REGULATION OF MEMBRANE TRAFFIC BY INTRINSIC AND EXTRINSIC MECHANISMS

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ABSTRACT

Chao-Wei Hung: Regulation of Membrane Traffic by Intrinsic and Extrinsic Mechanisms
(Under the direction of Mara Duncan)

Vesicular membrane traffic regulates many biological activities, such as cell growth, motility and the maintenance of the cell shape. One of the most important types of traffic is mediated by clathrin coated vesicles. To form a vesicle, clathrin must be recruited to the membranes where cargoes are located by clathrin adaptors. Although it is well established that clathrin adaptors are essential for the recruitment of clathrin and the initiation of traffic, not much is known about the mechanisms by which cells regulate the recruitment and activities of adaptors. Furthermore, the role of clathrin adaptors in traffic beyond simple clathrin recruitment remains largely unexplored. In this dissertation, I explored the regulation of adaptor activities and recruitment by intrinsic regulatory motifs and extrinsic energy dependent mechanisms. I revealed the motifs on adaptors Gga2 and Ent5 that are important for the temporal regulation of adaptor recruitment. I also demonstrated a multi-step, energy dependent mechanism that regulates the recruitment of adaptors in response to the energy availability. Finally, we revealed that in addition to recruiting clathrin and initiating traffic, clathrin adaptors are also involved in the late stage of traffic. These findings suggest the role of adaptors in traffic is more diverse.
This dissertation is dedicated to my family, especially my parents, sister and wife for their support and encouragement during my graduate stud
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CHAPTER 1. GENERAL INTRODUCTION

Section 1: Adaptor and Membrane Traffic

1.1 Biological Significance of Membrane Traffic

Proteins regulate many essential biological activities. However, they must be delivered to the right place at the right time in order to exercise their activities properly. One major mechanism by which cells regulate the localization and the distribution of proteins is through the process of vesicular membrane traffic. By delivering proteins to different cellular compartments, vesicular membrane traffic plays an important role in cell polarity, proliferation and motility (Reviewed in [1]). Disruption of membrane traffic may contribute to diseases such as heart attack, Alzheimer’s disease, type II diabetes and cancer [2-5].

The trans-Golgi network (TGN) and endosomes represent a major intracellular sorting station that mediates the traffic of plasma membrane proteins (Figure 1.1). For proteins to be sorted to the plasma membrane or targeted for secretion, proteins must first be delivered to TGN. Conversely, proteins or pathogens that are targeted for internalization must first be directed to endosomes. In conclusion, traffic at the TGN and endosomes allows proteins to gain entry into the cells or be targeted to other cellular compartments. Disruption of TGN and endosomal traffic can affect the distribution of proteins distribution and secretion and preventing cell from responding to extracellular signals.

TGN-endosomal traffic not only regulates the distribution of TGN and endosomal proteins, but is also responsible for the process and delivery of many plasma membrane proteins. Additionally, the TGN and endosomes can sequester plasma membrane proteins, preventing
delivery to plasma membranes. Glut4, the main glucose transporter in mammalian cells, is one of many plasma membrane proteins regulated by TGN-endosomal traffic. Under non-stimulating conditions, Glut4 is packaged into Glut4 storage vesicles (GSV) and resides in the TGN and endosomes. Glut4 is only targeted to plasma membranes when cells are stimulated by insulin [6-8]. One of the major mechanisms by which Glut4 is trapped in its intracellular compartments is a futile cycle between GSV and the TGN and/or recycling endosomes. This futile cycle prevents Glut4 translocation to plasma membranes prior insulin stimulation (Reviewed in [8]). Defects in the regulation of Glut4 transporter translocation is linked to the onset of type II insulin resistance diabetes (Reviewed in [8]). Other important plasma membrane proteins, such as Aqp2, a water channel in renal epithelium, and Gap1, a general amino acid permease found in yeast, are also cycled through the TGN-endosomes constantly. Their targeting to the plasma membrane is triggered only when stimuli are present [9, 10]. By regulating the distribution of plasma membrane proteins, the TGN and endosomes play an essential role in cellular response to environmental signals.

TGN-endosomal traffic is regulated by clathrin. The next section will discuss clathrin mediated traffic (CMT) and clathrin adaptors in detail.

1.2 Introduction to Clathrin Adaptors

CMT is one of the most important cellular traffic machineries. It is involved in endocytosis and intracellular traffic between organelles (review in[11]). To recruit and assemble clathrin into clathrin coated vesicles (CCV), cells require a group of proteins known as clathrin adaptors. In addition to clathrin recruitment, adaptors can bind to lipid membrane, recognize cargoes, recruit and induce clathrin polymerization, and recruit other factors that are essential for the maturation of coated vesicles (Reviewed in[12, 13]) (Figure 1.2). Studying the regulation of adaptors is thus necessary for us to understand the traffic mediated by CCVs.
In *Saccharomyces cerevisiae*, there are three major types of clathrin adaptors that function at the TGN and endosomes: the heterotetrameric AP-1 complex; the monomeric Golgi localized Gga proteins; and epsin family proteins, which can be divided into ANTH family protein and ENTH family protein.

**AP-1:**

The AP-1 heterotetrameric complex consists of γ, β1, μ1 and σ1 subunits. The globular core domain includes μ1 and σ1. The N terminal of γ and β1 are also part of the core domain. The appendages of AP-1 include the C terminals of γ, β1 subunits which are consisted of unstructured hinge domains that are followed by small globular domains known as γ and β1 ear. The core domain is important for AP-1 attachment to the membrane of Golgi, while the appendage regions are indispensable for AP-1 to recruit clathrin and other factors that regulate trafficking [14-16].

**Gga:**

The Gga proteins (Golgi-localized, ear containing, ADP-ribosylation factor binding), consist of a VHS, a GAT, a hinge and an ear domain, which is a homolog to the γ ear found in AP-1. Gga proteins play roles in mannose 6-phosphate trafficking, cargo recognition, ubiquitin binding, and interaction with other clathrin adaptors [16-20]. Humans have three Golgi proteins, while yeasts only have Gga1 and 2; the expression of Gga2 is substantially higher than that of Gga1 [21].

**Epsins:**

Ent3 and Ent5 are two epsin family proteins that function on yeast TGN and endosomes. Ent3 and Ent5 contain lipid binding ENTH and ANTH domains, respectively [22-24]. In addition to membrane binding, the ENTH domain of Ent3 can induce membrane curvature. Both
Ent3 and Ent5 contain multiple gamma ear binding motifs [23]. Unlike Ent5, Ent3 does not possess canonical clathrin binding motifs; however, evidence has shown that Ent3 is capable of binding to clathrin directly in vivo, although with less efficiency than Ent5 [25]. Both Ent5 and Ent3 have been shown to interact with cargo [25, 26].

Roles of Adaptors in Traffic

Clathrin adaptors form a complicated interaction network. Many clathrin adaptors share structural similarities and functional redundancies. However, evidences suggest that many adaptors have specific functions or mediate a specific route of traffic. For example, Ent3 plays a role in the Gga2 mediated trafficking pathway, while Ent5 and AP-1 mediate an alternative trafficking pathway [27]. Additionally, adaptors seem to be directional specific: AP-1 can function in traffic from TGN to endosomes and retrieving cargo from endosomes to TGN, while Gga proteins strictly regulate TGN to endosome traffic [12, 27].

In the Chapter 1 and Chapter 2 of this dissertation, I investigated the role of interactions between clathrin, Ent5 and Gga2 on traffic regulation. I discovered a dual function, autoregulatory motif on Gga2 that mediates the interaction between Gga2, Ent5 and clathrin. This novel autoregulation plays an important role in the temporal and spatial regulation of CMT.

I also explored the role Ent5 in traffic. We revealed that, unlike many clathrin adaptors, Ent5 is not involved in the initiation or recruitment of clathrin. Rather, it plays an important role during the late stage and promotes the maturation of clathrin traffic structures.

Section 2: Regulation of Membrane Traffic by Energy Metabolism

2.1 Energy Availability and Traffic Regulation during Energy Stress

Eukaryotes adopt different kinds of metabolism during different stages of development. For example, glycolysis is the preferred metabolic pathway by human pluripotent stem cells,
while their differentiated counterparts prefer energy production by pathways that involve mitochondria [28]. Metabolic remolding not only occurs in response to developmental signals, but is also triggered in response to changes in nutrients. When glucose is present, yeasts, *Saccharomyces cerevisiae*, produce ATP via glycolysis and undergo exponential growth. Additionally, mitochondrial activity is actively inhibited by cell signaling response to glucose. During glucose starvation, yeast will activate mitochondrial activities and use oxidative phosphorylation to metabolize alternative carbon sources [29].

Glucose is the preferred carbon source for many eukaryotes from yeasts to human. Additionally, glucose is the regulatory molecule to many cellular pathways. For example, translation is inhibited during glucose starvation [30]. In many cell types and organisms, glucose starvation represents a major survival challenge that cells must deal immediately. Knowing the immediate responses and the long term cellular adaptations to glucose starvation can provide insight into understanding mechanisms by which cells survive under stressful conditions.

Studying metabolic remodeling in response to the availability of nutrients and growing conditions is also beneficial for improving the yield of pharmaceutical proteins. Metabolic remodeling is a common approach to trigger the synthesis of pharmaceutical proteins. For example, to produce insulin analogue precursor (IAP), genetically modified yeast is grown in low glucose, aerobic conditions. This condition, coupled with the burden to produce a large amount of heterologous protein, forces yeast to adapt a metabolic shift. However, this metabolic shift often leads to the reduction in the secretion of fully processed IAP [46]. This finding suggests that secretion is tightly coordinated with the metabolism. Metabolic remodeling likely influences the yield of pharmaceutical proteins by affecting traffic at the TGN and endosomes. Traffic of proteins to these cellular compartments is not only necessary for their secretion but also essential for the synthesis of fully functional proteins through the correct post translational
modifications [47]. Understanding the metabolic regulation on traffic at the TGN and endosomes will improve the yield of pharmaceutical proteins.

Our lab has discovered evidence that suggests that metabolism affects cellular behavior through remodeling traffic. We have demonstrated that traffic remodeling is one of the immediate cellular response to starvation [31]. Upon an acute glucose starvation, many adaptors that function on the TGN and endosomes are mislocalized from the membranes. However, adaptors relocalize to membranes during a prolonged starvation [31]. This observation suggests that membrane traffic is highly sensitive to change of metabolism.

One possible mechanism by which glucose regulates traffic is by influencing cytosolic ATP levels. When glucose is present, glycolysis occurs efficiently, and ATP is produced. The presence of ATP activates the recruitment of adaptors and enables traffic. During acute glucose starvation, the cellular ATP reservoir is quickly depleted. The depletion of ATP inhibits the additional recruitment of adaptors and adaptors rapidly dissociate from the membranes. This process also shuts down membrane traffic. If the starvation persists, cells will attempt to restore ATP production by using oxidative phosphorylation to metabolize alternative carbon sources. This metabolic shift restores ATP production. As the consequence, adaptors will be recruited back to membranes and the traffic will resume. In summary, when ATP levels drop during acute glucose starvation, adaptors are mislocalized; when ATP levels are restored by oxidative phosphorylation during prolonged starvation, adaptors are recruited again. This phenomenon strongly indicates that ATP is the key signaling molecule. In the Chapter 2 of this dissertation, we used several biochemical assays to support this model. First we show that the addition of antimycin A, a cytochrome C reductase inhibitor, prevent the recovery of adaptors during the prolonged starvation. This result strongly supports that the production of ATP via oxidative phosphorylation is necessary for the recruitment of adaptors during the prolonged glucose
starvation. I then developed a permeabilized cell assay to demonstrate that ATP and GTP work synergistically to recruit clathrin adaptors. This chapter shows that energy and metabolism play an important role in the regulation of membrane traffic. We proposed a multi-steps energy dependent mechanism that regulates traffic in response to the availability of energy.

Our lab demonstrated that clathrin adaptors localization is highly sensitive to cellular ATP levels. However, clathrin adaptors do not have ATP binding domains or ATPase activities. Thus, ATP must control adaptor recruitment by activating other traffic factors. The following section will describe several ATPases that are known to play essential role in traffic

2.2 Roles of ATPases in Traffic

Roles of Flippases in Traffic

One possible ATPase that is important for energy dependent regulation of traffic is flippases. Flippases are essential for catalyzing the trans-bilayer movement of lipid molecules. This movement, also known as “flip-flop”, of lipid molecules between two leaflets maintains the asymmetry of lipid bilayers. The energy independent flip-flop catalyzed by scramblases, which is a flippases that functions in ER, is a very fast process with half times range from seconds to minute [32]. At the plasma membrane and the Golgi, flip-flop is catalyzed by energy dependent flippases, a subfamily of P-type ATPases [33].

Yeast flippases are known to regulate many trafficking pathway that include: endocytosis, TGN to endosomes, and TGN to ER. Yeast have five flippases: DNF1 and DNF2 works in the endocytic pathway, DNF3 and DRS2 works in the TGN trafficking pathway, while NEO1 is involved in Golgi to ER trafficking [33-35]. The deletion of DRS2 is synthetically lethal with arflΔ [36]. This result is significant because Arf1 is a small GTPase that functions at the TGN, and it involved in the recruitment of AP-1, Gga2 and can affect vesicles formation by inducing
membrane curvature [14, 18, 37]. Additionally, $drs2A$ delays the processing of CPY, which is a yeast protease that has to travel from the TGN to endosomes and then the vacuoles, in order to be fully processed [35]. Finally, Drs2 has a role in the formation of secretory clathrin coated vesicles, and the formation of these coated vesicles is dependent on the hydrolysis of ATP by Drs2 [38]. Thus, flippases are lively involved in the regulation of membrane traffic by energy dependent mechanisms.

**Roles of V-ATPase in Traffic**

Another ATPase that is important for the traffic is V-ATPase. The V-ATPase is a major proton pump that is comprised of a peripherally associated $V_1$ domain and a membrane associated $V_0$ domain. Hydrolysis of ATP by V-ATPases enables $H^+$ to be transported to the lumen of endosomes against the gradient. This process induces the luminal acidification, which appears to be important for the trafficking from endosomes to vacuoles. However, it is unclear whether the acidification is required for early to late endosome traffic or late endosome to vacuole trafficking [39-40]. The luminal acidification is required for the recruitment of $\beta$COP, which is a coat protein that is involved in the formation of vesicles that mediate transport from early to late endosomes [41]. Finally, the V-ATPase has been shown to interact directly with ARNO, a GEF for ARF6 [42], which is a small GTPase that mediate endocytosis, further highlighting its role in trafficking. By using ATP and maintaining the pH gradient across membranes, V-ATPases likely plays a role in traffic regulation in response to the availability of energy.

**Roles of Phosphatidylinositol 4-Kinase in Traffic**

Phosphatidylinositol kinase is another candidate that plays a heavy role in the regulation of traffic by energy dependent mechanisms. One mechanism for phosphatidylinositol kinase to
mediate the trafficking is by synthesizing phosphoinositides (PIs) on the surface of membranes. PIs are important membrane phospholipids that are required for organelles functions from yeast to humans. There are seven kinds of PIs that differ by their sites of phosphorylation [43]. Many proteins are targeted to a specific membrane by recognizing the PIs that are present on the membrane. Essentially, PIs define the identity of membranes, allowing the right proteins to be targeted to the right compartment.

Traffic at TGN and endosomes is regulated by phosphatidylinositol-4 phosphate (PI4P), a subspecies of PIs that is found on the membrane of the TGN and is synthesized by phosphatidylinositol 4-kinase (PI-4K). The level of PI4P seems to play a heavy role in the recruitment of clathrin adaptors. A recent study shows that, when PI4P level is low, the Golgi membrane recruits only Gga2 and Ent3. The recruitment of Gga2 is accompanied by the recruitment of Pik1 (one of the three PI-4Ks in yeast [44]), which will then elevate the level of PI4P on the membrane. As the level of PI4P increases, Ent5 and AP-1 will be recruited [45].
Figure 1.1: The TGN and endosomes are major proteins sorting stations. The TGN and endosomes controls the exist and the entry of proteins. TGN and endosomal traffic is also involved in the degradation of proteins by maintaining the traffic to the vacuole.
Figure 1.2: Clathrin adaptors are important for traffic. Clathrin adaptors are involved in binding cargoes and lipid molecules. Adaptors are essential for recruiting clathrin and other traffic accessory proteins. Finally, some adaptors are involved in generating membrane curvature.
REFERENCES


CHAPTER 2. ADAPTOR AUTOREGULATION PROMOTES COORDINATED BINDING WITH CLATHRIN COATS

Introduction

Clathrin acts in many processes, including endocytosis and transport between the trans-Golgi Network (TGN) and endosomes. (Reviewed in[11]). Clathrin is a multimeric protein complex that forms a polyhedral lattice on the outer surface of some transport vesicles [46]. Formation of the clathrin lattices in vivo is controlled by a class of proteins called adaptors. In both endocytosis and transport between TGN and endosomes, adaptors perform three general functions: membrane association, clathrin binding and assembly, and cargo collection. The adaptors that function in endocytosis and at the TGN are encoded by different genes; however the adaptors play the key role in directing formation of clathrin-coated vesicles in both of the processes.

Clathrin dependent traffic requires seemingly redundant adaptors [25, 26, 47]. At least three different classes of adaptors act at the TGN and endosomes, the heterotetrameric AP-1 complex, the GGA proteins Gga1 and Gga2 in yeast and Gga1, Gga2 and Gga3 in mammals and the epsin-like proteins Ent3 and Ent5 in yeast, and EpsinR in mammals [15, 23, 48-54]. Multiple adaptors can be recruited to the same transport event ([55] and reviewed in [56]). The involvement of multiple adaptors is a hallmark of clathrin dependent traffic, although the functional significance of this complexity remains unclear. Initially it was proposed that adaptor

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redundancy allows the transport of distinct subsets of cargo. Recent work in endocytosis also suggests that potentially redundant adaptors play different mechanical roles within a single endocytic event [57].

In addition to shared function, different adaptors utilize the same molecular interfaces to interact with clathrin. One common mechanism relies on an interaction with the globular domain at the N-terminus of clathrin known as the terminal domain (reviewed in [58]). Many adaptors contain “clathrin box motifs” which bind to a pocket in the terminal domain of clathrin. Clathrin box sequences are characterized by the consensus L φX φ [D/E](where X is any amino acid and φ is a hydrophobic amino acid). Some adaptors contain an additional motif with the consensus sequence [D/E]LL. This DLL-type motif was first characterized in the endocytic adaptors, where, in multiple copies, it mediates the interaction of adaptors with the clathrin triskelion and clathrin cages [59]. Importantly, DLL-type motifs interact with cages formed of triskelia that lack the terminal domain, suggesting that the DLL-type motifs can bind to a different region of clathrin than the clathrin box[60]. The presence of different adaptors, each capable of binding to clathrin at the same sites, adds to the complexity of clathrin coats.

Supporting the possibility that different adaptors cooperate in function, adaptors interact with one another. At the TGN and endosomes, the three different classes of adaptors interact with one another. Ggas and AP-1 share a C-terminal homologous domain termed the γ-ear [47, 48, 61-63]. The γ-ears mediate interactions with a motif in Epsin-like adaptors with the consensus DFXXφ ([64] and reviewed in [58]). Additionally, some isoforms of Gga proteins interact directly with the γ -ear of AP-1 via a motif within a large flexible central domain of Gga known as the hinge [65, 66]. The functional significance of these adaptor-adaptor interactions at the TGN and endosomes is largely unknown.
The physical interactions between TGN/endosome adaptors raises the possibility that adaptors all act coincidently, however genetic results in yeast suggest they function in separate events. In particular, analysis of deletion mutants provides evidence that, although Ent5 cooperates with both AP-1 and Gga proteins in vivo, AP-1 and Gga proteins seem to play roles in distinct transport functions [27]. Only in the case of Ent3 has an adaptor-adaptor interaction been shown to influence function. In this case, Ent3 requires Gga proteins for recruitment to clathrin rich structures. Thus, although evolutionary conservation implies that adaptor-adaptor interactions are important in TGN/endosome traffic in yeast, in most cases the functional significance of such interactions has not been addressed.

In the present study, we describe a previously unrecognized auto-regulatory sequence within yeast Gga2 that controls interaction with Ent5. This sequence encodes overlapping motifs that can bind to clathrin or to the Gga2 γ-ear. We find that in vivo the auto-regulatory sequence modulates interactions with both clathrin and Ent5 and is important for establishing a temporal delay between recruitment of Gga2 and Ent5 to clathrin-rich structures. These findings reveal a highly specialized mechanism that regulates the location/timing of the interaction of Gga2 with Ent5.

Results

Binding of Gga2 to clathrin requires Ent5

We and others previously demonstrated that Ent5 can bind both AP-1 and Gga2 and all three bind to clathrin [23, 67]. Our previous studies did not investigate whether adaptors compete for or cooperate in clathrin binding. Ent5, Gga2, and AP-1 all contain clathrin box motifs and in principle can compete for the same binding pocket on a single clathrin terminal domain. However, clathrin coats have multiple terminal domains, allowing simultaneous binding to
clathrin box motifs in different adaptors. In addition, Gga2 and AP-1 contain multiple DLL-type clathrin interaction surfaces that do not bind to the terminal domain. To determine whether one adaptor influences clathrin binding to other adaptors, we performed clathrin immunoprecipitations from non-denatured lysates of different deletion mutants.

In wild-type cells, both Gga2 and Ent5 co-immunoprecipitated with native clathrin (Figure 2.2.1a). In cells lacking Ent5, substantially less Gga2 immunoprecipitated with clathrin (Figure 2.2.1a & b). In contrast, deletion of the gamma subunit of AP-1 enhanced the amount of Gga2 associated with clathrin. We observed a minor effect of GGA2 deletion on the interaction of Ent5 with clathrin in some immunoprecipitation reactions. However in other reactions, deletion of GGA2 did not produce an effect, suggesting that the effect of Gga2 on Ent5 binding to clathrin is at most minor (Figure 2.2.1a). These results suggest that maximal interaction of Gga2 with clathrin requires Ent5 and also that AP-1 may compete with Gga2 for clathrin. Due to the strong influence of other adaptors on Gga2 binding, we investigated clathrin binding of Gga2 in more detail.

**The clathrin box motif in Gga2 is the major contributor to its interaction with clathrin**

Gga2 contains a single canonical clathrin box motif in the hinge region that is the major site of clathrin interaction [67]. We generated three mutations, each converting different pairs of residues to alanine within or near the clathrin box motif (Figure 2.2a). We first tested the effects of these mutations on interactions of the hinge fragment of Gga2 with purified clathrin. Mutation of the first two residues (ΔCB) of the motif reduced the interaction with purified clathrin to undetectable levels (Figure 2.2b lane 5). Mutation of the third and fourth residues (Δcore) also severely reduced the interaction (Figure 2.2b lane 3). Mutation of the two residues just after the canonical clathrin box (ΔEB) had no effect on the binding to purified clathrin (Figure 2.2b lane 4). In cell lysates, the ΔCB and Δcore mutations ablated the hinge interaction with clathrin.
whereas the ΔEB mutations reduced but did not eliminate clathrin binding (Figure 2.2c). These results confirm previous studies demonstrating that the clathrin box residues are required for direct interactions with clathrin [67]. Furthermore, these results demonstrate residues just after the clathrin box are not required for clathrin binding but that these residues contribute to clathrin affinity in the context of a complex cytosol.

We next investigated the effect of the mutations in the context of a larger Gga2 C-terminal fragment that contains the hinge and ear of Gga2. The Δcore and ΔCB mutations prevented interaction with clathrin while the ΔEB mutation had no effect on clathrin interaction. Thus in cell lysates, only the canonical clathrin box residues are required for clathrin binding (Figure 2.2d). Within the hinge and ear fragment are three DLL-type motifs in addition to the clathrin box motif. The inability of this fragment to bind clathrin when the clathrin box motif is non-functional confirms that stable interaction between Gga2 and clathrin requires the clathrin box.

**The clathrin box motif in Gga2 regulates Ent5-clathrin interaction**

To investigate the effects of the mutations on interactions of full length Gga2 and Ent5 with clathrin, we replaced the endogenous *GGA2* locus with mutant alleles encoding each of the three mutations. We then immunoprecipitated clathrin from cell lysates and probed for associated Ent5 and Gga2. The ΔCB mutation, which alters the first two residues of the clathrin box, dramatically reduced the interaction of Gga2 with clathrin (Figure 2.3a lane 8). At the same time, less Ent5 co-immunoprecipitated with clathrin (Figure 2.3a). Surprisingly, the ΔEB mutation of residues adjacent to the clathrin box enhanced the amount of Gga2 co-immunoprecipitating with clathrin but did not alter the levels of clathrin-associated Ent5 (Figure 2.3a lane 7). The Δcore mutation did not affect either Gga2 or Ent5 levels co-immunoprecipitating with clathrin (Figure 2.3a, lane 6). Together these results provide evidence
that sequence elements in the clathrin box region of Gga2 influence clathrin interaction with both Ent5 and Gga2.

We also investigated the effects of the mutations on Gga2 interactions detected by co-immunoprecipitations of GFP-tagged alleles of Gga2. Similar to the results from the clathrin immunoprecipitations, gga2-ΔCB-GFP exhibited a severe reduction in interaction with clathrin, however Ent5 binding to Gga2 was not significantly changed (Figure 2.3b lane 9). Also similar to the clathrin immunoprecipitations, the ΔEB mutation augmented interaction of Gga2p with both clathrin and Ent5 (Figure 2.3b lane 8). gga2-Δcore-GFP displayed a possible increase in clathrin binding and a clear elevation in Ent5 binding. Together our results provide evidence for a network of pairwise interactions between Gga2, Ent5 and clathrin that can influence assembly of the adaptors with clathrin. In particular, the evidence for increased binding of Ent5 and clathrin to ΔEB and Ent5 to Δcore suggest that the clathrin box region is involved in an inhibitory interaction.

An auto-regulatory motif in Gga2 modulates binding to both clathrin and Ent5

In light of these findings, we reviewed residues in and around the clathrin box. The canonical clathrin box motif partially overlaps with a sequence similar to the characterized γ-ear binding motif from Ent5 and other related proteins, DFXX□ (Figure 2.2a). The residues targeted in the ΔCB mutation are specific for the clathrin binding motif, the ΔEB residues are specific for the γ-ear binding motif, and the Δcore residues are shared by the two motifs. Importantly, because both binding motifs share the central residues, γ'-ear interaction could prevent binding to clathrin and vice versa.

To determine whether the γ-ear binding motif in the clathrin box region of Gga2 can interact with the Gga2 γ-ear domain, we performed chemical crosslinking with a GST-tagged
Gga2 ear fragment and a 6x-histidine fragment of the Gga2 hinge containing the clathrin box motif. Upon crosslinking, a slow migrating species was detected by antibodies against Gga2 (Figure 2.4a lane 8). This species was not observed in reactions containing only the Gga2 ear fragment, only the Gga2 hinge fragment, or in reactions lacking the cross-linker. Furthermore, when reactions were performed with a hinge fragment encoding the Δcore mutation, the level of the specific crosslinked product was substantially reduced compared to wild-type reactions (Figure 2.4a lane 10). These results provide evidence that the clathrin box region of Gga2 also encodes a sequence that can interact with the Gga2 γ-ear domain.

Next, we tested whether the clathrin box region in Gga2 mediates an intramolecular interaction. Gga2 fragments encompassing the hinge and ear with or without the Δcore mutation were subjected to gel-filtration followed by quasi-elastic and multi-angle-light scattering. Both fragments eluted as a single peak, however the mutant form eluted earlier than the wild-type indicating a slightly larger size (Figure 2.4b). The molecular mass of both the wild-type and mutant fragments were determined to be that of a monomer by light scattering. These results are consistent with a model in which the core residues mediate an intramolecular interaction that compacts the structure of the hinge-ear fragment. Without this intramolecular interaction, the Δcore protein assumes a more extended form.

Together our results demonstrate that the clathrin box region of Gga2 can bind to both clathrin and the Gga2 γ-ear. The γ-ear binding can occur as an intramolecular interaction. Mutation of the residues important for γ-ear binding enhances the interaction of full-length Gga2 with both Ent5 and clathrin in co-immunoprecipitation reactions. This suggests that the clathrin box region acts as an auto-regulatory motif that, through binding to the γ-ear, modulates interaction with both clathrin and Ent5. Due to its dual roles, we will refer to the larger sequence...
encompassing both the clathrin box and γ-ear binding motif as the clathrin and adaptor binding motif (CAB).

**Mutation of an auto-regulatory motif in Gga2 alters Ent5 recruitment in vivo**

We tagged Gga2 alleles with S65T-GFP and Ent5 with mCherry at the endogenous loci to assess the function of the Gga2 CAB in cells. Ent5 and Gga2 co-localize on a subset of structures in wild-type cells (Figure 2.5a). Because both Ent5 and Gga2 structures are highly motile within the cell, we fixed cells and analyzed the area of co-localization in >100 individual cells for each genotype to obtain a quantitative measure of the effect of mutations on co-localization (Figure 2.5b). In the ΔCB mutant, in which Gga2 shows a reduced interaction with both clathrin and Ent5, co-localization of Ent5 and Gga2 is unaffected. In contrast, in the Δcore mutant, in which the CAB does not bind to the γ-ear or to clathrin and Ent5 binding is enhanced, there was a statistically significant increase in the co-localization of Ent5 with Gga2. Furthermore, in the ΔEB mutant, in which the CAB retains some ability to bind to clathrin, and interactions with both clathrin and Ent5 are elevated, the extent of colocalization between Ent5 and Gga2 was even greater. Indeed, very few structures could be identified that did not contain both Ent5 and Gga2. Thus when Gga2 has enhanced binding to Ent5, the two proteins almost always co-localize. These results suggest that the auto-regulatory motif of Gga2 limits co-localization of Ent5 and Gga2 in vivo, consistent with the inhibitory role detected by biochemical assays.

To better understand the effects of Gga2 autoregulation on Ent5 and Gga2 localization in vivo, we monitored adaptor recruitment dynamics in live cells. Similar to previous reports, Gga2 and Ent5 exhibit a stereotypical recruitment order in wild-type cells (Figure 2.6a,b)[68]. When monitored by single-plane confocal imaging, numerous events were observed where structures rich in Gga2 initially lack detectable Ent5. Over time many of these Gga2-rich structures
acquired significant Ent5 fluorescence. In contrast, very few events were observed where structures rich in Ent5 but lacking Gga2 then acquired Gga2. In wild-type cells, Ent5 and Gga2 co-localized at some point in approximately 60% of events. Events were observed where Gga2 disappeared without ever recruiting Ent5 and vice versa. To describe the relationship between Ent5 and Gga2 in live cells, we determined the time differential between recruitment of Gga2 and Ent5. In events where Gga2 was recruited first the value is positive, where Gga2 was recruited second the value is negative. Gga2 rich structures that did not recruit Ent5 were omitted from this analysis and made up 23% of all events. In wild-type cells the median recruitment differential is 8 seconds with a broad standard deviation (Figure 2.6 b).

In contrast to wild-type cells, in the gga2-ΔEB-GFP cells Gga2 was rarely recruited before Ent5. Instead the majority of events observed were co-incident appearance of Gga2 and Ent5. Only 7% of Gga2 events failed to recruit Ent5 in gga2-ΔEB-GFP cells. The median differential recruitment interval was 0 with low standard deviation. However, we cannot distinguish co-incident recruitment from migration of a structure with both Gga2 and Ent5 into the plane of focus. Consequently, it was possible that the apparent decrease in differential recruitment time could result from enhanced stability of Gga2/Ent5 structures in the gga2-ΔEB-GFP cells rather than an actual change in the time between Gga2 and Ent5 recruitment. To investigate whether Gga2 and Ent5 structures are more stable in the gga2-ΔEB-GFP cells, we analyzed the life span of Gga2 foci observed by single plane imaging. In both wild-type and gga2-ΔEB-GFP mutant cells, the median lifespan of a Gga2 structure was 20 and 18 seconds respectively with a large variability (Figure 2.6c). The loss of Gga2-first events together with no change in event life-time suggests that the absence of Gga2 auto-regulation leads to a loss in sequential recruitment of Gga2 and Ent5. Thus, the auto-regulatory motif of Gga2 ensures a temporal delay of Ent5 recruitment to Gga2 rich structures.
Discussion

Our study reveals a complex interplay of interactions between Ent5, Gga2 and clathrin. In particular, we have identified a novel bifunctional motif in Gga2p, the CAB, which consists of overlapping sequences for clathrin and γ-ear binding. The evidence supports formation of an intramolecular interaction between CAB and the Gga2 γ-ear that inhibits binding of Gga2p to clathrin and Ent5. Based on these results we propose that CAB acts as an autoinhibitory element to restrict Gga2 interaction with Ent5 and impose a temporal delay in recruitment of Ent5 relative to Gga2 during formation of clathrin-coated vesicles.

Most, but not all, of the effects of CAB mutations can be explained by the finding that the motif binds to the γ-ear of Gga2. The two CAB mutations that prevent interaction with the γ-ear, ΔEB and Δcore, enhance binding of Gga2 to Ent5. Furthermore, the ΔEB mutation, which leaves the clathrin box intact, also stimulates binding to clathrin. Binding of CAB to the γ-ear domain would occlude both the CAB clathrin box and the DFxxφ binding pocket in the γ-ear. The ΔEB mutation prevents interaction between the CAB and γ-ear which allows clathrin unrestricted access to the clathrin box motif in CAB and frees the γ-ear to interact with Ent5, thereby enhancing the interaction of Gga2 with both proteins. In a similar fashion, the Δcore mutation releases the γ-ear for Ent5 binding but clathrin binding would not be enhanced because of the change in the clathrin box. It is not evident why this mutant retains clathrin binding; perhaps the enhanced binding to Ent5 allows an increase in indirect binding to clathrin that compensates for the loss of direct binding or supplements the weak interactions provided by the DLL-type interaction surfaces on Gga2.

Some effects of the ΔCB mutation can be explained by interaction of the CAB with the γ-ear. The ΔCB mutation reduces Gga2 and Ent5 binding to clathrin. The ΔCB mutation
inactivates the clathrin box, accounting for the significant decrease in clathrin binding. Because the \( \Delta CB \) retains residues required for \( \gamma \)-ear binding, interaction of the CAB with the \( \gamma \)-ear occludes interaction with Ent5 thus reducing interaction with Ent5 as well. However, the effects of the \( \Delta CB \) mutation on the interactions of Ent5 with clathrin and Gga2 are not as easily explained by a simple three protein interaction scheme. This suggests that a more complicated network involving clathrin, Ent5 and Gga2 may contribute to the core interactions defined in our studies.

Our findings strongly suggest that the Gga2-CAB is a key motif that limits the interaction of Ent5 and Gga2 \textit{in vitro} and \textit{in vivo}. However, deletion of Gga2 or Ent5 does not influence recruitment of the other protein to clathrin rich structures \textit{in vivo} (20), indicating that most Gga2 and most Ent5 are recruited independently of one another in wild-type cells. Nevertheless, loss of regulation due to CAB mutation resulted in coordinate assembly and increased colocalization of Ent5 with Gga2. This finding suggests that interaction of Gga2 with Ent5 can either promote Ent5 recruitment to Gga2-containing structures or stabilize an Ent5-Gga2 complex once it forms.

The Gga2 auto-regulatory mechanism described here provides insight into the basis of sequential clathrin adaptor recruitment to the TGN described in a recent report[68]. In agreement with our results, this study presented evidence that Gga2 and Ent5 are recruited in sequence, with Ent5 recruitment peaking together with AP-1. Notably, apparent colocalization of Gga2 and Ent5 (or AP-1) in wild-type cells observed by wide-field fluorescence microscopy was resolved in many cases into separate closely-spaced structures visualized by super-resolution methods. Thus, the delay in Ent5 recruitment relative to Gga2 can be attributed to Ent5 assembly into a later-forming class of AP-1-containing clathrin coats distinct from Gga2-enriched coats (Figure 2.7a). However, about 20% of Ent5 was recruited concurrently with Gga2 and some colocalization could be observed by super-resolution microscopy [68]. The co-assembly and colocalization in
small structures suggests that Ent5 and Gga2 act together in some instances in vivo. Indeed a functional role of Ent5 in Gga2 mediated traffic was previously indicated by genetic analysis [27]. Based on these findings and the results presented here, the role of CAB-mediated autoregulation is likely to be two-fold: first, to prevent premature interaction between Gga2 and Ent5 in the cytoplasm prior to assembly into clathrin coats; second, to limit levels of Ent5 recruited to Gga2-enriched clathrin coats at the TGN so that most Ent5 is free to assemble independently into later-forming AP-1-containing coats.

In this model, the intramolecular interaction between Gga2-CAB and Gga2 γ-ear domain limits Gga2 interaction with Ent5 outside of the context of assembling coats. Once Gga2 associates with the membrane to initiate coat formation, clathrin can be recruited through interactions with DLL motifs and limited interaction with the CAB clathrin box. In this nascent coat, higher concentrations of clathrin can compete with the γ-ear for binding to the CAB. Binding of clathrin to the clathrin box would release the γ-ear from the CAB, allowing Ent5 to be recruited to Gga2 structures through cooperative binding to the Gga2 γ-ear and clathrin. Ent5 recruitment would stabilize the coat assembly, both by binding directly to clathrin and by promoting the “open” Gga2 conformation with exposed clathrin box (Figure 2.7b). Supporting this view, deletion of Ent5 reduced co-immunoprecipitation of Gga2 with clathrin. In addition to the interactions described here it is also possible that interactions with other proteins and post-translational modifications contribute to the auto-regulatory process.

The functional significance of the limited association of Ent5 and Gga2 in vivo is currently unclear. We found no major defect in the maturation of alpha factor in cells carrying any of the Gga2 mutations (data not shown). Maturation of alpha factor depends on clathrin-dependent TGN-endosome trafficking of the processing protease Kex2. However, this assay may not capture cargo specific, subtle or kinetic defects in traffic. A more general compression
of the delay between Gga2 and both AP-1 and Ent5 recruitment resulted in subtle alpha factor maturations defects, providing evidence that the temporal regulation of assembly is important for optimal function of the TGN[68]. We therefore speculate that the function of Gga2 autoregulation of Ent5 recruitment is most important for TGN function in the complex natural environment of yeast growing in the wild.

**Materials and Methods**

**Yeast strains**

Yeast strains are listed in Table 2.2.1[69, 70]. Replacement of the genomic alleles used a full gene replacement strategy, in which a full gene deletion was replaced by a DNA fragment excised from a plasmid carrying the desired allele. Clones were then screened by PCR followed by restriction digestion to confirm integration of the desired alleles.

Fluorescent tags were added using a PCR-based strategy with either the pFA6a-S65TGFP-HIS3Mx plasmid or pKS390 (pFA6a-mCherry-KanMx) as described previously [71, 72].

**Plasmids**

Plasmids used in this study are described in Table 2.2. Point mutations were generated with the Quik-change site directed mutagenesis kit (Stratagene) according to manufacturer’s instructions.

**Antibodies**

Polyclonal antibodies against clathrin light chain (Clc1) and monoclonal antibodies against clathrin heavy chain (Chc1) are described elsewhere [73-75]. Polyclonal antibodies were generated in rabbits against full-length GST-tagged Ent3, Ent5, Gga2 and GFP in rabbits. Gga2, Ent3, Ent5 and Clc1 antibodies were affinity purified by passing serum first over 2 mls of
Affigel-10 (Biorad) cross-linked to purified GST to deplete GST-signal. Depleted serum was bound to 2mls of Affigel-10 cross-linked to purified GST-tagged full-length protein and eluted from the matrix with glycine according to manufacturer’s instructions.

**Protein purification**

Protein expression was induced in BL21-DE2 pLysS (Promega) in mid-log phase at 30°C in LB-Amp (ISC bioexpress) for four hours with 0.1mM IPTG. Cells were pelleted and resuspended in minimal water and frozen. Pellets were quick thawed in 1x PBS with 1:100 protease inhibitor cocktail (Sigma) and sonicated in 0.5 sec pulses for 1 min on ice. Lysates were incubated with 1% Triton-X100 for 30 min at 4°C and pelleted at 12krpm for 20min in a ss34 rotor at 4°C. For GST-tagged proteins, supernatants were incubated with glutathione sepharose (GE Life sciences) for 1 hour at 4°C, washed with PBS and eluted with HSE (100mM Tris pH9, 200mM NaCl, 5mM DTT, 20mM reduced glutathione), or cleaved in place with Factor X (New England Biolabs) in 50mM Tris pH 7.5, 150mM NaCl, 1mM CaCl. Factor X was removed with p-aminobenzamidine agarose (MP Biomedicals). For 6-His tagged proteins, supernatants were incubated with Talon Resin (Clonetech) and eluted with 150mM imidazole in PBS final pH 7.0. For light scattering experiments, cleaved proteins were further purified by passage over tandem Hightrap Q and SP (GE Life-Sciences) 1ml columns. Buffer exchange was performed by several rounds of concentration in Ultracel 10 concentration device (Millipore) at room temperature or several rounds of dialysis with Spectra/Por dialysis tubing MWCO 3500 (Spectrumlabs).

Clathrin triskelia were purified from TVY614 transformed with a Yep24-CHC-CLC, a high copy plasmid carrying both clathrin heavy and light chain genes. Six liters of cells were grown to mid-log phase in selective media, pelleted, resuspended in minimal water and frozen in small pellets by pouring a slow stream into a liquid nitrogen bath. Lysates were generated by blending pellets in a prechilled metal canister on a warring blender until a light powder was formed. Powder was
thawed with equal volume to weight in 2x Triskelia buffer (100mM TRis pH 7.5, 100mM NaCl, 2mM EDTA with protease inhibitors). High speed supernatants were generated at 4C in a Ti 70 at 60Krpm for 30 min. 20% weight to volume ammonium sulfate was added and high speed pellets were generated by centrifugation at 60Krpm for 15 min in a Ti70 rotor. Pellets were resuspended in 1X Triskelia buffer (50mM Tris pH 7.5, 50mM NaCl, 1mM EDTA) and dialyzed against two buffer changes of Triskelia buffer. Medium speed supernatants were generated by spinning at 13Krpm for 10 minutes and were fractionated on a Superose 12 column pre-equilibrated with 10mM KPO4 pH 7.5 1mM EDTA. Clathrin fractions were identified by SDS-PAGE analysis followed by coomassie blue staining and found to be free of adaptors (Ent5, Ent3, Gga2, AP-2 and AP-1) by immuno-blotting.

**Yeast cell lysates and binding studies**

Cell lysates for GST-tagged protein interaction studies were generated by culturing TVY614 in yeast rich media (10g/L yeast extract, 20 g/L peptone, 2 g/L glucose, 20mg/L Uracil, Adenine sulfate and L-tryptophan) followed by liquid nitrogen blending as described for clathrin purification. Powder was thawed with an equal volume to weight in Buffer A (100mM MES pH 6.5, 0.5mM MgCl2, 1mM EGTA). The lysate was clarified by centrifugation at 13 Krpm for 10min and resulting supernatants were used for binding studies. For binding studies, GST-tagged proteins were prebound to glutathione sepharose in 1ml of PBS for 1 hour at room temperature, washed twice with Buffer A and resuspended in lysate. Lysate binding was allowed to proceed for 1 hour at 4°C and beads were washed twice with Buffer A and once with Triskelia buffer. Beads were resuspended in HSE and incubated for 20 minutes at room temp. Supernatants were taken as the bound fraction.

Cell lysates for immunoprecipitations were generated by a combined spheroplast, bead lysis protocol. Spheroplasts were generated by first reducing the cell wall in 100mM Tris pH
9.4, 2% glucose and 5mM DTT. Cells were then resuspended in digestion buffer (Yeast rich media without additional amino acids or glucose and 0.5% glucose, 10mM Tris, 1.2M Sorbitol and 120 U lyticase) and gently agitated for 30 min at 30°C. Cells were washed in 1.2M sorbitol and lysed with the addition of Buffer A with protease inhibitors followed by glass bead lysis and addition of 1% Triton X-100. Lysates were clarified by centrifugation at 13Krpm for 10 min. Lystates were incubated overnight with 100 uls 20% protein A-agarose slurry and 3 ul of clc1 antibody or 0.5ul of GFP antisera. Lysates were washed three times with Buffer A and bound proteins were eluted in SDS-sample buffer.

For crosslinking studies, reactions were performed at room temperature in Buffer B (100mM HEPES, 50mM NaCl, pH 7.5)[15]. GST-Gga2 460-end was added to 3.78µM, wild-type 6-His hinge fragments to 55.42µM ΔEB fragment to 57.64µM and DTSSP (Thermo Scientific) was added to 1mM. After 30 min, crosslinking was quenched by the addition of 37mM final concentration of Tris pH 7.5. 14% of each reaction was loaded for immuno-blot analysis and for coomassie staining.

**Microscopy**

For fixed images, cells grown to mid-log phase were fixed by rapid mixing with equal volume of fixative (100mM KPO4 pH 6.5, 2mM MgCl2, 8% formaldehyde) and incubating for 1h in the dark. Cells were washed twice with PBS. Acid-washed 22x22mm coverslips were treated with 5 mg/ml Concanavalin A and allowed to dry. Six microliters of fixed cells were spotted onto a treated coverslip, placed on a clean glass slide and immediately sealed with VALAP (equal parts Vaseline, Lanolin and paraffin).

The images were acquired on a spinning disk confocal microscope described by Maddox et al[76]. Well-separated cells were first selected using bright-field microscopy. Twenty-one Z-stack images were collected at 0.2 μm intervals through each field of cells. Integration times for
the GFP and mCherry channels (1200 ms integration per image for each mCherry image and 400 ms integration per EGFP image in that order) were set so that relatively high and similar signal to noise ratio was maintained for both channels.

All non-overlapping, centrally located cells within a field were analyzed on a custom graphical user interface written in MatLAB R2009b (Natick, MD). We then conducted morphometry analysis on this down-sampled stack using functions from the Image Processing Toolbox in MatLAB. Briefly, a single cell from a Z-stack was selected manually by drawing a region of interest of arbitrary dimensions. This cropped area was then used for further analysis for the selected cell. First, a 5x5 pixel background region was manually selected from the maximum intensity projection image of all 21 planes in the cropped region to ensure that the dimmest region inside the cell (usually the vacuole) was specified as the background region. Using a nominal Z-depth of the confocal point spread function as 600 nm, we projected the maximum intensity pixels from a set of three successive image stacks into a single image (a stack of 21 images thus gets compressed into a stack of seven images). The coordinates of the background region were transferred to each of the seven images in the down-sampled stack to obtain background pixels for each plane. The background was estimated by using the relation: background = (mean pixel value) + (6 x standard deviation of pixel values). If one assumes that the background pixel values are normally distributed, this background estimate rejects 99% of the background pixels in our analysis. This background value was used as the threshold to define features within each plane of the cell. For the calculation of co-localization coefficients, we further discarded any patches smaller than the XY PSF of the microscope (less than 3 pixels, 1 pixel ~ 66 nm). Colocalization coefficient calculation was based on the method of Manders et al(22). In the analysis, we broadly categorized the cells as large-budded (late mitosis) and small-budded (G1 or S). The data reported in the figure provides measurements from large-budded...
cells, although analysis of small-budded cells obtained similar results.

For live cell imaging, the fixation and PBS washing steps were omitted. A single central plane was imaged with a 2 second interval. Analysis was performed in ImageJ, first the threshold command was applied to all images uniformly to generate a mask. Structures smaller than 2 squared pixels or that persisted less than five frames were omitted from analysis. Using the threshold masked Gga2 images, non-overlapping Gga2 structures were identified. The first frame in which a structure was visible was considered T=0. Preceding and subsequent frames from the Ent5 masked images were analyzed to identify if and when Ent5 showed above threshold fluorescence within the defined Gga2 mask. The data reported in the figure are from >70 individual events from at least 10 cells.

**Gel filtration and light scattering**

Purified Gga2 fragments were separated in Buffer A on a WTC-030s5 column (Wyatt) and subjected to multi-angle light scattering and quasi-elastic light scattering on a DAWN EOS light scattering instrument (Wyatt) interfaced to an Akta FPLC (GE life Sciences). Weighted molar mass was calculated with Astra software. Data shown is intensity from detector at position 11.

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### Table 2.1. Yeast strains used in this study.

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### Table 2.2. Plasmids used in this study

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Figure 2.1. Gga2 interaction with clathrin is altered in adaptor deletion strains. Clathrin was immunoprecipitated from indicated strains with a poly-clonal clathrin light chain antibody and interacting Ent5 and Gga2 were detected by immuno-blot analysis. Left panel shows a representative immunoprecipitation reaction. Right panel shows data from 4 independent immunoprecipitation experiments. Intensity measurements of adaptors were normalized to intensity in wild-type sample of the same experiment and then normalized to the intensity of clathrin heavy chain bands (Chc1) within the same sample. * indicates a p-value <0.05 as determined by a two-tailed students t-test.
Figure 2.2. Mutations of the clathrin box region alter interaction of Gga2 with clathrin in vitro.  

**a.** (Top) Schematic of Gga2 and interacting partners. Black dotted lines indicate previously defined interactions. The green dotted line indicates an intramolecular interaction defined in this study. (Middle) Schematic of fragments used in in vitro assays. (Bottom) Schematic of mutation generated. 

**b.** Indicated mutations were generated in GST-fusion constructs containing 60 a.a. of the Gga2 hinge, purified and incubated with purified clathrin. Interacting clathrin was detected by immuno-blot analysis. 

**c.** Indicated fusion proteins were incubated with cell lysates and interacting clathrin was detected by immuno-blot analysis. 

**d.** Indicated mutations were generated in GST-fusion constructs containing the Gga2 hinge and ear, purified and incubated with cell lysates. Interacting clathrin was detected by immuno-blot analysis.
Figure 2.3. Mutations of the clathrin box region alter interaction of Gga2 with clathrin in cell lysates.  

a. Effect of Gga2 mutations on clathrin binding to Gga2 and Ent5 in lysates. Indicated mutations were generated at the endogenous Gga2 locus. Clathrin was immunoprecipitated from indicated strains with a poly-clonal clathrin light chain antibody and interacting Gga2 and Ent5 were detected by immuno-blot analysis. Left panel shows representative immunoprecipitation result. Right panels show intensity analysis as described in Figure 1.

b. Effect of Gga2 mutations on Gga2 binding to clathrin and Ent5 in lysates. Indicated mutations were generated at the endogenous Gga2 locus and GFP-tagged at the C-terminus. Gga2-GFP alleles were immunoprecipitated with a mono-clonal GFP antibody and interacting clathrin and Ent5 were detected by immuno-blot analysis.
Figure 2.4. The clathrin box region of Gga2 interacts with the Gga2 $\gamma$-ear.  

a. The clathrin box region binds to the $\gamma$-ear. A 6-histidine tagged 60aa fragment containing the wild-type (CAB) or $\Delta$core (CAB-$\Delta$core) clathrin box region were incubated with a GST-tagged fragment encoding the $\gamma$-ear ($\gamma$-ear) were incubated with or without crosslinker. Identical samples were processed for western-blot analysis and coomassie staining. Note uncrosslinked CAB-wt does not adhere to membrane in western-blot but is present in coomassie stained gel. Arrowhead indicates migration of a band present only in reactions containing the CAB, $\gamma$-ear and crosslinker. 

b. A fragment containing the $\Delta$core mutant adopts an extended conformation. Elution profiles of Gga2 aa 340-end and Gga2 $\Delta$core mutant aa 340-end in gel filtration. Weight-averaged solute molar mass for the wild-type fragment was 25.18 KDa and for the mutant fragment was 26.02 KDa with an error of 1% for both mass values.
Figure 2.5. CAB mutations alter co-localization of Ent5 and Gga2. A. Representative images of cells analyzed. Indicated mutations were generated at the endogenous Gga2 locus and GFP-tagged at the C-terminus, Ent5 tagged at the C-terminus with mCherry was introduced into these strains through crosses. Cells were imaged as described in the Experimental Procedures section.

B. Colocalization analysis of Gga2-alleles with Ent5. Box-plot diagrams of Manders’ coefficients for analyzed data. On each box, the central mark is the median, the edges of the box are the 25th and 75th percentiles, the whiskers extend to the 95th percentile, and outliers are
plotted individually. Outliers are indicated as circles. P-values reflect Mann-Whitney test with Null hypothesis (h=0) that the two samples have the identical median with 95% confidence interval. * indicates a p-value value greater than $1 \times 10^{-3}$ for samples compared to wild-type.
Figure 2.6. Clathrin box region mutations alters the recruitment differential between Gga2 and Ent5. A. Representative images of events analyzed. Green arrow indicates the time-point utilized for t=0. Red arrow indicates the time point used to calculate the differential between Gga2 and Ent5 recruitment. Black arrow indicates time-point used to calculate event lifespan. Frames of time lapse are 2 seconds apart for each channel. Approximately 800ms separates channels at a single time point. b. Box-plot diagrams of analyzed recruitment differential data. c. Box-plot diagrams of analyzed lifespan data. On each box, the central mark is the median, the edges of the box are the 25th and 75th percentiles, the whiskers extend to the 95th percentile, and outliers are plotted individually. Outliers are indicated as circles. P-values reflect Mann-Whitney test with Null hypothesis (h=0) that the two samples have the identical median with 95% confidence interval.
Figure 2.7. Schematic of possible adaptor recruitment models. A. Different types of adaptor vesicle form in a temporal order on an organelle. Event type 1 which utilizes only Gga2 (orange) forms early on an organelle, later in the lifespan of the same organelle Event type 2 vesicles form which utilizes both Gga2 and Ent5 (purple), finally in late stage organelles only Event type 3 vesicles form which contain Ent5 and AP-1 (blue). Mutations of the autoregulatory domain of Gga2 may cause the loss of Event type 1 resulting in the observed increase in Ent5 and Gga2 co-localization and the loss of temporal order of recruitment between Gga2 and Ent5. B. Model of possible role of CAB in Ent5 recruitment in Event type 2 vesicles. Gga2 in its autoregulated form becomes concentrated at a cellular membrane. DLL-type motifs of Gga2 loosely recruit clathrin. Now in local high concentration, clathrin can bind to the CAB thus promoting the open form of Gga2. In the open form, Gga2 helps to stabilize Ent5 in the clathrin coat.
REFERENCES


CHAPTER 3. CLATHRIN BINDING BY THE ADAPTOR ENT5 PROMOTES LATE STAGES OF CLATHRIN COAT MATURATION²

Introduction

Clathrin dependent traffic is a central facet of all eukaryotic cell biology. It mediates traffic at multiple locations including endocytic traffic that originates at the plasma membrane and endosomal traffic that originates at trans-Golgi Network (TGN) or at the endosomes (reviewed in [11]). It regulates nearly every aspect of cellular behavior through effects on the localization of transmembrane, extracellular and organellar proteins (reviewed in [77]). To perform these many different functions, the clathrin coat must bind to many different protein cargo and package these into transport carriers. This cargo selection is performed by clathrin adaptors.

There are more than a dozen different clathrin adaptors encoded in the genomes of most eukaryotes (reviewed in [58]). Each clathrin adaptor acts as a complex interaction hub. In addition to binding to transmembrane cargo, most bind phospholipids, small GTPases or other membrane associated proteins that confer specificity to adaptor recruitment. Adaptors also directly interact with clathrin, the major structural component of the clathrin coat. This interaction links transmembrane cargo to the forming transport carrier. This function is the minimum definition of a clathrin adaptor. However, many adaptors perform additional

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mechanistic roles in the coat such as bending membranes or stimulating clathrin polymerization [59, 78-83]. However, it is unclear whether all adaptors perform such central mechanistic roles or whether some act solely as linkers between cargo and clathrin. Determining which adaptors perform mechanistic roles and what those roles are is important to understanding the regulation of clathrin function in vivo.

Elucidating the roles of clathrin adaptors at the TGN, early and late endosome has been particularly challenging due, in part, to difficulties in imaging individual transport events, and because cells can adapt to disruption in clathrin dependent traffic by upregulating other pathways [84]. In the yeast Saccharomyces cerevisiae, five clathrin adaptors are known to function at the TGN and endosomes: the heteromeric AP-1 complex; the Golgi localized gamma-adaptins, which are encoded by the paralogs GGA1 and GGA2; Ent3, a protein belonging to the ENTH-A sub-family of epsins; and Ent5, a protein belonging to the ENTH-D sub-family of epsins [23, 27, 48, 53, 61, 62, 85]. Genetic studies suggest that AP-1 and Gga proteins act in distinct pathways [61]. Ent3 appears to act exclusively with Ggas [27, 86]. In contrast, the role of Ent5 has been unclear. Deletion of Ent5 causes only minor defects in traffic, suggesting its role may be minor or cargo specific [27]. However, it localizes to every Gga2 or AP-1 structure in vivo, and is required for maximal Gga2 interaction with clathrin [86, 87]. These data suggest that it has a more central role.

To clarify the function of Ent5, we re-examined the role of Ent5 in endosomal/TGN traffic using new approaches. Using a quantitative assay of endosomal traffic, we find that loss of Ent5 impairs endosomal traffic. In addition, we report that removal of Ent5 prolongs the lifespan of clathrin coats after Gga2 and clathrin are recruited, indicating a defect after coat assembly initiates. We find that the direct interaction of Ent5 with clathrin is required for its role in coat behavior and cargo traffic, whereas direct interaction of Ent5 with Gga2 or AP-1 is
important for Ent5’s function but not the turn-over of Gga2 containing structures. Together these results suggest that clathrin binding by Ent5 plays a key mechanistic role in the maturation of Gga2 containing transport carriers, whereas adaptor binding by Ent5 does not.

**Results**

**Ent5 provides a central function in endosomal clathrin dependent traffic**

Previous research implicated Ent5 functions in endosomal/TGN traffic, however, it was unclear whether Ent5 was a specialized cargo-specific adaptor or if it played a central mechanistic role in endosomal/TGN clathrin dependent traffic (ECT) [25, 27]. To better understand its role in ECT, we tested if the deletion of *ENT5* impaired ECT using a quantitative calcofluor white (CFW) sensitivity assay. This assay measures the fidelity of ECT by making intracellular retention of the chitin synthase Chs3 dependent on ECT. When ECT is defective, some Chs3 is found at the cell surface in cell lacking *CHS6* whereas, in otherwise wild-type cells, all Chs3 is retained intracellularly when *CHS6* is deleted [88]. Cell surface Chs3 makes the cells sensitive to CFW. We found that deletion of *ENT5* increased the CFW sensitivity of cells lacking *CHS6* (Fig. 3.1A). As a further test of the role of Ent5 in ECT, we examined the localization of the SNARE Tlg1 in cells lacking Ent5. In wild type cells, Tlg1-mCherry was found in the vacuole and in punctate structures that co-localized with Gga2-GFP, consistent with the known localization of Tlg1 at the TGN. In contrast, in cells lacking Ent5, Tlg1-mCherry puncta were rarer and dimmer than in wild-type cells (Fig. 3.1B). Furthermore, steady state levels of Tlg1 are lower in cells lacking Ent5, suggesting Tlg1 is mis-sorted to the vacuole and degraded in these cells (Fig. 3.1C). These result are consistent with the loss of Ent5 causing a defect in ECT but does not distinguish between cargo specific or central mechanistic roles for Ent5.

To distinguish between a cargo-specific or central mechanistic role, we investigated if
Ent5 alters the maximal recruitment of Gga2 or clathrin heavy chain (Chc1). To do this, we monitored the intensity of Clc1-GFP and Gga2-GFP expressed from their endogenous loci in wild type and cells lacking Ent5. Surprisingly, the intensity of Clc1-GFP was unaffected by deletion of ENT5, suggesting that Ent5 is not required for the maximal recruitment of clathrin (Fig. 3.1D). Similarly, Gga2-GFP was recruited to punctate structures in the absence of Ent5. However, the intensity of Gga2 structures was increased by 1.4 fold in cells lacking Ent5. These results suggest that Ent5 is not required for the association of clathrin and Gga2 with membranes.

The increased intensity of Gga2 caused by loss of Ent5 is similar to increased intensity of endocytic proteins caused by loss of endocytic epsins [89]. In endocytosis, this increased intensity is caused by the stalling of endocytic events after the initiation of coat formation. To test if the increased intensity of Gga2 could be explained by a stalling of endosomal coat formation, we monitored the kinetics of coat assembly. To do this, we performed time lapse microscopy on fluorescently labeled Chc1 and Gga2. In wild-type cells, these structures show a relatively stereotypical behavior: fluorescence intensity of Gga2 and Chc1 increased steadily to a maximum point and then decreased rapidly (Fig. 3.2B, C). This behavior is thought to reflect the assembly of one or coordinated assembly of multiple clathrin coats on an endosomal organelle followed by the rapid disassembly of the coat(s) or the rapid movement of the vesicle(s) after the complete formation of the vesicle(s) [86, 87]. The mean life-span of such structures, defined as the time point when the structure was first visible over background to when it was no longer visible, was 24 seconds for Gga2-GFP and 23 seconds for Chc1-RFP (Fig. 3.2 D). In contrast, in cells lacking Ent5, this stereotypical behavior was perturbed. The rates of fluorescence intensity increase was less uniform and the mean lifespan of the structures was 11 seconds longer for Gga2 and 14 seconds longer for Chc1. This increase in lifespan was largely due to an increase in the fraction of events with very long life-span (>30 sec) (Fig. 3.2E). The increased lifespan is
unlikely to be caused by reduced movement of the structures into or out of the plane of focus because the mean speed of movement of structures determined from mean square displacement measurements were indistinguishable from wild-type (data not shown). These results suggest that loss of Ent5 causes a defect in coat formation after clathrin and Gga2 recruitment but before coat disassembly.

**Multiple domains of Ent5 contribute to its function and localization**

The extension in lifespan of Gga2 and Chc1 structures in cells lacking Ent5 suggests that Ent5 acts as more than a cargo linker. To better understand the role of Ent5 in ECT, we investigated the importance of different Ent5 domains and motifs in Ent5 function. To do this, we mutated each of the known domains and/or motifs in Ent5 (Fig. 3.3A). *ENT5* encodes an N-terminal ANTH domain. This domain is thought to bind cargo and/or lipids. To disrupt the function of the ANTH domain, we mutated several positively charged residues that are predicted to lie on the surface of the ANTH domain and are similar to residues that interact with lipids in other ANTH domains to generate a Ent5 ANTH domain charge reversal mutant (Ent5-CR) [80, 90]. Ent5 also binds the γ-ear of clathrin adaptors Gga2 and AP-1. We disrupted this activity by mutating key acidic and hydrophobic residues of the highly conserved γ-ear interaction motif to generate an Ent5 adaptor binding mutant (Ent5ΔAB) [15, 23, 91]. Finally, Ent5 contains a pair of clathrin box motifs which mediate interaction with clathrin in many proteins [92]. We disrupted clathrin binding by mutating key residues of each the clathrin box to generate an Ent5 clathrin binding mutant (Ent5ΔCB, Figure 3.4A). When expressed from the endogenous *ENT5* locus, each of the mutant proteins was expressed at the same level as wild-type Ent5 (Supplemental Figure 3.1A-C). However, each of the mutant alleles reduced the functional activity of Ent5 as assessed using the quantitative CFW assay (Fig. 3.3B), suggesting that each activity contributes to Ent5 function in ECT.
We next investigated the effects of each mutation on the localization of Ent5. To do this, we expressed GFP fusion proteins from the endogenous loci. We first confirmed that the addition of the GFP tag did not interfere with protein function using the quantitative CFW assay (Supplemental Figure 3.1D). We then assessed Ent5 localization by counting the number of GFP puncta per cell in a central plane (Fig. 3.3C and D). Each mutation altered the number of Ent5 puncta per cell in the central plane; however, the magnitude of the effect differed substantially. The ent5-CR mutation had the strongest effect. It increased the percent of cells with no central plane Ent5 puncta from 1.4% in wild-type to 69% in the mutant cells (Fig. 3.3C). Furthermore, the mean number of puncta per cell in a central plane was reduced to less than one in the mutant cells from 3 in wild-type cells. To determine if the loss of Ent5 puncta reflected a loss of endosomes, we monitored endosomes using clathrin as an endosomal marker. Clathrin structures were abundant in these cells suggesting the mutation prevents endosomal localization of Ent5 rather than disrupts endosomal structures.

Similar to the effects of ent5-CR, the ent5ΔCB mutation increased the percent of cells with no puncta to 32% and reduced the mean number of puncta per cell to 1 without altering clathrin localization. The ent5ΔAB mutation, which mutates the two motifs that interact with Gga2 and AP-1, had the weakest defect. It did not significantly alter the percent of cells with no puncta and only reduced the mean number of puncta per cell slightly, from 3 to 2.6. Together these results demonstrate that the ANTH domain and clathrin binding motifs play important roles in Ent5 localization to clathrin rich structures.

We next analyzed the effect of the mutations on the amount of Ent5 recruited to each puncta (Fig. 3.3E). We were unable to perform this analysis on the Ent5-CR cells due the low fluorescence intensity of this mutant allele. We found that the mean intensity of Ent5ΔCB structures was reduced by 4.8 fold compared to wild-type. In contrast, the mean intensities of
Ent5ΔAB structures were only modestly reduced by 1.6 fold. Together these results demonstrate that the ent5-CR and ent5ΔCB mutations perturb either the recruitment or persistence of Ent5 in clathrin rich structures whereas the ent5ΔAB mutation does not substantially perturb its localization.

The strong effect of the ent5ΔCB mutation on Ent5 localization suggests that clathrin binding plays a key role in Ent5 localization. The finding that the effect of ent5ΔAB mutation was less severe than the effect of the ent5ΔCB mutation is surprising because the affinity of Ent5 for Gga2 is predicted to be at least five times higher than the affinity of Ent5 for clathrin [93-95]. Therefore, based on just the predicted protein interaction affinity, the ent5ΔAB allele should have a stronger effect than the ent5ΔCB allele. However, we previously reported that clathrin binding by Gga2 enhances Gga2 binding to Ent5, suggesting clathrin binding can promote or stabilize the interaction between adaptors [87]. To test if clathrin stabilizes the interaction between Ent5 and Gga2, we performed co-immunoprecipitation analysis. We found that Ent5ΔCB co-immunoprecipitated less Gga2 than wild-type Ent5. This suggests that the ent5ΔCB allele reduces the interaction of Ent5 with Gga2, in addition to reducing the interaction with clathrin (Fig. 3.4A-C). In contrast, the ent5ΔAB allele only reduced the interaction with Gga2 and did not interfere with the interaction with clathrin. The stronger effect of the ent5ΔCB allele on localization is thus consistent with a stronger effect of this allele on physical interactions with the coat.

Surprisingly, we found Ent5ΔCB co-immunoprecipitated a small amount of clathrin, whereas Ent5ΔAB co-immunoprecipitated a small amount of Gga2. Since these alleles were designed to ablate each activity entirely, we suspected that this interaction might be indirect. For example, Ent5ΔAB could interact with Gga2 indirectly via clathrin. To test this possibility, we investigated the interactions of Ent5 that lacked both adaptor and clathrin binding motifs
(ABCB) with clathrin and Gga2. This allele reduced clathrin and Gga2 binding to undetectable levels, suggesting that, for the Ent5ΔAB and Ent5ΔCB proteins, the unexpected interactions are indirect.

The strong effect of the clathrin binding on Ent5 localization is not consistent with a previous report that the N-terminal ANTH domain of Ent5 was sufficient for localization [27]. We investigated this apparent contradiction in two ways. First, we reanalyzed the localization of the ANTH domain. We found the ANTH domain was found in fewer puncta per cell in a central plane and that these puncta were dimmer than wild-type Ent5 (Fig. 3.3C-E). This suggests that the ANTH domain is not sufficient for maximal Ent5 localization. Second, as an independent confirmation of the importance of clathrin binding in Ent5 localization, we monitored Ent5 localization in cells expressing a version of clathrin that lacks the interaction site for proteins like Ent5 (chc1Δbox) as the only version of clathrin [96]. We first confirmed that the chc1Δbox protein does not interact with Ent5 by co-immunoprecipitation (Fig. 3.4D). We then investigated the localization of Ent5 in this strain. We found that Ent5 was localized to fewer puncta in the chc1Δbox cells and that the puncta were dimmer (Fig. 3.4E). This reduction of Ent5 localization was not due to a gross defect in endosomal structures since Gga2 structures were abundant in these cells. Together these results strongly suggest that ANTH domain functions and clathrin binding play a pivotal role in Ent5 function and localization whereas adaptor binding is important for Ent5 function but is less important for localization.

**Ent5 clathrin binding promotes the turn-over of Gga2 structures in vivo**

To better understand how the Ent5 mutations perturb ECT, we monitored the effect of ENT5 mutations on the behavior of endosomal clathrin coats using Gga2-mcherry. The ent5ΔCB allele extended the life-span of Gga2 structures by a mean of 5 sec, which is indistinguishable from life-span extension observed in cells lacking Ent5 entirely (Fig 3.5 A and B). In contrast,
the ent5ΔAB allele did not alter the life-span of Gga2 structures. These results indicate that the interaction between Ent5 and clathrin regulates the behavior of endosomal clathrin coats, whereas the interaction between Ent5 and Gga2 is dispensable for the normal life-span of clathrin coats.

We next investigated the recruitment of the Ent5 mutant proteins in relation to Gga2. In wild-type cells, Ent5 is recruited after Gga2 with a mean “recruitment differential” of 4 sec (Fig. 3.5C). Because the Ent5-CR mutant protein is barely detectable in these structures, we did not perform this analysis for this allele. The ent5ΔCB mutation extended the mean of recruitment differential by 6 sec whereas the ent5ΔAB mutation did not alter the recruitment differential. Because the ent5ΔCB mutation increased both the life-span of Gga2 structures and recruitment differential between Ent5 and Gga2, we asked if the life-span extension of Gga2 structures could be explained exclusively by the delay in Ent5 recruitment. However, even after Ent5 was recruited, the life-span was extended as reflected in an increase in the average time that Ent5 and Gga2 colocalize from 20 sec in wild-type cells to 28 sec in mutant cells, and an increase in the lifespan of Ent5 structures (Fig. 3.5D & E). Together these results demonstrate that the life-span extension of the Gga2 structures is due to both a delay in the recruitment of Ent5 and a delay in the maturation of the structure after Ent5 recruitment. This suggests that even after Ent5 is recruited, the inability of Ent5 to bind clathrin delays endosomal coat formation. This suggests that in addition to regulating recruitment of Ent5 to the endosomal clathrin coat, clathrin binding of Ent5 performs an important function that promotes ECT.

Discussion:

Epsins are an ancient family of proteins that are found in every known eukaryote. They are important for both endosomal and endocytic clathrin coats. This conservation suggests that epsins perform a critical function that has been retained during evolution. However, the nature of
this function is unknown. Initial studies of epsins either failed to reveal a strong defect in membrane traffic upon depletion or loss of endocytic or endosomal epsins or revealed a cargo-selective function [23, 89, 97-99]. This led to the suggestion that epsins act as cargo specific adaptors and do not play a key role in the formation of the transport carrier. Recent work has clearly established that endocytic epsins play fundamental mechanistic roles in the formation of the transport carrier [78, 79, 89, 100]. We have now revealed that Ent5 plays a fundamental mechanistic role at the endosome. Based on the life-span extension observed in cells lacking Ent5, we propose that Ent5 plays a pivotal role in the formation of endosomal clathrin coats, rather than a cargo specific function. These data further suggest that Ent5 plays a role in the late stages of coat assembly, after the recruitment of Gga2 and clathrin. A late acting function is also supported by the observation that wild-type Ent5 is recruited after clathrin [86].

We propose that the key function performed by Ent5 is the stimulation of clathrin assembly (Fig. 3.6). This is consistent with the extended life span of coats containing Ent5, Gga2 and clathrin in cells expressing Ent5-CB. In contrast, despite reduced Ent5 recruitment, Ent5-AB containing coats do not stall. We speculate that in wild-type and in Ent5-AB expressing cells, Ent5 binding to clathrin promotes a conformational organization of clathrin that enables clathrin polymerization. This suggests that Gga2 on its own is not capable of promoting this conformational organization. This may be because Gga2 contains only one copy of the clathrin binding motif known as a clathrin box [101]. A single clathrin box can bind clathrin, however two clathrin boxes appear to be required for clathrin assembly in vitro [102]. Although Gga2 contains many clathrin binding sites in addition to the clathrin box, these additional sites are not clathrin boxes. They are low affinity DLL-type binding sites, which may not promote rapid clathrin polymerization [95, 103]. Thus, assembly may rely on Ent5 alone, which contains two clathrin boxes, or on the cooperation of the three clathrin-boxes available when both Ent5 and
Gga2 are present. Our findings are similar to a recent study showing that acute inhibition of EpsinR, the closest ortholog of Ent5 in mammalian cells, inhibits clathrin coat assembly at a stage after clathrin recruitment but before disassembly [104]. However, these results and ours are also consistent with other models such as a role of Ent5 and EpsinR in disassembling clathrin coats. Therefore, resolving the exact molecular mechanism by which Ent5 and EpsinR interfere with traffic will require an *in vitro* system that monitors assembly and organization directly using these proteins.

A pivotal late-acting role may be a universal characteristic for epsin-related proteins. Loss of all three mammalian endocytic epsins (Eps1-3) stalls coat maturation at a stage after clathrin recruitment [100]. Similarly, loss of yeast endocytic epsins stall endocytosis at a stage long after clathrin is normally recruited [89]. This suggests epsins may have a universal role in the clathrin coat after clathrin recruitment. However, it is unclear if Ent5 and endocytic epsins perform the same molecular activity to promote coat maturation. Mammalian endocytic epsins appear to promote endocytosis by linking actin to the clathrin coat [100]. However, Ent5 lacks the sequences important for actin interaction, suggesting Ent5 is unlikely to act via this mechanism. On the other hand, endocytic epsins could promote a late stage by both actin binding and by stimulating clathrin assembly. Indeed, recent *in vitro* work demonstrates that isolated endocytic epsins are potent stimulators of clathrin assembly, suggesting that clathrin assembly is a primary function of epsins [102, 103, 105].

Although the data presented here clearly indicate a late acting and pivotal role for the clathrin binding motifs of Ent5, the functional importance of adaptor binding is less clear. The loss of adaptor binding by Ent5 impairs Chs3 traffic as much as loss of clathrin binding, yet loss of adaptor binding does not alter the lifespan of Gga2 structures. This means that although Ent5-
Gga2 interaction is important, it does not play a pivotal role in the maturation of the coat at the level detectable by fluorescence microscopy. One possible function of the Ent5-Gga2 interaction is to dictate how much Ent5 is recruited. Because Gga2 can bind both clathrin and Ent5 at the same time, it may act as a bridge to recruit more Ent5 than clathrin could recruit unaided. In support of such a model, substantially less Ent5ΔAB is recruited to Gga2 structures than wild-type Ent5. This reduced recruitment of Ent5 could impair traffic of Chs3 by reducing the amount of Ent5-Chs3 complexes in the coat. Alternatively, Ent5 binding to Gga2 may be required to productively couple clathrin polymerization to membranes. The requirement for productive coupling in coat assembly was demonstrated with in vitro assays of endocytic adaptors [105]. In these assays, the endocytic adaptor AP180 could promote clathrin cage assembly, but these cages lacked membrane. Intriguingly, Ent5 and AP180 share an N-terminal ANTH domain, which differs from the ENTH domain in epsin in its ability to bind lipids with an extended interface [24]. Thus, Ent5 may require Gga2 to productively couple clathrin assembly to the membrane. Resolving the molecular requirement for Ent5-Gga2 interaction will require examination of the formation of Ent5-Gga2 coats on liposomes.

These results also confirm a crucial role for the ANTH domain in Ent5 localization, consistent with previous reports [27]. Mutation of the ANTH domain disrupts Ent5 localization, suggesting that the ANTH domain is required for localization. However, how the ent5-CR mutation disrupts Ent5 localization is currently unclear. Although many ANTH domains bind phosphoinositides, they can also bind proteins [106, 107]. Thus, the ent5-CR allele could interfere with either lipid or protein interaction. We have been unable to show specific phosphoinositide binding defects caused by the ent5-CR mutation. However, in our hands Ent5 shows weak non-specific binding to many phosphoinositides, consistent with previous surface plasmon resonance and vesicle centrifugation analysis of Ent5 [108]. This may be because in
Resident Ent5 binds lipids only when associated with a co-factor similar to some endocytic ANTH domains, which require hetero-dimerization for efficient lipid binding [78]. Alternatively, the ent5-CR mutation may abolish an unknown protein interaction. However, the ability of the ANTH domain alone to localize further confirms that the ANTH domain plays a major role in Ent5 localization.

In summary, these results demonstrate that Ent5 plays a pivotal role late in clathrin coat formation. All known domains and motifs of Ent5 contribute to the function of Ent5 in Chs3 traffic. Furthermore, all three activities are required for maximal Ent5 recruitment to membranes, although the ANTH domain is the most important for localization. Clathrin binding appears to be uniquely important for the maturation of clathrin coats, whereas adaptor binding appears dispensable for coat maturation, but may be important for linking cargo to coats. Together with recent reports of key mechanistic roles for endocytic epsins, these results suggest that epsin family members are key mechanistic drivers of clathrin coat function.

**Materials and Methods**

**Yeast Strains and Plasmids**

Yeast strains are listed in Fig 3.1. Fluorescent tags and gene deletions were introduced by a standard PCR-based method [72]. Strains containing multiple genomic modifications were generated by standard yeast genetics. For point mutations, plasmids containing the genomic region of ENT5 were mutated using Quik-change mutagenesis (Stratagene). The resulting plasmids were linearized and transformed into SEY 6210, SEY 6211 and ent5Δ cells to replace the ENT5 locus. pSL6 and pSL6-box encoding wild-type or chc1-box mutant were described previously [96].
Quantitative Calcofluor White (CFW) Sensitivity Assay

To determine the effect of CFW on cells growth, log phase cells were diluted 100 fold into YPD media supplemented with different concentrations of CFW and then incubated at 30°C for 10 hours before measuring the optical intensity. Growth inhibition was calculated by normalizing to the absorbance reading of untreated cells. IC50 values, the half way between the maximal and the minimal inhibition, were derived by a sigmoidal dose-response curve (variable slope, four parameters) using GraphPad Prism.

Medias, Antibodies and Reagents

Yeast cells were grown in yeast/peptone (YP) media supplemented with 2% glucose (D) or synthetic media (SM) supplemented with 2% glucose and an amino acid mix [109]. Antibodies against Ent5 and Gga2 were described previously [110]. Antibodies against GFP and mRFP were from Santa Cruz. Alexa Fluor secondary anti-bodies were from Invitrogen (Carlsbad, CA). Peroxidase conjugate anti-bodies were from Sigma. ECL plus reagent was from Advansta. Calcofluor white fluorescent brighter and lyticase were obtained from Sigma.

Whole-Cell Yeast Extracts

To generate lysates, log phase cells were pelleted, resuspended in Laemmli sample buffer (2% SDS, 1% 2-mercaptoethanol), boiled, and lysed by glass-bead mechanical disruption. The lysates were collected after centrifugation. Following SDS–PAGE, samples were transferred to nitrocellulose, blocked with 4% milk in TBS-T(NaCl 137 mM, Tris-HCl 15.2 mM, Tris 4.54 mM, Tween 20 0.896 mM), and then probed with primary and fluorescent secondary antibodies. Fluorescence signals were detected on a Typhoon imaging system (Amersham Biosciences, Piscataway, NJ). Protein intensities were quantitated by ImageJ. To determine the correction factor for the antibody against Ent5, lysates of cell expressing GFP-Ent5 were collected. Following SDS-PAGE and immunoblotting procedures described above, parallel samples were
detected with antibody against Ent5 and GFP. The correction factor was calculated by determining the ratio between GFP and Ent5 signal.

**Immunoprecipitation**

For immunoprecipitation, spheroplasts were first generated by resuspending cells in 100 mM Tris-SO₄, pH = 9.5, 2% glucose and 5 mM DTT for 10 minutes. Cells were then resuspended in YP media supplemented with 0.5% glucose, 10 mM Tris-HCl, 1.2 M sorbitol and 120 units of lyticase. Cells were gently agitated for 30 min at 30 °C and then washed in 1.2 M sorbitol and resuspended in Buffer A (100 mM MES, pH 6.5, 0.5 mM MgCl₂, 1 mM EGTA) with protease inhibitors cocktail (Sigma). Cells were lysed by glass bead lysis, followed by the addition of 1% Triton X-100. The lysates were clarified by centrifugation at 13,000 rpm for 10 min at 4°C. The lysates were incubated overnight at 4°C with 100 ul of 20% protein A-agarose slurry and 3 ul of antibody. The lysates were washed three times with ice cold Buffer A, and the bound proteins were eluted and mixed with SDS sample buffer.

**Microscopy**

Prior to imaging, cells were grown to log-phase in SM supplemented with 2% glucose and amino acids. Cells were mounted on untreated coverslides for imaging. Images were collected as previously described with a Nikon Ti-E inverted microscope with a 1.4 NA, 100 x, oil immersion objective. The Lumencor LED light engine (472/20 nm for GFP and 543/20 nm or 575/20 nm for mCherry) was used for fluorophore excitation. Filters used for imaging (Chroma) – (1) FRET and high-resolution colocalization: dual-band excitation filter ET/GFP-mCherry (59002x) and excitation dichroic (89019bs), emission-side dichroic (T560lpxr), emission filters: ET525/50 m and ET595/50 m. The number of puncta per cell was quantitated by counting the number of foci in a single central plane as described previously [111].
Measurement of fluorescence intensities of endosomal puncta were conducted using a previously described method [112]. Briefly, a 6 x 6 pixel box was centered on the brightest four pixels of a manually selected spot in the micrograph. The pixels within this box were treated as the signal region. To estimate the background fluorescence, a larger 8 x 8 pixel box was placed concentrically with the signal region. The median value of pixel intensities in the outermost ring of this box was used as the background intensity, and subtracted from each pixel in the signal region. The fluorescence signal was then calculated as the sum of all 36 pixels in the 6 x 6 box. The above operations were accomplished using a custom graphical user interface written in MATLAB. Intensity analysis was performed on structures from a minimum of 50 cells. Data shown comes from analysis performed on a single day, intensity variance between days was minimal.

For time lapse microscopy, single plane images were taken every second for a total duration of 2 minutes. Non-overlapping structures from multiple cells were identified. The first frame in which structures were visible was considered T = 0. The lifespan was monitored until the median intensity dropped to background levels. Analysis was performed on structures from a minimum of 10 cells per genotype. Data shown comes from analysis performed on a single day. This data is representative of lifespans determined on more than three different days.

Acknowledgments

We thank A. P. Joglekar, K. S. Bloom and E. D. Salmon for access to and help with microscopes used in this study; S. K. Lemmon for reagents. We acknowledge members of Duncan laboratory for helpful comments and technical support. This work was supported by National Institutes of Health Research Grant GM-092741 (MCD)
FIGURES AND TABLES

Table 3.1. Strains and plasmids used in this study

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Figure 3.1: Loss of Ent5 disrupts traffic at the TGN and/or endosomes. A) Loss of Ent5 disrupts traffic of Chs3. Loss of Ent5 increases the sensitivity of cells lacking Chs6 to the cell wall binding toxin CFW. Chart shows IC50 for indicated cells. Error bars indicate standard deviation; p-values were determined by a student’s t-test. B) Loss of Ent5 disrupts localization of Tlg1-mcherry. Micrographs show Z-stack projections of Gga2-GFP, expressed from its endogenous locus, and Tlg1-mcherry, expressed from its own promoter on a 2-micron plasmid in wild-type and ent5Δ cells. Arrows indicate vacuoles visible in phase-contrast image. C) Loss of Ent5 reduces steady-state expression levels of Tlg1-mcherry. Lysates prepared from cells expressing Tlg1-mcherry from its own promoter on a 2-micron plasmid in wild-type cells and ent5Δ cells were subjected to immunoblot analysis. D) Loss of Ent5 does not alter the fluorescent intensity of Chc1-GFP structures and increases the fluorescent intensity of Gga2-GFP. Z-stack projections of indicated cells expressing Chc1-GFP and Gga2-GFP from their endogenous loci. E) Fluorescence intensity measurements of structures in C. Scatter-plots display mean value and SEM; p-values were calculated using a two-tailed Mann-Whitney U test for the null hypothesis.
that the medians were equal. Horizontal bars indicate median and interquartile ranges. Scale bars=5\(\mu\)m.
Figure 3.2: Loss of ENT5 prolongs coat lifespan. A) Representative images of structures analyzed by live cell microscopy. Boxed regions indicate representative structures selected for analysis. B) Kymographs of representative structures. Each time-frame is 3 seconds. C) Intensity vs. time plots of selected structures. Gray lines are the traces of each individual structure. Red lines correspond to spot1 and blue lines correspond to spot2 in B). Black lines are the average of 10 independent structures. D) Quantification of the lifespan of Gga2 containing structures (top) and classification of structures based on their lifespan (bottom). Scatter charts display mean value and SEM, horizontal bars indicate median and interquartile ranges. Data is representative of two independent experiments. p-values reflect a two-tailed Mann-Whitney test. Scale bar = 5µm
Figure 3.3: Schematic of Ent5 and mutations generated. A) Top. The ANTH domain is indicated in grey, charged patches predicted to be important for ANTH function are indicated as CP1 and CP2, adaptor binding sites are indicated as AB1 and AB2 and clathrin boxes are indicated as CB1 and CB2. Bottom. Residues mutated are underlined. For ent5-CR alignment with rat AP180 and CALM is shown. Residues important for phosphoinositide binding residues in AP180 and CALM are colored red, underlined residues were mutated to glutamic acid. For ent5ΔAB and ent5ΔCB alignment, the consensus sequence is shown (cons), underlined residues were mutated to alanine. B) CFW sensitivity of indicated mutants. p = 0.0034 for chs6Δ vs. ent5Δ, p = 0.0004 for chs6Δ vs. LB, p = 0.0374 for chs6Δ vs. AB, p = 0.0174 for chs6Δ vs. CB,
p = 0.0005 for chs6Δ vs. ABCB, p < 0.0001 for chs6Δ vs. WT. Error bars indicate standard deviation; p-values were determined using a student’s t-test. C) Z-stack projection of Ent5-GFP and Chc1-RFP in indicated mutants. D) Quantification of the number of Ent5-GFP puncta per cell in a central plane. E) Fluorescence intensity measurements of individual puncta in indicated cells. Scatter-plots display mean value and SEM, horizontal bars indicate median and interquartile ranges. Scale bar = 5 um. p-values indicated a two-tailed Mann Whitney test.
Figure 3.4: Clathrin is required for maximal interaction of Ent5 with Gga2 and localization of Ent5 to membranes. A) A representative immunoprecipitation reaction. GFP-tagged Ent5 was immunoprecipitated from cell lysates, and the immunoprecipitates were probed with antibodies to Ent5, Gga2, and clathrin to monitor the effect of Ent5 mutations on the Ent5-Gga2 and Ent5-clathrin interactions. Apparent size shift in mutant proteins may be due to differences in surfactant binding as previously observed for other mutations that alter charge [113-115] B) Quantification of the effects of Ent5 mutations on Ent5-Gga2 interaction or C) Ent5-Chc1 interaction n=3. Error bars indicate standard deviation; p-values determined with students t-test. D) Chc1-box mutation blocks interaction of Ent5 with clathrin. Clc1 was immunoprecipitated
from lysates of cells lacking *CHC1* transformed with plasmids that contain wild type *CHC1* or *chc1Δbox*. E) Live cell imaging of *CHC1* deleted cells transformed with plasmids that contain wild type *CHC1* or *chc1Δbox* mutant. Scale bar is 5 µm.
Figure 3.5: Mutation of clathrin-binding or adaptor-binding domains affect the lifespan of Ent5-Gga2 structures and the timing of the recruitment of Ent5. A) Representative images of the events analyzed. RD = recruitment differential. The region highlighted by the box indicates area in associated kymograph. Each frame is 1s. Bi) Quantification of the lifespan of Gga2 in indicated Ent5 mutants. Bii) Each event was classified based on its lifespan. C) Quantification of the recruitment differential and D) colocalization time in indicated Ent5 mutants. Ei) Quantification of the lifespan of Gga2 in indicated mutants. Eii) Each event was classified based on its lifespan. Scatter-charts display mean value and SEM, horizontal bars indicate median and interquartile ranges. p-values reflect a two-tailed Mann-Whitney test.
Figure 3.6: Model of coat assembly. 1) Gga2 accumulates on membranes and recruits clathrin. 2) Ent5, initially recruited via its ANTH domain, is stabilized by interaction with clathrin and Gga2. This allows the recruitment of Ent5-specific cargo. 3) Ent5 stimulates the formation of the transport carrier possibly by inducing clathrin polymerization or through forming a sub-clathrin coat. Gga2 helps increase the amount of Ent5 in the carrier either through initial recruitment of more Ent5 or, as illustrated, by Gga2 indirectly linking Ent5 to the clathrin coat.
Supplemental Figure 3.1: Mutations in Ent5 do not affect the steady state protein expression levels of Ent5. A) Antibody raised against Ent5 has reduced affinity for AB mutation. Cell lysates were prepared from cells expressing GFP-tagged Ent5 or Ent5 mutations from the endogenous locus. Lysates were analyzed by immunoblotting with antibodies to Ent5, GFP or Adh1 as a lysis control. B) Relative antibody affinity correction determined from ratio of signal from GFP antibody compared to Ent5 antibodies from three independent extraction samples. C) When correction factor is applied and normalized to extraction control Adh1, all mutants are expressed at near wild-type levels (bottom). D) Quantitative calcofluor white assay revealed that GFP tag does not interfere with protein function.
REFERENCES


CHAPTER 4. ENERGY METABOLISM REGULATES CLATHRIN ADAPTORS AT THE TRANS-GOLGI NETWORK AND ENDOSOME

Introduction

Many cells use nutrients as both the building blocks for new growth and as signaling molecules to regulate developmental programs [1-4]. The yeast *Saccharomyces cerevisiae* responds to glucose with a distinct developmental program that maximizes biomass increase at the expense of surrounding cells (reviewed in [5]). A key facet of this program is aerobic glycolysis. In aerobic glycolysis, glucose metabolism stops at the end of glycolysis and glycolytic products are not metabolized to carbon dioxide in the mitochondria (reviewed in [6]). This program has two goals. First, cells use the carbon derived from glucose to promote biomass increase. Second, cells secrete the end product of glycolysis, ethanol, to inhibit growth of other microorganisms (reviewed in [7]). To achieve these goals, yeast must actively inhibit mitochondrial respiration when glucose is abundant. However, upon glucose starvation, yeast must shift out of this exploitative developmental program, reactivate the mitochondria, and produce proteins required to survive in the new environment of scarcity [8].

The adaptation to glucose starvation is particularly challenging because the developmental program induced by glucose inhibits the synthesis of proteins that are required to use other energy sources. Furthermore, the cell does not retain reserve pools of glucose [9].

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Therefore, immediately upon glucose starvation, the cell experiences a severe energy deficit [10]. Yet it must synthesize new proteins and activate inhibited pathways in order to be able to harness available energy sources such as ethanol, proteins, and lipids. The cell’s immediate responses to glucose starvation must therefore facilitate the escape from of this energy deficit.

A transient inhibition of endosomal protein traffic likely contributes to energy conservation to counteract the energy deficit immediately following acute glucose starvation [11]. In proliferating cells, membrane trafficking pathways consume large amounts of energy to maintain the steady state localization of proteins within the endosomal system (reviewed in [12]). Such trafficking pathways work via multiple rounds of anterograde traffic followed by retrograde traffic to retrieve components back to the original organelle. Clathrin is a major player in this repeated cycling of proteins between the trans-Golgi network (TGN) and endosomes [13-15]. In this function, clathrin depends on clathrin adaptors that recruit it to the membrane. In yeast, there are at least five adaptors at the TGN and endosomes: the monomeric Golgi localized γ-adaptin proteins 1 and 2 (Gga1 and Gga2), the epsin related proteins Ent3 and Ent5 and the clathrin adaptor protein complex 1 (AP-1) (reviewed in [16]). We previously found that clathrin adaptors immediately but transiently disassociate from membranes upon glucose starvation [11]. This delocalization causes a transient stoppage of clathrin dependent traffic, which conserves energy. Although the functional significance of this transient stoppage of membrane traffic is apparent, it is unclear how the cell elicits it.

Protein Kinase A (PKA) is the primary effector of the glucose induced developmental program in yeast [17]. Our previous studies implicated PKA in the transient stoppage of endosomal traffic. However, its role appears to be indirect [11]. In cells with defects in PKA, adaptors remain membrane associated during glucose starvation. Surprisingly, acute chemical
inhibition of PKA does not alter endosomal traffic. Only if PKA is inhibited for a prolonged period prior to starvation, is the adaptor localization response altered. These results suggest that PKA acts indirectly on endosomal traffic [11]. Thus an unknown mechanism that is downstream of PKA must direct the transient stoppage of membrane traffic during glucose starvation.

To investigate the responses of endosomal traffic to glucose starvation in yeast, we took a genetic approach. This approach revealed that the AMP-activated kinase (AMPK) and glucose repression machinery are required for normal responses of clathrin adaptors to glucose starvation. We used chemical perturbations and an in vitro recruitment assay to demonstrate that ATP levels directly or indirectly regulate clathrin adaptor localization. Our findings suggest that endosomal traffic rates are coordinated with available cellular energy during the transition from proliferation to quiescence.

Results:

AMP-activated kinase (AMPK) is required for adaptor localization in prolonged starvation.

In response to glucose starvation, clathrin dependent traffic at the TGN and endosomes is transiently blocked. All clathrin adaptors at the TGN and endosomes immediately redistribute to the cytosol. Only after 30 minutes or more of continuous starvation, do the adapters partially regain their normal punctate localization reflecting association with membranes of the TGN and endosomes ([11] and Supplemental figure 4.1). We also demonstrated that these changes in localization reflect loss of adaptor association with membranes and not disassembly of the organelles [11]. Delocalization of adaptors prevents clathrin dependent traffic and thus prevents energy consumption by the clathrin dependent cycling of proteins between the TGN and endosomes. However, our previous findings did not reveal the molecular mechanism by which glucose starvation regulates adaptor function.
We previously found that Ent5 becomes hyper-phosphorylated at the same time that adaptors relocalize to membranes during prolonged starvation [11]. This hyper-phosphorylation is easily identified with immuno-blot analysis (Figure 4.1A arrowhead). Although the functional significance of this phosphorylation is still unknown, mutations that prevented the immediate delocalization of adaptors during glucose starvation also prevented hyper-phosphorylation [11]. Therefore, to identify additional regulators of adaptors, we screened for gene deletions that prevented Ent5 hyper-phosphorylation during glucose starvation using a focused library of strains lacking kinases or kinase co-factors. We performed immuno-blot analysis of lysates from cells from this library to identify gene deletions that altered Ent5 hyper-phosphorylation after prolonged starvation (not shown).

Using this approach, we found that mutation of several proteins in the AMP-activated kinase (AMPK) pathway gave strong defects in Ent5 hyper-phosphorylation (Figure 4.1A). AMPK consists of a core catalytic alpha-subunit and one of three partially redundant beta-subunits which contribute substrate specificity (reviewed in [18]). AMPK also requires activating phosphorylation by one of three upstream kinases. In the screen, we found that deletion of GAL83, a beta subunit, blocked Ent5 hyper-phosphorylation. We also found that deletion of the primary upstream activating kinase, SAK1, caused partial defects (Figure 4.1A arrowhead). In our initial screen, the core alpha-catalytic subunit (SNF1) was not identified as required for hyper-phosphorylation. However, strains from deletion library collection that we used to make the focused library may contain errors or have second site suppressor mutations. Therefore, we generated a strain carrying a complete deletion of SNF1. In this strain, Ent5 hyper-phosphorylation was inhibited (Figure 4.1A). Thus, the AMPK pathway is required for Ent5 hyper-phosphorylation.

To determine if Snf1 regulates adaptor localization response, we investigated adaptor
localization in snf1Δ cells. We chose Gga2 and Ent5 as representatives of the two functional modules of clathrin dependent TGN-endosome traffic: the Gga/Ent3 module and the AP-1/Ent5 module. These two modules have different requirements for localization and act at different stages in transport within the TGN and endosomes [19-24]. Furthermore, the Gga/Ent3 module promotes the recruitment of the Ent5/AP-1 module [22, 23]. Deletion of SNF1 did not dramatically alter adaptor localization in the presence of glucose or adaptor redistribution to the cytosol in the acute phase of glucose starvation (Figure 4.1B, C). However, deletion of SNF1 caused dramatic changes in adaptor localization after prolonged starvation. In wild-type cells, Gga2 and Ent5 accumulate in large puncta after prolonged starvation. In cells lacking Snf1, Gga2 accumulated in many very small puncta and Ent5 was found in only a few small puncta (Figure 4.1B, C). These results indicate that Snf1 is required for proper localization of Gga2 and Ent5 during the prolonged phase of glucose starvation.

Snf1 is a major regulator of cell physiology during glucose starvation. It can phosphorylate several proteins involved in membrane traffic, raising the possibility that Snf1 may directly regulate adaptor localization [25]. However, Snf1 should be activated rapidly upon glucose starvation, but, relocalization of Gga2 and Ent5 is delayed until 30 minutes after the onset of starvation [11, 26, 27]. A temporal difference in Snf1 activation and adaptor relocalization could mean that Snf1 acts indirectly in adaptor localization. In order to determine whether Snf1 activation coincided temporally with adaptor relocalization in our starvation conditions, we monitored Snf1 activation during glucose starvation. To monitor activation, we used an antibody that recognizes the active phosphorylated form of Snf1. Prior to glucose starvation, no active Snf1 was detected (Figure 4.1D). Within 5 minutes of starvation, Snf1 was phosphorylated and remained phosphorylated during the two hour time course. Because Snf1 is activated substantially before adaptors relocalize to punctate structures, the effect of Snf1 on adaptors is likely indirect.
Glucose repression is required for adaptor relocalization in acute starvation

We previously identified another glucose responsive kinase, PKA, as an indirect regulator of adaptor localization during glucose starvation [11]. PKA and Snf1 converge on the regulation of several glucose responsive pathways, including opposing regulation of glucose repression [17]. Glucose repression is the suite of changes in transcription and cell physiology that optimizes the cell to exclusively derive energy from the glycolysis of glucose or fructose [28]. Glucose repression is induced by the activity of PKA [29]. During glucose starvation, glucose repression is inactivated by Snf1[30]. We previously discovered that PKA activity in the presence of glucose is required for adaptor delocalization during the acute phase of glucose starvation [11]. Our finding that Snf1 is required for adaptor relocalization during the prolonged phase of glucose starvation, suggested that the two pathways were playing opposing roles in adaptor localization. Because PKA and Snf1 play opposing roles in both adaptor localization and in glucose repression, we hypothesized that adaptor localization was linked to the glucose repression program.

To investigate this hypothesis, we grew cells in carbon sources that activate glucose repression (repressing) or inactivate glucose repression (derepressing). In cells grown in the repressing carbon sources, glucose and sucrose, carbon starvation caused an immediate delocalization of Gga2 and Ent5 to the cytosol (Figure 4.2A, B). In contrast, in cells grown in the derepressing carbon sources, galactose, raffinose and glycerol/ethanol, starvation did not dramatically alter the localization of Gga2 or Ent5 (Figure 4.2 A, B). This result suggests that the starvation induced redistribution of adaptors depends on glucose repression.

To further test the role of glucose repression in adaptor redistribution, we investigated adaptor redistribution in cells lacking proteins required to establish glucose repression. The phosphatase regulatory subunit, Reg1, and the 14-3-3 protein, Bmh1, are required to establish
glucose repression [31]. We found that cells in lacking Reg1 or Bmh1 the number of punctate structures containing adaptors was unchanged by glucose starvation (Figure 4.3). This result further demonstrates that glucose repression is a necessary prerequisite for starvation induced redistribution.

**Energy metabolism is required for adaptor localization**

One of the major effects of glucose repression upon glucose starvation is on ATP production. The glucose repression program maintains aerobic glycolysis as the only pathway for energy generation—inhaling all other modes of energy generation [32]. In the absence of parallel ATP generation pathways, ATP levels drop precipitously immediately following glucose starvation [33]. Because of the link between glucose repression and adaptor localization, we hypothesized that adaptor redistribution was linked to energy generation. To test this hypothesis, we inhibited respiration with the cytochrome C reductase inhibitor antimycin A in cells grown under different conditions. In the absence of mitochondrial function, cells can produce ATP via glycolysis with six carbon sugars (glucose, galactose or raffinose). In cells grown in glucose, galactose or raffinose, antimycin A had no dramatic effect on adaptor localization (Figure 4.2A, B). This suggests that antimycin does not influence adaptors in cells that can generate energy via glycolysis. In cells pre-adapted to glycerol/ethanol, addition of antimycin A did not cause redistribution of adaptors to the cytosol (Figure 4.2A, B). This result may reflect the ability of these cells to store and metabolize carbohydrates such as glycogen and trehalose [34]. Together these results suggest that continued energy production is required for adaptor localization.

As a further test of this hypothesis, we treated cells that were pre-adapted and then starved for galactose or raffinose with antimycin A. Cells starved for galactose or raffinose generate ATP via respiration, thus the addition of antimycin A reduces ATP levels in these cells. In cells that had been pre-adapted and then starved for either galactose or raffinose, adaptors
redistributed to the cytosol immediately upon addition of antimycin A (Figure 4.2A,B). In cells
pre-adapted to glycerol/ethanol then starved and treated with antimycin, we saw a reduction in
the number of adaptor puncta, however delocalization was not as complete as with other
conditions. This may reflect the ability of these cells to generate ATP by glycolysis of glycogen
and trehalose. These results suggest that adaptor localization is closely correlated to steady-state
maintenance of energy levels.

To verify that changes in ATP concentration paralleled the changes in adaptor
localization, we monitored ATP concentration in cells grown in different carbon sources and
then starved. As expected, ATP concentration dropped more than 50% in cells grown in glucose
or sucrose and then starved for the carbon source (Figure 4.4A). In contrast, ATP concentration
remained high upon starvation of cells grown in galactose or raffinose. This observation is
consistent with the known effects of carbon source on oxidative phosphorylation.

In cells grown in glycerol/ethanol, measured ATP concentrations were lower than in the
other carbon sources. Furthermore, the levels after starvation were substantially lower than in
unstarved cells. This was unexpected based on the continued localization of adaptors under these
conditions. One possible explanation is that glycerol/ethanol grown cells may have a lower
volume of cytoplasm and this causes an underestimation of actual ATP concentrations.
Cytoplasmic volume depends on both the size of the cell and the volume of the cytoplasm
occupied by the vacuole and other ATP impenetrable organelles. We approximated total cell
volume with optical density. Glycerol/ethanol grown cells are smaller overall and have more
mitochondria and larger vacuoles, thus they are expected to have a lower cytoplasmic volume
than glucose grown cells [35, 36]. The ATP levels measured therefore likely underestimates ATP
concentrations for the glycerol/ethanol grown cells. However, even with this underestimation,
the measured ATP concentration of glycerol/ethanol starved cells were still 10% higher than
those in cells starved for repressing sugars (Figure 4.4 A, p<0.01). Thus, overall these results show that adaptor delocalization coincides with low ATP concentration in cells.

We next examined ATP concentrations in cells with characterized defects in adaptor localization during glucose starvation. Cells lacking functional PKA or Reg1 fail to induce adaptor delocalization in the acute phase of glucose starvation ([11] and Figure 4.3A). To modulate PKA activity, we used a strain carrying Tpk1-as, an analog-sensitive allele of the PKA catalytic subunit Tpk1, in which PKA activity can be inhibited by the kinase inhibitor analog 1NM-PP1. In this strain two of the three alternate catalytic subunits (TPK2 and TPK3) are deleted, and the third (TPK1) is mutated such that it can be inhibited by the kinase inhibitor analog 1NM-PP1. When Tpk1-as cells were treated with 1NM-PP1 for 1 hour prior to starvation, the ATP concentration did not change upon starvation (Figure 4.4B). This is in contrast to control DMSO treated cells, where the ATP concentration dropped upon glucose starvation. Similarly, in cells lacking Reg1, ATP concentration dropped only 30%. This change was significantly smaller than the 50% reduction of ATP concentration in wild-type cells (Figure 4.4C, p<0.01). These findings are consistent with the ability of 1NM-PP1 TPK1-as treated cells and reg1Δcells to generate ATP by mitochondrial respiration using non-glucose substrates. In contrast, in cells lacking Snf1, ATP concentration dropped 80% after glucose starvation, a level that was significantly lower than that of wild-type cells (Figure 4.4D, p<0.01). Together these results show that low ATP concentrations parallel changes in adaptor delocalization and that mutations that prevent adaptor delocalization prevent low ATP concentrations in the acute phase of glucose starvation.

To further investigate the coincidence between ATP concentration and adaptor localization, we monitored ATP concentration during a time course of starvation. In the first five minutes of glucose starvation, ATP concentration dropped to 20% of the level before starvation. The concentration rose to 35% within 10 minutes and continued to rise over the next 60 minutes
(Figure 4.5A). By 30 minutes, the time-point when adaptors return to membranes, ATP concentration was up to 40% of the pre-starved level. In contrast, in cells lacking Snf1, ATP concentration dropped to 10% of pre-starved and never rose above 30% in the 60 minute time course (Figure 4.5A). Thus the adaptor recruitment is coincident with ATP concentrations above 40% of pre-starved cells.

Next, we directly manipulated cellular ATP concentration and monitored adaptor localization. In de-repressed starved cells, antimycin A caused a concentration dependent reduction in ATP concentration (Figure 4.5B). Reduction of ATP concentration to less than 20% of replete concentration caused a nearly complete redistribution of adaptors to the cytosol (Figure 4.5C, second column). When ATP concentrations were at or above 40% of replete, adaptors were recruited to membranes. Interestingly, the effect was dose dependent. At lower concentrations of ATP, fewer punctate structures were observed than at higher concentrations of ATP (Figure 4.5D). Thus adaptor localization is closely correlated with cellular ATP concentration.

**Addition of ATP is sufficient for adaptor localization**

ATP is a ubiquitous molecule in cells. It is required for the proper functioning of motors, kinases and other enzymes, for pH and ion homeostasis and to maintain levels of GTP. Any one of these functions may directly or indirectly impact adaptor localization. To explore possible energy dependent mechanisms, we developed a permeabilized cell assay of adaptor localization. To prevent ATP generation, we antimycin A treated glucose starved cells prior to permeabilizing the cells. After permeabilization, Gga2-GFP could be seen as many small puncta, similar to its appearance in glucose starved snf1Δ cells. Ent5 was diffusely localized. Addition of ATP to permeabilized cells caused both adaptors to rapidly redistribute into a few large puncta that resembled their localization on organelles in intact cells (Figure 4.6A). Thus ATP can induce adaptor redistribution. Furthermore, because permeabilized cells cannot maintain pH or ion
gradients, cytosolic pH or ion concentrations can be excluded as the mechanism by which ATP regulate adaptors.

To determine whether adaptors were recruited to organelles, we performed co-localization analysis. Due to difficulties visualizing the weak mCherry fluorescence of mCherry-tagged proteins in permeabilized cells using a confocal microscope, we used total internal reflection microscopy (TIRF) for this analysis. Although TIRF only allows visualization of structures within close proximity of the cell surface, some Ent5 positive organelles are close enough to the cell surface to be captured via this method. Organelles were marked with Sec7-mCherry. Sec7 shows partial co-localization with Ent5 in intact cells in the presence of glucose and during prolonged starvation ([22] and Supplemental Figure 4.1). Importantly, Sec7 does not delocalize upon glucose starvation, therefore it is a reliable marker of organelles even when adaptors are delocalized [11]. When observed with TIRF microscopy, Ent5 appeared diffuse in starved permeabilized cells. In contrast, Sec7 was apparent in bright puncta in the permeabilized cells (Figure 4.6B). Upon addition of ATP, Ent5 appeared in punctate structures. As in intact cells, some of the Ent5 punctate structures co-localized with Sec7. These results indicate that ATP directs adaptor recruitment to organelles in permeabilized cells.

The analysis of adaptor redistribution in intact cells suggested that Snf1 acts indirectly to regulate adaptor localization via energy metabolism. To further test this model, we performed ATP add back experiments in permeabilized cells lacking Snf1. We found that ATP induced adaptor puncta even in permeabilized cells lacking Snf1 (Figure 4.6C). Because ATP can bypass the requirement for Snf1, this finding further supports our earlier conclusion that Snf1 regulates adaptor localization indirectly, via its roles in energy metabolism.

We next examined the ability of other nucleotides to induce adaptor localization in permeabilized cells (Figure 4.6A). Addition of GTP caused minor changes in Gga2 and Ent5
localization. However, ATP-γ-S and GTP-γ-S caused little change in either Gga2 or Ent5 localization. This suggests that full adaptor localization requires ATP hydrolysis and suggests a possible role for GTP in adaptor localization.

To further explore the role of nucleotides on adaptor localization, we examined adaptor localization in cells treated with different concentrations of ATP. For this analysis, we classified cells as having no puncta, small puncta or large puncta. In cells expressing Gga2-GFP without exogenous ATP, no cells contained large puncta, 75% of cells had small puncta and 25% had no puncta (Figure 4.7A). Addition of 5 or 10mM ATP caused an increase in the number of cells with large puncta, and in the number of cells with small puncta and a decrease of the number of cells with no puncta of Gga2. Increasing ATP from 10mM to 15mM caused a dramatic increase in the number of cells containing large puncta of Gga2. Thus Gga2 shows a dose-dependent response to ATP.

Ent5 also shows a dose-dependent response to ATP. In cells expressing Ent5-GFP without exogenous ATP, no cells contained large puncta, 38% of cells had small puncta and 62% had no puncta (Figure 4.7B). Addition of 5 mM ATP caused an increase in the number of cells with large puncta, and in the number of cells with small puncta and a corresponding decrease in the number of cells with no puncta. In the presence of 10 mM ATP, 65% of cells contained large puncta and only 4 percent contained small puncta. Increasing ATP from 10 mM to 15 mM ATP further increased the number of cells containing large puncta to 95%. Thus similar to Gga2, Ent5 shows a dose-dependent response to ATP. Furthermore, ATP induces more cells to contain large puncta of Ent5 than large puncta of Gga2 at all concentrations of ATP tested. This difference suggests that, similar to intact cells, Gga2 and Ent5 have different requirements for localization in permeabilized cells.
We next examined the effects of different concentrations of GTP on adaptor localization. We found that addition of 5, 10 or 15 mM GTP caused 10-16% of cells to contain large puncta of Gga2 (Figure 4.7A). This increase in large puncta compared to cells with no nucleotide was coincident with a slight decrease in the number of cells with small puncta and in the number of cells with no puncta. In contrast, for Ent5 addition of 5 or 10 mM GTP only increased the number of cells with small puncta, it had no effect on the number of cells with large puncta of Ent5 (Figure 4.7B). However, increasing GTP from 10 mM to 15 mM caused 10% of the cells to contain large puncta of Ent5. This increase was coincident with a decrease in the number of cells with small puncta. Thus although Gga2 and Ent5 show dose-dependent responses to GTP, the effect of GTP is weaker than the effect ATP on both adaptors.

The finding that GTP can induce weak adaptor localization could be explained by a role of GTP in adaptor localization or secondary production of ATP by the transfer of the gamma phosphate from GTP to ADP in the permeabilized cells. To distinguish between these two possibilities, we examined whether GTP and ATP acted synergistically. We treated cells with combinations of 5 mM or 10 mM ATP and 5 mM or 10 mM GTP. Gga2 localization in large puncta was increased by the addition of both GTP and ATP (Figure 4.7A). This effect was most pronounced for cells treated with 10mM GTP and 10mM ATP. This combination increased the number of cells containing large puncta of Gga2 from 16% in the presence of 10 mM ATP alone to 47% in the presence of 10 mM of ATP and GTP. GTP-γ-S was more effective than GTP in inducing large puncta of Gga2. In cells treated with 10 mM ATP and 10 mM GTP-γ-S, 75% of cells had large puncta of Gga2 (Figure 4.7A chart, C). Because GTP-γ-S is a poor substrate for gamma transfer reactions, this finding argues that the generation of ATP does not contribute to the synergy of GTP and ATP on Gga2 localization [37]. Together these results suggest that ATP and GTP act together to promote Gga2 localization.
The effect of GTP on Ent5 localization in ATP treated cells was even more substantial. Addition of 10 mM GTP to cells treated with 5 mM ATP increased the number of cells containing large puncta of Ent5 from 34% to more than 80% (Figure 4.7B). Furthermore, 10 mM GTP-γ-S was as effective as 10 mM GTP on the formation of large Ent5 puncta in cells treated with 5 mM ATP (Figure 4.7B chart, C). Together these results suggest that as with Gga2, ATP and GTP act synergistically to promote Ent5 localization. Thus GTP and ATP both contribute to adaptor localization in permeabilized cells.

Although the concentration of nucleotide required for these effects were relatively high, the maximal ATP concentration used is only five times higher than current estimates of physiological ATP concentrations in yeast [38]. Furthermore, without an ATP/GTP regenerating system, nucleotides may be rapidly consumed. Thus, the ATP responses observed may be induced by ATP concentrations equal to endogenous ATP concentrations. Alternatively, high nucleotide levels may be required because key factors such as GTP exchange factors are more dilute in the permeabilized cells. If the substrates or cofactors are more dilute, this would require higher concentrations of nucleotide for the same effect.

Upstream regulators of adaptors are differentially regulated by energy metabolism

Neither Gga2 nor Ent5 has any obvious GTP or ATP sensing domains. Therefore, the regulation of adaptor localization is likely mediated by one or more upstream factors. To begin to characterize these factors, we first determined whether known regulators of Gga2 and Ent5 respond to ATP concentration. Several nucleotide binding proteins are known to act upstream of adaptors, including the functionally interchangeable small GTPases Arf1 and Arf2, the PI4 kinase Pik1 and its product PI4p. Although Pik1 is known to redistribute in cells starved for glucose, the kinetics of this delocalization were unknown [39, 40]. We investigated the changes in localization of the major Arf isoform, Arf1, Pik1 and the PI4p binding domain from the
human GOLPH3 proteins during glucose starvation. In the presence of glucose, Arf1-GFP, GFP-Pik1 and GFP-GOLPH3 all localized to many bright punctate structures in cells (Figure 4.8). Immediately upon glucose starvation, all three lost bright punctate staining. GFP-Pik1 became completely diffuse, whereas both Arf1 and GOLPH3 retained localization to dim punctate structures. These results show that the majority of Arf1 and Pik1 are rapidly delocalized during glucose starvation and that the levels of PI4p at the TGN and endosomes drop dramatically.

Upon prolonged glucose starvation, when Gga2 relocalized to several bright punctate structures, Pik1 was not found in bright punctate structures. Arf1 and GOLPH3 punctate structures remained dim. Notably, the number of ARF went up only slightly and GOLPH3 puncta did not change upon prolonged starvation (Figure 4.8D). These results show that unlike the adaptors, the localization of Arf1, Pik1 and GOLPH3 do not show adaptation in the form of increased membrane localization during prolonged glucose starvation.

We next investigated the co-localization of Arf1 and PI4p with Gga2. In the presence of glucose, Arf1 and GOLPH3 show partial co-localization with Gga2. During prolonged starvation, Arf1 and GOLPH3 also partially co-localized with Gga2. We observed three types of Arf containing structures: those that contained both Arf1 and Gga2, those that contained only Arf1 and those that contained only Gga2 (Figure 4.8A). This partial colocalization is consistent with partial co-localization seen in in glucose replete cells. In contrast, for GOLPH3, we saw only two types of structures: those that contained both GOLPH3 and Gga2 and those that contained only Gga2 (Figure 4.8C). Gga2 is thought to activate the formation of PI4p in glucose replete cells [22]. Thus, the partial co-localization of GOLPH3 and Gga2 in glucose starved cells may indicate Gga2 plays a similar role in influencing PI4p levels in glucose starved cells. Taken together these results show that glucose starvation clearly reduces the levels of Arf1 and PI4p in
the endosomal system; however their ability to localize to organelles that recruit Gga2 is unchanged.

We next investigated whether ATP induces changes in the localization of Arf1, Pik1 or PI4p in permeabilized cells. In glucose starved permeabilized cells, Arf1 localized to a few dim puncta whereas GOLPH3 and Pik1 were diffuse (Figure 4.9A). Addition of ATP caused a slightly higher number of dim Arf1 puncta. In contrast, ATP immediate induced formation of many bright GOLPH3 or Pik1 puncta. Thus ATP addition does not have a prominent effect on Arf1 localization, but it can direct dramatic changes in PI4p and Pik1 localization.

We next investigated the effects of GTP and GTP-γ-S on Arf1, Pik1 and PI4p (Figure 4.9A). As with ATP, the localization of Arf1 was not substantially increased by either GTP or GTP-γ-S. In contrast, both Pik1 and GOLPH3 localized to puncta after the addition of GTP. However, the number of puncta per cell induced by GTP was less than the number induced by ATP (Figure 4.9 B). GTP-γ-S was even less effective at recruiting Pik1 and GOLPH3 than was GTP. Both Pik1 and GOLPH3 formed fewer puncta in the presence of GTP-γ-S than in GTP. Furthermore, the number of cells with large puncta were lower in the presence of GTP-γ-S than in GTP (Figure 4.9C). Together these results show that Arf1 localization is largely non-responsive to nucleotides. In contrast, Pik1 and GOLPH3 localization is induced by ATP, partially by GTP and weakly by GTP-γ-S.

**Arf1 and PI4p are required for adaptor localization during glucose starvation**

To investigate if adaptor localization depends on Arf, Pik1 and PI4p during glucose starvation, we first tested if Arf is required for adaptor localization in glucose starvation. To rapidly inhibit Arf function, we used the lactone antibiotic brefeldin A. Brefeldin A inhibits several Arf-GEFs. Inhibition of these GEFs leads to rapid loss of active Arf at select locations (reviewed in [41]). In cells treated with brefeldin A in the presence of glucose, both Gga2 and
Ent5 localized to a few large puncta per cell (Figure 4.10A, Supplemental Figure 4.2). This suggests that some Gga2 and Ent5 structures are independent of a brefeldin A sensitive GEF in the presence of glucose. In contrast, during prolonged starvation both Gga2 and Ent5 to became diffuse within minutes of brefeldin A treatment (Figure 4.10A). Thus the localization of both Gga2 and Ent5 depend on Arf during glucose starvation.

We next investigated whether Pik1 is required for adaptor localization during glucose starvation. To modulate Pik1 activity, we used a previously described temperature sensitive pik1-83 allele [42]. As previously described, in the presence of glucose, the pik1-83 allele did not alter Gga2 localization (Figure 4.10 B, Supplemental Figure 4.2 and [22]. In contrast, Gga2 became diffuse when cells were shifted to the non-permissive temperature during prolonged starvation. These results suggest Gga2 localization requires Pik1 activity during starvation.

Ent5 localization was highly dependent on Pik1 activity under all conditions tested (Figure 4.10 B, Supplemental Figure 4.2). In the presence of glucose, Ent5 became diffuse upon shift to the non-permissive temperature, confirming previous results that Ent5 requires Pik1 for localization [22]. Notably, even at permissive temperature, Ent5 did not localize to bright puncta in the pik1-83 cells during prolonged glucose starvation. This result suggests that pik1-83 is not fully functional in glucose starved cells and that Ent5 requires full Pik1 activity for localization during glucose starvation. Together these results suggest that Ent5 localization depends on Pik1 activity both in the presence and absence of glucose.

PI4p levels are also regulated by the lipid phosphatase Sac1 which redistributes to the TGN during glucose starvation [40, 43]. To investigate the role of Sac1 relocalization in adaptor localization during glucose starvation, we monitored Gga2 and Ent5 localization in cells lacking Sac1 (Figure 4.10 C, Supplemental Figure 4.2). In these cells, Gga2 mostly became diffuse following glucose starvation, although some dim Gga2 puncta persisted. Such dim puncta were
not seen in wild-type cells. In contrast, Ent5 became diffuse immediately after glucose starvation, and then relocalized to bright puncta during prolonged starvation. These results suggest Sac1 has a minor role in Gga2 relocalization but not Ent5 during glucose starvation.

**Discussion:**

**Glucose repression machinery regulates adaptors indirectly**

Glucose starvation causes immediate and transient responses in several cellular processes [10, 11, 33]. Until now, how the cell communicated glucose starvation to induce these transient responses. In this study, we characterized cellular energy as the mechanism leading to the transient delocalization of clathrin adaptors during glucose starvation. Our previous findings that PKA regulates adaptor localization in glucose starvation are explained by its role in cellular metabolism. Previously, we found that inhibition of PKA prevented adaptor redistribution in response to glucose starvation [11]. However, in order to alter adaptor redistribution, PKA had to be inhibited for an extended period prior to starvation. The need for extended inhibition suggested PKA acts indirectly. Based on our current findings, the extended PKA inhibition allows glucose de-repression and metabolic remodeling to occur even under glucose replete conditions. Therefore, ATP concentration does not drop low enough upon glucose starvation to cause adaptor delocalization. The role of Snf1 is similarly indirect. The delay between Snf1 activation and adaptor relocalization can be explained by the role of Snf1 in activation of respiration. Adaptors fail to relocalize in cells lacking Snf1 because, in the absence of both glycolysis and respiration, ATP concentration remain low. Thus the unknown “factor” that regulates adaptor localization downstream of PKA and Snf1 is mitochondrial function.
Energy regulates adaptor localization at multiple steps

A multistep model of adaptor recruitment emerges from these studies (Figure 4.11). Our current findings are consistent with these steps occurring concurrently or in various orders. We propose that Arf acts early. Our finding that a five minute treatment with Brefeldin A eliminates adaptor localization during glucose starvation, argues that Arf plays a key role in the process. However, Arf1 localization is not biphasic in response to glucose starvation and is unaffected by either ATP or GTP. This suggests that Arf localization is not regulated by energy levels. This finding is consistent with the ability of purified Arf1-GDP to associate with membranes [44]. We propose that during glucose starvation a small pool of Arf remains associated with membranes, when energy levels rise it is already positioned to help recruit adaptors.

A subsequent step is the Arf-GTP dependent recruitment and activation of Pik1 (Figure 4.11, Step 1). In permeabilized cells, we find that GTP induces robust recruitment of Pik1 in permeabilized cells. Although we do not see localization of GFP-Pik1 at membranes in starved cells, the requirement for Pik1 in intact cells strongly argues that Pik1 is active at the TGN and endosomes in glucose starved cells. This apparent contradiction may be explained by competition from endogenous untagged Pik1 in the strains used. We propose that Arf1 likely helps recruit and activate this pool of Pik1. This role is supported by reduced levels of PI4p in cells lacking Arf1 [45], and the GTP responsiveness of Pik1 localization in permeabilized cells.

The next step is the ATP dependent synthesis of PI4p (Figure 4.11, Step 2). In permeabilized cells, GTP induces GOLPH3 recruitment. We propose this reflects limited synthesis of PI4p by the GTP-activated Pik1 using the residual ATP in the permeabilized cells. Importantly, although GTP induces PI4p synthesis, it is not sufficient for adaptor recruitment in the absence of additional ATP. This suggests the existence of an additional ATP dependent step (Figure 4.11, Step 3). If Arf activation and PI4p synthesis were the only two processes required,
we would expect substantial adaptor recruitment in cells treated with 15mM GTP, in contrast we see only minor changes in adaptor recruitment in GTP treated permeabilized cells. The lack of adaptor recruitment in 15mM GTP suggests the existence of an additional ATP dependent factor. Alternatively, adaptor recruitment may require very high concentration of PI4p not achieved with GTP addition. Although our current analysis cannot distinguish between these two alternatives, additional ATPases are implicated in clathrin function in replete cells [46, 47]. These ATPases, Drs2 and the V-ATPase, could constitute additional ATP-dependent requirements during glucose starvation. It also remains to be determined which of these steps precisely coordinates adaptor recruitment with energy levels in intermediate starvation conditions and, how Arf1 localization is regulated by starvation.

**Glucose starvation changes adaptor localization requirements**

This study also reveals mechanistic changes in clathrin dependent traffic during glucose starvation. We found that during glucose starvation adaptor localization is more dependent on both Arf and Ptk1 than it is in the presence of glucose. The increased importance of Arf and Ptk demonstrates the mechanistic underpinnings of clathrin dependent traffic differs dramatically between glucose replete and starved cells.

The mechanistic change in adaptor recruitment may be functionally important to the cell during glucose starvation. In the presence of glucose, the cell can afford to be wasteful if the wasteful process provides a competitive advantage. The cell synthesizes membrane proteins that it cannot use only to degrade them in the vacuole. For example, when no siderophores are available, the cell makes siderophore transporters and then degrades them in the vacuole [48]. Presumably, this process provides a competitive advantage in the wild through providing an enhanced ability to exploit a rare encounter with scarce siderophores. In the presence of glucose, the cell also allows waste via non-selective traffic. Instead actively retaining proteins within an organelle, the cell
relies on numerous retrieval systems to maintain steady state localization of proteins in their correct localization [49]. The changes in the mechanism of adaptor recruitment during prolonged glucose starvation likely reduces overall traffic rates, to coincide with lower traffic volumes due to reduced synthesis of unneeded transmembrane proteins. This reduction in overall rates further reduces the volume of proteins requiring retrieval systems. The increased requirement for both Arf and Pik1 may be the mechanism that ensures the reduction in overall traffic rates to coincide with reduced volume. We can only speculate on whether the changes in adaptor recruitment mechanisms also act in increased surveillance to prevent the non-selective transport of cargo.

The regulation of adaptor localization by ATP explains how endosomal traffic rates are coordinated with available cellular energy. The productive traffic of proteins via clathrin dependent pathways requires a large amounts of ATP [50]. Importantly, much of this energy is consumed in the disassembly of the clathrin coat, which must occur prior to fusion of the transport carrier with the target membrane [51]. By linking adaptor recruitment to available energy, the cell stops coat assembly at the first step and thus limits the amount of ATP consumed by clathrin dependent traffic until energy levels rise. This mechanism preserves limited supplies of ATP for more critical activities such as reactivating the mitochondria. This regulatory mechanism is extremely rapid and reversible and, as our results in Figure 4.5 indicate, can tune traffic rates precisely with cellular ATP levels.

Energy appears to regulate diverse processes coordinately in yeast. In addition to endosomal traffic, translation initiation and cell polarity also transiently stop during glucose starvation and are dependent on the glucose repression machinery for this inhibition. These similarities suggest that these processes are coordinately regulated [10, 33]. However, at intermediate starvation levels, the cell appears to differentially regulate endosomal traffic and translation initiation. Translation initiation is strongly inhibited when cells are transferred to
0.5% glucose [8]. In contrast, we did not observe dramatic changes in adaptor localization in cells transferred to 0.5% glucose (data not shown). The inhibition of translation initiation at intermediate concentrations of glucose allows the cell to prepare for imminent starvation [8]. The higher sensitivity of translation initiation to glucose depletion could reflect an additional non-ATP dependent regulation of translation initiation or, alternatively, in cells cultured at 0.5% glucose, ATP levels may be reduced [52-54]. Regardless of the mechanism of translation inhibition at intermediate levels, it is clear that the cell uses energy metabolism as a global tuning mechanism for endosomal traffic and likely for cell polarity and translation.

The ATP concentration dependent changes in endosomal traffic are particularly striking. Intracellular ATP concentrations have long been correlated with growth rates in eukaryotic and prokaryotic cells. [55]. Furthermore, metabolism alone can reprogram a differentiated cell to adopt stem cell characteristics or drive a stem cell to differentiate [56]. Our results suggest that metabolism, acting through endosomal traffic, may globally regulate cell behavior. Global changes in endosomal traffic would enhance or suppress the signaling and proliferating capacity of the cell by altering the protein content of the plasma membrane and endosomal organelles. Such a role for metabolic regulation in endosomes of multicellular organisms is yet to be explored.

Materials and Methods:

Yeast strains and plasmids

Yeast strains, sources and plasmids are listed in Table 1 [57-60]. Fluorescent tags and gene deletions were introduced by standard PCR-based methods (Longtine et al., 1998). Strains containing multiple mutations were generated by standard yeast genetics. The GFP-GOLPH3 probe was graciously provided by Chris Burd (UPenn). To construct the GFP-Pik1 plasmid
pQA76, the genomic promoter and ORF of Pik1 was amplified and ligated into the Kpn1/Sac1 site of pRS316. The ORF for GFP was amplified with flanking Hind III sites and ligated into a Hind III site in the N-terminus of the Pik1 ORF.

**Media, Antibodies, and Reagents**

Yeast cells were grown in YP or supplemented synthetic media (SM) in the presence or absence of 2% glucose (dextrose), 2% galactose, 2% raffinose, or 2% glycerol/3% ethanol. Supplemented synthetic media (SM) without dextrose was used for all starvation experiments. Yeast peptone dextrose (YPD) media is 1% bacto-yeast extract (Difco, Detroit, MI) and 2% bacto-peptone (Difco) supplemented with 2% dextrose and 20 μg/ml adenine, uracil, and L-tryptophan. Synthetic media with dextrose (SD) is 0.67% of yeast nitrogen base without amino acids (Difco) and 2% dextrose. SD media with was supplemented with 100μg/ml adenine, L-leucine, L-lysine, L-tryptophan; 50 μg/ml L-histidine, L-methionine, and 20 μg/ml uracil. For cells expressing GFP-GOLPH or GFP-Pik1, uracil was omitted to maintain plasmid selection.

The following antibodies were used: anti-myc (UNC Immunology Core Facility, Chapel Hill, NC), anti-phospho AMPK (Cell Signaling, Danvers, MA), and anti-Ent5 (Aoh et al. 2011). Alexa Fluor secondary antibodies were from Invitrogen (Carlsbad, CA). Other reagents were obtained as follows: antimycin, ATP, ATP-γ-S, GTP, GTP-γ-S and DMSO (Sigma-Aldrich, St. Louis, MO) and 1NM-PP1 (Calbiochem, San Diego, CA).

**Immunoblotting**

For whole-cell extracts to analyse most proteins, 2 OD₆₀₀ of cells were resuspended in Laemmlı sample buffer, boiled, and subjected to glass-bead disruption. The extracts were cleared by centrifugation. Following SDS-PAGE, samples were transferred to nitrocellulose, blocked with 4% milk in TBS-T, and then probed with primary and fluorescent secondary antibodies.
Fluorescence signals were detected on a Typhoon imaging system (Amersham Biosciences, Piscataway, NJ). For analysis of Snf1 activation, extractions were performed as described by [61]. For kinase deletion library screen, individual cultures of each strain were grown to mid-logarithmic phase in SM with 2% glucose, washed three times into starvation media and cultured overnight. Lysates were generated and processed as described above.

**Growth conditions and carbon starvation**

For glucose starvations, yeast cells were grown overnight in YPD or SD media at 30°C and aerated by rotary shaking and then diluted into SD media and grown another 4-6 hrs to mid-log phase, as described previously in [11]. To remove the glucose, the cells were washed three times with supplemented synthetic media and then sampled immediately for acute starvation or after 1-3 hrs for prolonged starvation. For assays of cells in different carbon source, the cells were first pre-adapted to the carbon source by growing overnight in YPD, diluted into YP with 2% galactose, 2% raffinose, or 2% glycerol/3% ethanol, and grown overnight again. The cells were then diluted the next day and grown to mid-log phase (OD600 ~0.2-0.5). The cells were then starved as described above. Antimycin A or an equivalent amount of vehicle (ethanol) was added to a final concentration of 2 ug/mL as indicated. For experiments using the *tpk1-as tpk2Δ tpk3Δ* strain, cells were grown in YPD as described above and PKA was inhibited by incubating the cells with 2 uM 1NM-PP1 or an equivalent amount of DMSO following growth to mid-log phase for 1hr prior to starvation. To examine the effect of brefeldin A on adaptor localization, *erg6Δ* cells were grown in SD as described above and brefeldin A was added to a final concentration of 150 uM for 5min prior to imaging at the indicated times. For experiments in *pik1-83* cells, cells were grown overnight in YPD at room temperature on a rotary shaker and then diluted the next day into supplemented SD media and grown another 4-6 hrs at room temperature on a rotary shaker. The cells were shifted to the non-permissive temperature by
incubating them at 38°C with mixing in a heat block for 30min.

**Live microscopy, image processing, and quantitation**

For carbon starvations, the cells were grown as described above and then briefly pelleted and resuspended in 50-200uL media prior to imaging. For experiments using antimycin, the cells were grown as described above and antimycin was added for 5min before imaging. Cells were mounted onto a clear coverslip and then imaged. For each field, Z-stack images were captured using a 100x oil objective (numerical aperture 1.4) on a spinning disk confocal or epifluorescent microscope, as described previously [11]. The number of puncta per cells was quantitated by counting the number of foci in a single Z-stack from the middle of a cell (N>40) for each condition specified. In micrographs of intact cells, Z-stacks were compressed into a single maximum intensity image in ImageJ. Statistical significance was determined using a two-tailed Mann-Whitney U test for the null hypothesis that the medians were equal. For TIRF analysis, permeabilized cells were fixed using 4% paraformaldehyde for 15 minutes at room temperature. Cells were then washed three times with phosphate buffered saline. Samples were mounted in Aqua Poly/Mount (Polysciences Inc, Warrington, PA) and allowed to set at 4°C overnight before imaging. TIRF was performed using on a Nikon Eclipse Ti using a 100x oil objective (numerical aperture 1.49). Image collection was on a Roper Scientific Cascade 512B camera. 488 nm and 561 nm laser were used to excited GFP and mCherry, respectively. The exposure time for both GFP and mCherry was 500 ms.

**ATP Measurements**

ATP measurements were performed as described by Ashe *et al.* (2000). To measure ATP concentration in the presence of different carbons, cells were grown and washed as described above and ~ 1OD of cells was collected by centrifugation in the presence of the carbon source or
15 min after starvation. To measure ATP concentration in the presence of antimycin, the cells were grown in supplemented SD with 2% galactose as described above and then starved for 30 min. Serially diluted antimycin A or an equivalent amount of vehicle (ethanol) was added to the cells for 5 min before collection. For all experiments, the cells were then resuspended in 2.5% TCA. The OD of the cells was measured. The cells were then diluted 1:20 with 25 mM Tris pH 9.4. Five to ten microliters of the samples were assayed using the ATP Determination Kit (Life Technologies), according to the manufacturer’s instructions. Luminescence was measured at 570 nm by using a SpectraMax Luminesence L microplate reader. Duplicate samples were taken for each condition, and the experiments were performed in triplicate. Relative ATP concentration are expressed as the average % of control samples. Statistical significance was determined with a two tailed students’ T-test. Data is reported as ATP with cell concentrations normalized to OD$_{600}$.

**Permeabilized cell assays**

To generate permeabilized cells, approximately 50 OD$_{600}$ of cells grown to mid-log phase were pelleted and resuspended in supplemented SD media with 100 mM Tris-SO$_4$ (pH 9.4) and 10 mM DTT for 10 minutes at 30°C. The cells were then washed twice with supplemented SM and then incubated in supplemented SM with 1 M sorbitol, 10 mM Tris-HCl (pH 7.4), 2 ug/ml antimycin A and 120ug/ml lyticase for 15 min at 30°C. To permeabilized the cells, the cells were pelleted and resuspended in 0.2 M sorbitol, 50 mM potassium acetate, 2 mM EDTA, 20 mM HEPES (pH 6.0), 1 mM DTT and protease inhibitor cocktail (Sigma). The cells were then dounced 20 times with a tight-fitting pestle. Cells were kept on ice for no more than two hours prior to imaging. For the nucleotide treatments, nucleotides were added to the permeabilized cells and then incubated at room temperature without any agitation for 5 min before imaging.
Acknowledgements: We gratefully acknowledge A.P. Joglekar, K. Bloom, E. D. Salmon and K.C. Slep for access to and help with microscopes used in this study; G.S. Payne, C. Burd and V. Bankaitis for reagents; J.Y.Y. Martinez-Marquez, D. Buelto and M.J. Lang for generating strains used in this study; R.J. Duronio and A.P. Joglekar for comments and suggestions on the manuscript. This work was supported by National Institutes of Health Research Grant GM-092741 to M.C.D.
# FIGURES AND TABLES

## Table 4.1 Strains and plasmids used in this study

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Figure 4.1. Snf1 is required for localization of the TGN-endosomal clathrin adaptors Gga2 and Ent5 during prolonged glucose starvation. (A) AMPK pathway proteins are required for Ent5 hyper-phosphorylation. Cellular lysates were prepared from indicated cells prior to or 2 hours after glucose starvation. The cell lysates were then probe with an antibody to Ent5. Arrowhead indicates hyper-phosphorylated Ent5 (B) Snf1 is required for adaptor localization during prolonged glucose starvation. Wild-type (WT) and snf1Δ cells expressing Gga2-GFP (top) or Ent5-GFP (bottom) from their endogenous loci were imaged before (+glucose), within 10 minutes (acute starvation) and after 2 hours of glucose starvation (prolonged starvation). Scale bar is 5µm. (C) Quantification of the number of puncta per cell for cells in B. **p<0.01; n.s., not significant. Charts show box plots of the data. The line indicates the median, the edges of the box indicate the 25% and 75% percentile and the whiskers extend to the extremes of the
data not considered outliers. (D) Snf1 is activated within 5 minutes of glucose starvation. Cellular lysates were prepared from cells expressing Snf1-myc from its endogenous locus at the indicated time points before or after glucose starvation. Active Snf1 was detected with a phospho-AMPK, and total cellular Snf1 was detected with an anti-myc antibody.
Figure 4.2. Inhibition of cellular energy production induces adaptor redistribution. Wild-type cells expressing Gga2-GFP (A) or Ent5-GFP (B) from their endogenous loci were pre-adapted to different carbons sources by continuous logarithmic growth for 48 hours. Cells were imaged during logarithmic phase growth (column i), after treatment with 2 μg/mL antimycin (column ii), immediately after carbon source withdrawal (column iii) and immediately after carbon source withdrawal followed by treatment with 2 μg/mL antimycin (column iv). Scale bar is 5μm.
Figure 4.3. Glucose repression genes are required for glucose starvation induced redistribution of Ent5. (A,C). Indicated cells expressing Ent5-GFP from the endogenous locus were imaged before or immediately after glucose starvation. (B,D). Quantification of the number of puncta per cell for cells in A and C. ** P<0.01; n.s., not significant. Chart shows box-plot of data as described in Figure 1. Scale bar is 5µm.
Figure 4.4. Cellular ATP concentration decreases significantly during glucose starvation and is regulated by glucose repression pathways. (A) Cellular ATP was measured in wild-type cells pre-adapted to different carbon sources (2% glucose, 2% galactose, 2% raffinose, 2% glycerol/3% ethanol, or 2% sucrose) before and after carbon source withdrawal. (B) TPK1-as tpk2Δ tpk3Δ cells were grown to mid-log phase, incubated with 2 μM 1NM-PP1 or DMSO for 1 hr and then starved. Cellular ATP was measured in cells before and after starvation. ATP concentrations were normalized to cells grown in glucose and treated with DMSO. (C) Cellular ATP was measured in reg1Δ cells before or after glucose starvation. The relative ATP concentrations were normalized to wild-type cells grown in glucose. (D) snf1Δ cells were processed as in C. ** P<0.01, *P<0.05. Charts show average values and standard deviation.
Figure 4.5. Cellular ATP concentrations correlate with the association of adaptors to membranes. (A) Cellular ATP measured in indicated cells before and after glucose starvation as described in the materials and methods. (B) Wild-type cells were grown to mid-log phase in galactose and then starved. Varying amounts of antimycin were added to the cells. Cellular ATP measured as above. Charts show average values and standard deviation. (C) Indicated cells were processed as in B and imaged. Scale bar is 5μM. (D) Quantification of the number of puncta per cell for cells in (C). Chart shows box-plot of data as described in Figure 1.
Figure 4.6. **ATP is sufficient to recruit adaptors in permeabilized cells.** (A) Cells expressing Gga2-GFP or Ent5-GFP from the endogenous loci were permeabilized and incubated with or without indicated nucleotides for five minutes before imaging. (B) Ent5 is recruited to Sec7 containing organelles upon addition of ATP. Cells expressing Ent5-GFP and Sec7-mcherry were permeabilized an incubated with or without ATP. Cells were fixed, mounted for TIRF
microscopy and imaged. (C) Snf1 is not required for adaptor localization in the presence of exogenous ATP. Cells lacking Snf1 and expressing Gga2-GFP or Ent5-GFP from the endogenous loci were permeabilized and imaged with or without addition of exogenous ATP. Scale bar is 5µm.
Figure 4.7. ATP and GTP both contribute to adaptor recruitment. (A&B) (Top) Addition of GTP reduces the amount of ATP required to induce adaptor localization in permeabilized cells. Cells were prepared as in Figure 6 and incubated with indicated amounts of nucleotides for 5 minute prior to imaging. (Bottom) Quantification of adaptor localization in different conditions. Cells were classified as having no puncta, only small or dim puncta or large puncta. Scale bar is 5um. Charts show representative data from one of three replicate experiments. N>50 cells (C). GTP-γ-S reduces the amount of ATP required to induce adaptor localization.
Figure 4.8. Glucose starvation alters the localization of Arf1, Pik1 and PI4p. (A) Arf relocalizes to dim puncta during acute and prolonged starvation. Diploid cells heterozygous for ARF1-GFP and homozygous for GGA2-mCherry were imaged before, within 15 minutes or after 2 hours of glucose starvation. (B) Pik1 redistributes to the cytosol upon glucose starvation. Haploid wild-type cells expressing GFP-Pik1 from a plasmid were imaged before, within 15
minutes or after 2 hours of glucose starvation. (C) GOLPH3 relocalizes to dim puncta during acute and prolonged starvation. Haploid wild-type cells expressing Gga2-mCherry from the endogenous locus and the PI4P probe GOLPH3-GFP from a plasmid were imaged before, within 15 minutes or after 2 hours of glucose starvation. (D) Quantification of puncta per cell for cells grown in or acutely starved for glucose as described in A, B and C. ** P<0.01; n.s., not significant. Charts show box-plot of data as described in Figure 1.
Figure 4.9 Arf1, Pik1 and PI4p show differential responses to exogenous nucleotides in permeabilized cells. (A) Diploid cells heterozygous for ARF1-GFP or haploid wild-type cells expressing GFP-Pik1 GOLPH3-GFP from plasmids were prepared as described in Figure 6 A and imaged before or after addition of 15 mM indicated nucleotides. Scale bars are 5µm. (B) Quantification of puncta per cell. ** P<0.01; * P<0.05; n.s., not significant. Charts show box-plot of data as described in Figure 1. (C) Quantification of adaptor localization in different conditions. Cells were classified as in Figure 7. Black portion of the bars indicate percent of cells with large puncta, grey small puncta and white indicates no puncta.
Figure 4.10. Arf1, Pik1 and Sac1 modulate adaptor localization during glucose starvation.

(A) Brefeldin A induces adaptor redistribution only during glucose starvation. erg6Δ cells expressing Gga2-GFP or Ent5-mCherry were imaged before or after 2 hours of glucose starvation. Cells were treated with DMSO (control) or 150μM Brefeldin A for five minutes prior to imaging. (B) Adaptors show increased dependence on Pik1 during glucose starvation. pik1-83 cells expressing Gga2-mRFP or Ent5-GFP were imaged before or after 2 hours of glucose starvation at the permissive (25°C) or after a 30 minute shift to non-permissive (38°C) temperature. For temperature shifted starved cells, cells were first starved for 1.5 hours and then shifted to the non-permissive temperature. (C) Gga2 but not Ent5 requires Sac1 for rapid redistribution during acute starvation. Wild-type and sac1Δ cells expressing Gga2-GFP or Ent5-GFP were imaged before or after 2 hours of glucose starvation. Scale bar is 5μm.
Figure 4.11: Model of energy dependent steps in adaptor recruitment. The steps illustrated may occur concurrently or in a different sequence than illustrated. Prior to energy input, Arf is localized to membranes but Pik1 and adaptors are cytosolic. (1) Upon availability of GTP, Pik1 becomes localized and activated. (2) Upon availability of ATP, Pik1 synthesizes PI4p however this is not sufficient for adaptor recruitment. (3) Adaptors are only recruited when a third uncharacterized factor has access to sufficient ATP. All three activities, Arf1, Pik1 and this third factor are required for localization of adaptors to membranes during glucose starvation.
Supplemental Figure 4.1. Adaptors partially co-localize with Sec7 during prolonged glucose starvation. Wild-type cells expressing Sec7-mCherry, Gga2-GFP (A) or Ent5-GFP (B) or Apl4-GFP (AP-1) (C) from their endogenous loci were imaged before or after 2 hours of glucose starvation.
Supplemental figure 4.2. Arf1, Pik1 and Sac1 modulate adaptor localization during glucose starvation. Quantification of puncta per cell for samples described in Figure 10. ** P<0.01; n.s., not significant. Charts show box-plot of data as described in Figure 1.
REFERENCES


CHAPTER 5. GENERAL DISCUSSION

Vesicular membrane traffic is the fundamental machinery that delivers the right proteins to the right place at the right time. Traffic at the TGN and endosomes is especially important because it regulates the localization of plasma membrane proteins, which allow cells to respond to environmental signals.

Claathrin plays an essential role in mediating the traffic at the TGN and endosomes. For clathrin to mediate traffic, it requires clathrin adaptors to recruit it to membranes. Although the importance of clathrin is well established, the role of clathrin adaptors in TGN-endosomal traffic is not well understood. Clathrin adaptors are known to be important for recruiting clathrin and for traffic initiation. However, little has been done to elucidate the mechanisms which recruit clathrin adaptors to TGN and endosomal membranes. Another important question is why do cells have many different kinds of adaptors if all adaptors do is to recruit clathrin? Do cells diversify their adaptors so that they can accommodate every possible kind of cargo? This dissertation used yeast genetic and biochemical assays as well as quantitative microscopy to reveal the intrinsic and extrinsic mechanisms that regulate the recruitment and to explore functions of clathrin adaptors.

In Chapter 2, I discussed an autoregulatory motif on Gga2 we discovered. This novel autoregulatory motif has dual function as it regulates the interaction between Gga2 and clathrin and the interaction between the hinge domain and the γ-ear domain of Gga2. By regulating the self-interaction of Gga2, the autoregulatory motif allows Gga2 to adapt an open and a close conformation. Additionally, the disruption of this autoregulation alters the temporal and spatial
regulation of clathrin adaptors. Taken together we proposed that when Gga2 arrives at the traffic site, it exists in both an open and closed conformation. Clathrin binding at the autoregulatory motif promotes Gga2 to adapt an open conformation. When Gga2 is open, its ear domain can interact and recruit other clathrin adaptors such as Ent5, promoting the traffic structure to mature.

Although the autoregulatory motif on Gga2 is important for the temporal regulation of adaptor recruitment, it remains unclear if this regulation has any biological significance. I speculate that this autoregulation is important for Pi4P synthesis on TGN and endosomal membranes. Gga2 recruitment has been shown to coincide with the recruitment of Pik1 and an increase in local Pi4P levels. As the level of PI4P increases, late acting adaptors, such as Ent5 and AP-1 will be recruited [1]. Thus, the interaction between Gga2 and clathrin that promotes the open conformation of Gga2 may be essential for Pik1 recruitment. The recruitment of Pik1 subsequently leads to the arrival of late acting traffic factors by modifying membranes through Pi4P synthesis. Therefore, the autoregulation of Gga2 may be the first step to membrane modifications which lead to the maturation of the traffic structures.

One way to study the biological significances of Gga2 autoregulation is to use the techniques we developed in Chapter 3. I have developed a quantitative calcofluor white sensitivity assay to investigate the intracellular retention of Chs3. Additionally, I can also study the defect on traffic by monitoring the localization of Tlg1-mCherry. It will be interesting to use these techniques to quantitatively and qualitatively investigate the role of the autoregulatory motif on Gga2 in the traffic of Chs3 and Tlg1.

Our work demonstrated how interaction between Gga2 and clathrin can promote the interaction and recruitment of Ent5. The next question we examined was the role of Ent5 in traffic. If Gga2 is capable of recruiting clathrin, why do cells still need to recruit Ent5 to the same traffic structures? Does Ent5 has any unique function in traffic? In Chapter 3 of this
dissertation, I showed that Ent5 indeed has a non-redundant role in traffic as it mediates the localization of Chs3 and Tlg1. I next revealed that, although Ent5 is not required for the recruitment of clathrin and traffic initiation, it is important for the late stage of clathrin mediated traffic. I showed that the interaction between Ent5 and clathrin is necessary to maintain Ent5 functionality. First, the interaction between Ent5 and clathrin allows Ent5 to localize to membranes. Second, the interaction between Ent5 and clathrin stabilizes Ent5-Gga2 interaction. Finally, Ent5-clathrin interaction allows traffic structures to mature in a timely manner. Overall, this work expands the role of clathrin adaptors. Some adaptors are not just clathrin recruiters or traffic initiators. They have a role during different stages of traffic.

This dissertation shows that Ent5, or epsin family proteins in general, may have a housekeeping role in traffic, rather than just being a cargo specific adaptor. In agreement with our work, several studies also demonstrated that epsins have a much more diverse role in traffic. For example, the mammalian endocytic epsins are speculated to be important for the organization of actin cytoskeleton [2]. Another study used the EpinsR (a mammalian epsin) knocksideways method to sequester EpsinR in mitochondria and showed that the depletion of EpsinR from the TGN and endosomes drastically change the composition of intracellular clathrin coated vesicles. Not only is the expected cargo, Vit1, depleted from the vesicles in EpsinR knocksideways cells, many other coat accessory proteins and luminal proteins are reduced. Furthermore, many other cargoes, such as hydrolase receptors and carboxypeptidases D that were originally thought to depend on other clathrin adaptors for their traffic are also strongly depleted [3]. These findings strongly indicate that the role of epsins on traffic is far-reaching. They can affect the fundamental composition of clathrin coated vesicles.

Another important question we want to address in the future is how does the interaction between Ent5 and clathrin promote traffic maturation? We proposed that Ent5-clathrin
interaction is essential for clathrin polymerization. By promoting clathrin polymerization, Ent5 helps clathrin to reorganize into a cage-like structure to surround the vesicle. Future work will use an *in vitro* and an *in vivo* assay to investigate the role of Ent5 on clathrin assembly.

We next examined the regulation of traffic by energy-dependent mechanisms. Previously, our lab demonstrated that the localization of clathrin adaptors is highly sensitive to energy starvation induced by glucose removal. In this dissertation, we further confirmed that the localization of clathrin adaptors *in vivo* is correlated with the change of cellular ATP levels.

Combined with our unpublished data, we speculate that metabolism and energy regulate traffic. In return, traffic maintains the production of energy. When cellular ATP levels drop due to glucose removal, clathrin adaptors are removed from membranes. This process shuts down traffic at the TGN and endosomes and possibly allows cells to use the limited energy to maintain or activate other more essential activities. One essential activity that is activated during glucose starvation is oxidative phosphorylation. Oxidative phosphorylation can restore cellular ATP levels by metabolizing carbon sources such as lipid molecules and free amino acids. Our recent work showed that these lipids and amino acids likely come from the plasma membrane. We observed that plasma membrane proteins are internalized and delivered to the vacuole for degradation during glucose starvation. This process produces free lipids and amino acids for cells to metabolize during prolonged glucose starvation. Our current unpublished work shows that clathrin adaptors such as Gga2 and Ent5 are necessary for the delivery of plasma membrane proteins to the vacuole during glucose starvation. Essentially, adaptors deliver food to the vacuole so cells can restore energy. When ATP production is restored, increases in energy levels allow more adaptors to be recruited, resulting in maintenance of fully functional traffic during prolonged starvation.
We revealed a multi-step energy dependent mechanism that regulates the recruitment of clathrin adaptors in this dissertation. We showed that ATP and GTP work synergistically to recruit clathrin adaptors. Given that adaptors do not have ATP or GTP binding domains, there must be ATP and GTP sensing proteins that detect the availability of energy. The future goal will be to identify the ATP and GTP sensing agents that are involved in this process. Although we have evidence to suggest that Arf1 and Pik1 serve as the GTPase and ATPase, respectively, to regulate the traffic, there appear to be other ATPases that are essential for the maximal recruitment of clathrin adaptors. Given their known role in traffic, it is reasonable to assume that ATPases such as Drs2 and V-ATPases are involved in this process. In fact, the enzymatic activity of Drs2 depends on the synthesis of Pi4P by Pik1[4]. This evidence suggests that multiple ATPases work together in this process.

In conclusion, this dissertation reveals the regulatory mechanisms of clathrin adaptors that function at the TGN and endosomes. We also expanded the role of clathrin adaptors, showing that they are not just clathrin recruiters. They can serve as the finishers. Future work will focus on showing the biological significance of clathrin adaptors. We have evidence to show that clathrin adaptors that function at the TGN and endosomes are essential to maintain this traffic and ensure cell viability during a prolonged glucose and energy starvation. Our work will show that energy, metabolism and traffic form a network that allows cells to adapt, survive and proliferate in response to changes of environment.
REFERENCES


