLA1* gene (*sla1*^{AAA}) impedes physical interaction with clathrin and causes a defect in endocytosis of endogenous NPFxD-dependent cargo [30]. While this result underscored the importance of adaptor-clathrin interaction during CME, it is not clear if the endocytosis defect is the result of aberrant clathrin recruitment, Sla1 recruitment or both.

How does the adaptor-clathrin interaction impact progression of endocytosis and the ability of the CME machinery to bend the membrane and change the invagination shape? This question has gained particular relevance in light of recent findings that all the needed clathrin appears to be present at endocytic sites since early stages of CME when the membrane is still flat. This result was first reported in yeast cells, which necessitate actin polymerization to drive membrane bending and internalization [18]. Subsequently, the same correlative fluorescence and electron

microcopy approach applied to mammalian cells, which may have a more nuanced actin requirement, also showed the presence of a full clathrin coat before membrane bending [35, 36]. As a result, the traditional view of clathrin function in shaping the plasma membrane invagination has been challenged [20, 37, 38]. In this emerging new model, clathrin is being described as a passive player that adjusts to the changing curvature imposed by other components of the machinery such as actin and BAR-domain proteins [18, 35]. It is therefore unclear if clathrin cooperates with such membrane shaping forces of the CME machinery during progression of endocytosis.

Here we explored the functional meaning of the adaptor-clathrin interaction using Sla1 vCB mutant cells (*sla1*^{AAA}). We observed an overall decrease in clathrin recruitment and increase in Sla1 recruitment at CME sites, a significant delay in progression to later stages of endocytosis, an excess accumulation of the actin machinery and other CME proteins, and abnormal endocytic membrane profiles. The findings suggest that clathrin recruitment by adaptor protein contributes to transition to later stages of endocytosis and cooperates with other components of the CME machinery to shape the invaginating membrane.

2.3 Results

2.3.1 Impaired Sla1-clathrin binding results in higher levels of Sla1 at endocytic sites

Our previous work showed *sla1*^{AAA} cells constitute an ideal system to address the function of adaptor-clathrin interaction in CME [30]. First we wanted to establish if an impaired interaction between Sla1 and clathrin in *sla1*^{AAA} cells could cause a defect in Sla1 recruitment, clathrin recruitment or both. To investigate this question, a strain expressing both Sla1^{AAA}-GFP and Pan1-RFP from the corresponding endogenous locus was generated and analyzed by two-

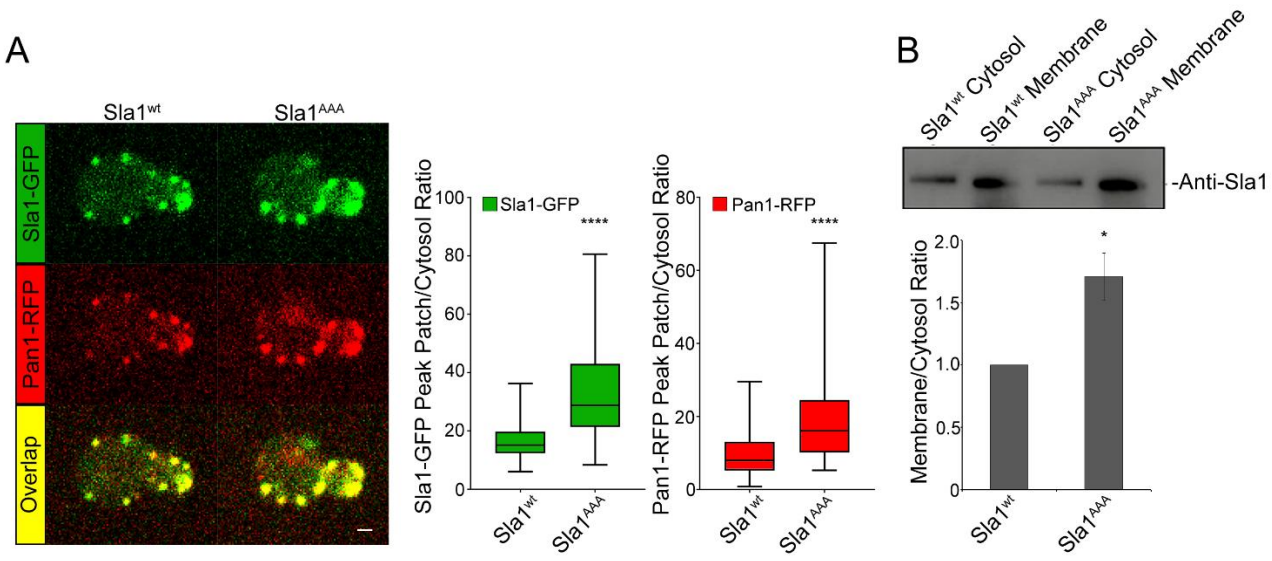

Figure 2.1: Defective Sla1-clathrin binding results in higher levels of Sla1 and Pan1 at endocytic sites. (A) Live cell confocal fluorescence microscopy analysis of yeast cells expressing both Sla1^{AAA}-GFP and Pan1-RFP from the corresponding endogenous locus (*sla1^{AAA}* cells). Wild type cells expressing Sla1^{wt}-GFP and Pan1-RFP were analyzed in parallel for comparison. Left, panels show one representative frame of a movie (scale bar, 1μm). Right, quantification represented as box and whisker (minimum-maximum) plots showed higher peak patch/cytosol fluorescence intensity ratio for Sla1^{AAA}-GFP relative to Sla1^{wt}-GFP ($P<0.0001$, N=50 patches). The black horizontal line inside each box indicates the mean. Pan1-RFP levels at endocytic sites were also enhanced in cells expressing Sla1^{AAA}-GFP relative to cells expressing Sla1^{wt}-GFP ($P<0.0001$, N=50 patches). (B) Yeast cell extracts from *sla1^{AAA}* cells and wild type cells were separated into membrane and cytosol fractions and subjected to immunoblotting analysis with anti-Sla1 antibodies. Representative experiment (top) and quantification (bottom) showing a higher proportion of membrane associated Sla1^{AAA} relative to Sla1^{wt} ($P<0.05$, N=6, bars represent mean ± SEM).

color, live cell confocal fluorescence microscopy. A wild type control strain expressing Sla1-GFP and Pan1-RFP was also generated and analyzed in parallel. Pan1 is an endocytic protein with a recruitment time similar to that of Sla1 that serves as a reference for endocytic sites [13, 39, 40]. Importantly, Sla1^{AAA}-GFP was present at all Pan1-RFP patches, paralleling results with wild type cells (Figure 2.1A). We then measured the Sla1 peak patch/cytosol fluorescent intensity ratio in both wild type and *sla1^{AAA}* cells. Results from these experiments demonstrated that not only were levels of Sla1^{AAA}-GFP not reduced at endocytic sites, but were actually enhanced to statistically significant higher levels relative to Sla1-GFP (Figure 2.1A). The number of Sla1^{AAA}-GFP patches per cell was also increased relative to Sla1-GFP (Figure 2.2, Supporting Information). Interestingly, Pan1-RFP levels at endocytic sites were also enhanced in *sla1^{AAA}* cells. As an independent method to assess Sla1^{AAA} levels at the plasma membrane, we performed a biochemical experiment. Total membrane and cytosol fractions from yeast extracts expressing wild type Sla1 or the Sla1^{AAA} mutant were separated by ultracentrifugation and subjected to immunoblotting analysis with anti-Sla1 antibodies (Figure 2.1B). Quantification of band intensities (Figure 2.1B) demonstrated higher membrane/cytosol ratio in *sla1^{AAA}* cells relative to wild type cells, a result that correlates with the live cell fluorescent microscopy data. These results indicate that the defects in endocytosis observed in *sla1^{AAA}* cells are not a result of reduced levels of Sla1^{AAA} protein at endocytic sites.

2.3.2 Deficient Sla1-clathrin binding results in lower clathrin levels during late stages of endocytosis

In order to determine the effects of abolished Sla1-clathrin binding on clathrin levels at endocytic sites, we took a live cell confocal fluorescence microscopy approach. A strain

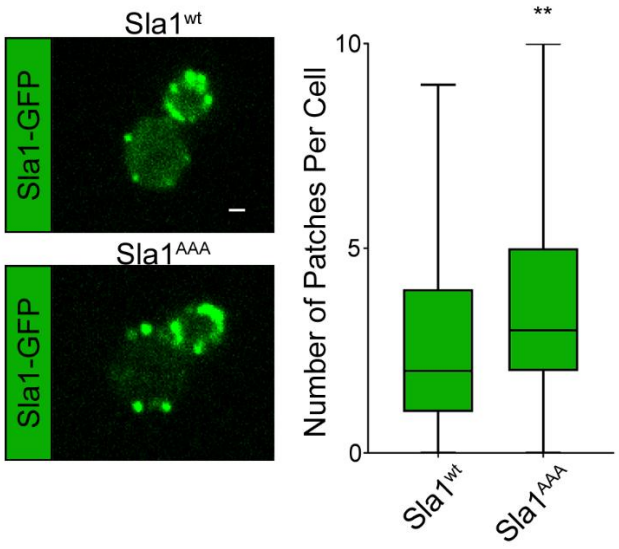

Figure 2.2: Defective Sla1-clathrin binding results in a higher number of endocytic sites labeled by Sla1-GFP. Live cell confocal fluorescence microscopy analysis of yeast cells expressing Sla1^{AAA}-GFP or Sla1^{wt}-GFP. Left, panels show one representative frame of a movie. Scale bar, 1μm. The number of endocytic sites was determined using a single random frame per cell analyzing the mother cell, where endocytic patches are more spatially separated. Right, quantification represented as box and whisker (minimum-maximum) plots showed higher number of patches per cell for Sla1^{AAA}-GFP relative to Sla1^{wt}-GFP (P=0.001, N=271 wild type cell patches and 335 *sla1^{AAA}* cell patches).

expressing both Sla1^{AAA}-RFP and clathrin heavy chain-GFP (CHC-GFP) from the corresponding endogenous locus was generated and analyzed. A wild type control strain expressing Sla1-RFP and CHC-GFP was also generated and analyzed in parallel. Since clathrin localizes to regions of the cell other than the plasma membrane (*trans*-Golgi Network, endosomes), Sla1-RFP was used as a marker for localization of clathrin to endocytic sites. Quantification showed lower levels of CHC-GFP at endocytic sites in *sla1^{AAA}* cells relative to wild type cells (Figure 2.3A). To corroborate this result, comparison of CHC-GFP levels at endocytic sites between wild type and *sla1^{AAA}* cells was also performed following a "blinded" approach in which the operator was unaware of the identity of the samples. Again, results demonstrated that reduced Sla1-clathrin binding causes lower absolute levels of CHC-GFP at endocytic sites as well as lower CHC-GFP patch/cytosol ratio (Figure 2.3B). To further examine clathrin levels at endocytic sites, we performed additional experiments in which we utilized both wild type and *sla1^{AAA}* strains with switched tags (expressing Sla1 and CHC tagged with GFP and RFP respectively). The cells were treated with 250uM Latrunculin-A to inhibit actin polymerization, thus stalling the endocytic process at late stages and allowing for the accumulation of coat proteins at endocytic sites [8, 12]. Confocal fluorescence microscopy analysis was performed to measure the levels of CHC-RFP at endocytic sites. Similar to the previous experiments, lower levels of CHC-RFP were found at endocytic sites in cells expressing the Sla1^{AAA} mutant (Figure 2.3C). These results indicate that interaction with Sla1 is important for either recruiting clathrin and/or maintaining clathrin levels at endocytic sites. Given that in wild type cells initial clathrin recruitment precedes Sla1 arrival [11, 12], the Sla1-clathrin interaction is likely important for maintaining normal clathrin levels at later stages but not at early stages of endocytosis. Consistent with this idea, we measured CHC-GFP levels at endocytic sites before the arrival of Sla1-RFP and found

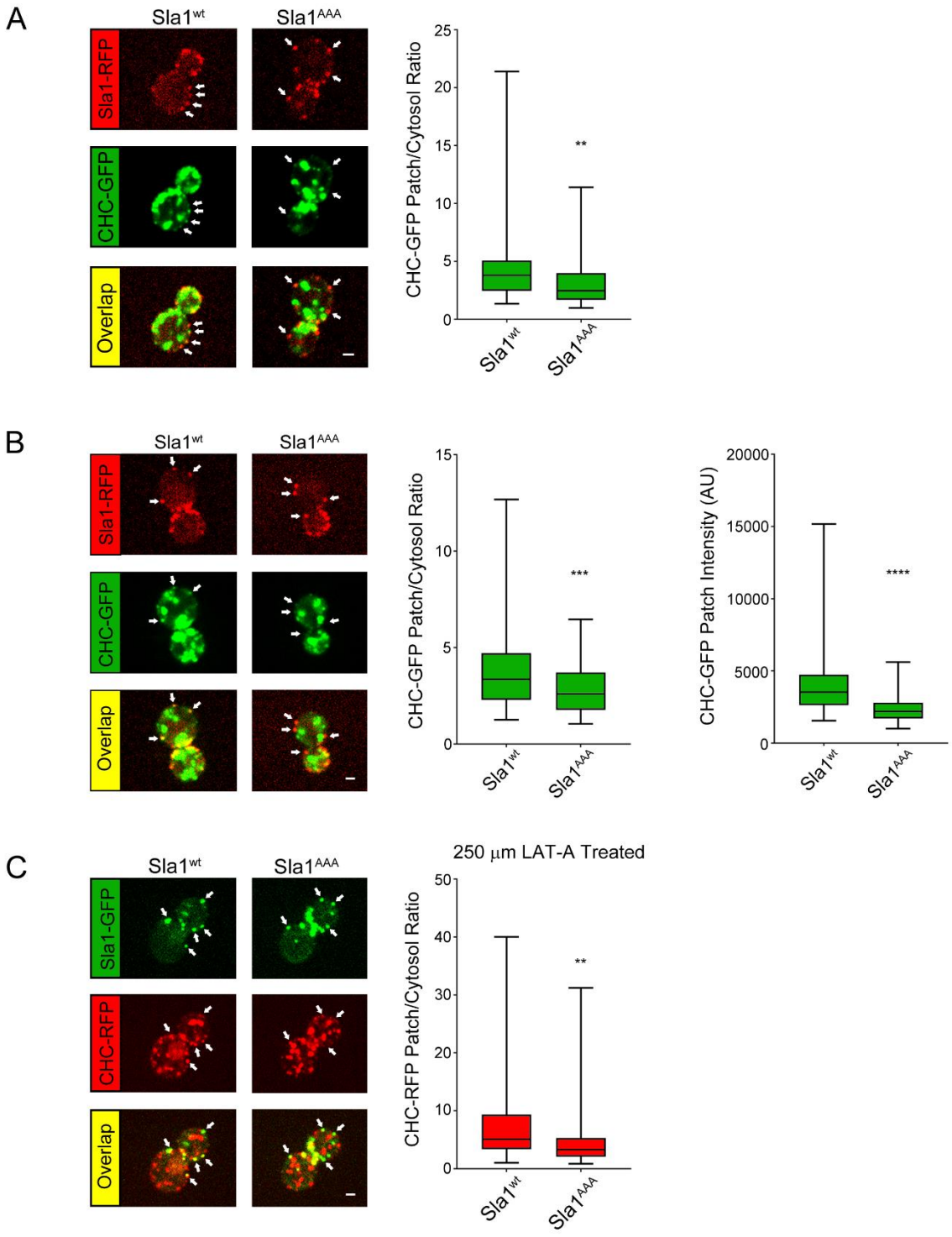

Figure 2.3: Defective Sla1-clathrin binding results in lower clathrin levels during late stages of endocytosis. (A) Live cell confocal fluorescence microscopy analysis of yeast cells expressing both Clathrin Heavy Chain (CHC)-GFP and Sla1^{AAA}-RFP from the corresponding endogenous locus (*sla1^{AAA}* cells). Wild type cells expressing CHC-GFP and Sla1^{wt}-RFP were analyzed in

parallel for comparison. Left, panels show one representative frame of a movie. Given that CHC-GFP also localizes to internal organelles, the presence of Sla1 was used as a reference to quantify CHC-GFP specifically at endocytic sites. White arrows indicate examples of endocytic sites. Scale bar, 1μm. Right, quantification represented as box and whisker (minimum-maximum) plots demonstrates lower levels of CHC-GFP at endocytic sites in *sla1*^{AAA} cells relative to wild type cells ($P<0.001$, N=83 wild type cell patches and N=112 *sla1*^{AAA} cell patches. (B) Similar CHC-GFP live cell imaging and quantification was performed in wild type and *sla1*^{AAA} cells as described in Figure 2.2A. The quantification of this experiment was performed in a blind fashion such that the identity of the samples was not known by the investigator until post imaging and fluorescent intensity analysis. Left, panels show one representative frame of a movie with white arrows indicating examples of endocytic sites. Right, quantification showed lower CHC-GFP patch/cytosol fluorescence intensity ratio ($P<0.001$, N=75 patches per cell type) and patch fluorescence intensity ($P<0.0001$, N=75 patches per cell type) in *sla1*^{AAA} cells compared with wild type cells. (C) Live cell confocal fluorescence microscopy analysis of yeast cells expressing both CHC-RFP and Sla1^{AAA}-GFP from the corresponding endogenous locus (*sla1*^{AAA} cells). Wild type cells expressing CHC-RFP and Sla1^{wt}-GFP were analyzed in parallel for comparison. Before imaging, cells were incubated for 20 min in media containing 250 μM Latrunculin A, an actin depolymerizing agent. Left, panels show one representative frame of a movie. Right, quantification demonstrates lower levels of CHC-RFP associated with endocytic sites ($P<0.01$, N=62 patches from wild type cells and N=64 patches from *sla1*^{AAA} cells).

no difference between wild type and *sla1^{AAA}* cells, suggesting deficient Sla1-clathrin binding does not affect initial clathrin recruitment (Figure 2.4). Reduced clathrin levels at endocytic sites during late phases of internalization explain the defects in endocytosis observed in *sla1^{AAA}* cells.

2.3.3 Defective Sla1-clathrin binding slows the rate and delays the timing of Sla1 and Pan1 recruitment to endocytic sites

In order to further examine the effects of impaired Sla1-clathrin binding on Sla1 dynamics, we measured the rate of Sla1 recruitment in wild type and *sla1^{AAA}* cells. Results from these experiments show that while peak endocytic site levels of Sla1^{AAA}-GFP are much higher than Sla1-GFP, the rate of recruitment is lower for Sla1^{AAA}-GFP (Figure 2.5A). This result along with lower levels of clathrin at endocytic sites in *sla1^{AAA}* cells suggests that the Sla1-clathrin interaction is necessary for proper recruitment of both proteins.

In order to determine if there was a delay in Sla1^{AAA}-GFP recruitment compared with wild type Sla1-GFP, we generated strains expressing either of these proteins and Ede1-RFP from the corresponding endogenous locus. Ede1 is an early marker that arrives at endocytic sites well before Sla1 [14]. Quantification of patch lifetimes and relative recruitment times between Ede1 and Sla1 gave two results of interest (Figure 2.6A). First, the timing of Sla1^{AAA}-GFP recruitment following Ede1-RFP (Δt) was delayed compared with the Δt between wild type Sla1-GFP and Ede1-RFP ($\Delta t = 111 \pm 9$ sec vs. $\Delta t = 71 \pm 7$ sec). Second, the patch lifetime of Ede1-RFP was significantly extended in *sla1^{AAA}* cells relative to wild type cells (168 ± 12 sec vs. 100 ± 7 sec). Together these results suggest that Sla1-clathrin binding is important for properly timed Sla1 recruitment to endocytic sites and progression of endocytosis.

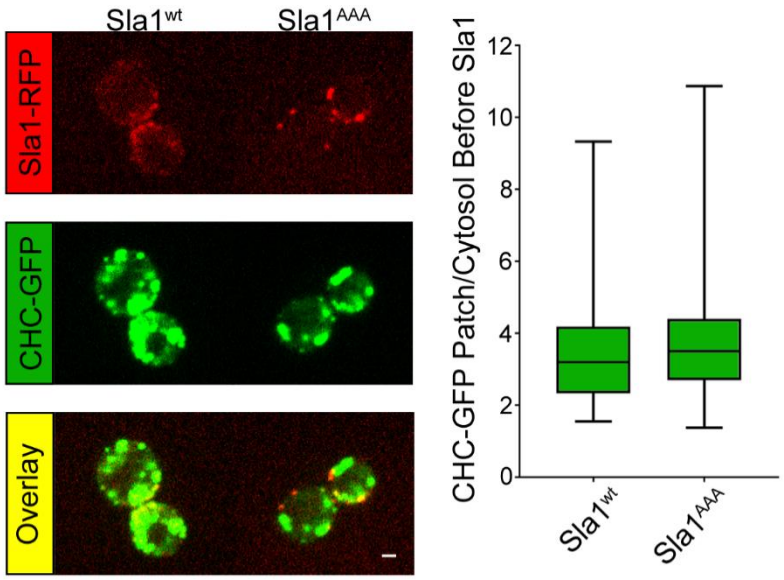

Figure 2.4: Defective Sla1-clathrin binding does not affect clathrin levels at early stages of endocytosis. Live cell confocal fluorescence microscopy analysis of yeast cells expressing both CHC-GFP and Sla1^{AAA}-RFP from the corresponding endogenous locus (*sla1^{AAA}* cells). Wild type cells expressing CHC-GFP and Sla1^{wt}-RFP were analyzed in parallel for comparison. Left, panels show one representative frame of a movie. Scale bar, 1µm. Given that CHC-GFP also localizes to internal organelles, the presence of Sla1 was used as a reference to identify CHC-GFP localized at endocytic sites. After endocytic patch identification, movies were rewound to analyze the CHC-GFP fluorescence during the period preceding the arrival of Sla1-RFP or Sla1^{AAA}-RFP. The CHC-GFP fluorescence intensity was recorded except for the time points in which internal structures interfered by overlapping with endocytic sites as determined by visual inspection of each patch frame by frame. Right, quantification represented as box and whisker (minimum-maximum) plots demonstrates the CHC-GFP levels at endocytic sites in *sla1^{AAA}* are indistinguishable from wild type cells (P=0.45, N=155 patches from wild type cells and N=133 patches from *sla1^{AAA}* cells).

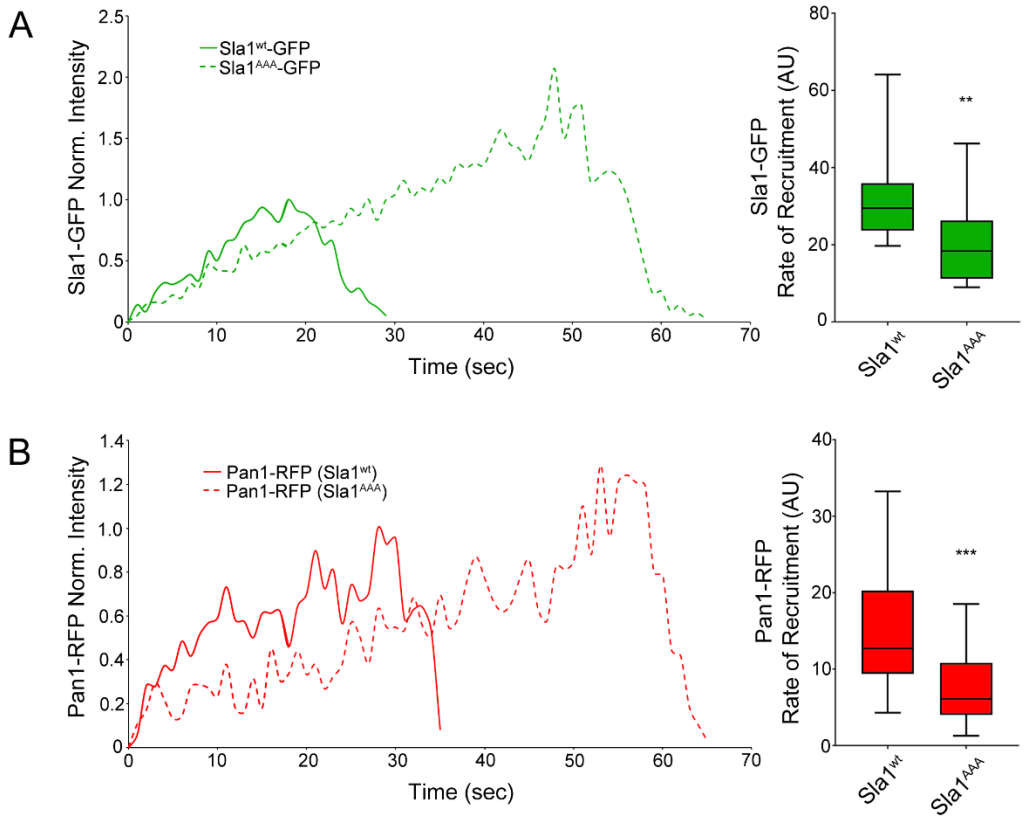

Figure 2.5: Impaired Sla1-clathrin binding slows the rate of Sla1 and Pan1 recruitment to endocytic sites. Live cell confocal fluorescence microscopy analysis of yeast cells expressing both Sla1^{AAA}-GFP and Pan1-RFP from the corresponding endogenous locus (*sla1^{AAA}* cells). Wild type cells expressing Sla1^{wt}-GFP and Pan1-RFP were analyzed in parallel for comparison. (A) Left, representation of patch fluorescence intensity over time shows that Sla1^{AAA}-GFP achieves a higher maximum value than Sla1^{wt}-GFP, but takes significantly longer time. Right, box and whisker (minimum-maximum) plots showing the rate of Sla1^{AAA}-GFP recruitment is significantly slower than Sla1^{wt}-GFP ($P<0.01$, N=23 patches per cell type). (B) Left, representation of patch fluorescence intensity over time shows that Pan1-RFP also takes significantly longer time to reach its maximum recruitment in *sla1^{AAA}* cells compared to wild type cells. Right, the rate of Pan1-RFP recruitment is significantly slower in *sla1^{AAA}* cells ($P<0.001$, N=23 patches per cell type).

Previous work showed Pan1 arrives at endocytic sites with similar timing as Sla1, and that the two proteins interact physically [13, 40]. To begin assessing if the Sla1-clathrin interaction is important for the dynamics of other endocytic machinery proteins, we investigated the dynamics of Pan1 in *sla1*^{AAA} cells. Just like Sla1, Pan1 also has a reduced rate of recruitment in *sla1*^{AAA} cells relative to wild type cells (Figure 2.5B), despite achieving higher overall levels at endocytic sites (Figure 2.1A). The recruitment timing of Pan1 to endocytic sites is closely associated with that of Sla1 both in wild type and *sla1*^{AAA} cells (Figure 2.6B), suggesting Pan1 recruitment should be delayed relative to Ede1 in *sla1*^{AAA} cells. To corroborate this idea, we generated cells expressing Pan1-GFP and Ede1-mCherry from the corresponding endogenous locus both in wild type and *sla1*^{AAA} background. Indeed the timing of Pan1-GFP recruitment following Ede1-mCherry (Δt) was delayed in *sla1*^{AAA} cells compared with wild type cells ($\Delta t = 136 \pm 11$ sec vs. $\Delta t = 87 \pm 7$ sec) (Figure 2.6C). This result suggests a more general defect in coat formation and progression from early to late stages of endocytosis when the Sla1-clathrin interaction is impaired.

2.3.4 Other endocytic clathrin binding adaptors are present at endocytic sites in Sla1^{AAA} *cells*

In addition to Sla1, the are other endocytic adaptors such as Yap1801/2 and Ent1/2 with the ability to bind clathrin [27]. Yap1801/2 and Ent1/2 have complementary/redundant functions, arrive to endocytic sites before Sla1, and remain until the patch internalizes [41]. To study their dynamics and overall recruitment levels, we generated cells expressing Yap1801-GFP and Sla1-RFP or Sla1^{AAA}-RFP from the corresponding endogenous locus. We also generated cells expressing Ent1-GFP and Sla1-RFP or Sla1^{AAA}-RFP from the corresponding endogenous locus. Quantification of patch lifetimes and relative recruitment times between Yap1801 and Sla1 gave

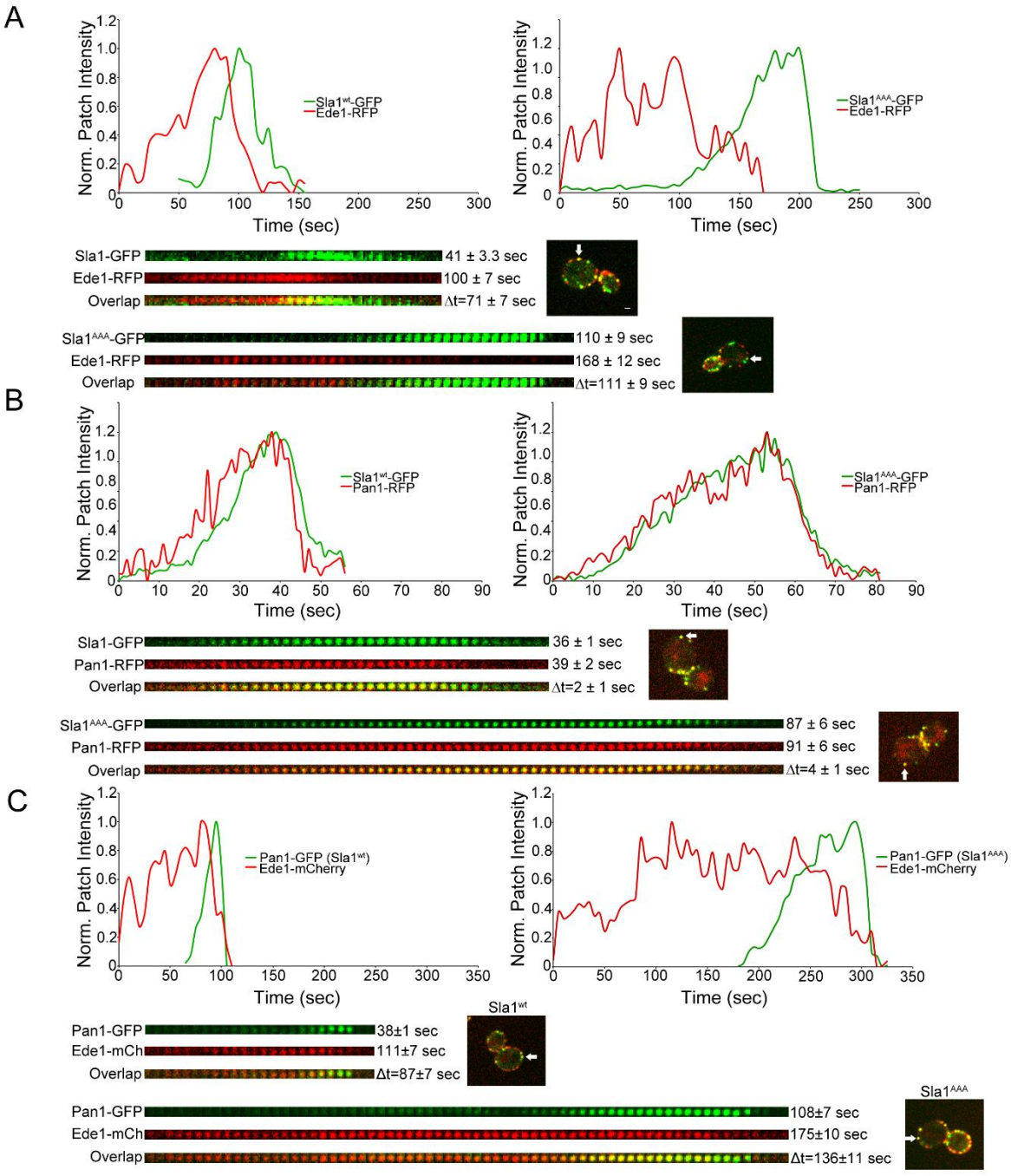

Figure 2.6: Impaired Sla1-clathrin binding delays the timing of Sla1 and Pan1 recruitment to endocytic sites. (A) Live cell confocal fluorescence microscopy analysis of yeast cells expressing both Sla1^{AAA}-GFP and Ede1-RFP from the corresponding endogenous locus (*sla1^{AAA}* cells). Wild type cells expressing Sla1^{wt}-GFP and Ede1-RFP were analyzed in parallel for comparison. Graphs and kymographs demonstrate a delay in Sla1^{AAA}-GFP recruitment following early arriving endocytic protein Ede1-RFP compared with Sla1^{wt}-GFP. White arrows indicate the endocytic sites used to construct the graphs and kymographs. Average patch lifetimes and

relative recruitment times are given next to each kymograph. The patch lifetime of both Sla1^{AAA}-GFP and Ede1-RFP were longer than the corresponding times in wild type cells ($P<0.0001$, $N=40$ patches per strain). The timing of Sla1^{AAA}-GFP recruitment following Ede1-RFP (Δt) was delayed compared with the control ($P<0.001$, $N=40$ patches per strain). Scale bar, 1μm. (B) Live cell confocal fluorescence microscopy analysis of yeast cells expressing both Sla1^{AAA}-GFP and Pan1-RFP from the corresponding endogenous locus (*sla1^{AAA}* cells). Wild type cells expressing Sla1^{wt}-GFP and Pan1-RFP were analyzed in parallel for comparison. Graphs and kymographs demonstrate closely timed recruitment to endocytic sites of Pan1 with both Sla1^{AAA}-GFP and Sla1^{wt}-GFP. White arrows indicate the endocytic sites used to construct the graphs and kymographs. Average fluorescent patch lifetimes and relative recruitment times are given next to each kymograph. Quantification therefore showed the Δt was not significantly different between *sla1^{AAA}* cells and wild type cells ($P=0.26$, $N=31$ patches per strain). The patch lifetime of both Sla1 and Pan1 was significantly longer in *sla1^{AAA}* cells relative to control ($P<0.0001$, $N=31$ patches per strain). (C) Live cell confocal fluorescence microscopy analysis of yeast cells expressing both Pan1-GFP and Ede1-mCherry from the corresponding endogenous locus in both *sla1^{AAA}* cells and wild type cells. Graphs and kymographs demonstrate a delay in Pan1-GFP recruitment following early arriving endocytic protein Ede1-mCherry in *sla1^{AAA}* cells compared with wild type cells. White arrows indicate the endocytic sites used to construct the graphs and kymographs. Average patch lifetimes and relative recruitment times are given next to each kymograph. The patch lifetime of both Pan1-GFP and Ede1-mCherry were longer in *sla1^{AAA}* cells than in wild type cells ($P<0.0001$, $N=40$ patches per strain). The timing of Pan1-GFP recruitment following Ede1-mCherry (Δt) was significantly delayed compared with the control ($P<0.001$, $N=40$ patches per strain).

two results of interest (Figure 2.7A). First, the timing of Sla1^{AAA}-RFP recruitment following Yap1801-GFP (Δt) was delayed compared with the Δt between wild type Sla1-RFP and Yap1801-GFP (Δt = 85 ± 6 sec vs. Δt = 56 ± 5 sec). Second, the patch lifetime of Yap1801-GFP was significantly extended in *sla1^{AAA}* cells relative to wild type cells (160 ± 7 sec vs. 88 ± 6 sec). Similar results were observed with Ent1-GFP. The Sla1^{AAA}-RFP recruitment following Ent1-GFP (Δt) was delayed compared with the Δt between wild type Sla1-RFP and Ent1-GFP (Δt = 22 ± 4 sec vs. Δt = 10 ± 7 sec) (Figure 2.7B). Likewise, the patch lifetime of Ent1-GFP was significantly extended in *sla1^{AAA}* cells relative to wild type cells (107 ± 39 sec vs. 39 ± 2 sec) (Figure 2.7B). Results with Yap1801 and Ent1 parallel those with Ede1 and Pan1, supporting the idea that Sla1-clathrin binding is needed for normal progression of endocytosis. Importantly, the fluorescence intensity of Yap1801-GFP and Ent1-GFP at endocytic sites was not decreased in *sla1^{AAA}* cells compared with wild type cells. Yap1801-GFP fluorescence intensity was somewhat increased in *sla1^{AAA}* cells relative to wild type, although the difference was not statistically significant (Figure 2.9A). The fluorescence intensity of Ent1-GFP was significantly increased in *sla1^{AAA}* cells relative to wild type cells (Figure 2.9B). This result indicates that the clathrin binding function of Yap1801/Ent1 is not redundant with that of Sla1 and supports the idea that Sla1-clathrin binding is necessary for maintaining a proper level of clathrin during late stages of endocytosis.

2.3.5 Sla1-clathrin binding is necessary for normal progression between coat formation and actin polymerization

Recruitment of Sla1 to endocytic sites is one of the final steps in coat formation. Shortly after Sla1 recruitment (~15 sec), Arp2/3-mediated actin polymerization takes place, which is

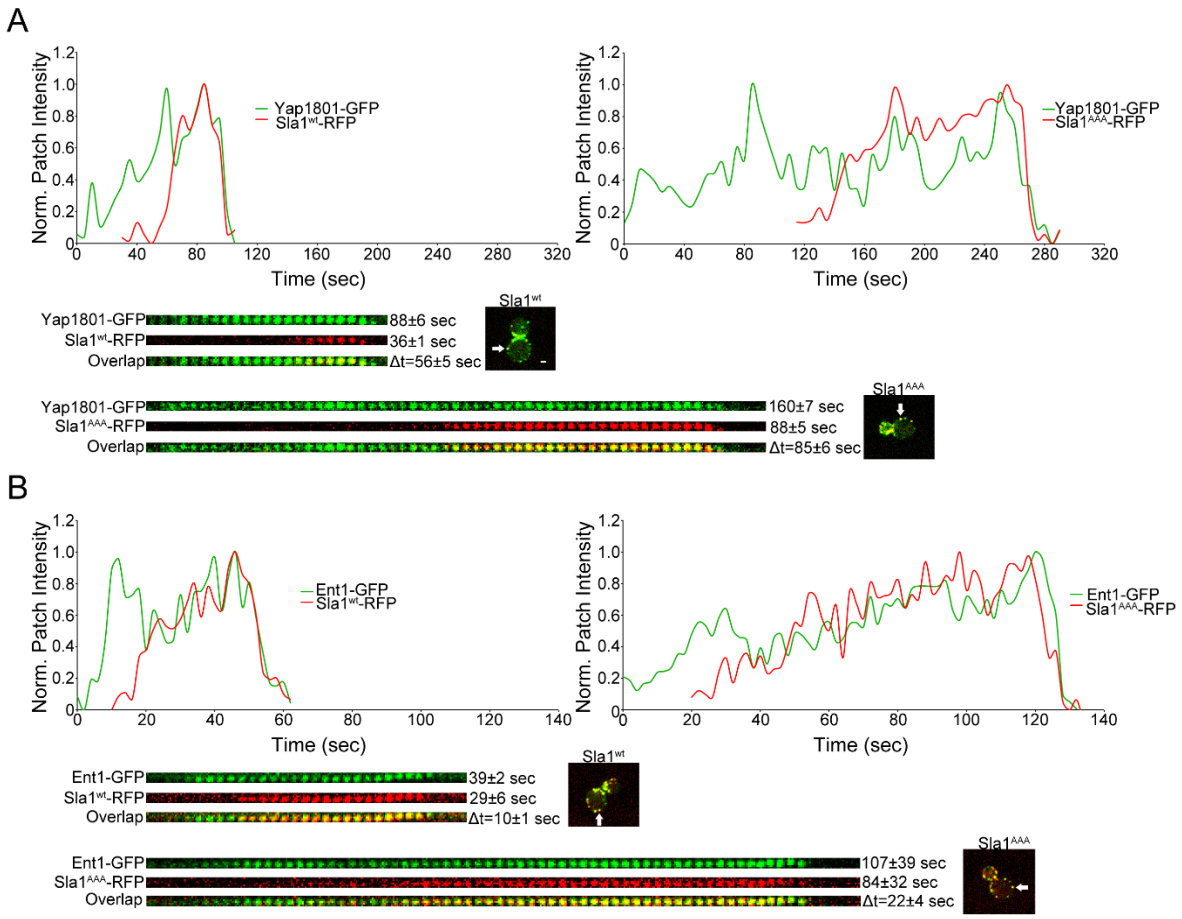

Figure 2.7: Yap1801 and Ent1 display an extended lifetime at endocytic sites and a delayed Sla1 recruitment in cells with abolished Sla1-clathrin binding. (A) Live cell confocal fluorescence microscopy analysis of yeast cells expressing both Sla1^{AAA}-RFP and Yap1801-GFP from the corresponding endogenous locus (*sla1^{AAA}* cells). Wild type cells expressing Sla1^{wt}-RFP and Yap1801-GFP were analyzed in parallel for comparison. Graphs and kymographs demonstrate a delay in Sla1^{AAA}-RFP recruitment following Yap1801-GFP compared with Sla1^{wt}-GFP. White arrows indicate the endocytic sites used to construct the graphs and kymographs. Scale bar, 1μm. Average patch lifetimes and relative recruitment times are given next to each kymograph. The patch lifetime of both Yap1801-GFP and Sla1^{AAA}-RFP were longer than the corresponding times in wild type cells (P<0.0001, N=40 patches per strain). The timing of Sla1^{AAA}-RFP recruitment following Yap1801-GFP (Δt) was delayed compared with the control (P<0.001, N=40 patches per strain). (B) Live cell confocal fluorescence microscopy analysis of yeast cells expressing both Sla1^{AAA}-RFP and Ent1-GFP from the corresponding endogenous locus (*sla1^{AAA}* cells). Wild type cells expressing Sla1^{wt}-RFP and Ent1-GFP were analyzed in parallel for comparison. Graphs and kymographs demonstrate a delay in Sla1^{AAA}-RFP recruitment following Ent1-GFP compared with Sla1^{wt}-GFP. White arrows indicate the endocytic sites used to construct the graphs and kymographs. Average patch lifetimes and relative recruitment times are given next to each kymograph. The patch lifetime of both Ent1-GFP and Sla1^{AAA}-RFP were longer than the corresponding times in wild type cells (P<0.0001, N=35 patches per strain). Also, the timing of

Sla1^{AAA}-RFP recruitment following Ent1-GFP (Δt) was delayed compared with control ($P<0.01$, N=35 patches for the wild type cells and N=31 patches for *sla1*^{AAA} cells).

typically detected in live cells by imaging Abp1 [8]. Las17 and Myo3/5 are the strongest activators of Arp2/3-mediated actin polymerization [13]. In wild type cells, Las17 arrives to endocytic sites at the same time as Sla1 and Myo3/5 arrive simultaneously with Abp1 [13, 42]. Vrp1 and Bzz1 are also part of the actin polymerization machinery and in wild type cells they arrive at endocytic sites just before Abp1 (~5 sec) [13, 43]. Here we examined the dynamics of Las17-GFP, Myo5-GFP, Vrp1-GFP and Bzz1-GFP relative to Abp1-mCherry in cells expressing each protein pair from the corresponding endogenous locus, both in wild type and *sla1*^{AAA} background. In these experiments it was determined that the patch lifetime of all of these four proteins was significantly extended in *sla1*^{AAA} cells beyond that found in wild type cells (Figure 2.8). Importantly, the time between their recruitment and Abp1-mCherry arrival (Δt) was significantly increased in *sla1*^{AAA} cells relative to wild type cells ($\Delta t = 80 \pm 6$ sec vs. $\Delta t = 23 \pm 1$ sec for Las17-GFP; $\Delta t = 24 \pm 2$ sec vs. $\Delta t = 1 \pm 1$ sec for Myo5-GFP; $\Delta t = 63 \pm 4$ sec vs. $\Delta t = 11 \pm 1$ sec for Vrp1-GFP; $\Delta t = 44 \pm 3$ sec vs. $\Delta t = 10 \pm 1$ sec for Bzz1-GFP). Furthermore, the maximal level of these four proteins at endocytic sites was also increased in *sla1*^{AAA} cells compared with wild type cells (Figure 2.9C-F), as was the case with Sla1, Pan1 and Ent-1 (Figure 2.1 and 2.9B). These results demonstrate that deficient Sla1-clathrin binding results in delayed actin polymerization, as marked by Abp1-mCherry, relative to the recruitment of the actin polymerizing machinery. Given that clathrin is present at reduced levels in *sla1*^{AAA} cells (Figure 2.3), the data suggests a link between formation of the endocytic clathrin coat and initiation of actin polymerization, one of the final stages in CME.

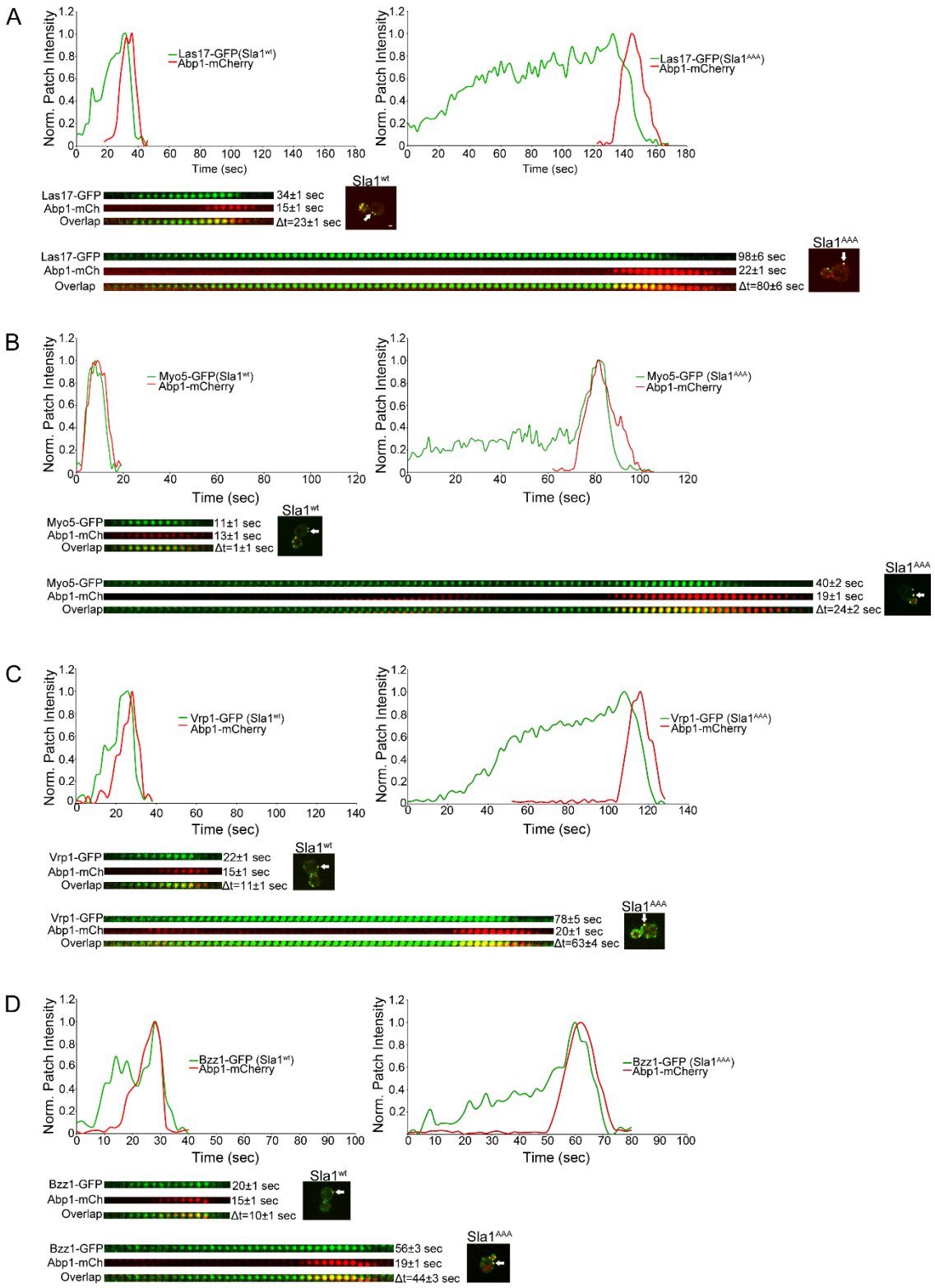
A
Norm. Patch Intensity
Time (sec)
Las17-GFP(Sla1wt)
Abp1-mCherry
Las17-GFP(Sla1AAA)
Abp1-mCherry
Las17-GFP
Abp1-mCh
Overlap
34±1 sec
15±1 sec
Δt=23±1 sec
Sla1wt
98±6 sec
22±1 sec
Δt=80±6 sec
Sla1AAA
B
Myo5-GFP(Sla1wt)
Abp1-mCherry
Myo5-GFP (Sla1AAA)
Abp1-mCherry
11±1 sec
13±1 sec
Δt=1±1 sec
40±2 sec
19±1 sec
Δt=24±2 sec
C
Vrp1-GFP (Sla1wt)
Abp1-mCherry
Vrp1-GFP (Sla1AAA)
Abp1-mCherry
22±1 sec
15±1 sec
Δt=11±1 sec
78±5 sec
20±1 sec
Δt=63±4 sec
D
Bzz1-GFP (Sla1wt)
Abp1-mCherry
Bzz1-GFP (Sla1AAA)
Abp1-mCherry
20±1 sec
15±1 sec
Δt=10±1 sec
56±3 sec
19±1 sec
Δt=44±3 sec

Figure 2.8: Normal transition between coat formation and actin polymerization depends on Sla1-clathrin binding. (A) Graphs and kymographs obtained by confocal fluorescent microscopy analysis of Las17-GFP and Abp1-mCherry expressed from the corresponding endogenous locus in wild type and *sla1*^{AAA} cells. White arrows indicate the endocytic sites used to generate the graphs and kymographs. The patch lifetime of both Las17-GFP and Abp1-mCherry were longer in *sla1*^{AAA} cells than in wild type cells ($P<0.0001$ for both proteins, N=50 patches per strain). The timing of Abp1-mCherry recruitment following Las17-GFP (Δt) was delayed in *sla1*^{AAA} cells compared with wild type cells ($P<0.0001$, N=50 patches per strain). Scale bar, 1μm. (B) Graphs and kymographs obtained by confocal fluorescent microscopy analysis of Myo5-GFP and Abp1-mCherry expressed from the corresponding endogenous locus in wild type and *sla1*^{AAA} cells. The patch lifetime of both Myo5-GFP and Abp1-mCherry were longer in *sla1*^{AAA} cells than in wild type cells ($P<0.0001$ for both proteins, N=70 patches per strain). The timing of Abp1-mCherry recruitment following Myo5-GFP (Δt) was delayed in *sla1*^{AAA} cells compared with wild type cells ($P<0.0001$, N=70 patches per strain). Also notice that in *sla1*^{AAA} cells the Myo5-GFP fluorescence intensity is present for a long time before Abp1-mCherry arrival but increases concomitantly with Abp1-mCherry recruitment (C) Graphs and kymographs obtained by confocal fluorescent microscopy analysis of Vrp1-GFP and Abp1-mCherry expressed from the corresponding endogenous locus in wild type and *sla1*^{AAA} cells. The patch lifetime of both Vrp1-GFP and Abp1-mCherry were longer in *sla1*^{AAA} cells than in wild type cells ($P<0.0001$ for both proteins, N=50 patches per strain). The timing of Abp1-mCherry recruitment following Vrp1-GFP (Δt) was delayed in *sla1*^{AAA} cells compared with wild type cells ($P<0.0001$, N=50 patches per strain). (D) Graphs and kymographs obtained by confocal fluorescent microscopy analysis of Bzz1-GFP and Abp1-mCherry expressed from the corresponding endogenous locus in wild type and *sla1*^{AAA} cells. The patch lifetime of both Bzz1-GFP and Abp1-mCherry were longer in *sla1*^{AAA} cells than in wild type cells ($P<0.0001$ for Bzz1-GFP and $P<0.05$ for Abp1-mCherry, N=30 patches per strain). The timing of Abp1-mCherry recruitment following Bzz1-GFP (Δt) was delayed in *sla1*^{AAA} cells compared with wild type cells ($P<0.0001$, N=50 patches per strain).

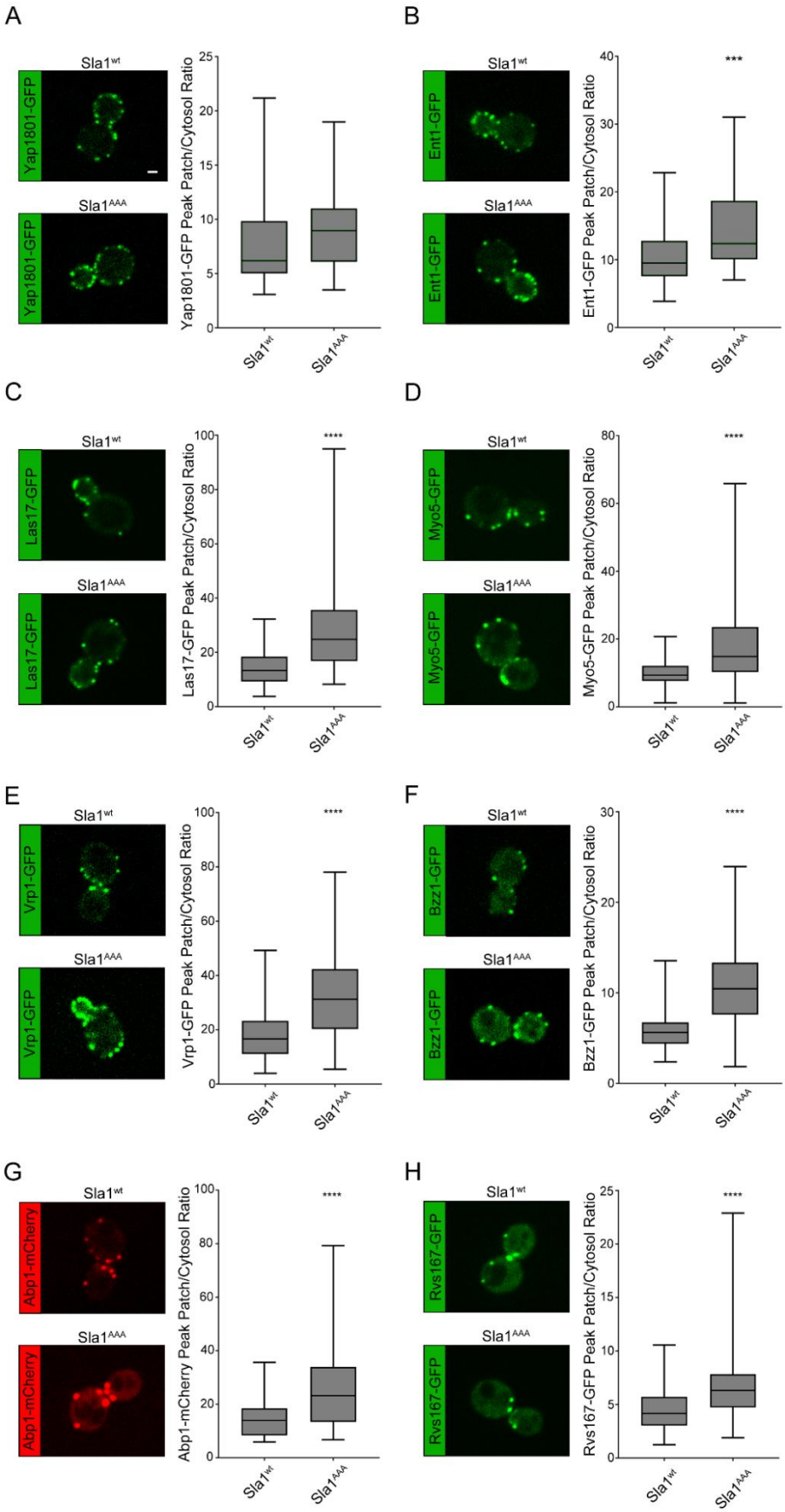
A
Sla1wt
Yap1801-GFP
Sla1AAA
Yap1801-GFP
Yap1801-GFP Peak Patch/Cytosol Ratio
Sla1wt
Sla1AAA
B
Sla1wt
Ent1-GFP
Sla1AAA
Ent1-GFP
***
Ent1-GFP Peak Patch/Cytosol Ratio
C
Las17-GFP
Las17-GFP Peak Patch/Cytosol Ratio
****
D
Myo5-GFP
Myo5-GFP Peak Patch/Cytosol Ratio
E
Vrp1-GFP
Vrp1-GFP Peak Patch/Cytosol Ratio
F
Bzz1-GFP
Bzz1-GFP Peak Patch/Cytosol Ratio
G
Abp1-mCherry
Abp1-mCherry Peak Patch/Cytosol Ratio
H
Rvs167-GFP
Rvs167-GFP Peak Patch/Cytosol Ratio

Figure 2.9: Deficient Sla1-clathrin binding results in higher levels of coat proteins, polymerized actin, the actin polymerization machinery and the scission machinery. Live cell confocal fluorescent microscopy analysis of various endocytic proteins expressed from the corresponding endogenous locus both in wild type and *sla1*^{AAA} cells. Scale bar, 1μm. The peak patch/cytosol fluorescence intensity ratio was determined for each protein in both *sla1*^{AAA} cells and control cells and is represented using box and whisker plots: (A) Yap1801-GFP (N=40 patches per strain); (B) Ent1-GFP (N=35 patches per strain); (C) Las17-GFP (N=50 patches per strain); (D) Myo5-GFP (N=70 patches per strain); (E) Vrp1-GFP (N=50 patches per strain); (F) Bzz1-GFP (N=50 patches per strain); (G) Abp1-mCherry (N=50 patches per strain); (H) Rvs167-GFP (N=70 patches for wild type cells and N=64 patches *sla1*^{AAA} cells). With the exception of Yap1801, the peak patch/cytosol fluorescence intensity ratio was significantly higher for each of these proteins in *sla1*^{AAA} cells compared with wild type cells ($P<0.001$ for Ent1-GFP and $P<0.0001$ in all other cases).

2.3.6 Impaired Sla1-clathrin binding results in higher levels of polymerized actin and scission machinery

In our analysis of the actin machinery we noticed that in *sla1^{AAA}* cells the Abp1-mCherry patches appeared to be much brighter and the lifetime of Abp1-mCherry was longer compared to wild type cells. Since actin polymerization is considered the main force-generating component of CME and Abp1 acts as a marker for polymerized actin, it was important to corroborate these observations. Quantification showed that indeed in *sla1^{AAA}* cells the Abp1-mCherry levels were significantly higher (Figure 2.9G) and the patch lifetime was slightly longer (~5 sec, Figure 2.8) compared with wild type cells. This data indicates defective Sla1-clathrin binding results in a larger network of polymerized actin at endocytic sites.

We then investigated the effects of impaired Sla1-clathrin interaction on the scission machinery. Rvs167 is one of two BAR domain-containing scission proteins recruited to the endocytic site neck right after actin polymerization begins to assist in vesicle release [11, 44]. We generated and examined cells expressing Rvs167-mCherry and Sla1-GFP or Sla1^{AAA}-GFP from the corresponding endogenous locus (Figure 2.10A). Rvs167-mCherry was significantly delayed in its recruitment following Sla1^{AAA}-GFP compared with wild type Sla1-GFP (Figure 2.10A). This result parallels the late recruitment of Abp1-mCherry in *sla1^{AAA}* cells (Figure 2.8). Also, similar to Abp1-mCherry, Rvs167-GFP fluorescent intensity appeared to be higher and patch lifetime longer in *sla1^{AAA}* cells relative to wild type cells. We thus generated and analyzed wild type and *sla1^{AAA}* strains expressing Rvs167-GFP and Abp1-mCherry from the corresponding endogenous locus. Quantification showed that indeed in *sla1^{AAA}* cells the maximal levels of Rvs167-GFP were significantly higher (Figure 2.9H) and the patch lifetime of Rvs167-GFP was extended by ~5sec (Figure 2.10B) when compared to wild type cells. Rvs167-GFP

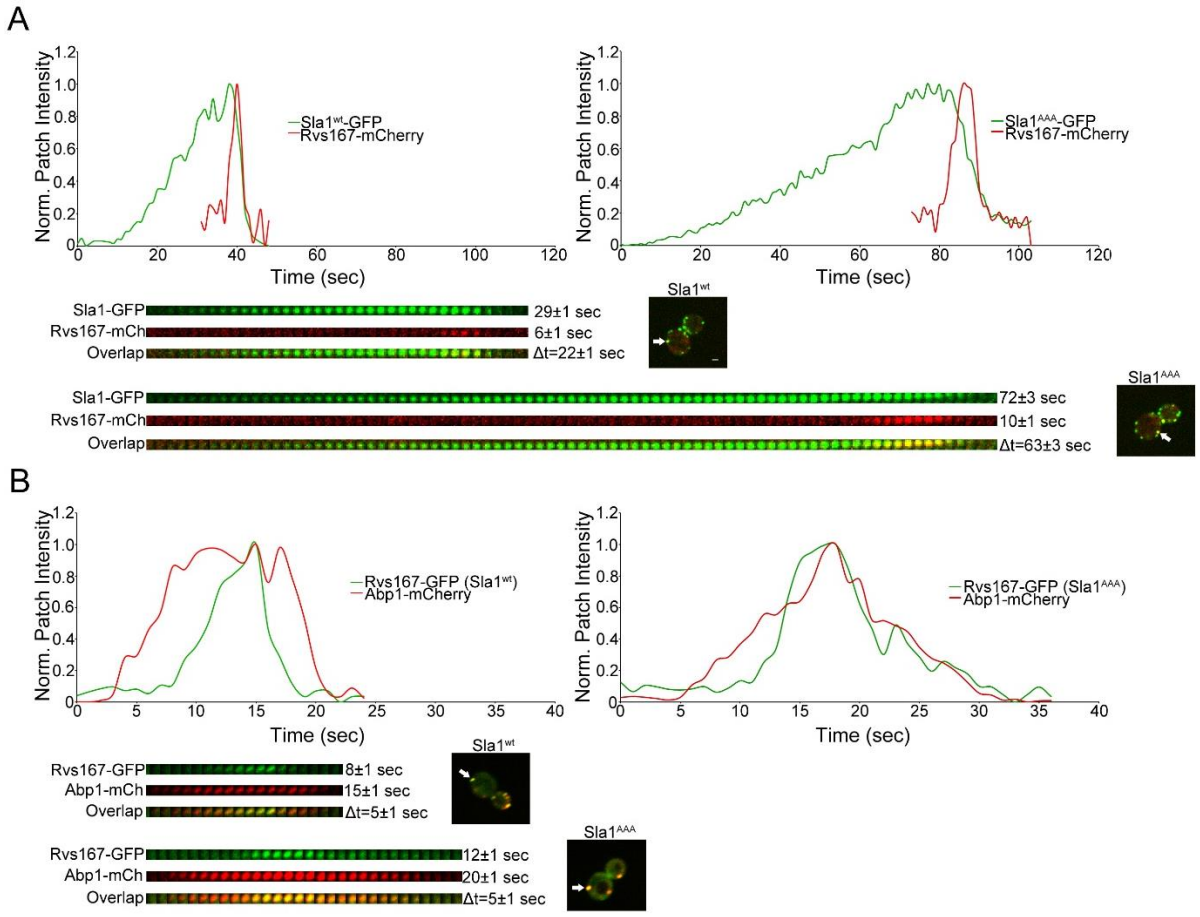

Figure 2.10: Normal transition between coat formation and recruitment of the scission machinery depends on Sla1-clathrin binding. (A) Live cell confocal fluorescence microscopy analysis of yeast cells expressing both Sla1^{AAA}-GFP and Rvs167-mCherry from the corresponding endogenous locus (*sla1^{AAA}* cells). Wild type cells expressing Sla1^{wt}-GFP and Rvs167-mCherry were analyzed in parallel for comparison. Graphs and kymographs are depicted and average patch lifetimes and relative recruitment times are given next to each kymograph. Quantification demonstrates a significant delay in Rvs167-mCherry recruitment following Sla1^{AAA}-GFP compared with control cells ($P<0.0001$, $N=70$ patches per strain). The patch lifetime of Rvs167-mCherry was longer in *sla1^{AAA}* cells than in wild type cells ($P<0.0001$, $N=70$ patches per strain). Scale bar, 1µm. (B) Live cell confocal fluorescence microscopy analysis of yeast cells expressing Rvs167-GFP and Abp1-mCherry from the corresponding endogenous locus in both *sla1^{AAA}* cells and control cells. Analysis confirmed both proteins have an extended patch lifetime in *sla1^{AAA}* cells relative to control cells ($P<0.0001$, $N=50$ patches per strain). The recruitment timing of Rvs167-GFP following Abp1-mCherry in *sla1^{AAA}* cells was the same as in wild type cells ($\Delta t=5\pm1$ sec in both strains, $N=50$ patches per strain).

recruitment occurs shortly after Abp1-mCherry both in *sla1*^{AAA} and wild type cell (Figure 2.10B). This data indicates defective Sla1-clathrin binding results in the recruitment of more molecules of Rvs167 to endocytic sites with a timing closely associated with actin polymerization.

2.3.7 Defective Sla1-clathrin binding enhances average membrane invagination length and the size of the ribosome exclusion zone

To analyze the effect of impaired Sla1-clathrin binding in endocytic membrane invagination shape with appropriate resolution, wild type and *sla1*^{AAA} cells were subjected to high pressure freezing and processed for thin-section electron microscopy. Both the endocytic membrane invagination and its surrounding ribosome exclusion zone, which is caused mainly by the endocytic coat and the actin network [18], appeared to be abnormally large in *sla1*^{AAA} cells. Quantitative analysis corroborated the average invagination length was longer in *sla1*^{AAA} cells compared to control cells (Figure 2.11). This result indicates that the Sla1-clathrin interaction contributes to proper shaping of the endocytic invagination likely through either recruitment or stabilization of the clathrin coat. Furthermore, the ribosome exclusion zone was quantified in wild type and *sla1*^{AAA} cells by measuring the distance from the membrane invagination tip to the closest ribosome found towards the cell interior (26 ± 2 nm vs. 35 ± 3 nm, n = 62 patches per strain, $p<0.01$) and the width of the exclusion zone at the base of the invagination (98 ± 3 nm vs. 129 ± 7 nm, n = 62 patches per strain, $p<0.01$). Quantification therefore corroborated a larger ribosome exclusion zone surrounds the endocytic invagination in *sla1*^{AAA} cells. These results are consistent with the fluorescent microscopy determinations showing increased levels of coat proteins other than clathrin (Sla1, Pan1, Ent1), polymerized actin and actin machinery (Abp1, Las17, Myo5, Vrp1, Bzz1), and scission machinery (Rvs167) in *sla1*^{AAA} cells. These results also

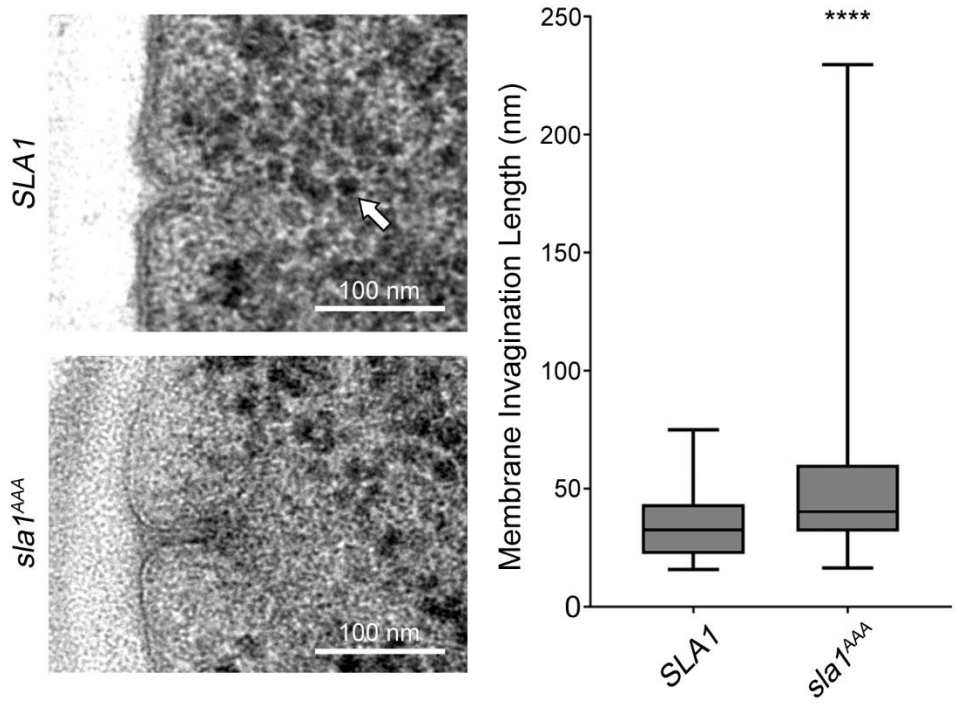

Figure 2.11: Impaired Sla1-clathrin binding results in longer membrane invagination and larger ribosome exclusion zone at endocytic sites. Upper left, electron micrograph demonstrating a typical membrane invagination of an endocytic site in wild type cells. Lower left, electron micrograph demonstrating a membrane invagination of an endocytic site in *sla1*^{AAA} cells. Notice the typical area devoid of ribosomes around the invagination is larger in *sla1*^{AAA} cells (quantification described in the text). The white arrow indicates a ribosome. Right, box and whisker plot quantification of membrane invaginations in both wild type and *sla1*^{AAA} cells showing a difference in the membrane invagination length ($P<0.001$, N=77 wild type cell patches and N=118 *sla1*^{AAA} cell patches).

reinforce the idea that Sla1-clathrin binding is needed for consistent progression to late stages of endocytosis, normal levels of actin polymerization and proper membrane shaping.

2.4 Discussion

While the function of the adaptor protein-clathrin interaction in CME is conceptually clear, studying its importance in live cells has not been straightforward. For example the existence of several clathrin adaptors can result in redundancy/compensation and lack of phenotype when only one is mutated [41, 45, 46]. Furthermore, deletion of the adaptor gene can complicate the interpretation of results because the other interactions that adaptors make with cargo and the CME machinery are also lost [3, 7, 23, 24]. On the other hand, overall clathrin deficiency results in defects beyond CME due to its function in the secretory pathway and endosomes [6, 20, 24]. For instance, if clathrin is mutated the CME cargo would not be normally delivered to the plasma membrane in the first place. The *sla1*^{AAA} allele is unique in demonstrating a defect in endocytosis of CME endogenous cargo with a mutation that specifically disrupts the clathrin-binding motif but preserves all other Sla1 domains [30]. Also Sla1 functions specifically in CME, thus avoiding confounding factors such as a role in other vesicle transport pathways that could indirectly affect CME.

The lower clathrin levels detected at late stages of endocytosis in *sla1*^{AAA} cells imply interaction with Sla1 is needed for normal clathrin recruitment even though other clathrin binding adaptors – Yap1801, Ent1 – are present. However, initial clathrin recruitment occurs before Sla1 arrival to endocytic sites [11, 12]. This suggests the Sla1-clathrin interaction is needed for later clathrin recruitment or maintenance of a full clathrin coat as endocytosis progresses but should not be needed at early stages. Supporting this idea, clathrin levels at

endocytic sites before Sla1^{AAA} arrival were not affected. It was recently suggested that while a complete clathrin coat already exists at early stages when the membrane is still flat, such membrane bound clathrin exchanges with soluble clathrin while transitioning from flat to curved membrane [18, 35]. This would allow adapting the clathrin configuration of hexagons and pentagons to the changing membrane curvature. Our results are consistent with a model in which interaction with other adaptors recruited at earlier stages bring initial clathrin triskelia to an incipient CME site [12, 41] and interaction with Sla1 is needed during exchange with soluble clathrin triskelia at later stages, perhaps during the flat-curved membrane transition.

While it is conceptually clear that clathrin recruitment requires binding to adaptor proteins, the converse relationship may also be true. In other words, adaptors may be recruited or stabilized at endocytic coats in part by binding to clathrin, in addition to interacting with cargo and other endocytic proteins. Even though Sla1^{AAA} achieved overall membrane recruitment levels higher than the wild type protein, the rate of recruitment was reduced compared to wild type Sla1. This result is consistent with the idea that binding to clathrin is important for proper adaptor recruitment to endocytic sites.

Endocytosis proceeds through various stages that take place in a reproducible and stereotypical manner [2-4, 8, 11]. Several experimental results obtained here underscore the need for adaptor-clathrin interaction and proper clathrin recruitment levels for normal progression of endocytosis:

First, the patch lifetime of early (Ede1, Yap1801), intermediate (Sla1, Pan1, Las17, Ent1) and late (Vrp1, Bzz1, Myo5, Abp1, Rvs167) CME machinery proteins was significantly extended in *sla1^{AAA}* cells. Moreover, the transition between the various endocytic machinery modules (Δt) was significantly delayed. It was particularly noteworthy to find that the key

components activating Arp2/3-mediated actin polymerization (Las17, Pan1, Myo5, Vrp1, Bzz1) were present for a significantly longer time before actin polymerization took place in *sla1*^{AAA} cells. This result highlights a previously unappreciated link between coat formation and activation of actin polymerization.

Second, with the exception of clathrin, the levels of all endocytic machinery proteins studied were higher than normal at the endocytic sites of *sla1*^{AAA} cells. In particular, the significantly higher levels of Abp1 and expanded ribosome exclusion zone around endocytic sites indicate a larger actin network in *sla1*^{AAA} cells. Given that actin polymerization is considered the main force driving membrane bending, this result suggests that the clathrin coat normally cooperates in shaping the membrane and that a higher level of actin polymerization compensates when clathrin contribution decreases. Another force believed to cooperate in membrane bending arises from the steric collision of coat proteins such as adaptors on the cytosolic side of the membrane, which is alleviated by invaginating the membrane [38, 47, 48]. Again, the higher levels of various proteins in *sla1*^{AAA} cells could represent a compensatory mechanism to allow endocytosis progression in a situation of reduced clathrin contribution. Alternatively, increased levels of coat and actin network proteins may be caused by the slower rate of vesicle formation that provides more time for additional recruitment of endocytic factors.

Third, the endocytic membrane profile was longer in *sla1*^{AAA} cells. Given that we only find a deficit of clathrin at CME sites, this result also suggests that a full coat is necessary to produce a normal invagination shape. Interestingly, cells carrying a deletion of the *RVS167* gene have shorter endocytic profiles [18]. Rvs167 is normally recruited to the endocytic site when the invagination transitions from dome shaped to developing parallel membranes ultimately aiding the scission step. Thus, the higher level of Rvs167 observed in *sla1*^{AAA} cells is consistent with a

longer endocytic profile that is able to accommodate more copies of Rvs167. The extra time provided by a slower process of vesicle formation could also allow for additional recruitment of Rvs167. The enhanced levels of Rvs167 could also represent another compensatory mechanism to alleviate the lack of clathrin coat contribution to membrane bending, perhaps at the invagination tip-neck transition. Interestingly, a recent superresolution microscopy analysis suggested Sla1 forms a ring at the invagination tip-neck transition[49]. Consequently, *sla1*^{AAA} cells may lack clathrin recruitment specifically at a key region where the clathrin cage meets the invagination neck. Such a mechanism would help explain clathrin contribution to the regularity of vesicle scission and the resulting vesicle size [50].

Several studies previously reported the phenotype of strains carrying a deletion of the clathrin heavy chain gene (*CHC1*) or clathrin light chain gene (*CLC1*). The fact that in yeast clathrin is not needed to produce endocytic invagination profiles as observed by electron microscopy was first reported using chemical fixation [19] and then high pressure freezing and freeze substitution of *chc1Δ* cells [50]. The first study also found that immunogold labeling of Sla1 decorated a higher proportion of longer endocytic invaginations in *chc1Δ* cells than in wild type cells [19]. The second study found that the endocytic invaginations in *chc1Δ* cells have approximately the same morphology as in wild type cells [50]. Importantly, fluorescence microscopy studies of live *chc1Δ* cells and *clc1Δ* cells demonstrated less endocytic sites labeled by Sla1-GFP, more cytosolic Sla1-GFP background and a shorter Sla1-GFP lifetime at endocytic sites compared with wild type cells [11, 51]. These results contrast with our findings of Sla1^{AAA}–GFP displaying more patches per cell, longer lifetime, higher membrane/cytosol proportion, and longer invagination profiles compared with wild type Sla1-GFP. While the reason for the different phenotypes is not entirely clear, several possibilities can be envisioned:

(i) As previously mentioned, vesicular transport in *chc1Δ* cells and *clc1Δ* cells is more globally affected than in *sla1*^{AAA} cells due to lost clathrin function at the *trans*-Golgi Network and endosomes. Endocytosis phenotypes observed in *chc1Δ* cells and *clc1Δ* cells may therefore be partly indirect and reflect factors such as a reduced flux of membrane and protein cargo requiring endocytosis/recycling. This would explain for instance the lower number of endocytic sites observed in *chc1Δ* cells and *clc1Δ* cells [11, 51].

(ii) In *sla1*^{AAA} cells early clathrin recruitment and endocytic site initiation is normal due to the function of other clathrin binding adaptors. Subsequently, the presence of assembled clathrin may impede progression to later stages of endocytosis. If as suggested above Sla1 normally functions in the remodeling of assembled clathrin during the flat-curved transition, the inability of Sla1^{AAA} to bind clathrin may delay such transition. Sla1 would still be recruited to endocytic sites (although at a slower rate) via interactions with various other machinery components and transmembrane protein cargo. Such a scenario with normal endocytic site initiation but slower progression would explain the longer Sla1^{AAA} lifetime and, consequently, the increased number of patches detected at steady state relative to wild type Sla1.

(iii) In addition to binding clathrin, the Sla1 vCB sequence (LLDLQ) binds intramolecularly to the Sla1 SAM domain [30]. Interestingly, the Sla1 SAM domain can homo-oligomerize thus driving Sla1 homo-oligomerization. The region of the SAM domain surface involved in binding vCB overlaps with the SAM domain homo-oligomerization surface. Accordingly, vCB was proposed to act as a switch that binds to clathrin or the Sla1 SAM domain thereby mediating clathrin recruitment or inhibiting Sla1 self-oligomerization [30]. Based on this model, the vCB mutation should increase the proportion of oligomeric Sla1, which is expected to occur while concentrated at endocytic sites [30], thus contributing to the higher Sla1^{AAA}

patch/cytosol ratio observed here. Considering Sla1 brings Las17 to endocytic sites [42] and that Sla1 and Las17 interact with many other endocytic proteins, a higher level of Sla1^{AAA} oligomerization/recruitment may then cause an increased recruitment of other coat and actin network components.

The phenotype of *sla1^{AAA}* cells is broader than the one found in cells with mutated canonical clathrin-box and W-box binding sites on the clathrin heavy chain N-terminal domain [52]. The more recent discovery of additional binding sites on the clathrin heavy chain N-terminal domain likely explain this difference and the mild phenotype of such mutant [53].

In summary, this work shows the clathrin-Sla1 adaptor protein interaction is important for recruitment of both proteins to endocytic sites, progression through various stages of endocytosis (especially actin polymerization), and normal shaping of the invagination membrane. The diagram depicted in Figure 2.10 encapsulates the role Sla1 plays in clathrin binding and the defects seen during clathrin-mediated endocytosis in *sla1^{AAA}* cells. This work advances our understanding of the function of the adaptor-clathrin interaction and also opens new questions. For example, in the future it will be important to further explore the mechanistic link between coat formation and actin polymerization.

2.5 Experimental procedures

2.5.1 Yeast strains

Standard methods were utilized to generate SDY1031 (*MATa ura3-52, leu2-3,112 his3-Δ200,trp1-Δ901, lys2-801, suc2-Δ9 GAL –MEL EDE1-RFP::HIS3*), which was then mated with SDY063 (*MATα ura3-52, leu2-3,112 his3-Δ200,trp1-Δ901, lys2-801, suc2-Δ9 GAL –MEL SLA1-GFP::TRP1*) or GPY4918 (*MATα ura3-52, leu2-3,112 his3-Δ200,trp1-Δ901, lys2-801,*

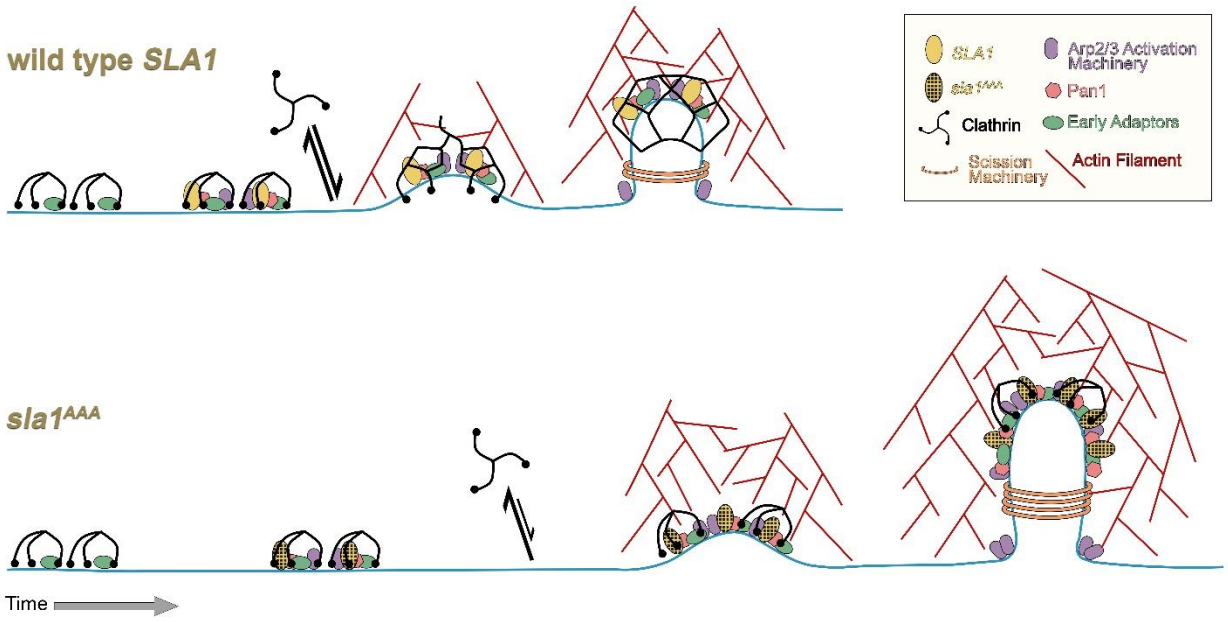

Figure 2.12: Sla1 plays an important role in clathrin coat formation during mid/late stages of endocytosis. Top, early arriving adaptor proteins establish the endocytic site and are involved in initial clathrin recruitment and coat formation. Sequential Sla1 recruitment and clathrin binding contributes to clathrin coat formation and progression to actin polymerization and vesicle scission stages of endocytosis. Bottom, mutation of the Sla1 clathrin binding domain (*sla1*^{AAA}) results in lower levels of clathrin at endocytic sites following Sla1 recruitment when compared to wild type cells. Disruption of the Sla1-clathrin interaction delays progression of endocytosis, actin polymerization, and scission. This defect results in increased levels of mid/late arriving components of the endocytic machinery and an increase in membrane invagination length.

suc2-Δ9 GAL –MEL CHC-RFP::KAN, sla1^{AAA}-GFP::TRP1)[30, 42, 54, 55]. The corresponding diploid cells were then subjected to sporulation and tetrad dissection to generate haploid segregant SDY1033 (*MAT ura3-52, leu2-3,112 his3-Δ200,trp1-Δ901, lys2-801, suc2-Δ9 GAL –MEL EDE1-RFP::HIS3, SLA1-GFP::TRP1*) and SDY1034 (*MAT ura3-52, leu2-3,112 his3-Δ200,trp1-Δ901, lys2-801, suc2-Δ9 GAL –MEL EDE1-RFP::HIS3, sla1^{AAA}-GFP::TRP1*). Standard methods were utilized to generate SDY1032 (*MATa ura3-52, leu2-3,112 his3-Δ200,trp1-Δ901, lys2-801, suc2-Δ9 GAL –MEL PAN1-RFP::HIS3*) which was then mated with SDY063 and the corresponding diploid cells were then subjected to sporulation and tetrad dissection to generate haploid segregant SDY1035 (*MAT ura3-52, leu2-3,112 his3-Δ200,trp1-Δ901, lys2-801, suc2-Δ9 GAL –MEL PAN1-RFP::HIS3, SLA1-GFP::TRP1*).

The vCB mutation (LLDLQ to AAALQ) was introduced into the endogenous *SLA1* gene in GPY1805 (*MAT*a *ura3-52, leu2-3,112 his3-Δ200,trp1-Δ901, lys2-801, suc2-Δ9 GAL –MEL ste2Δ::LEU2*) following a two-step approach similar to the one previously described [31, 32]. For the first step, sequences flanking the Sla1 SHD2 domain and vCB motif, nucleotides 1207–1410 and 2427–2589 were amplified by PCR and cloned into the NotI/BamHI and EcoRI/SalI sites of pBluescriptKS, respectively. A PCR fragment containing *URA3* was then subcloned into the BamHI/EcoRI sites. The resulting construct was cleaved with NotI/ SalI and the *URA3* fragment was introduced by lithium acetate transformation [54] into GPY1805 to generate SDY057 in which SHD2 and the vCB were replaced by *URA3*. In the second step, BsgI/AgeI fragments from pBKS-Sla1-1207-2589 (*SLA1* nucleotides 1207–2589 subcloned into the NotI/SalI sites of pBluescriptKS) containing the LLDLQ-to-AAALQ mutation was cotransformed with pRS313 (*HIS3*) [56] into SDY057. His+ colonies were replica-plated onto agar medium containing 5-fluorotic acid to identify cells in which the mutant sequence replaced

URA3, thus generating strain SDY059 (*sla1*^{AAA}). Standard methods were subsequently applied to strain SDY059 to generate SDY1027 (*MAT*a *ura3-52, leu2-3,112 his3-Δ200,trp1-Δ901, lys2-801, suc2-Δ9 GAL –MEL ste2Δ::LEU2, sla1*^{AAA}*, PAN1-GFP::TRP1, EDE1-mCHERRY::HIS3*) and to GPY1805 to generate wild type control strain SDY1025 (*MAT*a *ura3-52, leu2-3,112 his3-Δ200,trp1-Δ901, lys2-801, suc2-Δ9 GAL –MEL ste2Δ::LEU2, PAN1-GFP::TRP1, EDE1-mCHERRY::HIS3*).

The wild type control and vCB mutant yeast strains used for biochemical analysis shown in Figure 2.1 were previously described: TVY614 (*MAT*a *ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 pep4*::*LEU2 prb1*::*HISG prc1*::*HIS3*) and GPY4913 (*MAT*a *ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 sla1*^{AAA} *pep4*::*LEU2 prb1*::*HISG prc1*::*HIS3*) [30, 57].

The vCB mutation (LLDLQ to AAALQ) was also introduced into the endogenous *SLA1* gene in SDY358 (*MATα ura3-52, leu2-3,112 his3-Δ200,trp1-Δ901, lys2-801, suc2-Δ9 GAL –MEL SLA1-RFP::Kan*) following a similar two-step approach as described above. The first step generated SDY855 in which the third SH3 domain, SHD2 and vCB of *SLA1* were replaced by *URA3*. In the second step, the BsgI/AgeI fragment containing the LLDLQ-to-AAALQ mutation cotransformed with pRS314 (*TRP1*) into SDY855. Trp+ colonies were replica-plated onto agar medium containing 5-fluorotic acid to identify cells in which the mutant sequence replaced *URA3*, thus generating strain SDY878. This strain was then mated with strain expressing CHC-GFP from the endogenous locus *(MATa his3Δ1, leu2Δ0, met15-Δ0, ura3Δ0, CHC-GFP::HIS3)* and the resulting diploid cells were subjected to sporulation and tetrad dissection to generate haploid segregant SDY884 (*MATα his3Δ1, leu2Δ0, met15-Δ0, ura3Δ0, CHC-GFP::HIS3, sla1*^{AAA}*-RFP::Kan*). Wild type control SDY650 (*MATα his3Δ1, leu2Δ0, met15-Δ0, ura3Δ0,*

CHC-GFP::HIS3, SLA1-RFP::Kan) was generated by crossing the corresponding wild type strains. Strains GPY4916 (*MATα, ura3-52, leu2-3,112 his3-Δ200,trp1-Δ901, lys2-801, suc2-Δ9, CHC-RFP::Kan, SLA1-GFP::TRP1*) GPY4918 (*MATα, ura3-52, leu2-3,112 his3-Δ200,trp1-Δ901, lys2-801, suc2-Δ9, CHC-RFP::Kan, sla1^{AAA}-GFP::TRP1*) were previously described[30].

Standard methods were applied to wild type (SDY087) and *sla1^{AAA}* (SDY059) cells to tag the endogenous genes and generate strains expressing the following protein pairs: Las17-GFP and Abp1-mCherry (SDY1029, wt; SDY1023, *sla1^{AAA}*); Vrp1-GFP and Abp1-mCherry (SDY860, wt; SDY862, *sla1^{AAA}*); Myo5-GFP and Abp1-mCherry (SDY871, wt; SDY873, *sla1^{AAA}*); Bzz1-GFP and Abp1-mCherry (SDY864, wt; SDY870, *sla1^{AAA}*); Rvs167-GFP and Abp1-mCherry (SDY856, wt; SDY858, *sla1^{AAA}*). Standard methods were also applied to wild type (SDY358) and *sla1^{AAA}* (SDY878) cells to tag the endogenous *YAP1801* and *ENT1* genes with GFP and generate strains SDY1107 (*MATα ura3-52, leu2-3,112 his3-Δ200,trp1-Δ901, lys2-801, suc2-Δ9 GAL –MEL SLA1-RFP::Kan, YAP1801-GFP::TRP1*), SDY1108 (*MATα ura3-52, leu2-3,112 his3-Δ200,trp1-Δ901, lys2-801, suc2-Δ9 GAL –MEL sla1^{AAA}-RFP::Kan, YAP1801-GFP::TRP1*), SDY1103 (*MATα ura3-52, leu2-3,112 his3-Δ200,trp1-Δ901, lys2-801, suc2-Δ9 GAL –MEL SLA1-RFP::Kan, ENT1-GFP::TRP1*), SDY1105 (*MATα ura3-52, leu2-3,112 his3-Δ200,trp1-Δ901, lys2-801, suc2-Δ9 GAL –MEL sla1^{AAA}-RFP::Kan, ENT1-GFP::TRP1*)

2.5.2 Fluorescent microscopy

Fluorescent microscopy imaging was performed using an Olympus IX81 spinning disk confocal microscope as described [58-60]. Cells were grown to early log phase and imaged at room temperature. Time laps images where collected every 1, 2, 3 or 5sec according to the length of the patch lifetimes. Imaging software Slidebook6 (3I, Denver, CO) was used for

capturing and analysis of images. Data of endocytic patch lifetimes was obtained by drawing a trace around imaged endocytic sites and measuring the average fluorescent intensity for the masked area. Peak patch fluorescence intensities and patch/cytosol fluorescence intensities were measured by drawing a mask on endocytic sites and internal regions, and then normalizing the intensity to that of the background. Kaleidagraph software was used for patch-lifetime determination of fluorescently tagged proteins by integrating the intensities of each consecutive time point and establishing the minimum and maximum value of the integral as previously described [42]. Sla1-GFP and Pan1-RFP recruitment rates were determined by measuring the slope of the linear portion of fluorescence intensity over time plots in the region corresponding to 25% to 50% of the maximal fluorescence. For Figure 2.2C, cells expressing CHC-RFP and Sla1-GFP or Sla1^{AAA}-GFP were treated with 250uM Latrunculin A in SD complete media for 20 minutes before being imaged as described [12]. Statistical significance between wild type and *sla1^{AAA}* cells was determined using an unpaired student's t-test (Graph-pad Software) to determine the SEM and P values.

2.5.3 Biochemical assay

To obtain total cell extracts, 9mL of yeast were cultured in YPD media to an OD500 of 1.0, pelleted via centrifugation, washed 2 times with 2mL of sterile diH2O and placed on ice. 400uL of 0.5mm glass beads were then added to each cell pellet along with 100uL of Buffer A (10mm Hepes pH 7.4, 150mM NaCl, 1mM EDTA) supplemented with protease inhibitor cocktail. Cells were then broken by vortexing at 4°C for 1min. Samples were subjected to centrifugation at 16,000×*g*, 4°C, for 10 min to remove unbroken cells and nuclei. The supernatant was removed and placed into tubes for ultracenrifugation at 75,000rpm for 20

minutes using a Beckman TLA 100.3 rotor to separate the membrane fraction from the cytosol. The cytosolic fraction was removed and the pelleted membrane fraction was resuspended in RIPA buffer (150mM NaCl, 1mM EDTA, 0.1% SDS, 1%Triton X-100, 1% deoxycholate, 20mM Tris pH 7.4). The solubilized membrane fraction was then spun at 16,000×*g*, 4°C, for 15 min and the supernatant was removed and placed in a new culture tube on ice. Equivalent amounts of membrane and cytosol fractions were boiled in Laemmli sample buffer and analyzed by immunoblotting as described [61]. Imaging of immunoblots was performed by use of ImageQuant Las500 from GE and quantification of band intensity was performed using ImageJ software [62].

2.5.4 Transmission electron microscopy

Yeast cultures SDY063 (wild type) and GPY4918 (*sla1*^{AAA}) were grown in YPD media to an OD500 of 0.5, quickly concentrated by filtration, and subjected to high pressure freezing, freeze-substitution, and embedding in Lowicryl HM20 resin as described previously [63-65]. This was followed by collecting 90nm sections of the resin embedded cells onto formvar-coated copper grids. The grids were then stained with 2% uranyl acetate prepared in a 70% methanol 30% water mixture for 15 minutes in the dark. Grids were then washed in a 70% methanol 30% water mixture for 20 secs. This was followed by incubation in Reynolds lead stain for 3 minutes in the dark, followed by four 50 second washes in diH20. Grids were then subjected to transmission electron microscopy using a JEOL2000 microscope. Membrane invagination length and ribosome exclusion zone were measured using Adobe Photoshop software. Statistical significance between membrane invagination lengths and the size of ribosome exclusion zones

was determined using an unpaired student's t-test (Graph-pad Software) to determine the SEM and P value.

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CHAPTER 3

CARGO-INDUCED RECRUITMENT OF THE ENDOCYTIC ADAPTOR PROTEIN SLA1

3.1 Summary

Clathrin-mediated endocytosis (CME) requires the properly timed recruitment of adaptor proteins, assembly proteins, and accessory factors that function to drive membrane invagination and vesicle internalization. While a central function of endocytic adaptor proteins is the binding, concentration and internalization of membrane associated protein cargo, little is known about the function of endocytic cargo in the recruitment of the endocytic machinery and endocytic progression. Cargo-adaptor proteins have been shown to link membrane protein cargo to endocytic sites. Two classes of signals have been shown to target integral plasma membrane proteins by their corresponding adaptors. These include posttranslational ubiquitination and the presence of a cytoplasmic exposed protein sorting motif. In yeast, monoubiquitination and the endocytic protein sorting signal NPFxD have both been shown to function in targeting integral membrane proteins for endocytosis. The adaptor protein Sla1 binds the NPFxD motif through its SHD1 domain, and is necessary for the endocytosis of plasma membrane proteins. Furthermore, Sla1 and its mammalian homologue CIN85 have been shown to bind monoubiquitin through a conserved SH3 domain. To determine the role of cargo in adaptor recruitment, we mutated the Sla1 SHD1 domain at key residues that disrupt binding to the NPFxD sequence and demonstrate that disrupting cargo binding reduced recruitment of Sla1 to endocytic sites. Recruitment of Sla1 to endocytic sites was additionally disrupted upon deletion of its Pan1/End3 interacting SR region, implying Sla1 recruitment to endocytic sites is based upon multiple interactions.

Expression of different Sla1 fragments demonstrated that while the SHD1 domain alone could not be recruited to the plasma membrane, expression of any fragment containing both the SH3-3 domain, which binds ubiquitin, and the SHD1 domain resulted in robust membrane recruitment. In addition mutation of a SH3-3 residue that disrupts ubiquitin binding also reduced membrane recruitment of the SH3-3-SHD1 fragment. These results and the localization pattern of the SH3-3-SHD1 fragment uniformly across the plasma membrane suggest that the SH3-3 domain binds ubiquitinated endocytic cargo, and may contribute to Sla1 recruitment to the plasma membrane. Our results also imply that the C-terminal SR region of Sla1 may regulate the interaction of Sla1 with endocytic cargo, and this regulatory mechanism may target Sla1 to endocytic sites rather than diffusely binding the plasma membrane. In addition, reduced cargo binding by Sla1 also results in enhanced nuclear localization of Sla1, giving new insight and support to the concept of endogenous nuclear Sla1.

3.2 Introduction

Clathrin-mediated endocytosis (CME) is a primary mechanism by which eukaryotic cells internalize extracellular material and plasma membrane protein cargo, and additionally serves roles in regulating signaling pathways, membrane composition, and viral entry [1-6]. The regulated targeting of integral membrane proteins requires interactions with specific components of the endocytic machinery, termed cargo adaptors, that bind and incorporate endocytic protein cargo into clathrin-coated pits [7]. Sequential events of membrane invagination and vesicle scission results in internalization of a cargo loaded vesicle. The endocytic coat then dissociates from the vesicle, which is trafficked to endosomes, and the endocytic machinery is recycled back to the plasma membrane for repeated rounds of CME [8-11].

The model organism *Saccharomyces cerevisiae* has proven to be an extremely useful system for studying different aspects of CME. Through the use of genetic, biochemical, live-cell fluorescence microscopy, and electron microscopy imaging techniques, a more complete understanding of clathrin-mediated endocytosis and the significance of this process in all eukaryotes has become apparent. CME progresses through defined stages that involves the properly timed and well-orchestrated recruitment and disassembly of more than 60 endocytic proteins [1]. The process begins with recruitment of early components of the endocytic machinery, such as clathrin, Syp1, and Ede1, which establish the site of endocytosis [12-15]. This is followed by the progressive arrival of a variety of functionally diverse proteins, some of which form a coat on the plasma membrane [12]. These early stages of endocytosis are relatively immobile in terms of membrane dynamics, with no membrane bending occurring. This is followed by a short mobile phase (~15sec), in which Arp2/3-mediated actin polymerization drives invagination of the endocytic site and most protein coat components [16, 17]. The process then concludes with vesicle scission in which BAR domain containing proteins Rvs161/167 and the yeast dynamin Vps1 constrict the neck of the endocytic invagination until fission occurs and the vesicle is released into the cytosol [12, 18-20]. Shortly after scission, most components of the endocytic coat disassemble and the vesicle is trafficked towards endosomes. During this process various integral membrane protein cargos are targeted for internalization by cargo adaptors such as Syp1 and Sla1. It is, however, unclear whether cargo plays an active role in recruitment of the endocytic machinery, or if it is merely a passive player in progression of endocytosis.

Many endocytic protein cargos contain short amino acid sequences that function as a sorting signal for recognition by the endocytic machinery. In mammalian cells, signaling motifs Yxxφ and [DE]xxxL[LI], where φ is a hydrophobic residue and x is any residue, are targeted by

the tetrameric clathrin adaptor protein AP-2 [21-31]. Monomeric clathrin-adaptor proteins ARH and Dab2 target [YF]xNPx[YF] motifs through a conserved PTB domain [32-36]. In *Saccharomyces cerevisiae* two linear motifs have been established as being capable of inducing cargo internalization. The NPFxD motif was the first characterized endocytic cargo sorting signal in yeast [37]. NPFxD is utilized by integral membrane proteins Wsc1, Drs2, Dnf1, and Ste3 for endocytosis and is targeted by the SHD1 domain of the clathrin-adaptor protein Sla1 [38-41]. Second, the DxY motif present in endocytic cargos Mid2, Ptr2, Mep3, and the v-SNARE Snc1, is targeted by the early endocytic protein Syp1 through its μ-homology domain [15, 42]. Additionally, the mammalian Yxxφ sequence has been suggested to function as an endocytic signal in yeast, and may similarly be targeted by the AP2 μ-homology domain [43].

A second means by which plasma membrane proteins are targeted for endocytosis is posttranslational ubiquitination. Polyubiquitination has been shown to serve a number of functions, most notably in the targeting proteins for proteasomal degradation [44, 45]. Monoubiquitination modification of plasma membrane cargo, however, functions to target these proteins for CME [46-49]. Eps15 (Ede1) and Epsin (Ent1/2) proteins both contain ubiquitin binding domains [50, 51]. While work suggests these proteins target ubiquitinated cargo for endocytosis, it has additionally been proposed they may alternatively bind ubiquitinated components of the endocytic machinery [52-54]. The clathrin-adaptor protein Sla1 has been shown to be capable of binding mono-ubiquitin through its third SH3 domain [55, 56]. Interestingly, its mammalian homologue CIN85 was also shown to bind ubiquitin through its third SH3 domain, demonstrating a potentially conserved role for these two proteins [55]. A functional role for Sla1 in ubiquitin binding, however, remains to be demonstrated.

While it is clear that short linear motifs and monoubiquitination designates integral membrane cargo for internalization by the endocytic machinery, it is not clear if these signals play an active role in recruitment of the endocytic machinery, or progression of endocytosis. In mammalian cells, ligand stimulation of EGF receptors target them for ubiquitination and endocytosis, and also enhances Eps15 recruitment to endocytic sites [54, 57, 58]. Additionally, increased levels of ubiquitinated cargo in endosomes has been shown to drive Eps15 localization in a manner dependent upon its ubiquitin interacting motif [59]. In yeast, ubiquitinated cargo Ste2 arrives to endocytic sites after patch initiation, and it has been hypothesized that this arrival may act as a potential cargo checkpoint that must be satisfied before endocytosis is allowed to progress [60]. This work also suggests that Ede1 may not be recruited by endocytic cargo, since Ste2 arrived to endocytic sites after Ede1 recruitment. Additionally, it was shown in mammalian BSC1 cells that overexpression of an artificial NPxY containing cargo enhanced ARH and Dab2 recruitment to the plasma membrane in a manner that is dependent upon cargo binding [61]. While this work suggests a role for cargo in endocytosis, a number of questions remain. Does ubiquitinated cargo contribute to recruitment of the endocytic machinery? Do all endocytic cargo sorting signals contribute to adaptor recruitment and is this conserved across species? Does cargo contribute to adaptor recruitment in a native system in which proteins are expressed at endogenous levels? If cargo does recruit adaptors to the plasma membrane, how is this interaction regulated? These questions are fundamental to understanding the process of clathrin-mediated endocytosis, considering the primary function is the internalization of endocytic cargo.

In the work presented here we explore the role of endocytic cargo sorting signals in the recruitment of endocytic machinery by investigating the adaptor-cargo interaction between Sla1 and the NPFxD motif. In cells containing mutations to the Sla1 cargo binding SHD1 domain, we

observed a decrease in Sla1 recruitment to endocytic sites. Additionally, we found that while deletion of the C-terminal Sla1 repeat region did not prevent membrane recruitment, it did prevent proper Sla1 localization to endocytic sites. Our work further suggests that Sla1 binds ubiquitinated cargo, and that ubiquitin binding could contribute to proper Sla1 recruitment to the plasma membrane in general and endocytic sites in particular. Finally, our results suggest that when Sla1 is not properly recruited to endocytic sites it will display an enhanced nuclear localization.

3.3 Results

3.3.1 SHD1-cargo binding contributes to Sla1 membrane recruitment

Previous work has demonstrated that Sla1 is capable of targeting plasma membrane protein cargo for clathrin-mediated endocytosis [38-40]. Sla1 specifically binds the endocytic cargo sorting signal NPFxD through its SHD1 domain [41]. This interaction has been shown to be involved in targeting proteins that are completely dependent upon Sla1 for internalization such as Wsc1, or partially dependent upon Sla1 for their endocytosis, such as Ste2 and Ste3 [39, 62]. The interaction between the SHD1 domain and cargo occurs through a hydrophobic pocket of SHD1 where key residues were identified as playing a role in NPFxD binding [41]. This work presented us with an ideal system to determine if cargo binding contributes to adaptor Sla1 recruitment to the plasma membrane. To address this question strains expressing point mutations K525A, F507L, and I531E in the SHD1 domain of Sla1-GFP from the endogenous locus were imaged by confocal fluorescent microscopy. Importantly, these point mutations do not affect Sla1 overall stability but reduces NPFxD signal binding to different degrees [41]. The recruitment of each of these mutants to endocytic sites was compared to that of wild type cells.

The peak patch/cytosol ratio, as well as the total membrane/cytosol ratio, was analyzed for each strain (Figure 3.1). Disruption of NPFxD binding by the SHD1 domain resulted in reduced Sla1-GFP recruitment to the plasma membrane. This recruitment defect correlated with the reduced affinity each SHD1 mutant has been shown to have for the NPFxD motif [41]. Interestingly, imaging of the Sla1^{I531E}-GFP mutant strain showed a very noticeable Sla1 localization to an internal spherical structure that resembled the nucleus. Additionally, a less pronounced spherical structure additionally appeared in the Sla1^{F507L}-GFP cells. We further address this finding in our Figure 3.5 analysis.

3.3.2 Interactions with the Pan1/End3 complex contributes to Sla1 recruitment to endocytic sites

The Sla1 C-terminal repeat (SR) region is responsible for forming a complex with two components of the endocytic machinery, Pan1 and End3, and is capable of making distinct interactions with both Pan1 and End3 in a phosphorylation dependent manner [63-67]. It has previously been shown that deletion of the Sla1 SR region not only causes significant defects in progression of endocytosis, but also caused Sla1 to become more diffusely distributed across the plasma membrane, instead of the tightly localized punctate formation of typical endocytic sites [66, 67]. For these reasons, we decided to investigate whether the SR domain is capable of working cooperatively with the SHD1 domain to recruit Sla1 to endocytic sites. To examine this hypothesis strains were created containing the SR region deletion alone or in combination with the SHD1 point mutations described above in the endogenous SLA1 gene previously tagged with GFP (Figure 3.2). Fluorescence microscopy analysis and quantification of total membrane/cytosol GFP fluorescence intensity demonstrates that each of the SHD1 mutations

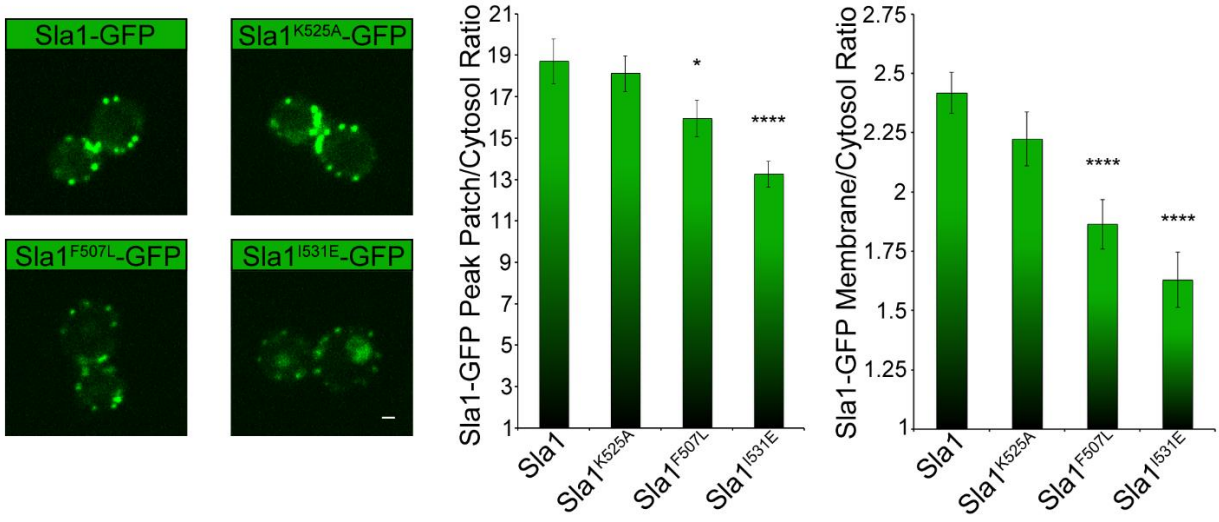


Figure 3.1: Clathrin adaptor protein Sla1 is actively recruited to endocytic sites by binding NPFxD signal containing cargo. Live-cell confocal fluorescent microscopy analysis of yeast cells expressing either Sla1^{wt}-GFP, Sla1^{K525A}-GFP, Sla1^{F507L}-GFP, or Sla1^{I531E}-GFP from the endogenous locus. Left, panels show representative frames of Sla1-GFP from each corresponding strain. Middle, quantification represented as bar graphs of the average peak patch/cytosol fluorescence ratio of wild type Sla1-GFP at endocytic sites and each of the Sla1 SHD1 domain mutants shows decreased membrane recruitment in SHD1 mutants (N=40 patches, P=0.6624 for Sla1^{K525A}, P=0.0477 for Sla1^{F507L}, P<0.0001 for Sla1^{I531E}). Right, quantification represented as bar graphs of the total membrane/cytosol ratio of Sla1-GFP in each of the SHD1 mutants and wild type cells reflects results seen in the quantification of peak patch/cytosol measurements (N=30 cells, P=0.1713 for Sla1^{K525A}, P<0.0001 for Sla1^{F507L}, P<0.0001 for Sla1^{I531E}).

had a significantly more dramatic effect on Sla1 recruitment to the plasma membrane when combined with a deletion of the SR region of Sla1 (Figure 3.2). Furthermore, plasma membrane recruitment was completely lost for Sla1^{I531E}ΔSR-GFP, which contains the most severe SHD1 mutation. These results suggest that Sla1-cargo binding contributes to general recruitment of Sla1 to the plasma membrane, while interactions with the endocytic machinery components Pan1/End3 specifically contribute to recruitment of Sla1 to endocytic sites. This concept is supported by previously published results that show expression of the fluorescently tagged SR region alone resulted in punctate membrane recruitment and internalization [67]. It would thus appear that both the SHD1 domain and SR region contribute to Sla1 recruitment to endocytic sites, however in a unique fashion in which the SHD1 recruits Sla1 based on cargo interactions at the membrane and the SR domain recruits Sla1 specifically to endocytic sites.

3.3.3 A minimal NPFxD and ubiquitin-binding Sla1 fragment is capable of plasma membrane recruitment

We next wanted to examine whether the SHD1 domain itself was capable of plasma membrane recruitment. To examine this we overexpressed SHD1-GFP from a high expression plasmid in wild type yeast cells (Figure 3.3, Frag 5, A&B). Fluorescent confocal microscopy imaging of the cells demonstrated no detectable recruitment of the SHD1 domain, which showed a cytosolic localization. We then decided to determine the minimal fragment of Sla1 that was necessary for recruitment to the plasma membrane. By taking a similar approach we expressed various fragments of Sla1 tagged with GFP and again performed fluorescent confocal microscopy imaging (Figure 3.3 A&B). The minimal fragment necessary for membrane

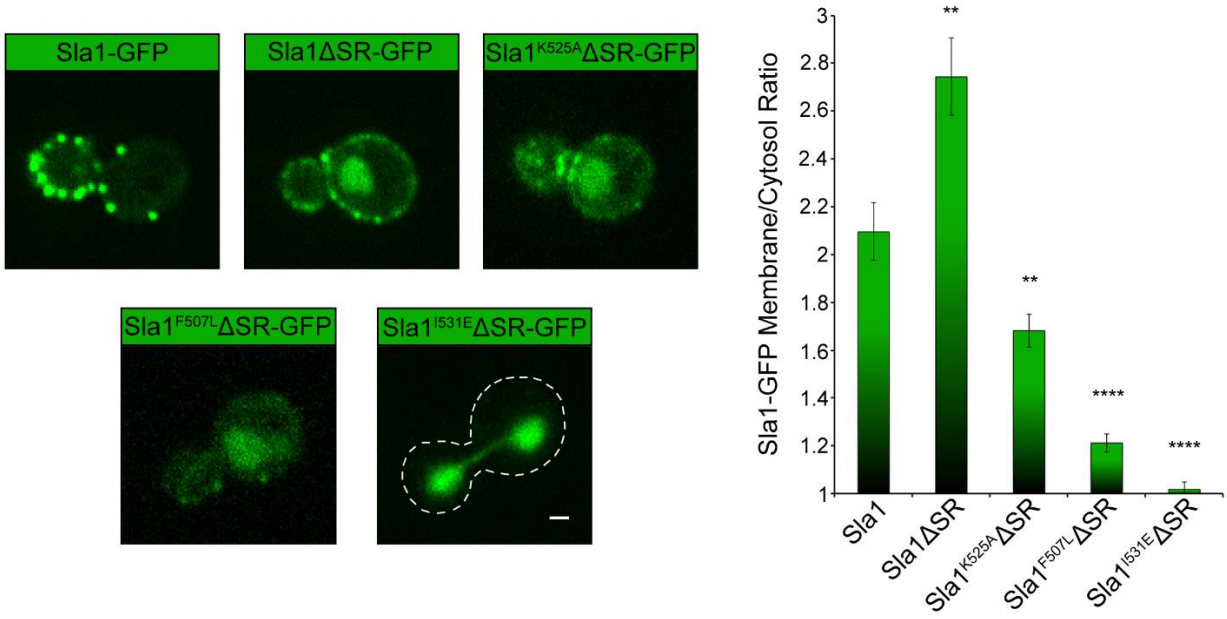


Figure 3.2: The Sla1 SR region contributes to Sla1 recruitment to endocytic sites in combination with cargo binding by the SHD1 domain. Live-cell confocal fluorescent microscopy analysis of cells expressing either Sla1ΔSR-GFP, Sla1^{K525A}ΔSR-GFP, Sla1^{F507L}ΔSR-GFP, or Sla1^{I531E}ΔSR-GFP from the corresponding endogenous locus. Left, panels show representative frames of Sla1-GFP from each of the corresponding strains. Right, quantification represented as bar graphs of the total membrane/cytosol ratio of Sla1-GFP in each of the ΔSR and SHD1 mutants as well as wild type cells demonstrates that deletion of the SR region in combination with mutation of the cargo binding surface of the SHD1 domain reduces Sla1 recruitment to the plasma membrane. The most severe mutant, Sla1^{I531E}, eliminates Sla1 membrane recruitment. White dotted line designates the cell membrane which is now barely visible (N=53 cells for Sla1^{wt}, N=34 cells P=0.0015 for Sla1ΔSR, N=46 Cells for Sla1^{K525A}ΔSR P=0.0054, N=47 Cells for Sla1^{F507L}ΔSR P<0.0001, N=42 cells for Sla1^{I531E}ΔSR P<0.0001).

recruitment was determined to be Fragment 3 (a.a. 350-560) that contains both the third SH3 domain and the SHD1 domain (Figure 3.3B). Furthermore, the SH3-3-SHD1-GFP poly-peptide showed a very diffuse and even distribution across the plasma membrane compared to the other Sla1 fragments or full length Sla1. This diffuse pattern of the Sla1 SH3-3-SHD1-GFP fragment suggests that it is recruited to the plasma membrane through interactions with ubiquitinated and NPFxD-containing cargo.

To further determine if the SH3-3-SHD1 fragment was specifically recruited to the plasma membrane through interactions with ubiquitin and the NPFxD motif, point mutations that have been shown to reduce ubiquitin or NPFxD binding were made to the expression plasmids. Mutation of residue W391A in the SH3-3 domain that has been shown to reduce ubiquitin binding diminished SH3-3-SHD1-GFP recruitment to the plasma membrane (Figure 3.3C) [55]. Recruitment was completely eliminated when mutation I531E of the SHD1 was made to the fragment, indicating that the SHD1 domain plays a more significant role in recruitment of Sla1. This is supported by our results demonstrating that the W391A mutation alone has no effect on recruitment of full length Sla1-GFP (data not shown).

Interestingly, Sla1 contains a C-terminal NPFGF* sequence at the tail region of the SR region. We hypothesized that the NPFGF* sequence may negatively regulate cargo binding and Sla1-membrane recruitment by competing for SHD1 binding. To test this theory, we fused the last 31 amino acids of Sla1, containing the NPFGF* sequence, to the SH3-3-SHD1-GFP fragment. Quantification of the SH3-3-SHD1-NPFGF*-GFP membrane recruitment demonstrated a significant reduction in membrane recruitment compared to the SH3-3-SHD1-GFP fragment (Figure 3.3C). While this result supports the idea that the NPFGF* sequence could

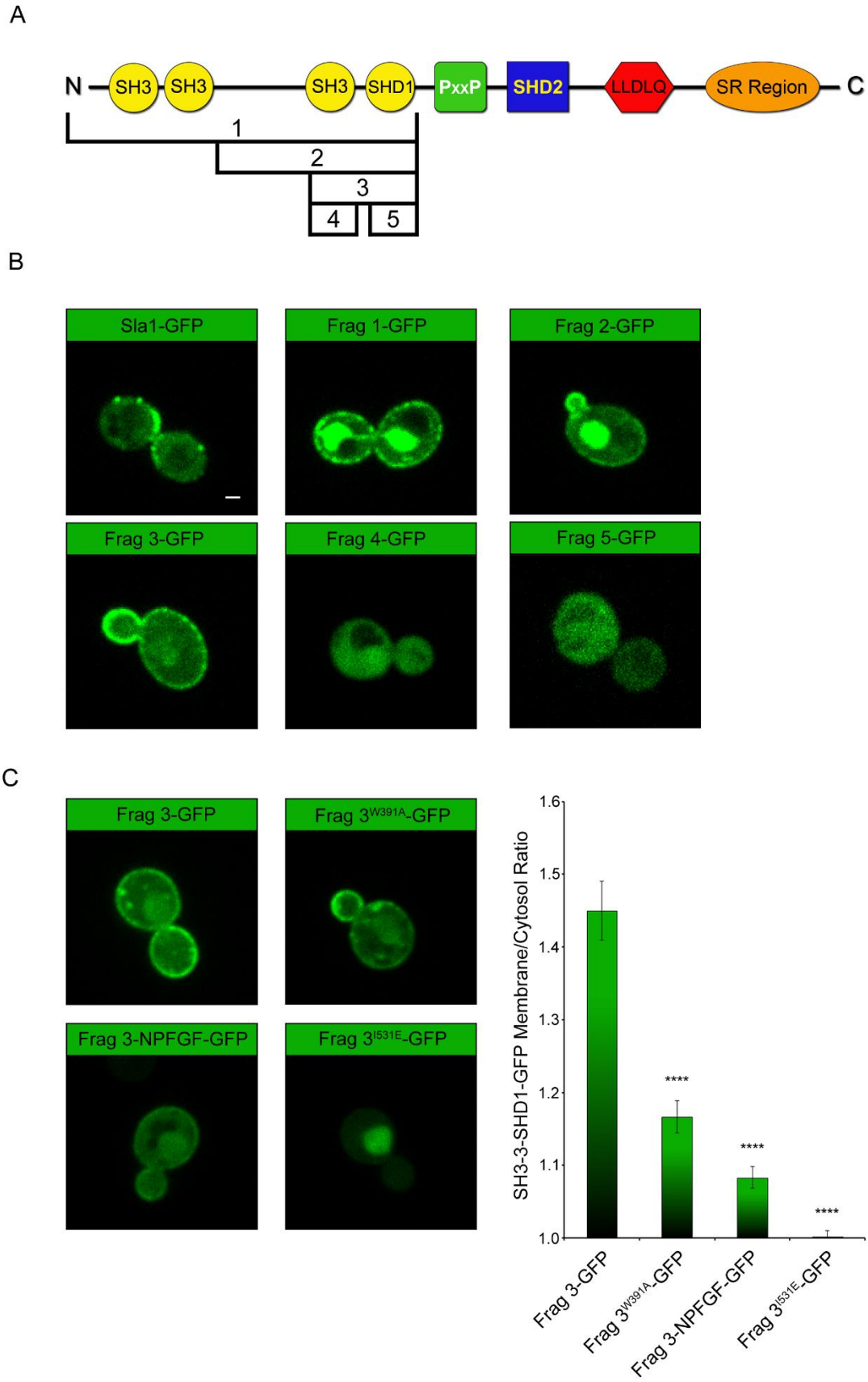

A
N
SH3
SH3
SH3
SHD1
PxxP
SHD2
LLDLQ
SR Region
C
1
2
3
4
5
B
Sla1-GFP
Frag 1-GFP
Frag 2-GFP
Frag 3-GFP
Frag 4-GFP
Frag 5-GFP
C
Frag 3-GFP
Frag 3W391A-GFP
Frag 3-NPFGF-GFP
Frag 3I531E-GFP
SH3-3-SHD1-GFP Membrane/Cytosol Ratio
1.6
1.5
1.4
1.3
1.2
1.1
1.0
****
****
****
Frag 3-GFP
Frag 3W391A-GFP
Frag 3-NPFGF-GFP
Frag 3I531E-GFP

Figure 3.3: The minimal fragment of Sla1 containing both the SH3-3 and SHD1 domains is capable of membrane recruitment. A, Diagram of Sla1 domains. Brackets indicate fragments of Sla1 used in Figure 3.3B for fluorescent imaging experiments. B, Live-cell confocal fluorescent microscopy analysis of yeast cells expressing GFP tagged fragments of Sla1. All fragments containing both the SH3-3 and SHD1 domains were shown to localize to the plasma membrane. C, Live-cell confocal fluorescent microscopy analysis of Sla1 SH3-3-SHD1 fragments. Top-Left is a representative frame of the Sla1 SH3-3-SHD1-GFP fragment. Top-Right is a representative frame of SH3-3-SHD1-GFP containing an SH3-3 mutation shown to reduce ubiquitin binding by SH3-3. Bottom-Left is a representative frame of SH3-3-SHD1-NPFGF-GFP where the last 31 residues of Sla1 where fused to the tail of the SH3-3-SHD1 fragment. Bottom-Right is a representative frame of SH3-3-SHD1-GFP containing the I531E mutation shown to reduce NPFxD binding by the SHD1 domain. Right, quantification of membrane/cytosol fluorescence ratio for the SH3-3-SHD1-GFP (Fragment 3) and fragment 3 modified versions shown in Figure 3.3C left (N=30 cells, $P<0.0001$ for Frag 3^{W391A}, $P<0.0001$ for Frag 3-NPFGF, $P<0.0001$ for Frag 3^{I531E}).

regulate the Sla1-NPFxD cargo interaction and membrane recruitment, deletion of the NPFGF* sequence in the context of full length Sla1did not result in diffuse cortical localization of Sla1-GFP, perhaps due to a dominant role of the SR region-Pan1/End3 interaction in localizing Sla1 to endocytic sites (data not shown). Nonetheless, these results further support the role cargo binding plays in Sla1 recruitment to the plasma membrane, and suggests an intramolecular interaction may regulate this recruitment.

3.3.4 Deletion of the Sla1 SR domain results in the cargo dependent recruitment of Sla1 diffusely across the plasma membrane

Previous reports have demonstrated that deletion of the Sla1 SR region causes significant defects in progression of clathrin-mediated endocytosis [66, 67]. It was additionally demonstrated that while Sla1ΔSR-GFP could appear at endocytic sites and partially co-localized with other components of the endocytic machinery, it did not appear to move inward upon membrane invagination [67]. One could interpret these results to suggest that Sla1 is being recruited to endocytic sites and then remaining on the membrane after invagination, resulting in the more diffuse cortical pattern of Sla1ΔSR-GFP. We, however, propose a different mechanism by which Sla1ΔSR is recruited directly to the membrane through interactions with membrane components not associated with endocytic sites. To test this theory we performed fluorescence recovery after photobleaching experiments. We generated cells expressing either Sla1ΔSR-GFP or the fluorescently tagged integral membrane and endocytic cargo protein Ste2-GFP. We then photobleached a portion of the membrane and measured the fluorescence recovery of each protein. Sla1ΔSR-GFP showed rapid membrane recovery to 23.4% of initial membrane

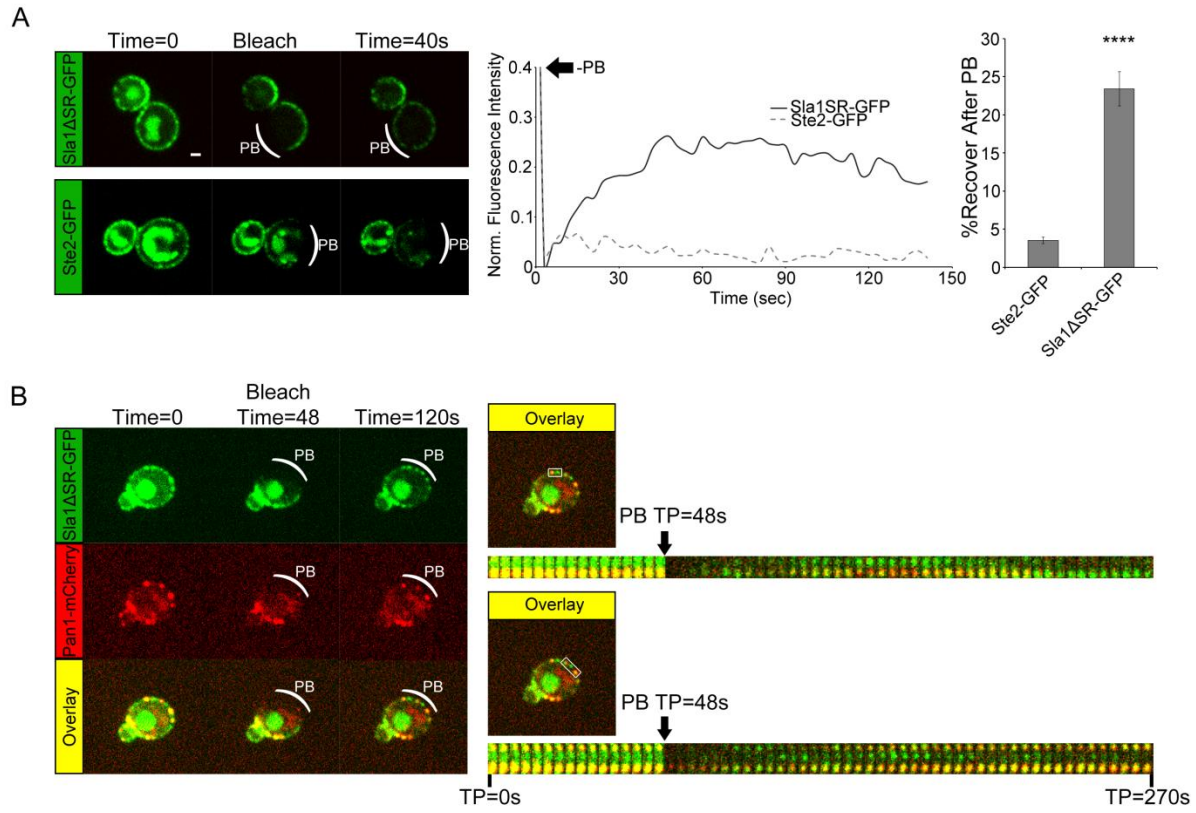


Figure 3.4: Sla1 is recruited to the plasma membrane at non-endocytic sites upon deletion of the SR region responsible for complex formation with Pan1 and End3. A, Fluorescence recovery after photobleaching (FRAP) confocal microscopy imaging in cells expressing either Sla1ΔSR-GFP or Ste2-GFP from the corresponding endogenous locus. Left, representative frames of Sla1ΔSR-GFP and Ste2-GFP before, immediately after, and 40sec after photobleaching. The area indicated by a curved white line was subjected to photobleaching (PB). Center, graphical representation of Sla1ΔSR-GFP and Ste2-GFP fluorescence recovery after photobleaching. Right, Quantification represented as bar graphs of the average maximal fluorescence recovery after photobleaching of Sla1ΔSR-GFP and Ste2-GFP (N=9 cells for Ste2-GFP and N=12 cells for Sla1ΔSR-GFP, $P<0.0001$). B, FRAP confocal microscopy imaging of cells expressing both Sla1ΔSR-GFP and Pan1-RFP from their corresponding endogenous locus. Left, representative frames of Sla1ΔSR-GFP and Pan1-RFP before, immediately after, and 40 after photobleaching. The area indicated by a curved white line was subjected to photobleaching (PB). Right, kymographs of Sla1ΔSR-GFP and Pan1-RFP photobleaching and recovery from cells represented to the left. Regions of cells used to make kymograph are labeled in white rectangles.

fluorescence, while Ste2-GFP show little to no membrane fluorescence recovery and an average 3.5% of initial fluorescence intensity (Figure 3.4A). These results can be interpreted to make a few conclusions. To begin, Sla1ΔSR appears to be recruited directly to the plasma membrane in a diffuse pattern, differing from the punctate formation that occurs at endocytic sites. Secondly, Sla1ΔSR recruitment appears to be highly dynamic, with Sla1Δ-GFP recovery occurring rapidly at the plasma membrane, unlike Ste2-GFP. This also indicates the Sla1-GFP recovery is likely not occurring through diffusion of membrane bound Sla1, since Ste2-GFP fluorescence recovery would control for this effect. The fact that Ste2-GFP showed such low levels of fluorescence recovery following photobleaching also suggests that integral membrane proteins are, at best, slowly diffusing through the yeast plasma membrane.

To further support the idea that Sla1ΔS-GFP is recruited to the plasma membrane in a fashion independent of binding to the endocytic machinery, we performed FRAP on cells expressing both Sla1ΔSR-GFP, and Pan1-RFP as a marker of endocytic sites. In these cells Sla1ΔSR-GFP recovered at various locations across the membrane, both with and without Pan1-RFP (Figure 3.4B). In combination with results from Figure 3.2, this suggests that Sla1ΔSR-GFP is recruited to non-endocytic membrane sites in a fashion that is cargo dependent. It also suggests that properties of the Sla1 SR domain contribute to maintaining cytosolic Sla1 in a state that is not capable of being recruited directly to the plasma membrane through cargo interactions.

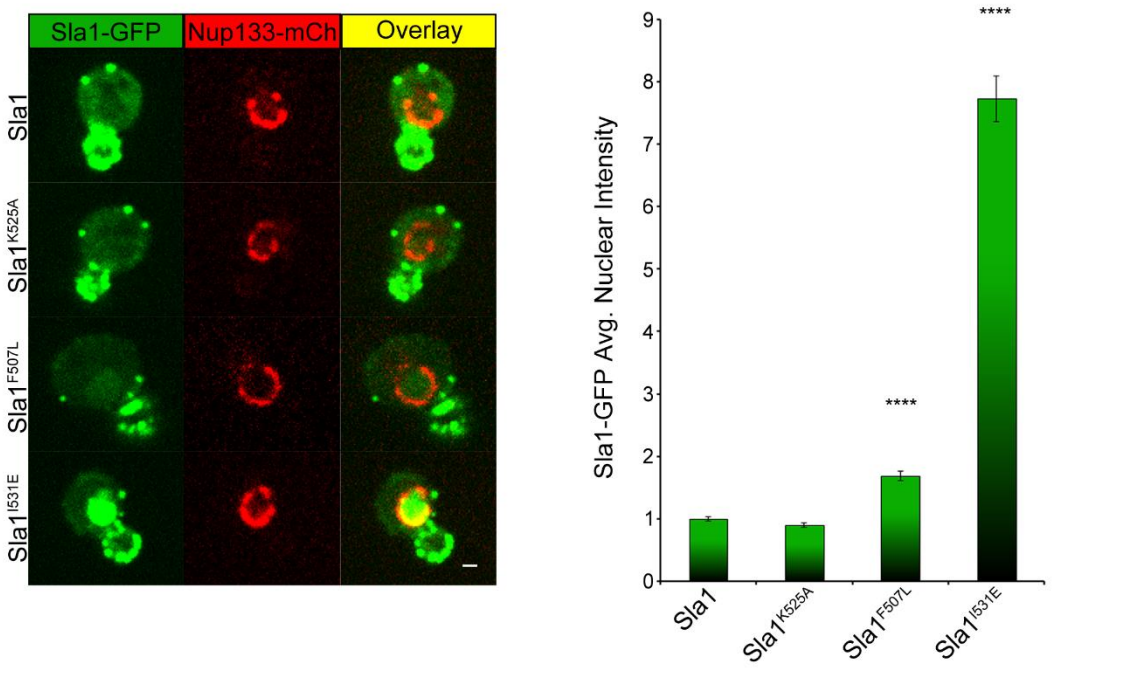
3.3.5 Sla1 localizes to the nucleus when endocytic site recruitment is disrupted

Previous publications have demonstrated that Sla1 may be targeted to the nucleus for reasons that are incompletely understood [68]. In this work truncated fragments of Sla1 were shown to localize to the nucleus [68]. Proteins of size greater than~40 kDa require the active

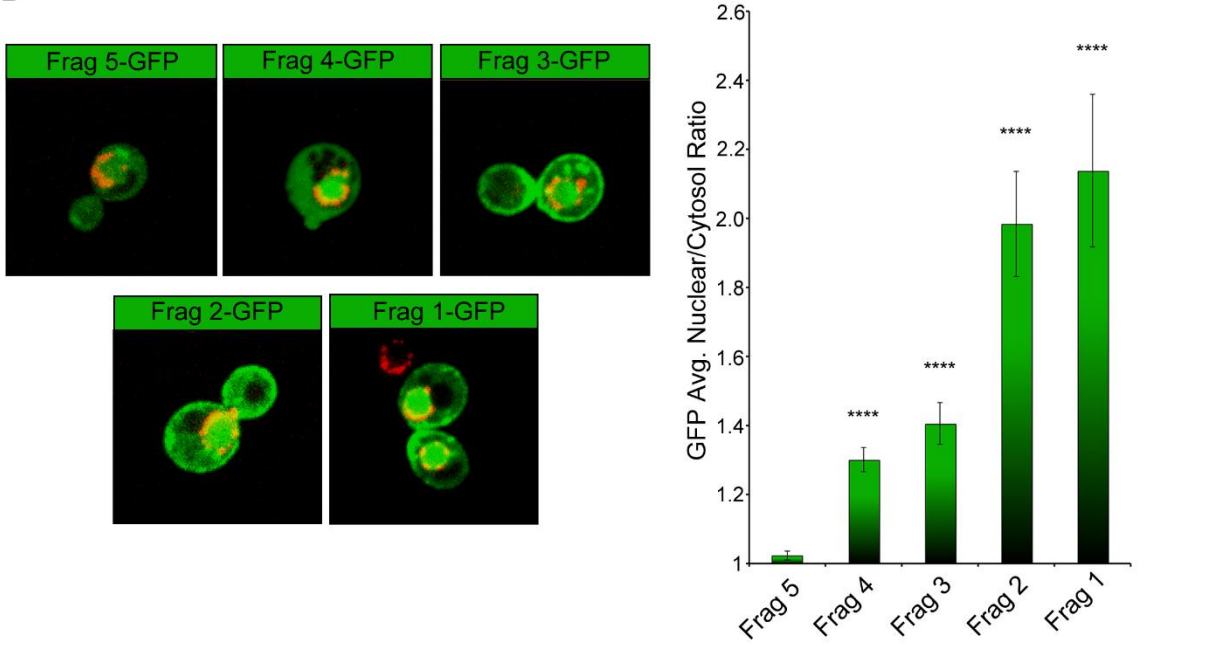
passage by nuclear transport factors, or carrier proteins called karyopherins, that recognize nuclear import signals [69, 70]. It was suggested that fragments of Sla1 were localizing to the nucleus when nuclear import signals were preserved and export signals were deleted [68]. Our work, however, suggests that Sla1 nuclear localization may merely be dependent upon its lack of recruitment to endocytic sites. Upon mutation of the Sla1 SHD1 domain, especially in regard to the I531E mutation, we observed that Sla1 localizes to an internal spherical structure, reminiscent of the nucleus. Nuclear localization was confirmed by generating and analyzing cells expressing Nup133-3xmCherry, a component of the nuclear pore complex, and Sla1-GFP containing the different SHD1 mutations [71]. Nuclear localization was demonstrated for two of the SHD1 mutations in a fashion in which enhanced nuclear localization correlated directly with reduced membrane recruitment (Figure 3.5A). It should be noted that the SHD1 mutations shown here do not overlap with any of the proposed nuclear import, or nuclear export signals of Sla1.

With this in mind we decided to investigate which region of Sla1 might contain the signal responsible for nuclear localization. Using the GFP tagged fragments of Sla1 from Figure 3.3 and expressing Nup133-3xmCherry, the minimal fragment necessary for nuclear localization was shown to contain just the SH3-3 domain (Figure 3.5B). While this fragment is hypothetically capable of diffusion through the nuclear pore complex, the similar sized SHD1-GFP fragment did not show nuclear localization above cytosolic levels, suggesting an active transport of the SH3-3 domain was occurring, in addition to any potential nuclear diffusion. NLS prediction algorithms and previous publications suggest a nuclear localization signal may exist in the SH3-3 domain [68]. All other fragments of Sla1, excluding the SHD1-GFP fragment 5, where shown to localize to the nucleus, hypothetically due to the presence of the SH3-3 domain. Larger

A


Sla1-GFP
Nup133-mCh
Overlay
Sla1
Sla1K525A
Sla1F507L
Sla1I531E
Sla1-GFP Avg. Nuclear Intensity
****
****
****
Sla1
Sla1K525A
Sla1F507L
Sla1I531E

B


Frag 5-GFP
Frag 4-GFP
Frag 3-GFP
Frag 2-GFP
Frag 1-GFP
GFP Avg. Nuclear/Cytosol Ratio
****
****
****
****
Frag 5
Frag 4
Frag 3
Frag 2
Frag 1

C

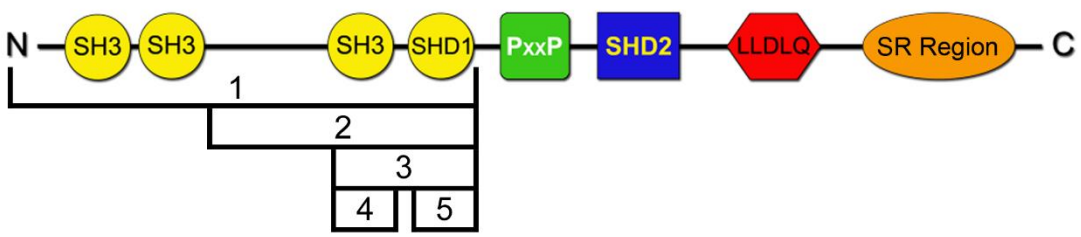

N
SH3
SH3
SH3
SHD1
PxxP
SHD2
LLDLQ
SR Region
C
1
2
3
4
5

Figure 3.5: Sla1 localizes to the nucleus when cargo binding and membrane recruitment is disrupted. A, Live-cell confocal fluorescent microscopy imaging of cells expressing Sla1-GFP wild type, or the indicated SHD1 mutant, and Nup133-3xmCherry expressed from the endogenous locus. Left, representative frames of Sla1-GFP and Nup133-3xmCherry in wild type and SHD1 mutant cells. Right, quantification represented as bar graphs of the Sla1-GFP nuclear/cytoplasm ratio in which a significant increase in Sla1-GFP nuclear localization was determined for F507L and I531E mutants (N=35 cells, P=0.0566 for $Sla1^{K525A}$, P<0.0001 for $Sla1^{F507L}$ and $Sla1^{I531E}$). B, Live-cell confocal fluorescent microscopy imaging of cells expressing GFP tagged fragments of Sla1 and Nup133-3xmCherry. All fragments containing the SH3-3 domain showed an increase in nuclear localization above the uniform cytosolic and nuclear localization seen in Fragment 5 (SHD1-GFP) (N=25 cells, P<0.0001 for all fragments). C, Diagram of Sla1 domains. Brackets indicate fragments of Sla1 used in Figure 3.5B for fluorescent imaging experiments.

fragments 1 and 2 were shown to have much higher levels of nuclear localization than fragments 4 and 5, likely due to the enhanced size that would prevent diffusion out of the nucleus.

3.4 Discussion

It is well established that adaptor protein binding is essential for transmembrane protein recruitment to endocytic sites and internalization. In this work we have addressed the converse relationship of whether cargo can recruit the endocytic machinery. We have demonstrated that proper Sla1 recruitment to endocytic sites is dependent, in part, upon binding of its SHD1 domain to endocytic cargo containing an NPFxD sequence. Mutations that disrupt NPFxD binding by the SHD1 domain also reduced Sla1-GFP localization to endocytic sites. The C-terminal Sla1 repeat region responsible for Pan1/End3 binding additionally contributes to Sla1 recruitment to endocytic sites, reflecting the phenotype seen by others [66, 67]. Interestingly, however, Sla1-GFP recruitment to the plasma membrane in general was actually enhanced in strains containing a deletion of the SR domain, with Sla1ΔSR-GFP recruitment no longer being targeted specifically to endocytic sites. Our results suggest that in the absence of the SR region, Sla1 membrane recruitment is now cargo binding dependent and this cargo-dependent recruitment occurs more diffusely at the cell cortex. These results are supported by others in which deletion of C-terminal portions of Sla1 resulted in Sla1-GFP membrane recruitment until both the SR repeat region and the SHD1 domain were removed [67]. Our results also suggest that interactions involved with the SR domain are responsible for regulating Sla1 membrane recruitment specifically to endocytic sites, as a diffuse pattern of Sla1-GFP membrane recruitment does not occur in the context of full length Sla1. Result from our experiments demonstrate that the C-terminal tail of Sla1, which contains the NPFGF* sequence, can compete

with NPFxD-cargo for SHD1 binding as seen in our SH3-3-SHD1-NPFGF*-GFP fragment (Figure 3.3C). It should be noted that others have shown degron depletion of either Pan1 or End3 does not prevent what appears to be punctate endocytic sites formation by Sla1-GFP, and depletion of both Pan1 and End3 does not appear to result in diffuse Sla1-GFP membrane localization [67, 72]. In combination with our finding, this would suggest that a unique regulatory property of the Sla1 SR region regulates Sla1-cargo binding that is separate from its role in Pan1/End3 binding. Considering our work indicates the Sla1 SR C-terminus contains an NPFGF* sequence capable of competing for SHD1 binding by NPFxD cargo, we hypothesize that the SR region may be preventing cargo binding through an intramolecular interaction.

Traditionally SH3 domains are known for their binding to specific polyproline motifs. Sla1 has been shown to bind ubiquitin through its third SH3 domain [55]. Truncations of Sla1 demonstrated that a minimal fragment necessary for highly diffuse membrane recruitment contains just the SH3-3 and SHD1 domains (Figure 3.3B Fragment 3). Similarly, just the C-terminal repeat region is also capable of membrane recruitment, although it localizes specifically to endocytic sites in a punctate fashion [67]. Mutations that disrupt NPFxD or ubiquitin binding also reduce membrane recruitment of Fragments 3. Since cargo has been shown to be internalized by either NPFxD expression or monoubiquitin modification, we interpret the diffuse SH3-3-SHD1 localization on the plasma membrane to be a result of binding cargo with an exposed NPFxD sequence and sites of monoubiquitination.

Lastly, our work demonstrates that disrupting proper Sla1 recruitment to endocytic sites results in its nuclear localization. It is becoming increasingly evident that certain components of the endocytic machinery serve a nuclear function [73]. A role for Sla1 in nuclear localization, however, remains to be demonstrated. One publication does seem to suggest that regulation of

Sla1 function and phosphorylation occurs in the nucleus, as it additionally occurs during endocytosis [68]. Our work suggest that the nuclear localization signal of Sla1 likely resides in the SH3-3 domain as this portion was shown to localize to the nucleus and contains a potential NLS, while the SHD1 domain of similar size did not. Additionally we have performed mutagenesis of one potential nuclear localization signal located upstream of the SH3-3 domain that showed no defect in nuclear localization of Sla1 fragments (data not shown).

These results indicate that Sla1 recruitment to the plasma membrane is dependent upon its binding with endocytic-cargo sorting signals. Considering the internalization of integral plasma membrane protein is one of the core function of the process it is logical that protein cargo would be a driving factor in recruitment of the endocytic machinery. Our results suggest that this may be a typical mechanism utilized by endocytic cargo-adaptor proteins for their active recruitment. Furthermore, our work suggests that Sla1 may be the first identified protein capable of binding cargo sorting motifs as well as ubiquitinated cargo, and that this function is regulated. It would be of significant interest to not only further investigate this process, but to also apply it to other endocytic machinery components, not only in yeast, but other organisms as well.

3.5 Experimental procedures

3.5.1 Plasmids

Plasmids encoding SLA1 and various truncations and mutations were generated using traditional PCR amplification techniques and In-Fusion cloning. Vector PCR fragments were generated from a pGBT9 plasmid that contained a deletion of the GAL4 DNA binding domain. Insert fragments of varying SLA1 regions and GFP were additionally PCR amplified with 15bp 5’ and 3’ DNA sequences homologues to regions of the 3’ and 5’ ends of the vector respectively.

SLA1 fragments were then inserted into the modified pGBT9 vector using In-Fusion cloning. Plasmid SDP904 (pGBT9 ΔGal4BD a.a.495-560 Frag 5 SHD1-GFP) was generated by In-Fusion Cloning of SLA1 fragment region a.a. 495-560 into the modified pGBT9 ΔGAL4BD vector, and sub sequential infusion cloning of the GFP sequence onto the tail end of the Sla1 Fragment. SDP978 (pGBT9 a.a.350-420 Frag 4 SH3-3-GFP) was generated by In-Fusion cloning replacement of the SLA1 residues in SDP904 with SLA1 residues 350-420. SDP962 (pGBT9 a.a. 350-560 Frag 3 SH3-3-SHD1-GFP) was generated by In-Fusion cloning replacement of the SLA1 residues in SDP904 with SLA1 residues 350-560. SDP963 (pGBT9 a.a.120-560 Frag 2 Undefined-SHD1-GFP) was generated by In-Fusion cloning replacement of the SLA1 residues in SDP904 with SLA1 residues 120-560. SDP964 (pGBT9 a.a.1-560 Frag 1 SH3(1/2)-SHD1-GFP) was generated by In-Fusion cloning replacement of the SLA1 residues in SDP904 with SLA1 residues 1-560. SDP965 (pGBT9 Full Length Sla1-GFP) was generated by In-Fusion cloning replacement of the SLA1 residues in SDP904 with full length SLA1. SDP979 (pGBT9 a.a.350-560 SH3-3^{W391A}-SHD1-GFP) was generated by point mutation of a.a.W391A using In-Fusion cloning. SDP997 (pGBT9 a.a.350-560 SH3-3-SHD1-NPFGF-GFP) was generated by In-Fusion cloning and attaching SLA1 residues 1214-1244 to c-terminal SLA1 residues in SDP962. SDP1054 (pGBT9 a.a.350-560 SH3-3-SHD1^{I531E}-GFP) was generated by point mutation of a.a.I531E using In-Fusion cloning. Plasmid SDP839 (Nup133-3xmCherry::URA3) was donated from the Steven Markus Lab.

3.5.2 Yeast strains

SDY063 (MATα ura3-52, leu2,112 his3-Δ200,trp1Δ901, lys2-801, suc2-Δ9 GAL-MEL SLA1-GFP::TRP1) cells used as a wild type control for microscopy imaging were generated as

previously described by our lab. The SHD1 mutations were introduced into the endogenous SLA1 gene in GPY1805 cells as previously described for fluorescent microscopy imaging in Figures 3.1 and 3.2. Standard methods were utilized to generate SDY542 (MATa ura3-52, leu2-3,112 his3-Δ200,trp1-Δ901, lys2-801, suc2-Δ9 GAL-MEL, Ste2::Leu, sla1^{I531E}-GFP::TRP). Standard methods were utilized to generate SDY545 (MATa ura3-52, leu2-3,112 his3-Δ200,trp1-Δ901, lys2-801, suc2-Δ9 GAL-MEL, Ste2::Leu, sla1^{F507L}-GFP::TRP). Standard methods were utilized to generate SDY546 (MATa ura3-52, leu2-3,112 his3-Δ200,trp1-Δ901, lys2-801, suc2-Δ9 GAL-MEL, Ste2::Leu, sla1^{K525A}-GFP::TRP).

Standard methods were utilized to generate SDY712 (MATα ura3-52, leu2-3,112 his3-Δ200,trp1-Δ901, lys2-801, suc2-Δ9 GAL-MEL, Ste2::Leu, sla1ΔSR-GFP::TRP). Standard methods were used to generate SDY718 (MATα ura3-52, leu2-3,112 his3-Δ200,trp1-Δ901, lys2-801, suc2-Δ9 GAL-MEL, Ste2::Leu, sla1^{I531E}ΔSR-GFP::TRP). Standard methods were utilized to generate SDY736 (MATα ura3-52, leu2-3,112 his3-Δ200,trp1-Δ901, lys2-801, suc2-Δ9 GAL-MEL, Ste2::Leu, sla1^{F507L}ΔSR-GFP::TRP). Standard methods were utilized to generate SDY738 (MATα ura3-52, leu2-3,112 his3-Δ200,trp1-Δ901, lys2-801, suc2-Δ9 GAL-MEL, Ste2::Leu, sla1^{K525A}ΔSR-GFP::TRP).

Yeast strains expressing SLA1-GFP in wild type and SHD1 mutant cells were used to generate strains also expressing Nup133-3xmCherry::URA3. Plasmid SDB839 was digested with BamHI restriction enzyme and introduced by lithium acetate transformation into yeast strains SDY063, SDY542, SDY545, SDY546 to generate yeast strains SDY704 (MATα ura3-52, leu2,112 his3-Δ200,trp1Δ901, lys2-801, suc2-Δ9 GAL-MEL SLA1-GFP::TRP1, Nup133-3xmCherry::URA3), SDY705 (MATα ura3-52, leu2,112 his3-Δ200,trp1Δ901, lys2-801, suc2-Δ9 GAL-MEL sla1^{I531E}-GFP::TRP1, Nup133-3xmCherry::URA3), SDY706 (MATα ura3-52,

leu2,112 his3-Δ200,trp1Δ901, lys2-801, suc2-Δ9 GAL-MEL sla1^{F507L}-GFP::TRP1, Nup133-3xmCherry::URA3), SDY707 (MATα ura3-52, leu2,112 his3-Δ200,trp1Δ901, lys2-801, suc2-Δ9 GAL-MEL sla1^{K525A}-GFP::TRP1, Nup133-3xmCherry::URA3).

Standard methods were utilized for generating SDY1181 (MATα ura3-52, leu2,112 his3-Δ200,trp1Δ901, lys2-801, suc2-Δ9 GAL-MEL, Nup133-3xmCherry::URA3). Subsequently, standard methods of yeast transformation were performed on strain SDY1181 using one of either plasmid SDP904, SDP978, SDP962, SDP963, or SDP964. This generated yeast strains SDY1183 (MATα ura3-52, leu2,112 his3-Δ200,trp1Δ901, lys2-801, suc2-Δ9 GAL-MEL, Nup133-3xmCherry::URA3, SDP904), SDY1187 ((MATα ura3-52, leu2,112 his3-Δ200,trp1Δ901, lys2-801, suc2-Δ9 GAL-MEL, Nup133-3xmCherry::URA3, SDP978), SDY1184 (MATα ura3-52, leu2,112 his3-Δ200,trp1Δ901, lys2-801, suc2-Δ9 GAL-MEL, Nup133-3xmCherry::URA3, SDP962), SDY1185 (MATα ura3-52, leu2,112 his3-Δ200,trp1Δ901, lys2-801, suc2-Δ9 GAL-MEL, Nup133-3xmCherry::URA3, SDP963), SDY1186 (MATα ura3-52, leu2,112 his3-Δ200,trp1Δ901, lys2-801, suc2-Δ9 GAL-MEL, Nup133-3xmCherry::URA3, SDP964)

3.5.3 Fluorescent microscopy

Fluorescent microscopy imaging was performed using an Olympus America Inc., Melvile, NY. IX81 spinning disk confocal microscope as described. Cells were grown to early log phase and imaged at room temperature. Imaging software Slidebook6 (3I, Denver, CO) as used for capturing and analysis of images. Peak patch fluorescence/cytosol fluorescence intensities were measured by drawing a mask on endocytic sites and internal regions when peak patch fluorescence intensity was reached, and then normalized to that of the background. Total

membrane fluorescence/cytosol fluorescence intensities were measured by drawing a mask around the plasma membrane and internal regions, and then normalized to that of the background. Nuclear/Cytosol ratio fluorescence intensities were measured by drawing a mask in the region encompassed by Nup133-3xmCherry for measuring nuclear intensities, and then the area outside that region for cytosol intensities, and then normalizing to the background and taking the ratio of the two. Statistical significance between wild type and Sla1 SHD1 mutant cells, or between full length Sla1 and Sla1 fragments, was determined using an unpaired student's t-test (Graphpad Software Inc. La Jolla, CA) to determine the SEM and *P* values.

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CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS FOR THE ROLE OF SLA1 IN CLATHRIN-MEDIATED ENDOCYTOSIS

4.1 Summary

While numerous studies in eukaryotes have established a variety of molecules and dynamic mechanisms involved in clathrin-mediated endocytosis (CME), the process remains incompletely understood. Various screens indicate a number of unknown endocytic proteins may still exist, and the functions of many established endocytic proteins remains to be fully characterized. The mechanism of CME follows the typical progression by which endocytic sites are initiated and the growth of a protein coat eventually leads to induced actin polymerization, membrane bending, and finally scission of an endocytic vesicle from the plasma membrane. Studies in *Saccharomyces cerevisiae* have presented society not only with a greater understanding of CME in humans, but also a greater understanding of how biological processes of essential function can be conserved across distant species. While CME is defined by the formation of a clathrin coat at the plasma membrane, it is equally as important to recognize the essential role clathrin adaptor proteins play in the process. In yeast, the role of the adaptor Sla1 in clathrin-mediated endocytosis appears to follow that of typical monomeric clathrin associated sorting proteins (CLASPS) [1]. This includes the ability of Sla1 to interact with components of the endocytic machinery such as clathrin, scaffolding proteins, actin regulatory machinery, and integral membrane proteins. Here we have uncovered novel roles for the Sla1-clathrin and Sla1-

cargo interactions that not only demonstrate newly identified functions for Sla1, but also point to what appear to be conserved mechanisms for CLASP protein interactions.

Sla1 has been shown to bind clathrin through a conserved vCB motif, which may potentially be regulated through its SHD2 domain in endocytosis [2]. Binding of Sla1 to clathrin was additionally demonstrated to be important for normal Sla1 dynamics, as well as the proper internalization of endocytic cargo [2]. While insightful, these results did not address the role Sla1-clathrin interactions play in overall endocytic site dynamics. Mutation of the Sla1 vCB motif appears to cause a greater defect in yeast CME than mutation of any other single, or even multiple clathrin adaptor proteins, suggesting a substantially important function for this interaction [2-4]. Our work presented here demonstrated that Sla1 contributes to proper clathrin coat formation at mid/late stages of endocytosis. We also demonstrated that this interaction is necessary for proper recruitment of the endocytic machinery and transition to later stages of endocytosis such as actin polymerization and vesicle scission. This suggests that clathrin coat formation is an important precursor for transitioning to late stage endocytic processes. This interaction also seems to be important for normal vesicle formation and membrane invagination at endocytic sites. It also demonstrates what we and other researchers are seeing as an important property post-clathrin arrival of clathrin-adaptor proteins play in progression of endocytosis. Considering clathrin is an early arriving component of the endocytic machinery it is becoming increasingly interesting to find that late arriving adaptor proteins orchestrate clathrin coat formation and remodeling.

The internalization of integral membrane protein cargo is a basic and fundamental function of CME [5, 6]. It is thus reasonable to suggest that endocytic cargo is a fundamental component of endocytic sites, and serves important functions in the progression of endocytosis.

While previous studies have demonstrated this may in fact be the case, direct evidence for cargo serving a specific function in endocytosis is lacking. As a result, the textbook description assigns cargo a passive role in the endocytic mechanism. Cargo is primarily targeted for CME through the presence of an internalization sorting motif or the post-translational mono-ubiquitination of lysine residues [7, 8]. Sla1 has previously been shown to bind endocytic cargo with cytosolic exposed NPFxD motifs [9, 10]. Sla1 binds NPFxD through a hydrophobic pocket located in its SHD1 domain, and this interaction has been shown to be important for the internalization of endocytic cargo [10]. Additionally, the Sla1 SH3-3 domain has been shown to bind monoubiquitin *in vitro* [11]. A role for ubiquitin binding by Sla1, however, has yet to be demonstrated. A role for cargo binding in Sla1 recruitment to endocytic sites has also not been addressed. Our work indicates NPFxD-cargo contributes to Sla1 recruitment to endocytic sites, thus providing evidence of an active role for cargo in endocytosis. These results change the more traditional view of endocytosis, in which endocytic adaptors are recruited to endocytic sites first and then contribute to cargo recruitment. Furthermore, a minimal fragment of Sla1 containing just the SH3-3 and SHD1 domains fused to GFP decorated the plasma membrane in a uniform fashion. This membrane distribution is reflective of what has been seen for various endocytic cargos that have been shown to be targeted for endocytosis by NPFxD related motifs, monoubiquitination, or both. Point mutations in SHD1 and SH3-3 domains shown to disrupt NPFxD or ubiquitin binding respectively reduced recruitment of this fragment to the plasma membrane. We also demonstrated that the Sla1 repeat (SR) region contributes to Sla1 recruitment specifically to endocytic sites, in combination with cargo binding. The properties of the SR region also appear to perform a regulatory function that prevents full length Sla1 from being recruited diffusely across the plasma membrane through cargo binding interactions.

Lastly, our work supports previous publications for the nuclear localization of Sla1. Various studies have demonstrated that components of the endocytic machinery are targeted to the nucleus in various conditions, cell lines, and stages of the cell cycle [12]. In our work we demonstrated Sla1 localized to the nucleus upon disruption of the Sla1-NPFxD interaction. Furthermore, we demonstrate that a nuclear localization signal likely resides in the SH3-3 domain, as Sla1-GFP fragments where shown to localize to the nucleus only when the SH3-3 domain was present.

While our findings contribute to clarifying the role clathrin-adaptors and cargo play in progression of endocytosis, a number of questions still remain. The first of which is, how exactly do clathrin-adaptors help orchestrate the clathrin coat in both time and space? Our work suggests that a proper clathrin coat is necessary for transitioning to later stages of clathrin-mediated endocytosis. If that is the case, what are the molecular interactions that facilitate this transition? In terms of the Sla1-cargo interaction, it is likely that other unidentified NPFxD-containing Sla1-targeted cargos exist and remain undefined. Furthermore, undetermined endocytic sorting signals may remain undiscovered. It is also unclear as to when cargo is recruited to endocytic sites, especially with respect to cargo that is dependent upon linear sorting motifs such as NPFxD. These and many other questions will continue to be addressed by further analysis of the known endocytic machinery and cargo, as well as by performing screens that can identify any missing components involved in the process [13].

4.2 A novel role for Sla1 in clathrin recruitment and progression of endocytosis

4.2.1 Previously identified functions for adaptors in clathrin coat formation

Studies with clathrin and adaptor proteins have demonstrated that clathrin has no established membrane binding domain and requires adaptor proteins for its recruitment to endocytic sites. The first endocytic protein to be characterized for its clathrin binding property was the mammalian AP2 adaptor [14-16]. Subsequent studies identified a variety of clathrin-adaptor proteins that have since been shown to function in clathrin binding, coat formation, and linking clathrin to other components of the endocytic machinery [17].

In mammalian cells, AP2 is believed to function in the recruitment of the first few molecules of clathrin to endocytic sites [18]. This early recruitment of clathrin seems to initially generate a flat clathrin sheet composed primarily of hexagons. Upon induction of membrane bending, however, the clathrin coat begins to bend and take a conformational pattern that now consists of a higher population of pentagons [19, 20]. It has been proposed that this conformational change requires disassembly and recruitment of individual clathrin triskelia at sites of endocytosis [20-22]. Furthermore, FRAP experiments indicate that a dynamic exchange of clathrin subunits occurs at endocytic sites, as does AP2, likely reflecting the exchange with cytosolic proteins that has been predicted [23, 24]. Other clathrin adaptors have additionally been shown to contribute to clathrin recruitment. Their arrival after initial clathrin coat formation, however, indicates that they may contribute to aspect of coat formation including stabilization of the coat, organization of the coat, and later events of clathrin recruitment. It would be interesting to see if clathrin exchange is enhanced or reduced in various clathrin-adaptor knock out (KO) or knock down (KD) cells. It would also be interesting to determine if disrupting clathrin binding by these adaptors also disrupts the geometry of the vesicle protein coat and size of endocytic

vesicles. Electron microscopy imaging and quantitative analysis of endocytic vesicles in yeast and mammalian cells after adaptor KD or KO could answer this question. Additionally other clathrin binding proteins do not appear to serve a role in clathrin recruitment and coat formation, but function to link clathrin to other components of the endocytic machinery. Identification of which adaptors contribute to coat formation and which contribute to other aspects of endocytosis is an additional area of research that requires further investigation.

In mammals clathrin adaptors other than AP2 have been shown to arrive to endocytic sites after AP2, and similarly contribute to clathrin coat formation [25]. This includes later arriving Epsin, AP180, and CALM proteins that all contain clathrin binding motifs and are capable of inducing clathrin coat formation, at least in *vitro* [26, 27]. Interestingly it has been proposed that Epsin functions in intermediate stages of endocytosis and clathrin coat formation and that this may be regulated through its membrane binding ENTH domain that can sense membrane curvature and contribute to recruitment [28]. In yeast it is not known which protein is responsible for recruitment of the first few molecules of clathrin to endocytic sites. Adaptors Ent1/2 and Yap1801/2 proteins arrive to endocytic sites at different stages of endocytosis, after clathrin recruitment, and contribute to clathrin coat formation [29, 30]. However, it has not been shown as to whether any of these endocytic proteins have unique functions for organizing the geometry of the clathrin coat, or have roles in late stage clathrin recruitment vs stabilization. It would be interesting for researchers to identify whether these proteins have specific organizational properties that help position individual clathrin triskelion in a way that attenuates the changing shape of the membrane invagination.

4.2.2 Sla1 contributes to late stage clathrin recruitment and progression of endocytosis

By investigating the functional role of Sla1-clathrin binding we have been able to demonstrate that Sla1 contributes to clathrin recruitment at intermediate/late stages of endocytosis. Additionally, it appears that proper Sla1-clathrin binding also contribute to normal recruitment of Sla1 and its interacting partner Pan1. Disrupting the Sla1-clathrin interaction and proper coat formation also stalled progression of endocytosis, the activation of actin polymerization, and vesicle scission. The delay in actin polymerization does not appear to be a result of disrupting recruitment of the WASP/Myo module responsible of activation of actin polymerization. Previous reports indicate that Las17, Vrp1, and either Myo3 or Myo5 are the minimal components necessary for activation of actin polymerization [31]. In our work, however, Las17 and Vrp1 appear to be recruited to endocytic sites with normal timing relative to Sla1, with increased peak levels and patch lifetimes. Myo5 was shown to be recruited to endocytic sites and maintained a low site level until actin polymerization occurred, at which point Myo5 recruitment was enhanced to levels above those in wild type cells. This poses us with a potentially novel concept in which generation of a well formed clathrin coat, or simply proper Sla1-clathrin binding, is needed before the endocytic site can initiate actin polymerization. Future investigation into why this is the case in yeast, and potentially other organisms, requires further investigation.

It would be interesting to test if the Sla1-clathrin interaction somehow contributes to the release of Las17 actin nucleation promoting factor (actin-NPF) activity. Pyrene actin polymerization experiments indicate that endocytic proteins Bzz1 and Sec4 relieve Las17 inhibition by disrupting the Sla1-Las17 interaction [32-34]. Gold particle immuno-EM experiments that looked at different components of the endocytic machinery indicate that the

inhibitory effects of Sla1 on Las17 actin-NPF activity may additionally be relieved by their physical separation, as Sla1 and Las17 were shown to have unique localizations along endocytic invaginations during internalization [35, 36]. Our work demonstrates that the mere presence of Las17, Vrp1, Myo3/5, Bzz1, and the Pan1 actin-NPF is not enough to trigger actin polymerization at endocytic sites. There appears to be specific interactions among the actin nucleation promoting factors that are disrupted in cells in which proper Sla1-clathrin binding does not occur. Interestingly, the Myo3/5 proteins have traditionally been shown to arrive to endocytic sites at the onset of actin polymerization, and its recruitment is correlated with the presence of actin filaments [34]. Our results suggest that low levels of Myo3/5 can be recruited to endocytic sites before actin polymerization and this recruitment is heavily increased upon actin polymerization.

While eukaryotes appear to perform a generally conserved mechanism of CME, distinct differences clearly exist between species. Cultured mammalian cells are capable of performing CME in the absence of actin polymerization, while in yeast actin polymerization is required for vesicle internalization [37-39]. In mammalian cells membrane bending appears to not only occur before actin polymerization, but interestingly may contribute to stimulation of actin polymerization [40-42]. While in yeast it appears that membrane bending and invagination does not occur until after actin polymerization, work in other labs seems to indicate that membrane bending can occur at endocytic sites when actin polymerization is disrupted [22, 35]. This raises the question of just how many components of the endocytic coat, such as clathrin and adaptors, contribute to membrane bending and deformation of the plasma membrane. Our work shows that an increase in the frequency of longer membrane invaginations is a result of disrupting the Sla1-clathrin interaction. This suggests that clathrin coat formation may in fact contribute to proper

shaping of the endocytic invagination and likely contributes to membrane bending in the presence of actin polymerization. This result is consistent with observations seen in *Δsla1Δbbc1* cells that produce extremely long membrane invagination profiles labeled by FM-464 [43].

4.2.3 The Sla1 SHD2 domain may regulate the Sla1-clathrin interaction

Previously our lab demonstrated that the Sla1 SHD2 domain was in fact a sterile alpha motif or SAM domain [2]. Pulldown experiments demonstrated that the SHD2 domain was not only capable of binding the Sla1 variable clathrin box (vCB), but also competed for vCB binding by clathrin. Additionally the SHD2 domain, like other SAM domains, appears to facilitate oligomerization of Sla1 molecules [2, 44]. In combination with our recently published work, these findings would suggest that SHD2 oligomerization may regulate Sla1-clathrin binding and clathrin recruitment to endocytic sites. The idea being that Sla1 remains in a clathrin-binding auto-inhibited state by the SHD2 domain, and dimerization of the SHD2 domain at endocytic sites results in release of the vCB, clathrin binding and recruitment. This mechanism, however, is questionable as mutation of the SHD2 domain that prevents oligomerization does not enhance the lifetime of Sla1, as the Sla1 vCB mutant does, but instead shortened the patch lifetime of Sla1 [2]. It is clear the role of the Sla1 SHD2 domain in endocytosis requires further investigation. We additionally suggest that the SHD2 domain could be involved in coat disassembly as it could compete off the interaction between Sla1 and clathrin.

4.3 The Sla1-NPFxD interaction demonstrates a role for cargo in adaptor recruitment

4.3.1 Endocytic cargo sorting signals function in clathrin-mediated endocytosis

The study of endocytic cargo sorting signals and endocytic receptors are important aspects for researchers studying a variety of genetic and infectious diseases such as those related to heart disease, dementia, and viral entry [45-49]. The first endocytic cargo sorting signal described was the mammalian NPxY motif located on the LDL receptor, and has since been shown to be present in other endocytic cargos [50]. Interestingly, the first cargo sorting signal motif recognized in yeast was the NPFxD sequence, which shares both similar and unique properties [51]. Both contain an asparagine and proline residue in the 1^{st} and 2^{nd} position, followed in the 3^{rd} or 4^{th} position respectively by an important hydrophobic and aromatic residue. Additionally, while the aspartic acid of the NPFxD sequence does enhance Sla1 SHD1 domain recognition, it is significantly less important for receptor internalization and SHD1 binding than the asparagine, proline or phenylalanine residues [10, 51]. Interestingly, a phenylalanine NPxF motif can function in cargo recognition by the same endocytic machinery as NPxY [52]. While similar, these sequences appear to have unique properties as well. For instance the Tyrosine residue on NPxY motif can be phosphorylated, adding an additional form of regulation that is applied to certain cargo proteins [7]. The NPxY motif is also recognized by the structurally different PTB domain, while the NPFxD sequence is recognized by the Sla1 SHD1 domain [10, 52]. It would be a significant scientific discovery to determine if these sequences are truly related and how they may have evolved recognition by their associated adaptors.

While the “dileucine signal” [DE]xxxL[LI] has only been shown to function in mammalian cargo recognition, related versions of the Yxxϕ signal may function in clathrin-mediated endocytosis in yeast [7, 53]. The Yxxϕ motif is recognized by the mammalian AP2

clathrin adaptor. In yeast deletion of the AP2 subunit was shown to disrupt normal trafficking of the Mid2 receptor [53]. Mid2 endocytosis has additionally been shown to depend upon its C-terminal domain, which also contains a single Yxxϕ motif [54]. Mutation of this motif, however, failed to significantly disrupt Mid2 internalization, and mutation of the theoretical AP2 cargo binding region did little to disrupt endocytosis. Interestingly, the AP2 domain was shown to bind the Mid2 tail *in vitro* [53]. Additionally, Mid2 endocytosis was shown to be heavily dependent upon a C-terminal DxY motif and the adaptor Syp1 μ-homology domain [55]. Again, however, Mid2 endocytosis is not completely dependent upon interactions with Syp1, and it appears that Mid2 endocytosis and the role of endocytic sorting signals Yxxφ and DxY in yeast is incompletely understood. It would be interesting to further determine what other properties of the Mid2 tail contribute to its endocytosis, and what factors it interacts with. Additionally, it would be interesting to determine if the DxY motif can function in mammalian endocytosis or if the [DE]xxxL[LI] sequence functions in yeast endocytosis. All of these findings seem to indicate that potentially novel sorting signals may still exist in addition to variants of the established motifs. The fact that early arriving components of the endocytic machinery such as Syp1 and its mammalian homologs FCHo1/2 appear to be important for targeting cargo for clathrin-mediated endocytosis raises two additional question [53, 56]. To begin, Syp1 and FCHo1/2 appear to disassociate from the endocytic site before internalization, begging the question of how certain endocytic cargo would stay associated with endocytic sites after Syp1 and FCHo1/2 departure [57-59]. A second question is, does the accumulation of specific cargo occur at different stages of endocytosis as does their targeting adaptors? Perhaps through the combined use of fluorescent microscopy and immuno-EM techniques one could elucidate the recruitment times of endocytic adaptors and their targeted cargo proteins.

In addition to protein sorting motifs, monoubiquitination serves a function in targeting cargo for endocytosis [60]. While various cargos have been shown to be monoubiquitinated on multiple lysine residues, a single molecule of ubiquitin has been shown to be sufficient for receptor-mediated endocytosis [60, 61]. Currently, it appears that the endocytic adaptors responsible for targeting ubiquitinated cargo for endocytosis are distinct from the adaptors responsible for targeting endocytic cargo sorting signals, hinting at what may be a unique function for ubiquitin-mediated endocytosis [7]. Studies have demonstrated that rapid receptor ubiquitination and degradation occurs post ligand binding [7, 62-64]. Furthermore, a mechanism by which improperly folded plasma membrane proteins are targeted for removal and degradation by the vacuole was recently identified in yeast [65]. This mechanism required a variety of (arrestin-related trafficking adaptors) ART proteins that were specific for targeting specific misfolded membrane proteins and directing the ubiquitin ligase Rsp5 for receptor ubiquitination [65]. While current results suggest that ubiquitinated cargo is targeted by endocytic machinery proteins Ede1 (Eps15) and Ent1/2 (Epsin), a direct interaction between cargo and these proteins has yet to be demonstrated [60, 66]. Current results however, also indicate that Ede1 and Ent1/2 may mediate interactions with other components of the endocytic machinery, and additional ubiquitin binding adaptors likely exist [67].

Considering the third SH3 domain of Sla1 and its mammalian homologue CIN85 are able to bind ubiquitin *in vitro* it would be interesting to determine if they are involved in binding ubiquitinated cargo [68]. Our chapter 3 results indicate that Sla1 may in fact bind ubiquitinated cargo through its SH3-3 domain. Receptors Ste2 and Ste3 have been shown to not only contain what appears to be a modified, yet functional version of the NPFxD sequence, but they are additionally ubiquitinated and targeted for endocytosis following ligand binding [9, 51, 69].

Considering sites of ubiquitination in these proteins are located in close proximity to their NPFxD motifs in their cytoplasmic tails, and the Sla1 SHD1 and SH3-3 domains are located next to each other, a mechanism by which the interaction between Sla1 and cargo is increased or decreased based on receptor ubiquitination is conceivable. Demonstrating this would be the first example of an endocytic protein that binds both ubiquitin and protein sorting motifs of endocytic cargo, either individually or simultaneously.

In clathrin-mediated endocytosis it is not clear as to how endocytic sites are initiated and when membrane cargo is recruited to these locations? Fluorescent microscopy imaging of alexa488-alpha factor in yeast has demonstrated that the ubiquitinated alpha factor receptor Ste2 is recruited to endocytic sites after Ede1-GFP, one of the early arriving endocytic proteins and potential ubiquitinated-cargo adaptor [70]. This suggests that ubiquitinated cargo is recruited after site initiation. However, considering inducing membrane protein ubiquitination in mammalian cells enhances Eps15 localization at endocytic sites, one would think cargo is a driving factor in initiation of endocytic site formation [71]. These results, however, do not indicate when non-ubiquitinated cargo is loaded at endocytic sites. Our results demonstrate that NPFxD cargo binding contributes to Sla1 recruitment to endocytic sites, but we have not addressed whether NPFxD cargo such as Wsc1 is recruited to endocytic sites before or after Sla1 arrival [72]. If Wsc1 and similar NPFxD containing cargo were loaded to endocytic sites before Sla1 arrival, a new question would then be how this cargo is initially loaded into endocytic sites considering its internalization is Sla1 dependent. If Sla1 were to arrive before Wsc1, it would indicate that NPFxD dependent cargo is completely dependent upon Sla1 binding for localization to endocytic sites. It is also interesting that endocytic cargo is targeted by different components of the endocytic machinery that associates with endocytic sites at different stages of progression

and positions along the membrane invagination [35, 73]. Does this mean that cargo is spatially positioned at different locations in the endocytic vesicle? Considering Sla1 and Syp1 display unique localization patterns on membrane invaginations, one might expect endocytic cargo to be localized regionally in the endocytic vesicles. It was demonstrated that Sla1 forms a unique ring, vs a spherical coat around the plasma membrane of an internalizing vesicle before actin polymerization occurs [74]. This could hypothetically mean that NPFxD cargo is also distributed in a ring like fashion around the internalizing vesicle. Secondly, could these findings mean that ubiquitinated cargo is also distributed to a defined region in the endocytic vesicle? Ede1 and Eps15, like Syp1 and FCHo1/2, seem to depart from endocytic sites before vesicle scission, and we thus ask what this would mean for the fate of ubiquitinated cargo targeted by Ede1 [25, 58]. Does an Ede1/Syp1 handoff occur with other components of the endocytic machinery that internalize with the vesicle after scission?

Posttranslational lysine monoubiquitination and cytosolic exposed membrane protein motifs both function as cargo sorting signals. While both function in targeting cargo for clathrin-mediated endocytosis, they appear to be distinguishable not only by the endocytic machinery that targets them, but also their role in constitutive vs. ligand-induced targeting [6, 75]. Various kinds of membrane cargo are targeted for endocytosis, including signaling proteins, receptors, and permeases [73, 76, 77]. Considering that many of these proteins have different endocytic sorting signals, it would seem reasonable to argue that endocytic sights of varying cargo composition would also result in variations in the endocytic machinery and in turn the size of the endocytic vesicle. It would be interesting to determine if the clathrin coat structure, and hence vesicle size, additionally change in response to the levels of integral membrane cargo. Overexpression of NPxY cargo in mammalian cells resulted in enhanced clathrin-adaptor recruitment, and enhanced

clathrin recruitment, however vesicle size was not measured [78]. Electron microscopy imaging could potentially be used to measure vesicle size post scission, in relation to varying levels of integral membrane protein cargo, possibly through inducing cargo ubiquitination. Additionally, one could measure recruitment of potential cargo adaptors such as Sla1 under these conditions and determine if enhanced levels of cargo also contribute to enhanced adaptor recruitment.

4.3.2 A role for Sla1 nuclear localization?

A role for the nuclear localization of various endocytic proteins is quickly becoming more evident among researchers, even though the functional meaning is unclear. Fragments of Sla1 have previously been shown localize to the nucleus, and results from this work suggest that Sla1 is targeted to the nucleus for phosphoregulation [79]. Our work demonstrates that when Sla1 is no longer properly associated with endocytic sites through NPFxD-cargo binding it is targeted to the nucleus. While wild type Sla1 has not been shown to localize to the nucleus, our results are the best to date that support this occurrence because they were obtained with full length Sla1 only containing a point mutation in the NPFxD binding SHD1 domain. It would be interesting to determine if certain cellular treatments or conditions can trigger nuclear localization, as has been demonstrate for other endocytic proteins. Additionally, the nuclear localization of other endocytic proteins have been shown to serve a particular function associated with other cellular processes [12]. It is thus possible that Sla1 nuclear localization serves a currently unknown function. It is interesting that nuclear localization is significantly enhanced upon disruption of cargo binding. Is this localization additionally altered based upon cargo presentation on the plasma membrane? Identifying a triggering mechanism for Sla1 nuclear

localization would greatly enhance our understanding of Sla1 trafficking, and additionally may lead to a functional understanding for Sla1 nuclear localization.

4.3.3 A disease-correlated purpose for understanding receptor-mediated endocytosis in yeast

One of the most beneficial results of scientific research has been the significant enhancement in the quality of human life. It is for this reason that we often correlate the importance of our work with the potential it holds for the treatment of disease. While studies of clathrin-mediated endocytosis in mammalian cells have demonstrated a link with disease progression, research in yeast has resulted in the discovery of various CME machinery components and cargo proteins that have then lead to the identification of homologues mammalian proteins, while also bringing significant clarity to their function. It was discovered early on in the field of receptor-mediated endocytosis that patients with familial hypercholesterolemia contained mutations in the LDL receptor [80]. These mutations where generally found to be present in the NPxY motif required for LDL receptor endocytosis and regulation of intracellular and blood cholesterol levels [81]. Targeting of the LDL receptor to CME is performed through binding of the NPxY motif by the clathrin-adaptor proteins ARH and Dab2, mutation or deletion of which can disrupt LDL endocytosis [82-84]. Interestingly, the yeast Sla1 clathrin adaptor has been shown to bind the sequence similar NPFxD motif. Our work has demonstrated that Sla1 is actively recruited to endocytic sites through binding of its SHD1 domain to NPFxD containing cargo. These results support findings that suggest ARH and Dab2 binding of NPxY motifs regulate their recruitment [78]. It would appear that this cargo-induced recruitment of CLASP proteins is conserved across species, and it would be interesting to determine if other properties of Sla1 are additionally conserved in different yeast and

mammalian adaptor proteins. For example, if further investigation of Sla1 identified an intramolecular mechanism of regulating cargo binding, as our work suggests, it would be interesting to determine if this exists in ARH, Dab2, or other CLASP proteins. Studies involving the LDL receptor and ARH have led to the discovery of cholesterol regulating drugs, such as statins, that help to reduce blood cholesterol levels, plaque formation, and incidence of heart attack or stroke [81]. Additionally, investigation of their function has led to an increased understanding of receptor-mediated endocytosis. It would thus seem reasonable to assume that investigation of all CLASP proteins, such as Sla1, could result in better treatment of CME related diseases.

A hallmark in the pathology of Alzheimer's disease is the formation of extracellular neuronal plaques of amyloid-β (Aβ) [85, 86]. The accumulation and aggregation of Aβ requires the proper trafficking and processing of the amyloid-precursor protein (APP) [86]. This APP precursor is an integral membrane protein that serves a significant role in neuronal development and function [87, 88]. The conversion of APP to the toxic Aβ product is performed through APP cleavage by β-secretase and γ-secretase [89]. This processing event requires APP trafficking to the plasma membrane and sequential internalization through clathrin-mediated endocytosis [46, 86, 90, 91]. Disruption of APP endocytosis by expression of an endocytic deficient version of APP results in significantly less Aβ production [91, 92]. Furthermore, enhanced neuronal synaptic endocytosis has been shown to result in enhanced APP endocytosis and release of Aβ [93]. This work reflected what had been seen in brain interstitial fluid measurements that showed increased levels of Aβ correlated with increasing levels of synaptic activity [94]. Just as is the case with the LDL receptor, APP is targeted for endocytosis by a related NPxY motif in its cytosolic tail, mutation of which reduces Aβ production [91, 92]. This signal can be targeted by

the CME regulator Numb1 through its PTB binding domain. Interestingly, it was shown that different isoforms Numb1 with varying lengths of its PTB domain regulated targeting of APP for lysosomal degradation vs endosomal processing to Aβ [95]. Considering that expression of Numb1 isoforms can be altered through the introduction of cellular stress, it appears that regulation of the endocytic machinery may play a role in Aβ production [46]. More recently it has been demonstrated that the endocytic clathrin-adaptor protein CALM may play a role in Alzheimer's pathology [96]. CALM has been shown to be involved in targeting γ-secretase for endocytosis and its proper trafficking to endosomes/lysosomes where it generates Aβ [97]. Additional results suggest that CALM is interacting with the γ-secretase Nct subunit via its ANTH domain, a potentially novel interaction for ANTH domains in cargo selection [97]. These and other studies have clearly established that clathrin-mediated endocytosis and Aβ production are linked. These results suggest that disrupting CME of APP or its processing factors could be a therapeutic target in the treatment of Alzheimer's disease. In an interesting turn of events, experiments using yeast have been performed to determine cellular factors that contribute to Aβ toxicity. Interestingly, endocytic proteins, Sla1, Yap1801, and INP52 where all identified as suppressors of Aβ toxicity, and have mammalian homologues that are either confirmed risk factors in Alzheimer's disease or directly interact with confirmed risk factors [98]. This work, however, seemed to suggest that the Aβ peptide actually disrupts CME, and overexpression of Sla1, Yap1801, and INP52 could rescue this effect. A role for the *C. elegans* homologs of Sla1, Yap1801, and INP52 in rescuing the toxic effects of Aβ on loss of neuron development was also shown [98]. Further work with Sla1 could thus help in the understanding of Aβ-induced cell toxicity, and how regulation of endocytosis can help prevent this affect.

The progression of many forms of cancer has additionally been correlated with alterations in clathrin-mediated endocytosis and the expression of endocytic protein machinery and cargo proteins [99, 100]. Mutations in certain cancer associated genes have been shown to regulate the plasma membrane localization of proteins involved in cell migration, invasiveness, and cancer progression [99, 100]. The signaling events controlled by some of these proteins such as receptor tyrosine kinases can be downregulated through clathrin-mediated endocytosis. Overexpression of plasma membrane signaling receptors and their endocytic deficient mutants have additionally been found to be associated with certain types of cancer. This mechanism functions to overwhelm the clathrin endocytic pathway or decrease receptor targeting, both resulting in increased plasma membrane localization and signaling. Additionally, the altered expressions of various clathrin adaptors, including Dab2 and HIP1 proteins, have been shown to be associated with various cancer types [99, 101, 102]. A general understanding of clathrin-mediated endocytosis and clathrin adaptors such as Sla1 and NPFxD-cargo could potentially help us understand the factors contributing to cancer progression.

The work presented in this dissertation has contributed to a more comprehensive understanding of how we view clathrin-mediated endocytosis. It not only appears that the clathrin coat is very dynamic at endocytic sites, but that the sequential recruitment of clathrin during different stages of endocytosis is more complex than previously appreciated. It would appear that this dynamic interaction occurs during different stages of endocytosis and that proteins like Sla1 contribute to the organization of the clathrin coat. Additionally, it is becoming increasingly clear that endocytic cargo contributes to the recruitment of Sla1 and other clathrin-adaptors to endocytic sites. Our work demonstrates that Sla1 recruitment is partially dependent on NPFxD binding by its SHD1 domain, and that binding of ubiquitinated cargo may also be a

contributing factor. It also appears that the nuclear localization of Sla1 is regulated by a mechanism related to its specific localization to endocytic sites, and that this regulation can be tailored based on interactions with endocytic cargo. Lastly, we suggest that Sla1 cargo binding is regulated to prevent diffuse membrane binding. To the best of our knowledge, this is a potentially novel mechanism not described in other monomeric clathrin-adaptor proteins. We believe that our discoveries are applicable to other systems and have generated a greater understanding of conserved mechanisms in clathrin-mediated endocytosis. It is for the reasons presented here that further investigation into the role of Sla1 and other cargo binding clathrin-adaptors would benefit the fields of endocytosis, intracellular trafficking, and fundamental eukaryotic cell biology.

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LIST OF ABBREVIATIONS

ADFH: Actin-depolymerizing factor homology domain

ANTH: AP180 N-terminal homology

AP: Adaptor protein

ARF: ADP ribosylation factor

ARH: Autosomal recessive with hypercholesterolemia

Arp2/3: Actin related proteins 2/3

BAR: Bin/Amphiphysin/Rvs

CBM: Clathrin box motif

CC: Coiled coil

CH: Calponin homology domain

CHC: Clathrin-heavy chain

CLASP: Clathrin-associated sorting proteins

CLC: Clathrin-light chain

CLR: Calponin-like repeats

CME: Clathrin-mediated endocytosis

COP: Coat protein complex

CR: Complement receptor

CRISPR: Clustered regularly interspaced short palindromic repeats

C3b: complement component 3b

DH: Dbl homology

EEA1: Early endosome antigen 1

EGF: Epidermal growth factor

EH: Eps15 homology

EM: Electron microscopy

ENTH: Epsin-N-terminal homology

ER: Endoplasmic reticulum

FH: Familial hypercholesterolemia

FRAP: Fluorescence recovery after photobleaching

GFP: Green fluorescent protein

GPCR: G-protein coupled receptor

GTP: Guanosine Triphosphate

HFD: Helical folded domain

Igs: Immunoglobulins

ITAM: immunoreceptor tyrosine-based activation motif

LDL: Low-density lipoprotein

LGM: Las17 G-actin binding motif

LR1: Long repeat 1

PH: Pleckstrin homology

PI: Phosphatidylinositol

PRD: Proline rich domain

PS: Phosphatidylserine

PTB: Phosphotyrosine-binding domain

RFP: Red fluorescent protein

RTK: Receptor tyrosine kinase

SHD1: Sla1 homology domain 1

SHD2: Sla1 homology domain 2

SH3: Src homology 3

SLAC: Sla1 and Las17 regulates actin polymerization during clathrin-mediated endocytosis

SR: Sla1 repeat

SYK: Spleen tyrosine kinase

SR: Sla1 repeat

Tfr: Transferrin receptor

THATCH: talin-HIP1/R/Sla2p actin-tethering C-terminal homology

TH1: Tail homology 1

TH2: Tail homology 2

TIRF: Total internal reflection fluorescence

UBA: Ubiquitin associated domain

UBX: Ubiquitin regulatory X

UIM: Ubiquitin interacting motif

vCB: Variable clathrin box

WASP: Wiskott-Aldrich Syndrome protein

WAVE: WASP family verprolin-homologue

WH1: WASP-homology 1 domain

WH2: WASP-homology 2 domain