Multiple ArfGAPs regulate vesicular transport

by

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For my parents, Eileen & Addison,

And my wife, Marie-Eve

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ABSTRACT

Membrane and protein cargo is moved among intracellular compartments by small, protein-coated lipid carriers known as transport vesicles. The formation and function of transport vesicles is controlled by several proteins, of which one is the small GTPase Arf. Arf function for vesicular transport is regulated by a cycle of GTP binding and GTP hydrolysis, which in turn is regulated by Arf guanine exchange factors (ArfGEFs) and Arf GTPase-activating proteins (ArfGAPs). In the budding yeast *Saccharomyces cerevisiae*, the ArfGAPs Gcs1 and Glo3 share essential overlapping function for retrograde vesicular transport from the *cis*-Golgi apparatus to the endoplasmic reticulum.

I have addressed the role of ArfGAP function for retrograde transport by identifying proteins that, when present in increased abundance, can suppress the temperature-sensitive phenotype of *gcs1-28 glo3Δ* double-mutant cells. I have identified the novel ArfGAP Age1 as a suppressor of the effects of deficient Gcs1 and Glo3 ArfGAP function for retrograde transport. Moreover, a mutant allele of *AGE1* lacking the amino-terminal coding sequences (*age1-Δ164*) is able to suppress *gcs1-28 glo3Δ* temperature sensitivity, even in low copy number. These data provide *in vivo* evidence that the Age1 ArfGAP can function for vesicular transport. I have also identified the *SLY41* gene as a dosage suppressor of *gcs1-28 glo3Δ* temperature sensitivity. Sly41 has been previously implicated in vesicular transport, and has homology to a small-ion transporter from spinach chloroplasts. Analysis of Sly41 suppression has led to the identification of a cell-wall defect in *gcs1-28 glo3Δ* double-mutant cells.

In this study, I have also characterized the contribution of the Gcs1 and Glo3 ArfGAPs to the retrograde-transport process at the molecular level. I have found that Glo3 associates with coatomer *in vivo* and is a component of COPI vesicles, whereas Gcs1 is not. Furthermore, a novel mutant version of Glo3 (Glo3-R59K) exerts a negative effect on cell growth and vesicular transport, even in the presence of the Gcs1 ArfGAP. These data indicate that Glo3 is the primary ArfGAP required for retrograde vesicular transport. Finally, I find that intact ArfGAP function is required for the generation of COPI vesicles.

LIST OF ABBREVIATIONS

bp	base pair
COPI	coat protein I
COPII	coat protein II
CPY	carboxypeptidase Y
ddH_2O	double-distilled water
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
ER	endoplasmic reticulum
g	gram
GAP	GTPase-activating protein
GDP	guanosine diphosphate
GEF	guanine exchange factor
GTP	guanosine triphosphate
kbp	kilobase pair
M	molar
min	minute
ORF	open reading frame
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
SDS	sodium dodecyl sulfate
SNARE	soluble NSF-attachment protein receptor

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I. INTRODUCTION

1. An overview of vesicular transport

Eukaryotic cells are divided into several membrane-bound compartments known as organelles. Biochemical processes that are essential for life are carried out in each organelle compartment. However, the proteins that are necessary for these biochemical processes must be transported from the organelles where these proteins are produced and modified to the organelle where they carry out their functions. Therefore, eukaryotic cells move membrane and protein cargo among organelles using a process known as vesicular transport. Such vesicular transport requires small, membrane-bound compartments called transport vesicles into which the cargo is incorporated. A transport vesicle is then shuttled to the appropriate target organelle, where the vesicle fuses with the organelle membrane to deliver its cargo. Vesicular transport is highly regulated, requiring numerous proteins that control each step in the transport process: vesicle generation, cargo packaging, vesicle targeting, and vesicle fusion (reviewed in Bonifacino and Glick, 2004). Such regulation is required to maintain organelle integrity, as well as the fidelity of cargo delivery.

Cargo is moved along many pathways (or transport stages) within the cell by vesicular transport. Transport vesicles shuttle cargo from the endoplasmic reticulum (ER) to the Golgi apparatus (also known as anterograde transport), from the Golgi to the ER (also known as retrograde transport), among the stacks of the Golgi apparatus, out of the Golgi apparatus, among the compartments of the endocytic system, from the plasma

membrane into the cell, and from intracellular compartments to the plasma membrane itself. A unique set of proteins regulates each transport pathway. For example, one set of proteins controls vesicular transport from the ER to the *cis* Golgi, whereas a different set of proteins controls transport from the *cis* Golgi to the ER.

The protein sets that regulate each transport pathway are made up of members of three protein families: coat proteins, adaptor proteins, and regulatory proteins. The vesicle coats consist of multi-subunit complexes that perform essential functions for vesicle budding, contributing to the architecture of a transport vesicle. There are three main coat complexes operating for vesicular transport: coat protein I (COPI; also known as coatomer), coat protein II (COPII), and clathrin (reviewed in Kirchhausen, 2000). The COPI coat facilitates retrograde vesicular transport from the Golgi apparatus to the ER, and perhaps intra-Golgi transport (Letourneur et al., 1994; Gaynor et al., 1998), the COPII coat facilitates anterograde transport from the ER to the Golgi (Barlowe et al., 1994), and the clathrin coat mediates vesicular transport within the endocytic pathway (Goldstein et al., 1979; Seeger and Payne, 1992). Adaptor proteins regulate the association of the coat proteins with organelle membranes during vesicle generation. The small GTPase Arf is an adaptor protein that regulates the membrane association of the COPI coat and the clathrin coat (Serafini et al., 1991; Palmer et al., 1993; Stamnes and Rothman, 1993), and the Sar1 GTPase regulates the membrane association of the COPII coat (Barlowe et al., 1994). Thus, adaptor proteins regulate vesicle budding by controlling the recruitment of coat proteins to the membrane surface.

An additional level of regulation for vesicular transport is found in the proteins that exert control over the function of the adaptor proteins themselves. The Sar1 and Arf GTPases control vesicle transport *via* a cycle of GTP binding and GTP hydrolysis; that is, both Sar1 and Arf cycle between GTP- and GDP-bound states. GTP-Arf and GTP-Sar1 are the active forms of these GTPases and recruit coat proteins to the membrane, whereas GDP-Arf and GDP-Sar1 are inactive and are thought to be cytosolic (Regazzi et al., 1991; Barlowe and Schekman, 1993). The GTP cycle of Sar1 and of Arf is regulated by two types of regulatory proteins: guanine exchange factors (GEFs) and GTPase-activating proteins (GAPs). GEFs are required for the exchange of GDP for GTP to activate the Sar1 and Arf GTPases (Barlowe and Schekman, 1993; Peyroche et al., 1996), whereas GAPs stimulate GTP hydrolysis by Sar1 or Arf, and therefore inactivate these proteins (Yoshihisa et al., 1993; Cukierman et al., 1995). Thus, the GEFs and GAPs ultimately regulate vesicular transport by controlling adaptor-protein function.

As a transport vesicle is formed through the action of the coat proteins, adaptors, and regulatory proteins, the protein cargo itself must be packaged into the nascent vesicle. Cargo packaging is thought to be achieved *via* an interaction between signal sequences in the cargo proteins and the coat proteins (Cosson and Letourneur, 1994; Kuehn et al., 1998), or through the bridging of cargo and coat proteins by a cargo receptor (Bremser et al., 1998). Furthermore, some studies have shown that cargo packaging and vesicle coat assembly may in fact be coupled (Bremser et al., 1998; Goldberg, 2000). This issue is discussed more thoroughly in the following sections.

Once a vesicle has packaged the appropriate cargo and budded from an organelle, it must be targeted to the proper location to deliver its cargo. Two classes of proteins are involved in the fidelity of cargo delivery: soluble NSF-attachment protein receptors (SNAREs) and vesicle tethering proteins. SNAREs are thought to act as molecular "locks and keys" to target vesicles to the proper organelle (McNew et al., 2000). SNAREs located on the vesicle are known as v-SNAREs, whereas SNAREs located on the target membrane are known as t-SNAREs (reviewed in Gerst, 1999). Combinations of v- and t-SNAREs provide some of the specificity for vesicle delivery; that is, only certain v- and t-SNARE pairs can interact to permit vesicle fusion at the proper compartment (McNew et al., 2000). In addition to providing fusion specificity, SNAREs themselves are also thought to provide the molecular energy and mechanism to mediate the fusion of the vesicle and target membranes (Weber et al., 1998). The v- and t-SNARE pairs form a structure known as a "SNAREpin", which undergoes a conformational change that provides the energy to bring the opposing membranes of the vesicle and the target compartment together, thus driving vesicle fusion.

Vesicle tethering proteins contribute an additional level of specificity to vesicle targeting. These proteins form multi-subunit complexes that allow a vesicle to attach to a target membrane prior to SNARE engagement (reviewed in Whyte and Munro, 2002). Unique vesicle tethers operate at each stage of vesicular transport; for example, the conserved oligomeric Golgi (COG) complex tethers vesicles to Golgi compartments, whereas the exocyst tethers transport vesicles to the plasma membrane (Whyte and

Munro, 2001; TerBush et al., 1996). The details of vesicle tethering are discussed in more detail in the next section.

After the transport vesicle has fused with the target organelle and delivered its cargo, the transport process is complete. The protein cargo may then be modified by enzymes resident in the organelle and transported to another location, or the cargo may remain resident in the organelle where it may carry out its function. Nonetheless, vesicular transport permits the movement of proteins and lipids throughout eukaryotic cells without the mixing of subcellular compartments and their contents, thereby allowing the enzymatic processes that are essential for cell function to remain separate and intact.

2. COPI vesicle generation, targeting, and fusion

From this point on I narrow the focus of the Introduction to the retrograde vesicular-transport pathway, since retrograde transport is of particular interest in this study. As mentioned in the previous section, the COPI coat complex is required for the production of retrograde transport vesicles. The COPI vesicle coat is a heptameric complex that consists the α-, β-, β'-, γ-, δ-, ε-, and ζ-COP proteins (Wieland and Harter, 1999). In the budding yeast *Saccharomyces cerevisiae* (the model system in which I have chosen to investigate retrograde transport), the seven subunits of the COPI coat are encoded by the *RET1* (α-COP), *SEC26* (β-COP), *SEC27* (β'-COP), *SEC21* (γ-COP), *RET2* (δ-COP), *SEC28* (ε-COP), and *RET3* (ζ-COP) genes (Wieland and Harter, 1999). The COPI subunits require the Arf GTPase to regulate their assembly on the membrane surface of the Golgi apparatus, from which a vesicle will be generated (typically known

as a "donor" membrane). Yeast cells contain three versions of the Arf GTPase: Arf1, Arf2, and Arf3. However, 90% of the cellular Arf pool consists of Arf1 (Stearns et al., 1990), which has been shown to regulate vesicular transport at multiple transport stages, such as transport from the *trans*-Golgi network and COPI-mediated retrograde transport (Yahara et al., 2001). The function of Arf1 is regulated by Arf guanine exchange factors (ArfGEFs) and Arf GTPase-activating proteins (ArfGAPs).

The generation of a COPI-coated retrograde transport vesicle begins when GDP-Arf is recruited to the donor membrane. Recruitment of Arf may be dependent on several factors. In the mammalian system, the p23 member of the p24 family of cargo receptor proteins is thought to bind to GDP-Arf to recruit Arf to the site of vesicle budding (Gommel et al., 2001). However, the entire p24 protein family is dispensable for COPI-mediated vesicular transport in yeast (Springer et al., 2000). A recent report has shown that an interaction between the Arf GTPase and SNARE proteins present in the target membrane may contribute to GDP-Arf recruitment in yeast (Rein et al., 2002). Therefore, multiple mechanisms may act to recruit GDP-Arf to the site of vesicle budding on the donor membrane.

Once GDP-Arf has been recruited to the surface of the donor membrane, nucleotide exchange occurs to activate Arf and initiate vesicle budding. The nucleotide-exchange reaction is carried out by an ArfGEF protein located on the surface of the donor membrane (Peyroche et al., 1996). In the case of COPI vesicle budding in yeast, guanine nucleotide exchange is performed by the Gea1 and Gea2 proteins (Peyroche et al., 2001; Spang et al., 2001). Upon exchange of GDP for GTP, the Arf

protein undergoes a conformational change that exposes an amino-terminally linked myristate residue, which inserts into the donor membrane (Antonny et al., 1997; Goldberg, 1998). At this point, Arf is stably anchored to the donor membrane and thus can recruit coat proteins to, and retain coat proteins at, the site of vesicle budding. Coat recruitment causes deformation of the donor membrane, bending it into the curvature of a transport vesicle (Schekman and Orci, 1996).

As the COPI coat assembles at the membrane surface, the proper cargo is packaged into the nascent vesicle. In mammalian cells, members of the p24 protein family are thought to bind soluble cargo proteins and interact with components of the COPI vesicle coat to couple cargo packaging to vesicle coat assembly (Bremser et al., 1999; Goldberg, 2000; see section 3). Yeast ER-luminal proteins contain an HDEL signal sequence that allows their retrieval from the Golgi (Pelham et al., 1988). The HDEL sequence in the cargo protein interacts with the Erd2 protein to facilitate packaging of the cargo protein into a COPI vesicle (Lewis et al., 1990; Semenza et al., 1990). Many transmembrane cargo proteins destined for retrieval from the Golgi back to the ER contain a di-lysine signal sequence in the cytoplasmic domain (K-K-X-X, where K is lysine and X is any amino acid; Cosson and Letourneur, 1994; Townsley and Pelham, 1994). The di-lysine sequence in the cargo protein interacts with subunits of the COPI coat to mediate incorporation of the cargo protein into the developing vesicle (Cosson and Letourneur, 1994). Thus, packaging of cargo proteins into COPI vesicles is a regulated process that depends on accessory proteins and specific amino acid sequences in the cargo proteins themselves.

After a transport vesicle has budded from the donor membrane, it is then trafficked to the proper target compartment to deliver its cargo. The fidelity of cargo delivery depends on SNAREs and vesicle-tethering proteins. SNAREs are small transmembrane proteins containing an α-helix in the cytoplasmic domain that allows them to interact with their SNARE partners (reviewed in Gerst, 1999). Typically, three t-SNAREs on the target membrane interact with one v-SNARE on the vesicle (Fasshauer et al., 1998), and form a four-helix bundle through interaction of the α-helices in their cytoplasmic domains (Katz et al., 1998). Conformational changes within the four-helix bundle then provide the energy to bring the opposing membranes of the vesicle and organelle together, and thus drive membrane fusion (Weber et al., 1998). For retrograde transport from the Golgi to the ER, it is thought that the v-SNARE Sec22 targets COPI vesicles to the ER, where Sec22 interacts with the t-SNAREs Sec20, Slt1, and Ufe1 (Lewis and Pelham, 1996; Lewis et al., 1997; Burri et al., 2003). Thus, these proteins (Sec22, Sec20, Slt1, and Ufe1) form a four-helix bundle (or "SNAREpin") required to drive the fusion of COPI vesicles with the ER membrane.

Before v-SNARE–t-SNARE interactions facilitate the fusion of a vesicle with a target membrane, the vesicle itself is brought into close proximity with the target membrane by protein complexes known as vesicle tethers. Tethering complexes operate by attaching the transport vesicle to the target compartment, therefore allowing the SNARE proteins on the surface of the target membrane and on the vesicle to interact and facilitate membrane fusion (Whyte and Munro, 2002). Furthermore, since vesicle-tethering complexes physically interact with components of the vesicle coat

(Suvorova et al., 2002) they provide an additional level of specificity for cargo delivery. For example, COPI vesicles interact only with vesicle-tethering complexes that are capable of binding the COPI vesicle coat. Two vesicle-tethering complexes that operate for retrograde transport are the COG complex and a Dsl1-containing complex (Whyte and Munro, 2002). The COG complex tethers transport vesicles derived from late-Golgi compartments to the *cis* Golgi, and transport vesicles delivering cargo from an endosomal compartment to the *cis* Golgi (Whyte and Munro, 2001). Thus, the COG complex tethers vesicles at the *cis*-Golgi apparatus. The Dsl1-containing complex facilitates retrograde transport by tethering transport vesicles that originate at the *cis* Golgi to the ER (Reilly et al., 2001).

Prior to fusion with a target membrane, a vesicle must lose its protein coat so that the vesicle lipid bilayer may interact with the organelle lipid bilayer. Therefore, the association between the COPI coat proteins and the vesicle membrane must be destabilized to allow loss of the protein coat. As mentioned above, Arf is stably anchored to the vesicle membrane by an amino-terminal myristate residue, and insertion of this myristate residue into the lipid bilayer of the vesicle is dependent on GTP being bound by Arf. Hydrolysis of Arf-bound GTP to GDP causes a conformational change in Arf that buries the myristate residue within the Arf protein itself (Goldberg, 1998), thus removing the myristate residue from the vesicle lipid bilayer and causing dissociation of Arf from the vesicle. Therefore, Arf-bound-GTP hydrolysis is necessary for vesicle uncoating (Tanigawa et al., 1993). However, Arf has little intrinsic GTPase activity, and therefore requires the action of a GTPase-activating protein (GAP) to stimulate GTP hydrolysis by

Arf (Randazzo and Kahn, 1994). In yeast, the Gcs1 and Glo3 proteins perform this essential GAP activity for retrograde transport (Poon et al., 1996, 1999), and hence the function of Gcs1 and Glo3 is likely required for COPI-vesicle uncoating. Upon stimulation of Arf-bound-GTP hydrolysis by an ArfGAP, the vesicle sheds its protein coat, and is then competent to fuse with the target membrane to deliver its cargo. Exactly when the protein coat is shed from the vesicle during the vesicular-transport process remains a point of debate.

3. Multiple roles for ArfGAPs

The classical model for vesicular transport proposed by Tanigawa et al. (1993) states that GTP hydrolysis by Arf causes dissociation of Arf from the vesicle membrane, hence leading to vesicle uncoating. Therefore, ArfGAPs were initially thought to only be required for the uncoating process. However, several studies have found a requirement for ArfGAP function at steps of vesicular transport other than vesicle uncoating. For example, ArfGAP function has been implicated in the fidelity of cargo packaging (Nickel et al., 1998; Goldberg, 2000; Pepperkok et al., 2000; Lanoix et al., 2001). The role of ArfGAP activity in cargo packaging has been modeled in the "discard pathway" theory (Goldberg, 2000).

The "discard pathway" theory states that the formation of a transport vesicle is linked to the selection of appropriate cargo (Goldberg, 2000). The "discard pathway" depends on three main components: coatomer, ArfGAP, and members of the p24 protein family. Coatomer was found to induce a 1000-fold increase in ArfGAP activity upon

interacting with an ArfGAP (Goldberg, 1999), while members of the p24 protein family are able to bind both cargo proteins and coatomer (Sohn et al., 1996). If the correct cargo is packaged into the nascent vesicle, this cargo will interact with the p24 proteins, causing a conformation change in the p24 protein itself. This conformational change permits p24 to block a site on coatomer that interacts with the ArfGAP to stimulate ArfGAP activity. Hence, in the presence of correct cargo, little ArfGAP activity is exhibited, and GTP-Arf remains at the membrane surface to recruit additional coat proteins and eventually generates a transport vesicle. However, if improper cargo (or no cargo at all) is selected for incorporation into the budding vesicle, the p24 proteins will not undergo a conformational change, and therefore fail to interact with coat proteins that have been recruited to the site by GTP-Arf. Coatomer is free to stimulate ArfGAP activity, resulting in GTP hydrolysis by Arf and dissociation of Arf from the donor membrane, causing vesicle budding to be aborted before a mature transport vesicle can form. Thus, cargo exerts an influence over vesicle generation. Although the ArfGAP activity of the yeast Glo3 protein is induced by the presence of coatomer (Szafer et al., 2001), the p24 protein family is dispensable in *S. cerevisiae* (Springer et al., 2000), and thus the "discard pathway" may not mediate cargo packaging in yeast.

Recent evidence has suggested that ArfGAPs may also be required for the generation of transport vesicles (Yang et al., 2002). Yang et al. (2002) showed that mammalian ARFGAP1 is a component of COPI vesicles, and that ArfGAP activity might promote COPI-vesicle generation. However, the results of Reinhard et al. (2003) support the original findings of Tanigawa et al. (1993), demonstrating that ArfGAP activity is

only required for the uncoating of COPI vesicle. Therefore, there is conflicting evidence in the literature concerning the role of ArfGAPs in the generation of transport vesicles. Nonetheless, the observation that ArfGAP activity may be required for COPI-vesicle generation highlights an unexpected role for ArfGAPs in vesicular transport.

In addition to being directly implicated in vesicular transport, ArfGAPs have also been shown to be involved with the actin cytoskeleton (Randazzo and Hirsch, 2004). For example, the yeast ArfGAP Gcs1 and the mammalian ArfGAP Git2-short were both found to mediate actin cytoskeletal organization (Blader et al., 1999; Mazaki et al., 2001). In yeast cells, the actin cytoskeleton provides a scaffold that directs polarized growth and secretion (reviewed in Bretscher, 2003). During yeast cell division (a process known as "budding"), the majority of growth is directed into the nascent daughter cell (or "bud"), a process known as polarized growth (reviewed in Madden and Snyder, 1998). Protein and lipid cargo that is required for such polarized growth is transported to the bud by transport vesicles (Novick and Schekman, 1979). These transport vesicles require the myosin protein Myo2 for segregation into the bud tip (Johnston et al., 1991); Myo2 moves the secretory vesicle along actin cables projecting from the mother cell into the nascent daughter cell (Pruyne et al., 1998). Thus, the actin cytoskeleton directs secretion and polarized growth during cell division.

As mentioned above, the yeast ArfGAP Gcs1 has been recently implicated in actin cytoskeleton organization. *gcs1Δ* single-mutant cells were found to contain an increased number cortical actin patches, and although actin cables were present they were often misaligned (Blader et al., 1999). Furthermore, a *gcs1Δ* mutation was found to have

genetic interactions with mutations in genes that encode proteins that bind to actin, and the Gcs1 protein was found to stimulate actin polymerization *in vitro* (Blader et al., 1999). The recent finding that the yeast Arf3 protein may direct polarized growth (Huang et al, 2003) suggests that the Arf GTPase itself may be involved in Gcs1-mediated actin function. Thus, these data suggest a role for the Gcs1 ArfGAP in actin cytoskeleton organization, in addition to its role for vesicular transport.

4. Overlapping function of the yeast ArfGAPs

The budding yeast *Saccharomyces cerevisiae* contains an ArfGAP family with six members: Gcs1, Glo3, Age2, Age1, Gts1, and Sps18. Each of these proteins contains an "ArfGAP domain", which consists of a cysteine-rich zinc-binding domain (C-X-X-C-X_{16}-C-X-X-C, where C is cysteine and X is any amino acid) and a downstream invariant arginine residue (Goldberg, 1999), and is required for stimulating Arf GTPase activity. To date, the Gcs1, Glo3, Age2, and Age1 proteins have been shown to possess ArfGAP activity, as determined by an *in vitro* assay (Poon et al., 1996, 1999, 2001; Zhang et al., 2003). Because there are six ArfGAPs in yeast, yet only three Arf proteins, some specificity of Arf function for vesicular transport is likely provided by the ArfGAP proteins themselves. That is, the localization and regulation of ArfGAP activity likely regulates the function of the Arf GTPases for multiple vesicular-transport pathways (such as retrograde transport and transport from the *trans*-Golgi network).

The Gcs1 and Glo3 ArfGAPs were shown to provide essential overlapping function for retrograde vesicular transport from the Golgi apparatus to the ER (Poon et

al., 1999). Yeast cells that lack chromosomal copies of both the *GCS1* and *GLO3* genes are inviable, and depletion of Gcs1 and Glo3 ArfGAP function causes disruption of the secretory pathway (Poon et al., 1999). Furthermore, mutation of either the *GCS1* or *GLO3* gene in combination with a *sec21-1* mutation leads to reduced growth robustness or inviability, respectively (Poon et al., 1999). Since *SEC21* encodes a component of the COPI coat complex that mediates retrograde transport, the *GCS1* and *GLO3* genes encode proteins that are likely involved in retrograde transport based on their genetic interactions with *sec21-1*. Moreover, in a separate study, mutation of the *GLO3* gene was found to impair the retrieval of retrograde cargo from the Golgi to the ER (Dogic et al., 1999), providing further evidence that Glo3 mediates retrograde transport. Together, these data indicate that Gcs1 and Glo3 have overlapping function for retrograde transport from the Golgi to the ER.

The Gcs1 ArfGAP is also involved in vesicular transport from the *trans*-Golgi network, and shares an essential overlapping function with the Age2 ArfGAP for this transport process (Wang et al., 1996; Poon et al., 2001). Under certain conditions, single-mutant *gcs1Δ* cells are defective for the internalization of the lipophilic dye FM4-64 from the plasma membrane to the vacuole (Wang et al., 1996), indicating that Gcs1 is involved in endocytic processes. Yeast cells lacking chromosomal copies of both the *GCS1* and *AGE2* genes are inviable, and depletion of Gcs1 and Age2 function causes disruption of the secretory pathway (Poon et al., 2001). *gcs1-3 age2Δ* double-mutant cells display temperature-sensitive growth that is caused by defective transport from the *trans*-Golgi network at the non-permissive temperature (Poon et al., 2001). For example, *gcs1-3*

age2Δ double-mutant cells accumulate membranous structures called "Berkeley bodies" at 37°C (Poon et al., 2001), which are indicative of defective vesicle traffic within the Golgi apparatus (Novick et al., 1980). Furthermore, *gcs1-3 age2Δ* double mutants also display defects in transporting cargo proteins, such as carboxypeptidase Y (CPY) and alkaline phosphatase (ALP), from the *trans*-Golgi network to the vacuole at the non-permissive temperature (Poon et al., 2001). Together, these data indicate that the Gcs1 and Age2 ArfGAPs have overlapping function for transport from the *trans*-Golgi network.

Although the Gcs1, Glo3, and Age2 proteins have been clearly demonstrated to operate for vesicular transport, the vesicular-transport function (if any) of the remaining ArfGAP family members (Age1, Gts1, and Sps18) remains unknown. Age1 has been identified as a suppressor of the effects of a temperature-sensitive *arf1* mutant (Zhang et al., 1998), as well as a suppressor of the effects of deficient Gcs1 and Age2 ArfGAP function for transport from the *trans*-Golgi network (Auger, 2000). A follow-up study has inferred from subtle genetic interactions that the *AGE1* gene encodes a protein that may be involved in endocytic transport processes (Zhang et al., 2003), but the normal function of Age1 remains open to speculation. The Gts1 protein has been implicated in the regulation of heat tolerance (Yaguchi et al., 1996), and has recently been shown to interact both genetically and physically with the Snf1 kinase (Yaguchi and Tsurugi, 2003). Because the Snf1 kinase is a transcriptional activator of glucose-repressible genes (Celenza and Carlson, 1986), it is likely that the Gts1 protein acts in the nucleus, although the normal function of Gts1 is still unknown. Expression of the *SPS18* gene is controlled

by a promoter that is activated only during meiosis (part of a developmental process known as sporulation in yeast), and therefore the Sps18 protein is thought to play a role in meiotic processes (Coe et al., 1994). Thus, the roles (if any) for the Age1, Gts1, and Sps18 members of the yeast ArfGAP family in the vesicular-transport process await characterization.

5. Objectives of this study

The Gcs1 and Glo3 ArfGAPs have previously been shown to be involved in retrograde vesicular transport from the Golgi apparatus to the ER (Poon et al., 1999). However, the exact functions of ArfGAPs for retrograde vesicular transport remain unknown. In this study, I have used two different approaches to characterize ArfGAP function for retrograde transport. First, to identify proteins that are involved with ArfGAP function for retrograde transport I have isolated and characterized dosage suppressors of the effects of deficient Gcs1 and Glo3 ArfGAP function. This dosage-suppressor approach has led to the identification of the ArfGAP Age1 and the Sly41 protein, which was previously implicated in vesicular-transport processes mediated by the Rab GTPase Ypt1. Second, in collaboration with Dr. Anne Spang (Friedrich Miescher Laboratorium in Tuebingen, Germany), I have assessed the roles of the Gcs1 and Glo3 ArfGAPs in the retrograde-transport process at the molecular level. This analysis has allowed me to determine the individual contributions of the Gcs1 and Glo3 ArfGAPs to retrograde transport from the Golgi to the ER. I have found that Glo3 is the primary ArfGAP

required for retrograde vesicular transport, and that intact ArfGAP function is required for the generation of COPI transport vesicles.

II. MATERIALS & METHODS

1. Yeast strains and growth conditions

All yeast strains used in this study are isogenic to the wild-type diploid yeast strain W303 (*leu2-3,112 ura3-1 his3-11,15 trp1-1 ade2-1*) and its isogenic haploid derivatives W303-1A (*MAT***a**) and W303-1B (*MAT*α) (Archambault et al., 1992). A summary of yeast strains generated and/or used in this study is outlined in Table 1. All yeast strains were propagated in rich liquid growth medium (YM-1: 1% succinic acid, 0.6% sodium hydroxide, 1% bactopeptone, and 0.67% bacto-yeast nitrogen base without amino acids at pH 5.8, and 2% glucose), on rich solid growth medium (YEPD: 2% glucose, 2% bactopeptone, 1% bacto-yeast extract, and 2% agar), in synthetic dropout liquid growth medium (YNB: 1% succinic acid, 0.6% sodium hydroxide, 2% glucose, 0.67% bacto-yeast nitrogen base without amino acids or ammonium sulfate, and supplemented with 0.1% ammonium sulfate prior to use) containing the amino acids (40 μg/ml) and/or purine and pyrimidine bases (20 μg/ml) required to satisfy the auxotrophies of the yeast strain, or on synthetic complete solid growth medium (SC: YNB liquid medium with 40 μg/ml each of the following L-amino acids: arginine, aspartate, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, tryptophan, threonine, tyrosine, and valine, 20 μg/ml each of the bases adenine and uracil, and 2% agar) missing the appropriate amino acid(s) or purine and pyrimidine base(s) for plasmid selection.

Table 1. Yeast strains used in this study.

Strain	Description	Source
W303-1A	MAT**a** *leu2-3,112 ura3-1 his3-11,15 trp1-1 ade2-1*	Archambault et al., 1992
W303-1B	MATα *leu2-3,112 ura3-1 his3-11,15 trp1-1 ade2-1*	Archambault et al., 1992
GWK-9A	W303-1A *gcs1Δ::URA3*	Ireland et al., 1994
PPY51-A	W303-1A *glo3Δ::HIS3*	Poon et al., 1999
PPY51-B	W303-1B *glo3Δ::HIS3*	Poon et al., 1999
SL112	W303-1A *gcs1Δ::URA3 glo3Δ::HIS3* [pSL314.28]	This study
SL138	SL112 [pSL301]	This study
SL160.4C	W303-1A *gcs1Δ::LEU2 glo3Δ::HIS3 age1Δ::URA3* [pSL314.28]	This study
SL167	SL112 [pSL344]	This study
SL186	SL112 [pSL377]	This study
SL187	SL112 [pSL384]	This study
SL228	PPY51-B [pSL443]	This study
SL235	PPY51-A [pSL440]	This study
SL236	PPY51-A [pSL439]	This study
SL237	SL112 [pRS315-SSD1]	This study
SL238	SL112 [pSL483]	This study
SL242	SL112 [pSL489]	This study

2. Yeast transformation

Plasmid DNA was transformed into yeast cells using a lithium acetate/single-stranded DNA/polyethylene glycol procedure modified from Gietz et al. (1995). Cells were grown to a concentration of ~1 × 10^6 cells/ml, collected by centrifugation, and washed 2× in sterile ddH_2O. Cells were then washed in 1 ml of 100 mM lithium acetate (LiAc), and resuspended in 500 µl of 100 mM LiAc. For each transformation reaction, 50 µl of cell suspension was transferred to an eppendorf tube, the cells were pelleted and the supernatant removed, and then the following components were added in sequence:

240 µl polyethylene glycol (50% w/v)
36 µl 1 M LiAc
5 µl single-stranded DNA (10 mg/ml)
75 µl plasmid DNA diluted in sterile ddH_2O

The transformation reaction was mixed, and incubated at 30°C for 30 minutes with constant rotation. Transformation reactions were then incubated in a 42°C - 45°C water bath for 15 minutes. Cells were pelleted and resuspended in 150 µl sterile ddH_2O, spread on the appropriate solid growth medium to select for transformants, and incubated at 30°C until single colonies appeared (~2-3 days).

3. Yeast plasmid loss

To select for yeast cells that had failed to receive a plasmid during cell division and plasmid segregation, a "plasmid-loss" procedure was employed (Cormack and Castano, 2002). Yeast cells containing a plasmid to be lost were grown in liquid medium

containing the specific products of the prototrophy conferred by the plasmid. Cells were passaged through this growth medium for at least 20 generations (~3 days), and ~250 cells were transferred onto solid rich growth medium. Colonies that formed were then replica-plated to solid growth medium selective for cells containing the plasmid and to rich solid growth medium. Any colonies able to grow on both the selective and rich growth media were considered to harbour the plasmid, whereas colonies that grew only on the rich growth medium, but failed to grow on the selective growth medium, were considered to have lost the plasmid.

4. Routine molecular biology techniques

i) Isolation of plasmid DNA from *E. coli*

Plasmid DNA was isolated from *E. coli* cells using either a boiling lysis protocol (Holmes and Quigley, 1981) or using a Qiagen Mini-prep kit (Qiagen, Valencia, CA).

ii) Isolation of plasmid DNA from yeast

Plasmid DNA was isolated from yeast cells using a glass-bead disruption protocol as described (Hoffman, 1997).

iii) Restriction endonuclease digestion of DNA

DNA was digested using restriction endonucleases (Invitrogen, Carlsbad, CA; New England Biolabs, Beverly, MA) using the supplied buffers and under the conditions outlined by the supplier.

5. PCR amplification

DNA sequences were amplified for molecular cloning and analysis using the polymerase chain reaction (PCR). Platinum® *Pfx* DNA polymerase (Invitrogen, Carlsbad, CA) was used for reactions requiring high fidelity, whereas *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) was used for colony PCR.

i) High-fidelity PCR

To amplify DNA sequences for molecular cloning, reaction mixtures were set up as follows:

5 µl	10× *Pfx* amplification buffer
1 µl	50 mM $MgSO_4$
8 µl	1.25 mM dNTPs
2.5 µl	10 µM oligonucleotide primers (each)
1 µl	Platinum® *Pfx* DNA polymerase (2.5 units/µl)
5 µl	template DNA (~0.1 µg/ml)
ddH_2O to bring reaction volume to 50 µl	

Reactions were mixed in 0.5-ml thin-walled eppendorf tubes and placed in a heated-top thermocycler (MJ Research, Waltham, MA). Reactions were incubated at 94°C for 5 minutes, and then cycled 35 times under the following conditions:

94°C	30 seconds
50°C - 55°C	45 seconds
68°C	1 minute per kbp of DNA sequence to be amplified

After the final cycle, the reaction was incubated for an additional 5 minutes at 68°C.

ii) Colony PCR

To amplify DNA sequences from yeast colonies, reaction mixtures were set up as follows:

10 µl 10× PCR buffer (200 mM Tris-KCl [pH 8.4], 500 mM KCl)
10 µl 100 mM Tris-Cl, pH 9.4
3 µl 50 mM $MgCl_2$
8 µl 1.25 mM dNTPs
5 µl 10 µM oligonucleotide primers (each)
0.5 µl *Taq* DNA polymerase (5 units/µl)
5-10 µl volume of yeast cells from a single colony (template)
ddH_2O to bring volume to 100 µl

Reactions were mixed in 0.5-ml thin-walled eppendorf tubes, and then microwaved at maximum power for 1 minute to disrupt yeast cells. Reactions were placed in a heated-top thermocycler (MJ Research, Waltham, MA), and incubated at 94°C for 5 minutes. Reactions were then cycled 35 times under the following conditions:

94°C	1 minute
50°C - 55°C	45 seconds
72°C	1 minute per kbp of DNA sequence to be amplified

After the final cycle, reactions were incubated for an additional 5 minutes at 72°C.

6. DNA ligation

DNA ligation was performed using T4 DNA ligase (Invitrogen, Carlsbad, CA) essentially as described, with some modifications (Struhl, 1987). Briefly, ligation reactions were set up as follows:

13 µl "Insert" DNA

2 µl "Vector" DNA

4 µl 5× T4 DNA ligase buffer (250 mM Tris-HCl [pH 7.6], 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT, 25% [w/v] polyethylene glycol-8000)

1 µl T4 DNA ligase (1 unit/µl)

Reactions were incubated at room temperature overnight. 2 µl of each reaction was used to transform *E. coli* cells by electroporation as described (Seidman et al., 1997).

7. Plasmid construction

A summary of plasmids used in this study is outlined in Table 2. The plasmids generated in this study were constructed as follows:

a) pSL314.28: Plasmid pPP805.28 was digested with *Bam*HI and *Sst*I to excise a ~2.1-kbp fragment containing the *gcs1-28* allele along with upstream and downstream flanking sequence, which was subsequently ligated into the multiple cloning site of pRS314 to generate the pSL314.28 plasmid.

b) pSL301: The oligonucleotides 5'-CGGGATCCACCTTTTTGCGTTGTGCCTGAAT and 5'-CGAGCTCGGGATTGTAGCCATTGTTTTCG were used to a generate a PCR product containing the *SLY41* gene with ~0.5 kbp of upstream and downstream flanking sequence, which was then digested with *Bam*HI and *Sst*I, and ligated into the multiple cloning site of YEp351 to generate the pSL301 plasmid.

c) pSL315: Plasmid pSL314.28 was digested with *Bam*HI and *Sst*I to excise a ~2.1-kbp fragment containing the *gcs1-28* allele along with upstream and downstream flanking sequence, which was subsequently ligated into the multiple cloning site of pRS315 to

generate the pSL315 plasmid. This construction removes an *Xba*I site from the sequence flanking the *gcs1-28* allele found in plasmids pPP805.28 and pSL314.28.

d) pSL321: Plasmid pSH4 was digested with *Bam*HI and *Xba*I to excise a ~1-kbp fragment containing the carboxy-terminal coding sequence of the *GCS1* gene with downstream flanking sequence, which was subsequently ligated into *Bam*HI- and *Xba*I-digested pSL315 to generate the pSL321 plasmid.

e) pSL324: Plasmid pSH4 was digested with *Sst*I and *Xba*I to excise a ~0.9-kbp fragment containing the amino-terminal coding sequence of the *GCS1* gene with upstream flanking sequence, which was subsequently ligated into *Sst*I- and *Xba*I-digested pSL315 to generate the pSL324 plasmid.

f) pSL340: The oligonucleotides 5'-CGCCCTCGAGATGTTCGTTCACTTGTA and 5'-CGGGATCCTGAAGCAGTTGGAACCAAGA were used to generate a PCR product containing the *AGE1* gene with ~0.5-kbp of upstream and downstream flanking sequence, which was then digested with *Xho*I and *Bam*HI and ligated into the multiple cloning site of pRS315 to generate the pSL340 plasmid.

g) pSL344: Plasmid YEp352-GLL4 was digested with *Hin*dIII and *Bam*HI to excise a ~1.8-kbp fragment containing the *AGE2* gene with upstream and downstream flanking sequence, which was subsequently ligated into the multiple cloning site of YEp351 to generate the pSL344 plasmid.

h) pSL377: Plasmid pSL340 was digested with *Apa*I and *Bam*HI to generate a ~2-kbp fragment containing the *AGE1* gene with upstream and downstream flanking sequence,

which was subsequently ligated into the multiple cloning site of pRS425 to generate the pSL377 plasmid.

h) pSL384: Plasmid pSL377 was subjected to site-directed mutagenesis using the mutagenic oligonucleotides

5'-CAAATGTTCGGGCGTTCATGCATCTCTGGGTTCACATATCTCC and

5'-GGAGATATGTGAACCCAGAGATGCATGAACGCCCGAACATTTC to generate an *age1-R215A* mutant allele, resulting in the pSL384 plasmid.

j) pSL397: Plasmid pSL377 was subjected to site-directed mutagenesis using the mutagenic oligonucleotides

5'-CAAATGTTCGGGCGTTCATAAGTCTCTGGGTTCACATATCTCC and

5'-GGAGATATGTGAACCCAGAGACTTATGAACGCCCGAACATTTG to generate an *age1-R215K* mutant allele, resulting in the pSL384 plasmid.

k) pSL439: Plasmid p314-MET was digested with *Bam*HI and *Apa*I to excise a ~1-kbp fragment containing the *MET3* promoter region with the translation start codon for *MET3*, which was subsequently ligated into the multiple cloning site of pRS315 to generate the pSL439 plasmid.

l) pSL440: The oligonucleotides 5'-CCCAAGCTTAGTAACGATGAAGGAGAAACA and 5'-GGGGGCCCGAACCAAATGCTACCTCGTCT were used to generate a PCR product containing the coding sequence of the *GLO3* gene (from ORF nucleotide +4 to the stop codon) flanked by ~0.5-kbp of downstream sequence, which was then digested with *Hin*dIII and *Apa*I and ligated into the multiple cloning site of pSL439 to generate the pSL440 plasmid.

m) pSL443: Plasmid pSL440 was subjected to site-directed mutagenesis using the mutagenic oligonucleotides 5'-CAATGCTCTGCTGTGCATAAAAACATGGGTGTTCATATC and 5'-GATATGAACACCCATGTTTTTATGCACAGCAGAGCATTG to generate a *glo3-R59K* mutant allele, resulting in the pSL443 plasmid.

n) pSL460: Plasmid pPPL42 was subjected to site-directed mutagenesis using the mutagenic oligonucleotides 5'-CAATGCTCTGCTGTGCATAAAAACATGGGTGTTCATATC and 5'-GATATGAACACCCATGTTTTTATGCACAGCAGAGCATTG to generate a *glo3-R59K* mutant allele, resulting in the plasmid pSL460.

o) pSL483: The oligonucleotides 5'-CGGGATCCTCCACTTGCATCCCTCATTTT and 5'-CGAGCTCTGGCACCTTACAATTGAACG were used to generate a PCR product containing the *YJL193W* gene with upstream and downstream flanking sequence, which was then digested with *Bam*HI and *Sst*I and ligated into the multiple cloning site of YEp351 to generate the pSL483 plasmid.

p) pSL485: The oligonucleotides 5'-AAACTGCAGAGTTTAGAGACATTATACTCC and 5'-GCTCTAGAATCCATTATTGATGGAACATC were used to generate a PCR product containing the *AGE1* promoter region and the start codon for the *AGE1* gene, which was then digested with *Pst*I and *Xba*I and ligated into the multiple cloning site of YEp351 to generate the pSL485 plasmid.

q) pSL489: The oligonucleotides 5'-GCTCTAGATCCAATAGAGACGAACTGGATA and 5'-CGGGATCCTGAAGCAGTTGGAACCAAGA were used to generate a PCR

product containing the partial coding sequence for the *AGE1* gene (*age1-Δ164* allele: ORF nucleotide +497 to stop codon) flanked by ~0.5-kbp of downstream sequence, which was then digested with *Xba*I and *Bam*HI and ligated into the multiple cloning site of pSL485 to generate the pSL489 plasmid.

r) pSL494: Plasmid pSL489 was digested with *Pst*I and *Bam*HI to excise a ~1.5-kbp fragment containing the *age-Δ164* mutant allele with downstream flanking sequence, which was subsequently ligated into the multiple cloning site of pRS315 to generate the pSL494 plasmid.

8. Site-directed mutagenesis

Site-directed mutagenesis was performed using the Quickchange® XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). Each mutagenesis reaction was set up as follows:

5 µl	10× reaction buffer (100 mM KCl, 100 mM $[NH_4]_2SO_4$, 200 mM Tris-HCl [pH 8.8], 20 mM $MgSO_4$, 1% Triton X-100, 1 mg/ml BSA)
10 ng	plasmid template
125 ng	mutagenic oligonucleotide primers (each)
1 µl	dNTP mix
3 µl	QuickSolution
2.5 units	*PfuTurbo*® DNA polymerase

ddH_2O to bring the reaction to a final volume of 50 µl

The reaction was then incubated at 95°C for 1 minute, followed by 18 cycles under the following conditions:

Table 2. Plasmids used in this study.

Plasmid Name	Description	Source
pPP421	*GCS1 URA3* 2μ	Dr. Pak Phi Poon
pPP805.28	*gcs1-28 LEU2* CEN	Poon et al., 1999
pPPL42	*GLO3*-HIS_6 AMP^R	Poon et al., 1999
pPPL43	*GLO3 URA3* 2μ	Dr. Pak Phi Poon
pRS315-SSD1	*SSD1 LEU2* CEN	Allyson O'Donnell
pSH4	*GCS1 LEU2* CEN	Dr. Pak Phi Poon
pSL301	*SLY41 LEU2* 2μ	This study
pSL314.28	*gcs1-28 TRP1* CEN	This study
pSL315	*gcs1-28 LEU2* CEN	This study
pSL321	*gcs1-K105E LEU2* CEN	This study
pSL324	*gcs1-A227T, G249R LEU2* CEN	This study
pSL340	*AGE1 LEU2* CEN	This study
pSL344	*AGE2 LEU2* 2μ	This study
pSL377	*AGE1 LEU2* 2μ	This study
pSL384	*age1-R215A LEU2* 2μ	This study
pSL397	*age1-R215K LEU2* 2μ	This study
pSL439	*MET3pr-"empty" LEU2* CEN	This study
pSL440	*MET3pr-GLO3 LEU2* CEN	This study
pSL443	*MET3pr-glo3-R59K LEU2* CEN	This study
pSL460	*glo3-R59K*-HIS_6 AMP^R	This study
pSL483	*YJL193W LEU2* 2μ	This study
pSL485	*AGE1* promoter *LEU2* 2μ	This study
pSL489	*age1-Δ164 LEU2* 2μ	This study
pSL494	*age1-Δ164 LEU2* CEN	This study
YEp352-GLL4	*AGE2 URA3* 2μ	Auger, 2000

95°C 50 seconds

60°C 50 seconds

68°C 1 minute per kbp of plasmid length

After the final cycle, the reaction was incubated for an additional 7 minutes at 68°C. The reaction was then cooled to 37°C and 10 units of *Dpn*I restriction enzyme was added to the reaction, which was then incubated for an additional 60 minutes at 37°C. XL-10 Gold® ultracompetent *E. coli* cells, pretreated with β-mercaptoethanol, were transformed using the heat-shock method (Seidman et al., 1997) with 2 μl of the *Dpn*I-treated DNA. After heat shock, *E. coli* cells were allowed to recover in 500 μl of 2× YT broth (1.6% bactotryptone, 1% bacto-yeast extract, and 0.5% sodium chloride) for 1 hour at 37°C, and then were spread on YT + 100 μg/ml ampicillin solid growth medium (2× YT broth with 2% agar) and incubated at 37°C for at least 16 hours.

9. Electron microscopy

Examination of *gcs1-28 glo3Δ* yeast cells by electron microscopy was performed essentially as described (Byers and Goetsch, 1975; Poon et al., 1999). A 100-ml culture of yeast cells was grown to mid-log phase at 30°C, and half of the culture was shifted to 37°C and incubated for an additional 1 hour, while the remaining culture was incubated at 30°C. Cells were collected from 5 ml of culture by centrifugation and resuspended in 1 ml PBS. Glutaraldehyde was added to a final concentration of 2.5% and cells were incubated at room temperature for 2.5 hours. Fixed cells were washed 3× in PBS, and then postfixed in 2% osmium tetroxide in 0.1 M cacodylate, pH 6.8, and 5 mM $CaCl_2$,

washed with ddH_2O, and then incubated in 2% uranyl acetate for 1 hour. Cells were then processed for electron microscopic examination.

Examination of *glo3Δ MET3pr-glo3-R59K* and *glo3Δ MET3pr-GLO3* yeast cells by electron microscopy was performed as described (Eitzen et al., 1997), with some minor modifications. Briefly, cells were grown to mid-log phase at 23°C in medium containing methionine, collected by centrifugation and washed 3× in medium lacking methionine, then resuspended in 50 ml of medium lacking methionine and incubated at 30°C for 6 hours. To a 5-ml sample of culture, NaN_3 was added to a final concentration of 10 mM, and cells were collected by centrifugation. Cells were incubated in 1 ml of 1.5% $KMnO_4$ solution for 20 minutes with shaking. Cells were washed 2× in PBS and then incubated in 1 ml of 1% sodium periodate solution for 15 minutes with shaking. Cells were washed in PBS and then incubated in 1 ml NH_4Cl for 10 minutes with shaking. Finally, cells were washed and resuspended in PBS, and then processed for electron microscopic examination.

10. CPY transport assay

i) Radiolabeling of cells

Cells were grown to mid-log phase (~5×10^6 cells/ml) at 30°C in YNB without methionine and cysteine. Cells were collected by centrifugation and resuspended in YNB without methionine and cysteine at a concentration of 5×10^7 cells/ml, and then incubated at 37°C for 15 minutes (pre-incubation). 715 μCi of radiolabeled [^{35}S]-methionine and [^{35}S]-cysteine (Redivue Pro-Mix; Amersham) were added, and cells were

incubated for 7 minutes at 37°C ("pulse"). Pre-warmed 10× "chase" solution (50 mM methionine, 10 mM cysteine, 4% yeast extract, 20% glucose; Gaynor and Emr, 1997) was added to 1× final concentration, and cells were incubated at 37°C for an additional 1 hour, during which time 500-µl samples were collected at specific timepoints (e.g. 0, 30, 60 minutes). To each sample, ice-cold NaN_3 was added to 10 mM final concentration, cells were pelleted, and then resuspended in 100 µl of 20 mM NaN_3.

ii) CPY immunoprecipitation

Cell lysis and immunoprecipitation of CPY were performed essentially as described (Franzusoff et al., 1991; Gaynor and Emr, 1997). To each sample, 100 µl of 2× Laemmli buffer (100 mM Tris-Cl [pH 6.8], 10 mM EDTA, 10% glycerol, 4% SDS, 2% β-mercaptoethanol) was added along with ~200 µl of glass beads. Samples were then vortexed at 4°C for 15 minutes, incubated at 100°C for 10 minutes, then cooled on ice. Cell lysates were pre-adsorbed by adding 50 µl of pre-washed Pansorbin cells (Calbiochem, La Jolla, CA) in 800 µl of IP dilution buffer (60 mM Tris-Cl [pH 7.4], 6 mM EDTA, 190 mM NaCl, 1.25% [v/v] Triton X-100), which were then incubated with continuous rotation at room temperature for 30 minutes. Samples were spun in a microfuge at 15,000× g for 15 minutes, and 900 µl of supernatant was transferred to a new eppendorf tube.

Mouse monoclonal anti-CPY antibody (Molecular Probes, Eugene, OR) was added at 1:1000 titre, and samples were incubated at 4°C with continuous rotation overnight. Affinity-purified rabbit anti-mouse IgG (Jackson ImmunoResearch

Laboratories Inc., West Grove, PA) was then added at 1:1000 titre, and samples were incubated at 4°C with continuous rotation for 5 hours. 50 µl of pre-washed Protein-A-agarose beads (50% suspension; Invitrogen, Carlsbad, CA) were added, and samples were incubated an additional 5 hours at 4°C with continuous rotation. Samples were briefly spun in a microfuge at 6,200× g to pellet Protein-A-agarose beads, and the following washes were performed:

1. Wash 2× in 1 ml of IP buffer (50 mM Tris-Cl [pH 7.4], 5 mM EDTA, 150 mM NaCl, 1% [v/v] Triton X-100, 0.2% [w/v] SDS)
2. Wash in 1 ml Urea wash buffer (50 mM Tris-Cl [pH 7.4], 5 mM EDTA, 250 mM NaCl, 2 M urea, 1% Triton X-100, 0.2% SDS)
3. Wash in 1 ml High-salt wash buffer (50 mM Tris-Cl [pH 7.4], 5 mM EDTA, 500 mM NaCl, 1% Triton X-100, 0.2% SDS)
4. Wash in 1 ml Detergent-free wash buffer (50 mM Tris-Cl [pH 7.4], 5 mM EDTA, 150 mM NaCl)

After the final wash, Protein-A-agarose beads were resuspended in 40 µl of 1× Laemmli buffer (50 mM Tris-Cl [pH 6.8], 5 mM EDTA, 5% glycerol, 2% SDS, 2% β-mercaptoethanol, 0.02% bromophenol blue) and incubated at 100°C for 5 minutes. 20 µl of each sample was resolved by 7.5% SDS-PAGE, and radiolabeled CPY was detected using a Phosphoimager system (Bio-Rad Laboratories, Hercules, CA).

11. Co-immunoprecipitation

Co-immunoprecipitation of proteins was carried out essentially as described (Elion, 1999). Cells were grown to mid-log phase in YM1, NaN_3 was added to a final concentration of 10 mM, and then cells were collected by centrifugation. The cell pellet was resuspended in 500 µl of co-immunoprecipitation buffer (50 mM Tris-Cl [pH 7.4], 15 mM EGTA, 100 mM NaCl, 0.1% [v/v] Triton X-100, 1 mM PMSF, protease inhibitors) containing ~200 µl glass beads, vortexed at 4°C for 15 minutes, and the supernatant was collected and clarified by centrifugation at 12,000× g at 4°C for 15 minutes. Protein concentration was determined by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). 0.5 mg of total protein extract was incubated in co-immunoprecipitation buffer with anti-Sec21 polyclonal antibody at 1:250 titre at 4°C with continuous rotation for 90 minutes. 50 µl of pre-washed protein-A-agarose beads (50% suspension; Invitrogen, Carlsbad, CA) were added, and samples were incubated for an additional 60 minutes at 4°C with continuous rotation. Following incubation, protein-A-agarose beads were washed 3× in 1 ml of co-immunoprecipitation buffer, resuspended in 40 µl of 1× Laemmli buffer and boiled for 5 minutes. 20 µl of each sample was resolved by 10% SDS-PAGE, and proteins were detected by Western-blot analysis.

III. RESULTS

Part A: Characterization of the *gcs1-28* temperature-sensitive allele

1. Phenotypic analysis

The Gcs1 ArfGAP can mediate vesicular transport at two distinct transport stages (Poon et al., 1999, 2001), and in each case Gcs1 collaborates with a distinct ArfGAP to provide an overlapping essential function. Either member of the Gcs1 + Glo3 ArfGAP pair can mediate retrograde vesicular transport from the Golgi apparatus to the endoplasmic reticulum (ER; Poon et al., 1999), whereas either member of the Gcs1 + Age2 ArfGAP pair can mediate vesicular transport from the *trans*-Golgi network (Poon et al., 2001). To better characterize Gcs1 ArfGAP activity at these two distinct stages of vesicular transport, a collection of thermo-sensitive *gcs1* mutations was generated. These *gcs1* mutant alleles allow rapid loss of Gcs1 function upon shift to the non-permissive temperature, and thus permit phenotypic examination of the consequences of inadequate Gcs1 ArfGAP function at each transport stage (Poon et al., 1999, 2001).

Several *gcs1* mutant alleles were generated using PCR-mediated mutagenesis (Poon et al., 1999), and then tested for temperature sensitivity at 37°C in two situations: in cells lacking chromosomal copies of both the *GCS1* and *GLO3* genes, and in cells lacking chromosomal copies of both the *GCS1* and *AGE2* genes (Auger, 2000). Although many of the *gcs1* mutant alleles were found to be temperature-sensitive in both double-mutant situations, the *gcs1-28* mutant allele was found to confer a temperature-sensitive phenotype in *gcs1Δ glo3Δ* double-mutant cells but not in *gcs1Δ age2Δ* double-mutant

cells (Auger, 2000). These results indicate that the *gcs1-28* mutant allele encodes a Gcs1 protein that is temperature-sensitive for retrograde transport from the Golgi apparatus to the ER, but is still capable of providing function for transport from the *trans*-Golgi network at the elevated temperature. That is, the Gcs1-28 mutant ArfGAP is defective only for retrograde vesicular transport under non-permissive conditions. I undertook further analysis of the Gcs1-28 mutant protein to investigate the underlying reason for its temperature-sensitive retrograde-transport function.

2. The Gcs1-28 temperature-sensitive protein is stable at 37°C

Many temperature-sensitive proteins have been found to be unstable under non-permissive conditions (Prelich, 1999). Thus, it is possible that the Gcs1-28 mutant protein fails to function effectively at the elevated temperature because the mutant protein is unstable under this condition. This situation is unlikely, because the Gcs1-28 protein retains function for transport from the *trans*-Golgi network (in the absence of the Age2 ArfGAP) at the non-permissive temperature. Nonetheless, the stability of the Gcs1-28 mutant protein was directly assessed by Western-blot analysis.

gcs1Δ glo3Δ double-mutant cells carrying a low-copy plasmid harbouring the *gcs1-28* mutant allele (herein referred to as *gcs1-28 glo3Δ* cells) were grown to mid-log phase at 30°C, and half of the culture was incubated for an additional 2 hours at 30°C while the other half was incubated at 37°C for this 2-hour period. Cells were collected by centrifugation and proteins were extracted, resolved by SDS-PAGE, transferred to PVDF membrane and probed with anti-Gcs1 antibodies. The wild-type Gcs1 protein was

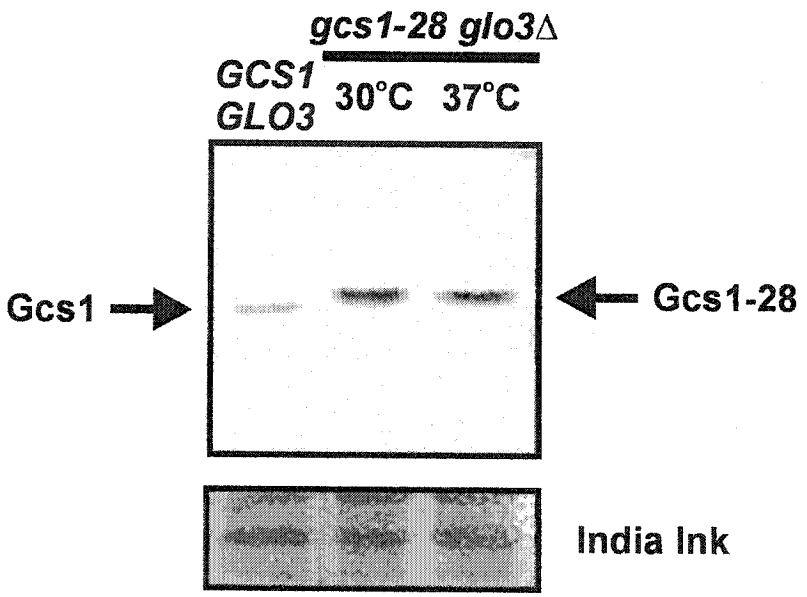

Figure 1. Gcs1-28 is a stable protein at 37°C. Wild-type cells were grown in rich growth medium at 30°C to mid-log phase, then shifted to 37°C and incubated for 2 hours; *gcs1-28 glo3Δ* cells were grown in rich medium at 30°C to mid-log phase, the culture was split, half was grown at 30°C for an additional 2 hours and half was grown at 37°C for 2 hours. Proteins were extracted, quantitated by Bradford assay, and equivalent amounts of protein were loaded in each lane of an acrylamide gel and separated by SDS-PAGE. Proteins were transferred to PVDF membranes, stained with India ink, and subjected to Western blot analysis using anti-Gcs1 antibodies.

detected in the extract from wild-type cells incubated at 37°C, and this sample serves as a positive control (Figure 1, lane 1). The Gcs1-28 mutant protein was detected in protein extracts from *gcs1-28 glo3Δ* cells incubated at either 30°C or 37°C at equivalent levels (Figure 1, compare lanes 2 and 3). Staining of the PVDF membrane with India ink showed that equivalent levels of protein were loaded and transferred to the membrane for each sample (Figure 1, lower panel). These results therefore indicate that the Gcs1-28 mutant protein is stable at 37°C, and that Gcs1-28 temperature sensitivity reflects a loss of Gcs1 function at the non-permissive temperature. Furthermore, since the stable Gcs1-28 ArfGAP retains function for transport from the *trans*-Golgi network under non-permissive conditions, this loss of function only affects retrograde transport.

3. The K105E substitution within Gcs1-28 causes temperature-sensitive loss of function for retrograde vesicular transport

The *gcs1-28* mutant allele was subjected to DNA sequencing to determine the base-pair changes in the *gcs1-28* mutant allele, and thus the resulting amino acid substitutions in the temperature-sensitive Gcs1-28 protein causing thermosensitivity for retrograde transport function. Three amino acid substitutions were identified in the Gcs1-28 mutant protein: a lysine residue is changed to a glutamate residue at position 105 (K105E), an alanine residue is changed to a threonine residue at position 227 (A227T), and a glycine residue is changed to an arginine residue at position 249 (G249R) (Figure 2A).

Since the *gcs1-28* allele contains multiple mutations, any one or a combination of mutations may be required for the temperature sensitivity of the Gcs1-28 mutant protein.

Figure 2. The K105E substitution within Gcs1-28 is responsible for temperature sensitivity. (A) Location of mutations in the *gcs1-28* allele. Shaded boxes indicate the location of the base-pair changes encoding the indicated amino acid substitutions. (B) *gcs1* hybrid alleles generated by fusion of the amino- or carboxy-terminal coding regions of the *gcs1-28* allele to the wild-type *GCS1* sequences. (C) *gcs1-28 glo3Δ* cells were transformed with plasmids carrying the *gcs1-28* gene, the *GCS1* gene, the *gcs1-28 (K105E)* gene, or the gcs1-*28 (A227T, G249R)* gene. The plasmid carrying the original *gcs1-28* allele was removed by plasmid loss. Cells were spread as patches on selective solid growth medium, incubated at 30°C for 2 days, and then replica-plated to a rich solid growth medium and incubated at 37°C for 3 days.

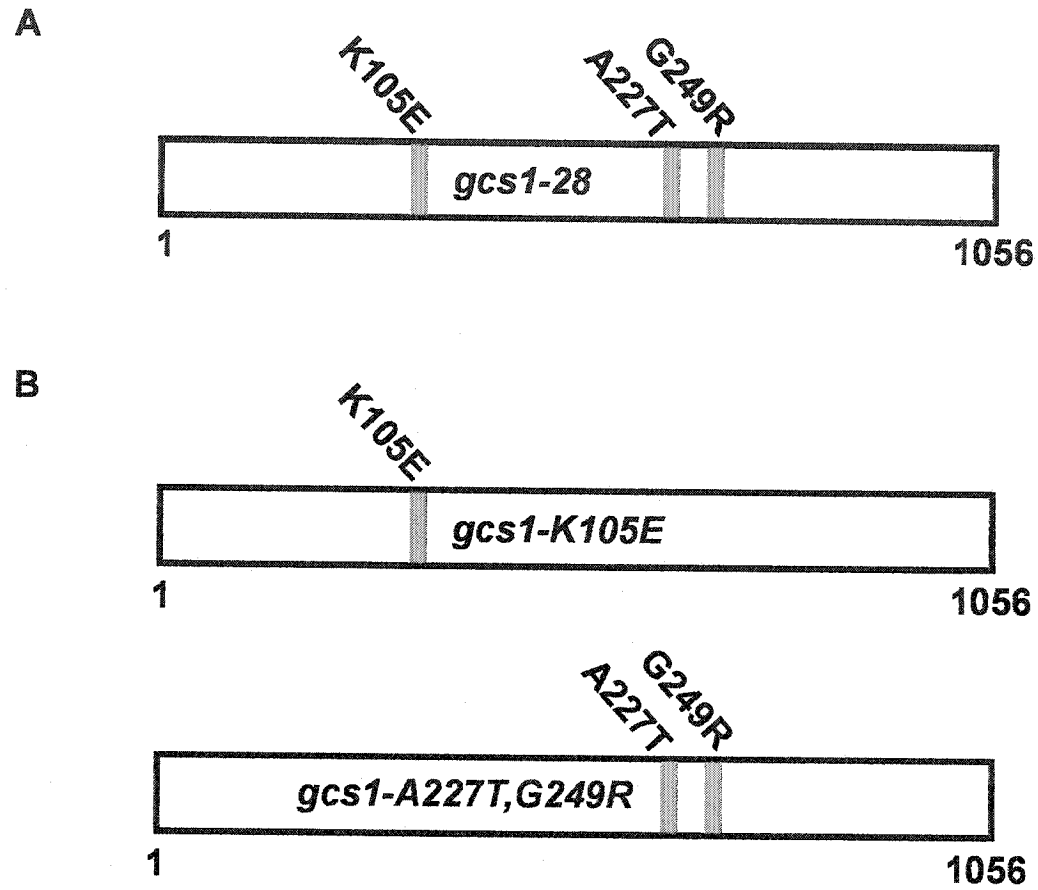
A
K105E
A227T
G249R
gcs1-28
1
1056
B
K105E
gcs1-K105E
1
1056
A227T
G249R
gcs1-A227T,G249R
1
1056
C
37°C
gcs1-28
GCS1
gcs1-K105E
gcs1-A227T,G249R

Figure 2

To determine which mutation or combination of mutations is required for the temperature-sensitive phenotype of the *gcs1-28* allele, hybrid *gcs1* alleles were generated and tested for temperature sensitivity in *gcs1Δ glo3Δ* double-mutant cells at 37°C. A convenient *Xba*I site within the *GCS1* and *gcs1-28* genes permitted separation of the sequence encoding the K105E substitution from the sequence encoding the A227T and G249R substitutions in the *gcs1-28* mutant allele.

The *gcs1-K105E* hybrid allele was generated by fusing the sequence encoding the amino terminus of the Gcs1-28 mutant protein to the sequence encoding the carboxy terminus of the wild-type Gcs1 protein; likewise, the *gcs1-A227T,G249R* hybrid allele was generated by fusing the sequence encoding the amino terminus of the wild-type Gcs1 protein to the sequence encoding the carboxy terminus of the Gcs1-28 mutant protein (Figure 2B). *gcs1-28 glo3Δ* cells were transformed with plasmids carrying these *gcs1* hybrid alleles, as well as a plasmid carrying the wild-type *GCS1* gene and a plasmid carrying the *gcs1-28* mutant allele. Derivatives of these transformants were then found that had lost the original *gcs1-28* plasmid (see Materials & Methods), leaving only the plasmid carrying a hybrid *gcs1* allele, the wild-type *GCS1* gene or the *gcs1-28* mutant allele on a different plasmid backbone. As expected, cells carrying only the plasmid with the wild-type *GCS1* gene were able to grow at 37°C, whereas cells carrying only the plasmid with the *gcs1-28* temperature-sensitive allele remained unable to grow at 37°C (Figure 2C). Similarly, cells carrying only the plasmid with the *gcs1-K105E* hybrid allele failed to grow at 37°C, whereas cells carrying only the plasmid with the *gcs1-A227T,G249R* hybrid allele were able to grow at 37°C (Figure 2C). These results indicate

that the K105E amino acid substitution is both necessary and sufficient for Gcs1-28 temperature sensitivity at 37°C.

Part B: Dosage suppressors of *gcs1-28 glo3Δ* temperature sensitivity

Although it is known that the Gcs1 + Glo3 ArfGAP pair mediates retrograde vesicular transport from the Golgi apparatus to the ER (Poon et al., 1999), little is known about exactly how either the Gcs1 or Glo3 ArfGAP functions in the retrograde vesicular transport process. I set out to characterize the role of ArfGAPs in retrograde transport by identifying gene-dosage suppressors of enfeebled Gcs1 function for this process in *gcs1-28 glo3Δ* double-mutant cells at 37°C. This analysis has led to the identification of a protein that can provide ArfGAP function *in vivo*, as well as another protein that has previously been implicated in the vesicular-transport process and a protein involved in cell-wall integrity.

1. Selection for dosage suppressors of *gcs1-28 glo3Δ* temperature sensitivity

Increased abundance of a protein that functions in the same or a related process as a mutant protein often allows the mutant cells to overcome the loss of function caused by the mutation (Prelich, 1999). In this situation, the gene encoding the protein that is present in increased abundance is known as a dosage suppressor. The identification of these dosage-suppressor genes provides insight into the mutant protein's role in a particular process. I undertook a selection for dosage suppressors of *gcs1-28 glo3Δ* temperature sensitivity to identify proteins involved with Gcs1 mediation of retrograde vesicular transport from the Golgi apparatus to the ER, and thus characterize the role of the Gcs1 ArfGAP in the retrograde transport process.

gcs1-28 glo3Δ cells were transformed with a high-copy yeast genomic library, and the resultant transformant colonies that formed at 30°C were then replica-plated to solid enriched medium and incubated at 37°C for 2 days. 134 colonies were identified that were able to grow at both 30°C and 37°C. These colonies are likely to harbour a library plasmid that suppresses *gcs1-28 glo3Δ* temperature-sensitivity. However, a colony may also regain the ability to grow at the non-permissive temperature because of a second-site chromosomal mutation or an intragenic suppressor mutation within the *gcs1-28* mutant allele itself. Since such mutational suppressor events were not of interest for this genetic study, suppressed colonies were subjected to a plasmid-loss procedure to determine if growth at 37°C was dependent on the presence of the library plasmid (see Materials & Methods). A total of 66 colonies were indeed dependent on the presence of the library plasmid for growth at 37°C. Therefore, each of the 66 colonies harboured a library plasmid that carries a potential dosage-suppressor gene, and thus these suppressed cells were subjected to further analysis.

I expected that the wild-type *GCS1* and *GLO3* genes would be identified by this dosage-suppressor approach, since either gene would be expected to eliminate *gcs1-28 glo3Δ* temperature sensitivity by providing a wild-type Gcs1 or Glo3 protein to the cell. To identify those colonies that harboured a plasmid carrying the wild-type *GLO3* gene, the 66 colonies that were dependent on the library plasmid for growth at 37°C were subjected to a "colony PCR" procedure (see Materials & Methods) using oligonucleotides that amplify the *GLO3* gene. Only colonies that harbour a library plasmid carrying the wild-type *GLO3* gene can produce a product by colony PCR using

these oligonucleotides, since chromosomal *GLO3* gene sequences have been deleted from *gcs1-28 glo3Δ* cells. 29 of the 54 colonies tested by colony PCR harboured a library plasmid carrying the wild-type *GLO3* gene; these colonies were not analyzed further.

To identify colonies that harboured a library plasmid carrying the wild-type *GCS1* gene, library plasmids were isolated from the remaining 25 colonies that did not harbour a library plasmid carrying the wild-type *GLO3* gene. The library plasmids, which carry a wild-type copy of the yeast *LEU2* gene, were separated from the plasmid carrying the *gcs1-28* temperature-sensitive allele by passage through the *E. coli* strain JF1754 (a *leuB* mutant strain), using the knowledge that the wild-type yeast *LEU2* gene complements mutant versions of the *E. coli leuB* gene (Ratzkin and Carbon, 1977; Storms et al., 1981). Those *E. coli* colonies that were able to grow in the absence of leucine in the growth medium contained a library plasmid. The library plasmids were then isolated from *E. coli* and used as templates for PCR amplification with oligonucleotides that amplify *GCS1* gene sequences. Only those library plasmids that contain a copy of the *GCS1* gene will generate a PCR product. 8 of the 25 library plasmids tested using this PCR-based approach carried the wild-type *GCS1* gene; these plasmids were not analyzed further.

Therefore, a total of 17 library plasmids carry confirmed dosage suppressors of *gcs1-28 glo3Δ* temperature-sensitivity. The results of the above experiments are summarized in Table 3. These library plasmids were then subjected to further analysis to determine the gene carried within the genomic insert responsible for suppression of *gcs1-28 glo3Δ* temperature sensitivity.

Table 3. Dosage suppressors of *gcs1-28 glo3Δ* temperature sensitivity

Plasmid	Genes present in library insert
YEp13-1	*GLO3*
YEp13-3	*EFR4, CFT2*
YEp13-4	*GCS1*
YEp13-6	*SLY41, SNU66, NOP58, YOR309C*
YEp13-8	*GLO3*
YEp13-9	*GLO3*
YEp13-10	*SEC18, SPT7*
YEp13-12	N.D.*
YEp13-13	N.D.
YEp13-14	*GLO3*
YEp13-16	*GLO3*
YEp13-17	*GLO3*
YEp13-18	*GCS1*
YEp13-19	*GCS1*
YEp13-21	*GLO3*
YEp13-22	N.D.
YEp13-24	*SLY41, SNU66, NOP58, YOR309C*
YEp13-25	*GCS1*
YEp13-26	*GCS1*
YEp13-28	N.D.
YEp13-30	*GLO3*
YEp13-31	*GLO3*
YEp13-33	*GLO3*
YEp13-35	*GCS1*
YEp13-36	*GLO3*
YEp13-40	*GLO3*
YEp13-41	*GLO3*
YEp13-42	*GLO3*
YEp13-46	*GCS1*
YEp13-47	*GLO3*
YEp13-48	*GLO3*
YEp13-51	*SLY41, SNU66, NOP58, YOR309C*
YEp13-53	*GLO3*
YEp13-54	N.D.
YEp13-58	*EPS1, BET1, YIL003W*
YEp13-59	*GLO3*
YEp13-60	*GCS1*
YEp13-61	N.D.
YEp13-65	*GLO3*
YEp13-69	*GLO3*
YEp13-72	*GLO3*

YEp13-74	*GLO3*
YEp13-75	N.D.
YEp13-76	*EFR4, CFT2*
YEp13-77	N.D.
YEp13-84	*SPS1, AGE1*
YEp13-86	*GLO3*
YEp13-87	*GLO3*
YEp13-90	*GLO3*
YEp13-91	N.D.
YEp13-94	*GLO3*
YEp13-95	*GLO3*
YEp13-101	*GLO3*
YEp13-102	*GLO3*
YEp13-103	N.D.
YEp13-104	N.D.
YEp13-105	N.D.
YEp13-107	N.D.
YEp13-117	N.D.
YEp13-119	N.D.
YEp13-120	N.D.
YEp13-121	N.D.
YEp13-122	N.D.
YEp13-123	N.D.
YEp13-125	N.D.
YEp13-134	N.D.

*N.D. = not determined

2. Identification of the *AGE1* gene as a dosage suppressor of *gcs1-28 glo3Δ* temperature sensitivity

The 17 library plasmids that were determined to carry dosage-suppressor genes for *gcs1-28 glo3Δ* temperature sensitivity were subjected to diagnostic restriction enzyme digestions to group plasmids according to the genomic insert carried. That is, library plasmids carrying the same genomic insert were expected to produce similar DNA fragments upon digestion with a restriction endonuclease. The restriction-digest pattern of each plasmid was then compared to the patterns of the other library plasmids, and those that were similar were grouped into a library-plasmid family. A representative plasmid from each group was then subjected to DNA sequencing to determine the region of the yeast genome carried by the library-plasmid family.

The library plasmid YEp13-84 was found to have a unique restriction-digest pattern. Furthermore, YEp13-84 is a very "strong" dosage suppressor of *gcs1-28 glo3Δ* temperature sensitivity, and was thus the first plasmid to be subjected to DNA sequence analysis to determine the region of the yeast genome carried within the library insert. DNA sequence analysis showed that the YEp13-84 library plasmid harbours a region of yeast chromosome IV (from co-ordinates 1,486,377 bp to 1,489,690 bp as displayed in Figure 3A). This region of yeast chromosome IV contains the entire *AGE1* gene and the 5' sequence of the *SPS1* gene.

It is unlikely that the *SPS1* gene is a dosage suppressor of *gcs1-28 glo3Δ* temperature sensitivity, because the *SPS1* gene is only expressed during meiotic cell division (sporulation), not during mitotic growth (Percival-Smith and Segall, 1986). In

contrast, the *AGE1* gene encodes a protein with ArfGAP activity that has been indirectly implicated in the vesicular-transport process (Zhang and Kahn, 1998; Zhang et al., 2003). Therefore, the *AGE1* gene was amplified by PCR, using the YEp13-84 library plasmid as a template, with oligonucleotides that amplify the *AGE1* open reading frame (ORF) flanked by 500 bp of upstream and downstream DNA sequence (Figure 3B). The resulting PCR product was then subcloned into a high-copy vector. *gcs1-28 glo3Δ* cells were transformed with the resulting high-copy *AGE1* plasmid, as well as a low-copy plasmid carrying the *GCS1* gene (positive control) and the high-copy vector itself (negative control). As expected, cells harbouring the low-copy *GCS1* plasmid were able to grow at 37°C, whereas cells carrying the "empty" vector failed to grow at 37°C (Figure 3C). Cells harbouring the high-copy *AGE1* plasmid were also able to grow at 37°C (Figure 3C), indicating that the *AGE1* gene is a dosage suppressor of *gcs1-28 glo3Δ* temperature sensitivity.

3. Increased dosage of the *AGE1* gene restores retrograde vesicular transport in *gcs1-28 glo3Δ* cells at 37°C

I have identified the *AGE1* gene as a dosage suppressor of the temperature-sensitive phenotype of *gcs1-28 glo3Δ* cells. To determine if the suppression of *gcs1-28 glo3Δ* temperature sensitivity by increased dosage of the *AGE1* gene is due to the restoration of retrograde vesicular transport in these cells, suppressed cells were first examined by electron microscopy (EM). Prior EM analysis of *gcs1-28 glo3Δ* cells incubated at 37°C showed that these cells cease proliferation and accumulate ER membranes (Poon et al.,

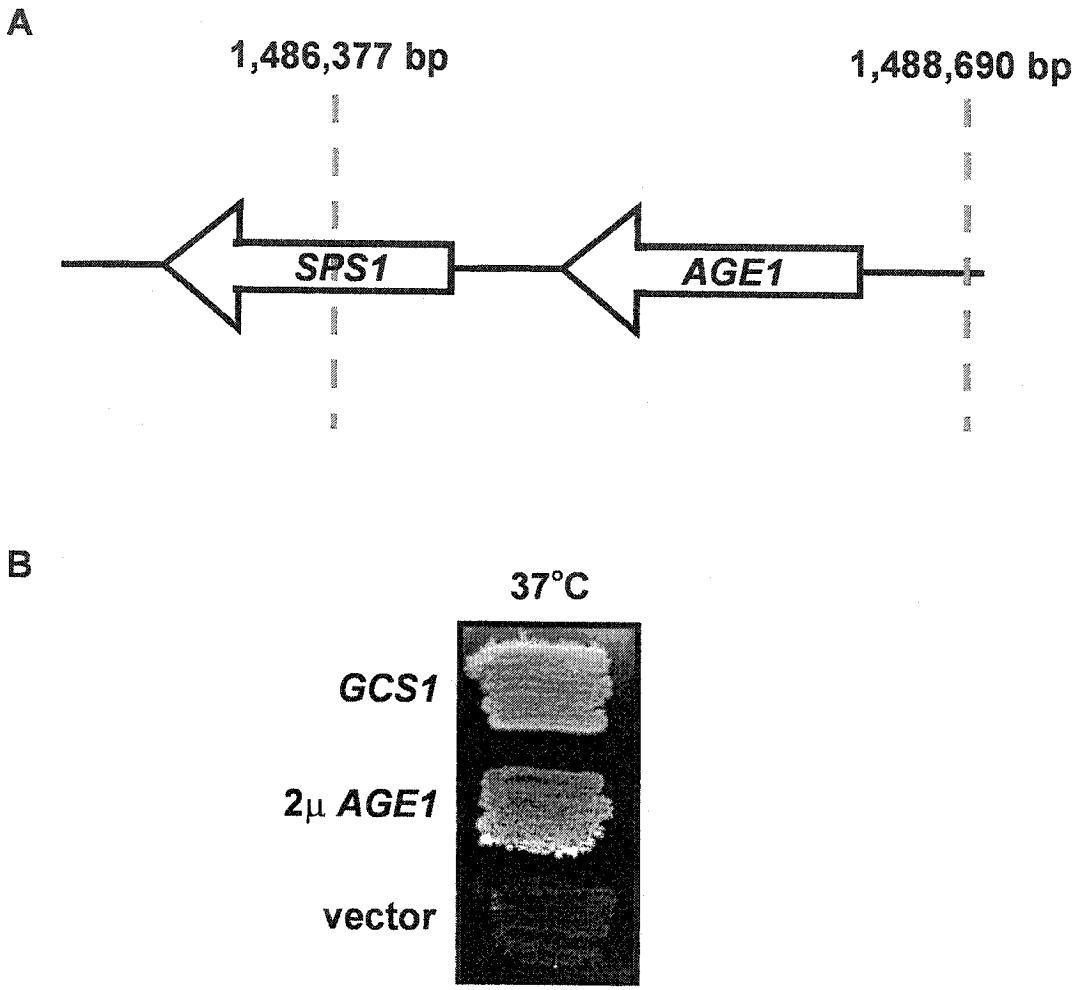

Figure 3. *AGE1* is a dosage suppressor of *gcs1-28 glo3Δ* temperature sensitivity. (A) The region of yeast chromosome IV carried by plasmid YEp13-84. (B) *gcs1-28 glo3Δ* cells were transformed with an "empty" high-copy (2 micron) vector, a plasmid carrying the *GCS1* gene, or a high-copy plasmid carrying the *AGE1* gene. Transformed cells were spread as patches on selective solid growth medium, incubated at 30°C for 2 days, and then replica-plated to rich solid growth medium and incubated at 37°C for 7 days.

1999). The accumulation of ER membranes by *gcs1-28 glo3Δ* cells at the non-permissive temperature likely reflects a breakdown in vesicular transport. Indeed, defects in retrograde transport from the Golgi to the ER affect anterograde transport out of the ER, since many of the components required for anterograde vesicular transport must normally be retrieved by the retrograde-transport machinery for use in subsequent rounds of anterograde vesicular transport (Gaynor and Emr, 1997). The resulting block of anterograde transport by defective retrograde transport leads to an expansion of the ER. Such expansion of the ER could be due either to accumulation of excess lipid in the ER membrane, or an expansion of the organelle to compensate for a buildup of anterograde protein cargo. Therefore, if increased dosage of the *AGE1* gene does indeed restore vesicular transport in *gcs1-28 glo3Δ* cells at 37°C, then less ER membrane accumulation would be expected at 37°C in *gcs1-28 glo3Δ* cells with increased *AGE1* gene dosage.

gcs1-28 glo3Δ cells carrying a low-copy *GCS1* plasmid, an "empty" vector, or a high-copy *AGE1* plasmid were grown to mid-log phase at 30°C, then transferred to 37°C and grown for an additional 2 hours. Cells were then collected and processed for EM analysis using standard techniques (Byers and Goetsch, 1975). *gcs1-28 glo3Δ* cells carrying the low-copy *GCS1* plasmid showed normal membrane morphology, with little to no accumulation of ER membrane (Figure 4A). In contrast, *gcs1-28 glo3Δ* cells carrying the "empty" vector showed a marked accumulation of ER membranes (Figure 4B; arrows indicate accumulated ER membranes). *gcs1-28 glo3Δ* cells carrying the high-copy *AGE1* plasmid showed significantly less accumulation of ER membrane compared

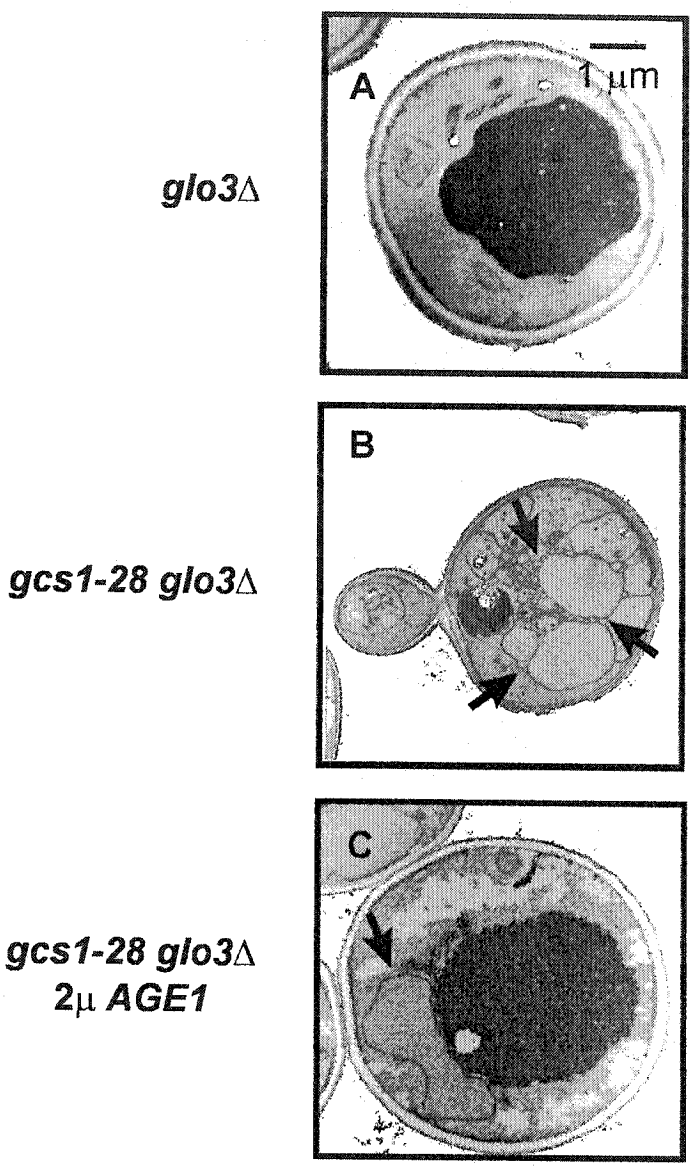

Figure 4. Increased dosage of *AGE1* partially restores ER morphology in *gcs1-28 glo3Δ* cells at 37°C. *gcs1-28 glo3Δ* cells carrying a plasmid with (A) the *GCS1* gene, (B) an "empty" vector, or (C) a high-copy plasmid with the *AGE1* gene were grown at 30°C in selective medium to mid-log phase, shifted to 37°C and incubated for 2 hours, and then fixed and processed for electron microscopy. Arrows indicate elaborated ER membranes.

to *gcs1-28 glo3Δ* cells carrying the "empty" vector (compare Figure 4C to 4B). These data indicate that increased *AGE1* gene dosage prevents the accumulation of ER membranes in *gcs1-28 glo3Δ* cells at 37°C, and thus likely restores vesicular transport in these cells.

To further characterize the effect of increased *AGE1* gene dosage on vesicular transport in *gcs1-28 glo3Δ* cells at 37°C, the processing of the transported cargo protein carboxypeptidase Y (CPY) was assessed. Processing of the CPY enzyme from the precursor forms (p1CPY and p2CPY) to the mature form (mCPY) requires vesicular transport to move the precursor forms among subcellular compartments, where different processing steps occur (Stevens et al., 1982). The p1 form of CPY is found in the ER, and is then transported to the *cis* compartment of the Golgi apparatus. Within the Golgi p1CPY becomes glycosylated to produce p2CPY, and p2CPY is then transported to the vacuole, where proteolytic processing of p2CPY leads to the production of the mature form, mCPY. Perturbation of vesicular transport among these subcellular compartments has been shown to cause the accumulation of the precursor forms of CPY (p1CPY or p2CPY), since these precursors cannot reach the proper compartment for processing due to a breakdown in the vesicular-transport process (Stevens et al., 1982). *gcs1-28 glo3Δ* cells were previously shown to accumulate p1CPY when incubated at 37°C, indicating a defect in vesicular transport from the ER to the Golgi apparatus, an indirect effect of blocked retrograde vesicular transport from the Golgi to the ER (Poon et al., 1999).

To assess the state of CPY, *gcs1-28 glo3Δ* cells carrying a low-copy *GCS1* plasmid, an "empty" vector, or a high-copy *AGE1* plasmid were grown to mid-log phase

at 30°C, then transferred to 37°C and subjected to pulse-chase labeling and immunoprecipitation of CPY as previously described (Poon et al., 1999). As shown in Figure 5, *gcs1-28 glo3Δ* cells carrying the low-copy *GCS1* plasmid process p1CPY to mCPY after only 30 minutes at 37°C (Figure 5A), whereas *gcs1-28 glo3Δ* cells carrying the "empty" vector process little p1CPY to mCPY even after 60 minutes at 37°C (Figure 5B). However, *gcs1-28 glo3Δ* cells carrying the high-copy *AGE1* plasmid process significant amounts of p1CPY to mCPY after 30 minutes, and are able to convert 55% of p1CPY to mCPY after 60 minutes at 37°C (Figure 5C). These results indicate that increased dosage of the *AGE1* gene in *gcs1-28 glo3Δ* cells at 37°C permits transport of p1CPY and p2CPY through the secretory pathway to the vacuole, where mCPY is produced. Therefore, increased *AGE1* gene dosage likely restores retrograde vesicular transport in *gcs1-28 glo3Δ* cells at 37°C.

4. Age1 ArfGAP activity is required for suppression of *gcs1-28 glo3Δ* temperature sensitivity

A previous study has shown that the Age1 protein has ArfGAP activity *in vitro* (Zhang et al., 2003). It is possible that increased abundance of the Age1 protein may provide sufficient ArfGAP activity for retrograde vesicular transport to allow *gcs1-28 glo3Δ* cells to compensate for decreased Gcs1-28 ArfGAP activity at 37°C. If this is indeed the case, then Age1 ArfGAP activity should be required for suppression of *gcs1-28 glo3Δ* temperature sensitivity. Conversely, increasing the abundance of the Age1 ArfGAP may

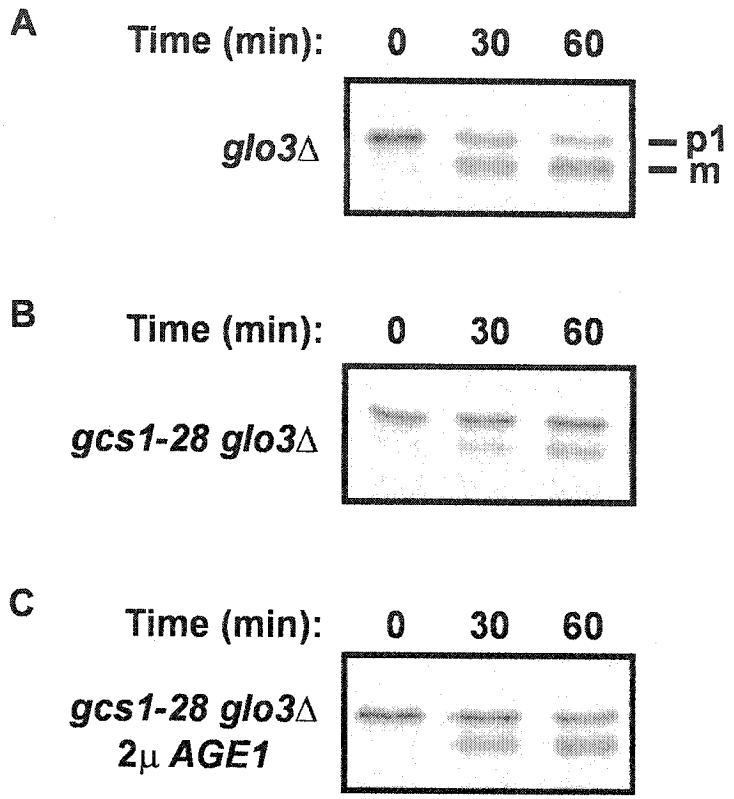

Figure 5. Increased dosage of *AGE1* partially restores CPY maturation in *gcs1-28 glo3Δ* cells at 37°C. *gcs1-28 glo3Δ* cells carrying (A) a plasmid with the *GCS1* gene, (B) an "empty" vector, or (C) a high-copy plasmid with the *AGE1* gene were grown to mid-log phase in selective medium. Cells were inoculated into growth medium lacking methionine and cysteine and grown at 37°C for 30 minutes, pulsed with [^{35}S]-methionine, [^{35}S]-cysteine for 7 minutes, and then chased for 60 minutes with excess unlabeled methionine and cysteine. At 0, 30 and 60 minutes samples were taken, cells were lysed, and CPY was immunoprecipitated from the extracts. Immunoprecipitants were resolved by SDS-PAGE, and CPY was detected by autoradiography. The p1 (ER-form) and m (mature form) species of CPY are indicated.

allow Age1 to titrate away a negative regulator of Gcs1 ArfGAP function, or Age1 may even provide a scaffold function in the absence of a functional Gcs1 (or Glo3) protein to facilitate important protein-protein interactions. In these situations, Age1 ArfGAP activity itself may not be required for suppression of *gcs1-28 glo3Δ* temperature sensitivity.

To test the requirement for Age1 ArfGAP activity for suppression of *gcs1-28 glo3Δ* temperature sensitivity, a mutant version of the Age1 protein was generated that should lack any detectable ArfGAP activity. It has been previously determined that an arginine residue downstream of the zinc-binding motif in the Gcs1 protein is absolutely required for Gcs1 ArfGAP activity (Yanagisawa et al., 2002). This arginine residue is invariant, since it is found in all members of the ArfGAP protein family. Therefore, to generate a mutant version of the Age1 protein that lacks ArfGAP activity, I employed site-directed mutagenesis to change the invariant arginine residue at position 215 in the Age1 ArfGAP to either an alanine residue or a lysine residue (Figure 6A). These mutant alleles of the *AGE1* gene are known as *age1-R215A* and *age1-R215K*, respectively.

gcs1-28 glo3Δ cells were transformed with a low-copy *GCS1* plasmid, an "empty" vector, a high-copy *AGE1* plasmid, a high-copy *age1-R215A* plasmid or a high-copy *age1-R215K* plasmid. As expected, cells carrying the low-copy *GCS1* plasmid or the high-copy *AGE1* plasmid were able to grow at 37°C, whereas cells carrying the "empty" vector were not (Figure 6B). *gcs1-28 glo3Δ* cells carrying the high-copy *age1-R215A* plasmid or high-copy *age1-R215K* plasmid were also unable to grow at 37°C (Figure 6B; data not shown). These data suggest that intact Age1 ArfGAP activity is

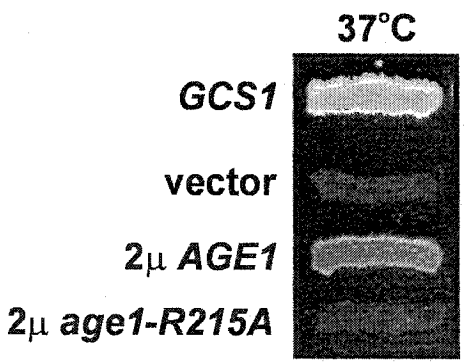

Figure 6. Age1 ArfGAP activity is required for suppression of *gcs1-28 glo3Δ* temperature sensitivity. *gcs1-28 glo3Δ* cells were transformed with an "empty" vector, a low-copy plasmid carrying the *GCS1* gene, a high-copy plasmid carrying the *AGE1* gene, or a high-copy plasmid carrying the *age1-R215A* allele. Transformed cells were spread as patches on selective solid growth medium, incubated at 30°C for 2 days, then replica-plated to rich solid growth medium and incubated at 37°C for 3 days.

indeed required for suppression of *gcs1-28 glo3Δ* temperature sensitivity.

5. Increased abundance of the Age1 ArfGAP cannot replace (bypass) the Gcs1 or Glo3 ArfGAP for retrograde vesicular transport

The finding that suppression of *gcs1-28 glo3Δ* temperature sensitivity by increased abundance of the Age1 protein requires Age1 ArfGAP activity raises the possibility that increased abundance of the Age1 ArfGAP may bypass the need for any Gcs1 or Glo3 ArfGAP activity for retrograde vesicular transport. In this situation, the Age1 ArfGAP may provide the required ArfGAP activity for retrograde vesicular transport when present at sufficiently high levels. Therefore, to determine whether increased abundance of the Age1 ArfGAP bypasses the need for the Gcs1 or Glo3 ArfGAP for retrograde vesicular transport, *gcs1-28 glo3Δ* cells carrying the high-copy *AGE1* plasmid were grown under conditions that allow the continued proliferation of cells that have lost the nutritional marker on the plasmid carrying the *gcs1-28* allele. Cells will only be able to survive this plasmid-loss event if increased abundance of the Age1 ArfGAP bypasses the need for either the Gcs1 or Glo3 ArfGAP for retrograde transport.

gcs1-28 glo3Δ cells lack chromosomal copies of the *GCS1* and *GLO3* genes, and the *gcs1-28* mutant allele is supplied on a low-copy plasmid that also contains the wild-type *TRP1* gene (*gcs1-28 TRP1* plasmid). The *AGE1* gene is harboured on a high-copy plasmid that also contains the wild-type *LEU2* gene (*AGE1 LEU2* plasmid). If *gcs1-28 glo3Δ* cells carrying the high-copy *AGE1* plasmid are able to grow in the absence of the

gcs1-28 mutant allele, these cells should be able to survive the loss of the *gcs1-28 TRP1* plasmid when grown under the appropriate "plasmid-loss" conditions. The resulting cells will be *gcs1Δ glo3Δ* double mutants with a high-copy *AGE1* plasmid, and such cells can easily be identified because the chromosomal copies of the *TRP1* and *LEU2* amino acid biosynthetic genes are mutated. Only those cells that harbour a plasmid carrying the wild-type *TRP1* gene and a plasmid carrying the wild-type *LEU2* gene will be tryptophan and leucine prototrophs. Therefore, cells that have lost the *gcs1-28 TRP1* plasmid, but retained the *AGE1 LEU2* plasmid, will be tryptophan auxotrophs and remain leucine prototrophs, because these cells have only the mutant *trp1* gene and carry a copy of the wild-type *LEU2* gene on the *AGE1 LEU2* plasmid.

When *gcs1-28 glo3Δ* cells carrying the high-copy *AGE1* plasmid were grown under the appropriate "plasmid-loss" conditions, I was unable to recover cells that were tryptophan auxotrophs and leucine prototrophs. Indeed, the over 5000 colonies examined were formed by cells that were both tryptophan and leucine prototrophs (the normal rate of plasmid loss in a population of yeast cells is ~ 1%), indicating that *gcs1-28 glo3Δ* cells carrying the high-copy *AGE1* plasmid still require the presence of the *gcs1-28* mutant allele (data not shown). Since increased abundance of the Age1 ArfGAP does not allow *gcs1Δ glo3Δ* double-mutant cells to grow, these data suggest that increased abundance of the Age1 ArfGAP does not bypass the need for either the Gcs1 or Glo3 ArfGAP for retrograde vesicular transport.

Table 4. Genotypes of meiotic segregants from *GCS1/gcs1Δ GLO3/glo3Δ* [2μ *AGE1*] diploid cells.

Wild-type segregants	*gcs1Δ* segregants	*glo3Δ* segregants	*gcs1Δ glo3Δ* segregants
24	24	11	0

59 meiotic segregants were examined

To confirm these results, heterozygous *gcs1* and *glo3* mutant diploids carrying only the high-copy *AGE1* plasmid were allowed to undergo meiosis, and meiotic segregants were tested for the presence of the *gcs1Δ* and *glo3Δ* mutations and the high-copy *AGE1* plasmid. Of the 59 meiotic segregants examined, no *gcs1Δ glo3Δ* double-mutant segregants carrying the high-copy *AGE1* plasmid were found (Table 4), suggesting that increased dosage of the *AGE1* gene does not allow *gcs1Δ glo3Δ* double-mutant segregants to grow. These findings, together with the results of the plasmid-loss experiment, indicate that increased abundance of the Age1 ArfGAP cannot replace (bypass) the need for the Gcs1 or Glo3 ArfGAP for retrograde vesicular transport.

6. Lack of genetic interactions between an *age1Δ* mutant and the *gcs1-28 glo3Δ* double-mutant situation

I have found that *gcs1-28 glo3Δ* cells carrying the high-copy *AGE1* plasmid must retain the *gcs1-28* mutant allele for viability. Therefore, increasing the abundance of the Age1 ArfGAP does not simply replace the Gcs1 and Glo3 ArfGAPs for retrograde vesicular transport. One explanation for this result is that the Gcs1, Glo3, and Age1 ArfGAPs may function together to facilitate retrograde vesicular transport, with the Age1 ArfGAP playing only a minor role. Increasing the abundance of Age1 may allow it to exert a stronger influence on retrograde transport, but functional retrograde transport would still require the presence of the enfeebled Gcs1-28 ArfGAP. To determine whether the Age1 ArfGAP plays a role in retrograde vesicular transport, genetic interactions between an *age1Δ* mutant and the *gcs1-28 glo3Δ* double-mutant situation were sought. If the Age1

ArfGAP does play a role in retrograde transport, a genetic interaction between the *age1Δ* mutant and the *gcs1-28 glo3Δ* double-mutant situation might be expected.

age1Δ mutant cells are viable, and display no obvious deleterious phenotype. An *age1Δ* mutant was mated to a *gcs1-28 glo3Δ* double-mutant to generate a *GCS1/gcs1-28 GLO3/glo3Δ AGE1/age1Δ* heterozygous diploid. These diploid cells were induced to undergo meiosis, and meiotic segregants were evaluated for the presence of *gcs1-28 glo3Δ age1Δ* triple-mutant segregants. Indeed, *gcs1-28 glo3Δ age1Δ* triple mutants were present, indicating that cells with the *gcs1-28 glo3Δ age1Δ* genotype are viable (data not shown). To determine if the *gcs1-28 glo3Δ age1Δ* triple-mutant situation exacerbates the temperature sensitivity of the *gcs1-28* allele, *gcs1-28 glo3Δ age1Δ* triple-mutant cells, *gcs1-28 glo3Δ* double-mutant cells, and *gcs1-28 glo3Δ* cells carrying a low-copy *GCS1* plasmid were incubated at various temperatures and examined for differences in growth capacity. At all temperatures tested (23°C, 30°C, 34°C, and 37°C), no differences in growth were seen between *gcs1-28 glo3Δ* double-mutant cells and *gcs1-28 glo3Δ age1Δ* triple-mutant cells (Figure 7), suggesting that the presence of the *age1Δ* mutation does not exacerbate the temperature sensitivity of the *gcs1-28* allele. I could not detect any obvious genetic interactions between an *age1Δ* mutant and the *gcs1-28 glo3Δ* double-mutant situation, suggesting that the Age1 ArfGAP may not normally play a role in Gcs1- and Glo3-mediated retrograde vesicular transport from the Golgi to the ER.

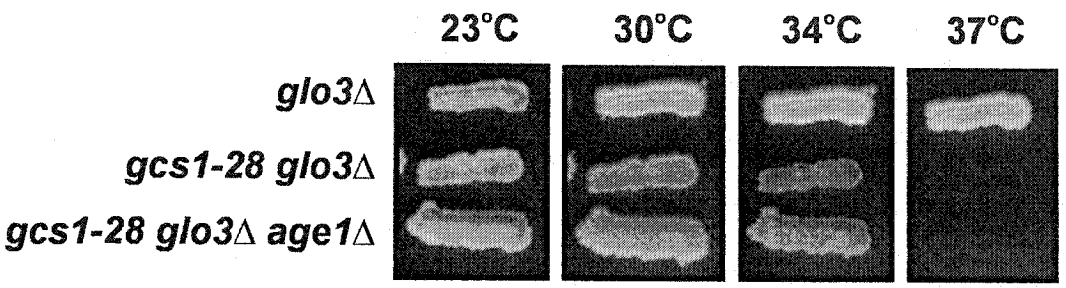

Figure 7. The Age1 ArfGAP does not normally function for retrograde vesicular transport. *glo3Δ*, *gcs1-28 glo3Δ*, and *gcs1-28 glo3Δ age1Δ* cells were spread as patches on rich solid growth medium and incubated at 23°C for 3 days, and then replica-plated to rich solid growth medium and incubated at 23°C, 30°C, 34°C, or 37°C for 3 days.

7. Increased abundance of the Age2 ArfGAP does not suppress *gcs1-28 glo3Δ* temperature sensitivity

I have no evidence that the Age1 ArfGAP normally functions for retrograde vesicular transport from the Golgi to the ER. Therefore, increasing the abundance of the Age1 ArfGAP may allow Age1 to perform a function that it does not normally carry out. The *Saccharomyces cerevisiae* genome encodes six proteins that are members of the ArfGAP family: Age1, Age2, Gcs1, Glo3, Gts1, and Sps18. The Gcs1, Glo3, Age1, and Age2 ArfGAP family members are known to possess ArfGAP activity *in vitro* (Poon et al., 1996; Poon et al., 1999; Zhang et al., 2003; Poon et al., 2001). It is therefore possible that ArfGAPs other than Age1 (and the Gcs1 and Glo3 ArfGAPs themselves) may facilitate retrograde transport, since increased abundance of such ArfGAPs may allow these proteins to perform a function not normally carried out. The only yeast ArfGAP family member other than Age1, Gcs1, and Glo3 with demonstrated ArfGAP activity that is not known to provide function for retrograde vesicular transport is Age2. The Age2 ArfGAP has been shown to function, along with the Gcs1 ArfGAP, for transport from the *trans*-Golgi network (Poon et al., 2001).

To test whether increased abundance of the Age2 ArfGAP suppresses *gcs1-28 glo3Δ* temperature sensitivity, the *AGE2* gene was first subcloned into a high-copy plasmid. *gcs1-28 glo3Δ* cells were then transformed with a low-copy *GCS1* plasmid, the high-copy *AGE2* plasmid, or an "empty" vector and tested for growth at 37°C. As expected, *gcs1-28 glo3Δ* cells carrying the low-copy *GCS1* plasmid were able to grow at

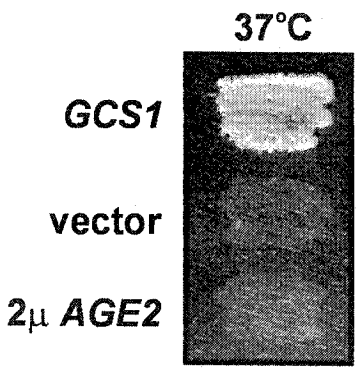

Figure 8. Increased abundance of the Age2 ArfGAP does not suppress *gcs1-28 glo3Δ* temperature sensitivity. *gcs1-28 glo3Δ* cells were transformed with an "empty" vector, a plasmid carrying the *GCS1* gene, or a high-copy plasmid carrying the *AGE2* gene. Transformed cells were spread as patches on selective solid growth medium, incubated at 30°C for 2 days, and then replica-plated to rich solid growth medium and incubated at 37°C for 3 days.

37°C, whereas *gcs1-28 glo3Δ* cells carrying the "empty" vector were not (Figure 8). Similarly, *gcs1-28 glo3Δ* cells carrying the high-copy *AGE2* plasmid also failed to grow at 37°C (Figure 8), suggesting that the Age2 ArfGAP cannot function for retrograde vesicular transport, even when present in increased abundance. Since increased abundance of the Age2 ArfGAP does not suppress the temperature sensitivity of *gcs1-28 glo3Δ* cells, this result suggests that suppression of the temperature-sensitive *gcs1-28 glo3Δ* retrograde transport defect cannot be mediated by a general increase in any ArfGAP protein, but appears to be specific to the Age1 ArfGAP.

8. The *age1-Δ164* mutant allele is a better dosage suppressor of *gcs1-28 glo3Δ* temperature sensitivity than the wild-type *AGE1* gene

I have shown that Age1 is the only yeast protein with demonstrated ArfGAP activity that can suppress *gcs1-28 glo3Δ* temperature sensitivity when present in increased abundance (other than the Gcs1 and Glo3 ArfGAPs themselves). Therefore, the Age1 ArfGAP may have unique features that allow it to facilitate retrograde vesicular transport, and thus suppress inadequate Gcs1 and Glo3 ArfGAP function. The Age1 ArfGAP differs from other members of the yeast ArfGAP family by possessing a long amino-terminal extension (Figure 9A). It is possible that this 164-residue amino-terminal region may contain sequences that allow the Age1 ArfGAP to function for retrograde vesicular transport when Age1 is present in increased abundance.

To determine the requirement for the Age1 amino-terminal extension for suppression of *gcs1-28 glo3Δ* temperature sensitivity, a mutant *age1* allele was

constructed that lacks the first 164 codons of the wild-type *AGE1* gene (*age1-Δ164*) and is expressed from the endogenous *AGE1* promoter (Figure 9B). The *age1-Δ164* allele was then subcloned into a high-copy plasmid, which was transformed into *gcs1-28 glo3Δ* cells. *gcs1-28 glo3Δ* cells carrying a low-copy *GCS1* plasmid, a high-copy *AGE1* plasmid, the high-copy *age1-Δ164* plasmid, or an "empty" vector were tested for growth at 37°C. As expected, *gcs1-28 glo3Δ* cells carrying the low-copy *GCS1* plasmid or the high-copy *AGE1* plasmid were able to grow at 37°C, whereas *gcs1-28 glo3Δ* cells carrying the "empty" vector failed to grow at 37°C (Figure 9C). Although *gcs1-28 glo3Δ* cells carrying the high-copy *age1-Δ164* plasmid were able to grow at 37°C, I noted that these cells actually grew much better than *gcs1-28 glo3Δ* cells carrying the high-copy wild-type *AGE1* plasmid (Figure 9C). The 164-residue amino-terminal extension of the Age1 ArfGAP is therefore not required for suppression of *gcs1-28 glo3Δ* temperature sensitivity, and indeed may even exert a negative effect on Age1 suppression of *gcs1-28 glo3Δ* temperature sensitivity.

9. Single-gene dosage of the *age1-Δ164* mutant allele suppresses *gcs1-28 glo3Δ* temperature sensitivity

I have found that the *age1-Δ164* mutant allele is a better dosage suppressor of *gcs1-28 glo3Δ* temperature sensitivity than the wild-type *AGE1* gene, raising the possibility that single-gene dosage of the *age1-Δ164* allele may also be able to suppress the temperature-

Figure 9. The *age1-Δ164* allele is a dosage suppressor of *gcs1-28 glo3Δ* temperature sensitivity. (A) Schematic alignment of the six ArfGAP family members in yeast; note the amino-terminal extension of Age1. (B) Schematic representation of the *age1-Δ164* allele. *AGE1* sequences from ORF nucleotides +2 to +496 were removed (indicated by arch). (C) *gcs1-28 glo3Δ* cells were transformed with an "empty" vector, a plasmid carrying the *GCS1* gene, a high-copy plasmid carrying the *AGE1* gene, or a high-copy plasmid carrying the *age1-Δ164* allele. Transformed cells were spread as patches on selective solid growth medium, incubated at 30°C for 2 days, and then replica-plated to rich solid growth medium and incubated at 37°C for 3 days.

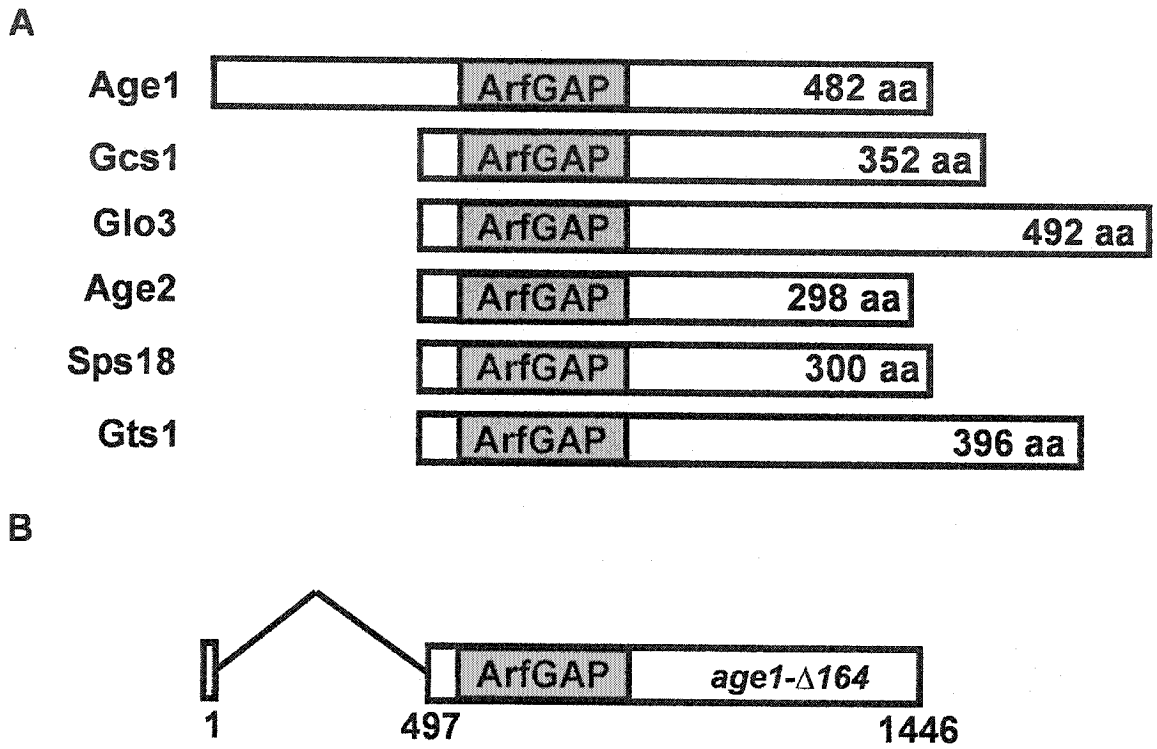
A
Age1
ArfGAP
482 aa
Gcs1
ArfGAP
352 aa
Glo3
ArfGAP
492 aa
Age2
ArfGAP
298 aa
Sps18
ArfGAP
300 aa
Gts1
ArfGAP
396 aa
B
ArfGAP
age1-Δ164
1
497
1446
C
37°C
GCS1
vector
2μ AGE1
2μ age1Δ164

Figure 9

sensitive phenotype of *gcs1-28 glo3Δ* cells. To test this hypothesis, the *age1-Δ164* allele was subcloned into a low-copy plasmid, which was subsequently transformed into *gcs1-28 glo3Δ* cells. *gcs1-28 glo3Δ* cells carrying a low-copy *GCS1* plasmid, the low-copy *age1-Δ164* plasmid, or an "empty" vector were then tested for growth at 37°C. As expected, *gcs1-28 glo3Δ* cells carrying the "empty" vector failed to grow at 37°C, whereas *gcs1-28 glo3Δ* cells carrying the low-copy *GCS1* plasmid were able to grow at this temperature (Figure 10). Remarkably, *gcs1-28 glo3Δ* cells carrying the low-copy *age1-Δ164* plasmid were able to grow at 37°C (Figure 10), whereas I had previously found that a low-copy plasmid carrying the wild type *AGE1* gene is unable to suppress *gcs1-28 glo3Δ* temperature sensitivity (data not shown). These results indicate that single-gene dosage of the *age1-Δ164* mutant allele suppresses the temperature sensitivity of *gcs1-28 glo3Δ* cells (as was noted for increased dosage of wild type *AGE1*, high-copy or low-copy *age1-Δ164* was unable to bypass Gcs1 or Glo3 function for retrograde transport; data not shown). Furthermore, these data support the possibility that the 164-residue amino-terminal region of the Age1 ArfGAP exerts a negative effect on Age1 function for retrograde vesicular transport.

10. Identification of the *SLY41* gene as a dosage suppressor of *gcs1-28 glo3Δ* temperature sensitivity

The library-plasmid family consisting of YEp13-6, YEp13-24, and YEp13-51 is the next most effective suppressor of *gcs1-28 glo3Δ* temperature sensitivity when compared to the

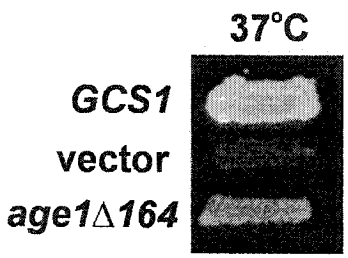

Figure 10. Single-gene dosage of the *age1-Δ164* allele suppresses *gcs1-28 glo3Δ* temperature sensitivity. *gcs1-28 glo3Δ* cells were transformed with an "empty" vector, a plasmid carrying the *GCS1* gene, or a low-copy plasmid carrying the *age1-Δ164* allele. Transformed cells were spread as patches on selective solid growth medium, incubated at 30°C for 2 days, and then replica-plated to rich solid growth medium and incubated at 37°C for 3 days.

YEp13-84 library plasmid harbouring the *AGE1* gene. Thus, one representative library plasmid from this family was subjected to DNA sequence analysis to determine the gene(s) carried within the genomic insert. DNA sequence analysis showed that library plasmids YEp13-6, YEp13-24, and YEp13-51 harbour a region of yeast chromosome XV (from co-ordinates 892,577 bp to 898,226 bp as displayed in Figure 11A). This region of yeast chromosome XV contains the *SLY41, SNU66, NOP58,* and *YOR309C* genes.

The *SNU66* gene encodes a pre-mRNA splicing factor, and the *NOP58* gene encodes a nucleolar protein involved in the pre-rRNA processing steps that lead to formation of 18 S rRNA (Stevens et al., 2001; Gautier et al., 1997). The *YOR309C* gene encodes a protein of unknown function, but has recently been reported as a dubious open reading frame, based on comparison with the genomes of closely-related yeast species, and therefore may not be transcribed and translated to produce a protein product (Kellis et al., 2003). The *SLY41* gene encodes a protein that has previously been implicated in the vesicular-transport process. The *SLY41* gene was first characterized as a dosage suppressor of the loss of *YPT1*, an essential gene that encodes a Rab protein thought to function at a tethering step prior to vesicle fusion with the target membrane at the ER-to-Golgi stage of vesicular transport (Daschler et al., 1991; Segev, 2001).

As a first approach, the *SLY41* gene was PCR-amplified, with the YEp13-24 library plasmid as a template, using oligonucleotides that amplify the *SLY41* open reading frame (ORF) flanked by 500 bp of upstream and downstream DNA sequence. The resulting PCR product was then subcloned into a high-copy vector. *gcs1-28 glo3Δ* cells

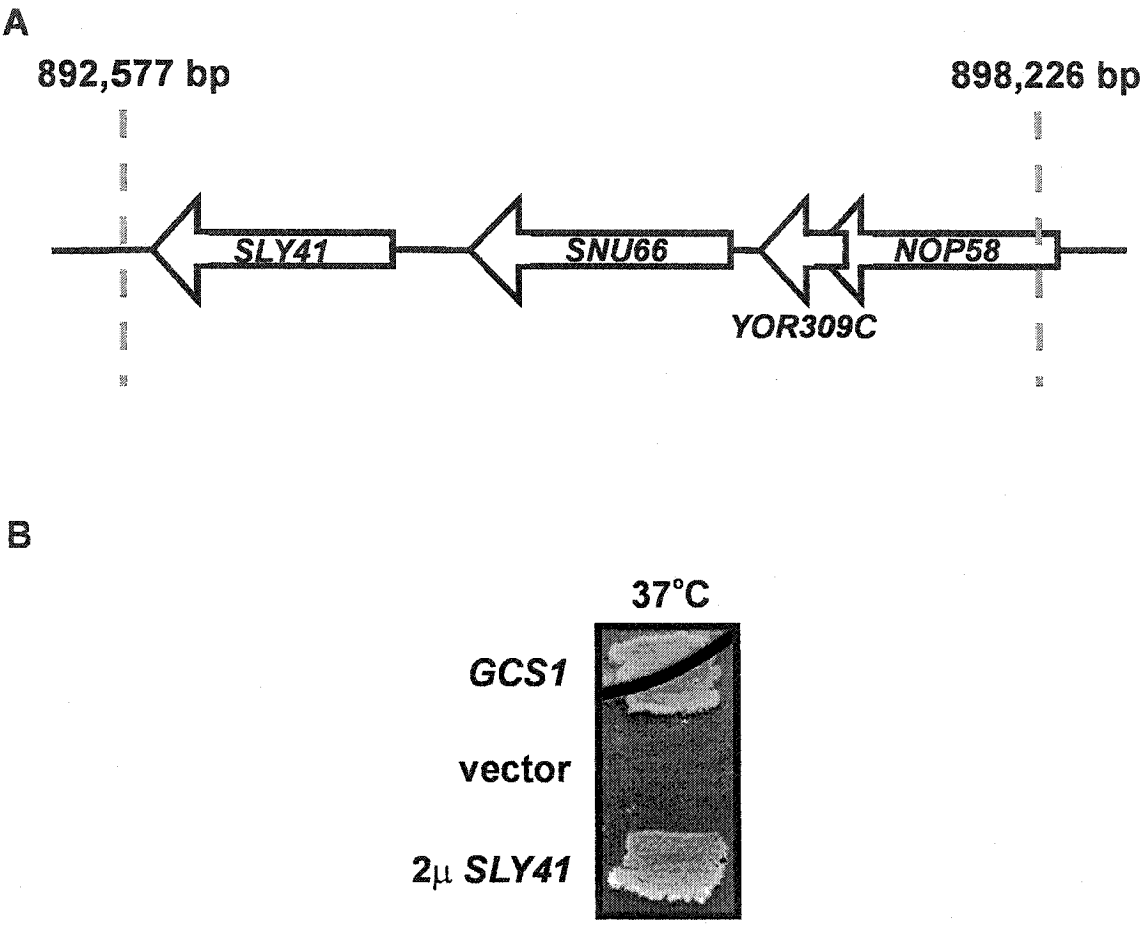

Figure 11. *SLY41* is a dosage suppressor of *gcs1-28 glo3Δ* temperature sensitivity. (A) The region of yeast chromosome XV carried by plasmids YEp13-6, YEp13-24, and YEp13-51. (B) *gcs1-28 glo3Δ* cells were transformed with an "empty" high-copy (2 micron) vector, a plasmid carrying the *GCS1* gene, or a high-copy plasmid carrying the *SLY41* gene. Cells were spread as patches on selective solid growth medium, incubated at 30°C for 2 days, and then replica-plated to rich solid growth medium and incubated at 37°C for 7 days.

were transformed with the resulting high-copy *SLY41* plasmid, as well as with a low-copy plasmid carrying the *GCS1* gene (positive control) and an "empty" high-copy vector (negative control). As expected, cells harbouring the low-copy *GCS1* plasmid were able to grow at 37°C, whereas cells carrying the "empty" vector failed to grow at 37°C (Figure 11B). In contrast, *gcs1-28 glo3Δ* cells harbouring the high-copy *SLY41* plasmid were able to grow at 37°C (Figure 11B), indicating that the *SLY41* gene is a dosage suppressor of *gcs1-28 glo3Δ* temperature sensitivity.

11. Increased dosage of the *SLY41* gene restores vesicular transport in *gcs1-28 glo3Δ* cells at 37°C

I have identified the *SLY41* gene as a dosage suppressor of *gcs1-28 glo3Δ* temperature sensitivity. To determine whether increased dosage of the *SLY41* gene restores vesicular transport in *gcs1-28 glo3Δ* cells at 37°C, the processing state of CPY was assessed. *gcs1-28 glo3Δ* cells carrying a low-copy *GCS1* plasmid, a high-copy *SLY41* plasmid, or an "empty" vector were grown to mid-log phase at 30°C , then transferred to 37°C and subjected to pulse-chase labeling and immunoprecipitation of CPY as previously described in Poon et al. (1999) and above in Section 3. *gcs1-28 glo3Δ* cells carrying the low-copy *GCS1* plasmid were able to process p1CPY to mCPY after 60 minutes of "chase" with excess unlabeled methionine and cysteine, whereas *gcs1-28 glo3Δ* cells carrying the "empty" vector failed to process p1CPY to mCPY during the same "chase" period (Figure 12, lanes 2 and 4). However, *gcs1-28 glo3Δ* cells carrying the high-copy

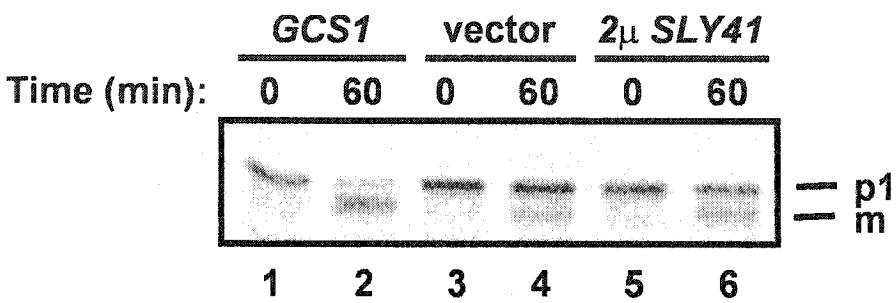

Figure 12. Increased dosage of *SLY41* partially restores CPY processing in *gcs1-28 glo3Δ* cells at 37°C. *gcs1-28 glo3Δ* cells carrying a *GCS1* plasmid (*GCS1*), a high-copy "empty" vector (vector), or a high-copy *SLY41* plasmid (2μ *SLY41*) were inoculated into growth medium lacking methionine and cysteine and grown at 37°C for 30 minutes, pulsed with [^{35}S]-methionine, [^{35}S]-cysteine for 7 minutes, and then chased for 60 minutes with excess unlabeled methionine and cysteine. At 0 and 60 minutes samples were taken, cells were lysed, and CPY was immunoprecipitated from the extracts. Immunoprecipitants were resolved by SDS-PAGE, and CPY was detected by autoradiography. The p1 (ER-form) and m (mature form) species of CPY are indicated.

SLY41 plasmid were able to process some p1CPY to mCPY after 60 minutes of "chase" with excess unlabeled methionine and cysteine (Figure 12, lane 6). These results show that increased dosage of the *SLY41* gene in *gcs1-28 glo3Δ* cells at 37°C permits transport of p1CPY and p2CPY through the secretory pathway to the vacuole, where mCPY is produced. Therefore, increased *SLY41* gene dosage likely restores vesicular transport in *gcs1-28 glo3Δ* cells at 37°C.

12. Increased concentration of cations in the growth medium suppresses *gcs1-28 glo3Δ* temperature sensitivity

Previous reports have implicated the *SLY41* gene in vesicular transport, since *SLY41* was originally characterized as a dosage suppressor of the loss of the Ypt1 Rab, an essential protein required for vesicular transport between the ER and the Golgi (Daschler et al., 1991). The *SLY41* gene was found to encode a protein with seven transmembrane-spanning domains that is homolgous to a small-ion transporter from spinach chloroplasts (Daschler et al., 1991). Furthermore, earlier experiments using a temperature-sensitive *ypt1* allele had shown that defective Ypt1 function could be suppressed by increasing the concentration of cations, such as calcium and magnesium, in the growth medium (Schmitt et al., 1988). Based on these observations, those authors hypothesized that increased abundance of Sly41, a putative small-ion transporter, may cause a redistribution of intracellular cations, leading to a bypass of Ypt1 function through some as-yet-unknown cation-mediated mechanism (Daschler et al., 1991). Since both the Gcs1 and Glo3 ArfGAPs are involved in vesicular transport between the Golgi and the ER, it is

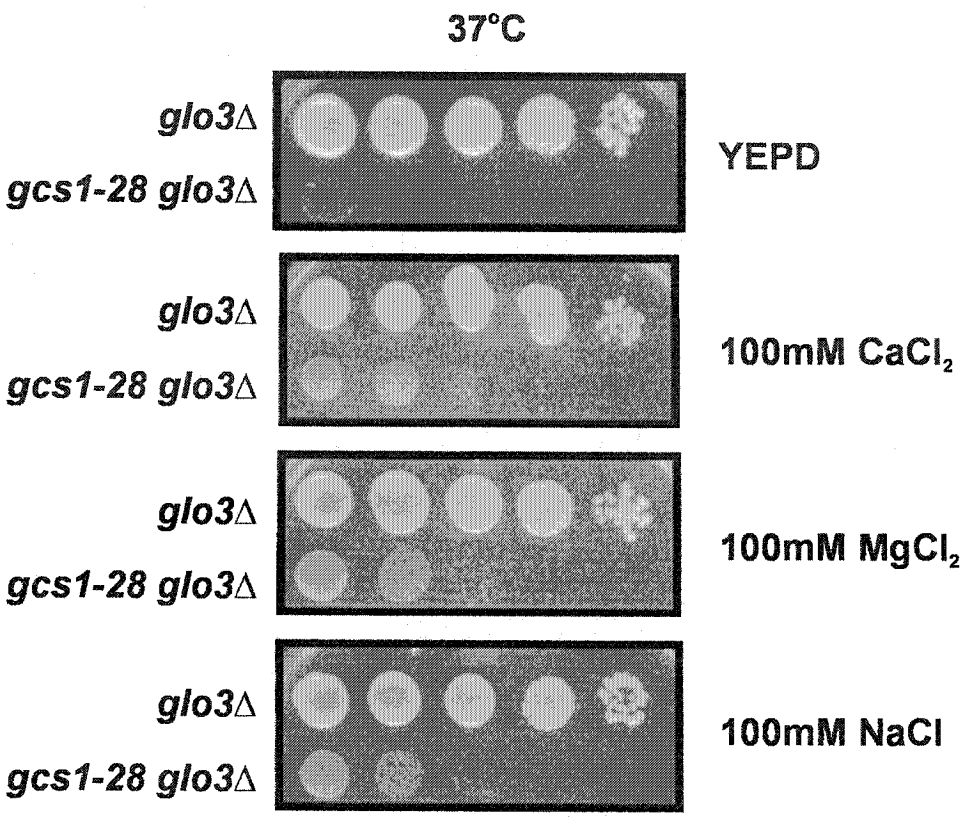

Figure 13. Increasing the concentration of cations in the growth medium suppresses the temperature-sensitive phenotype of *gcs1-28 glo3Δ* cells. *gcs1-28 glo3Δ* cells carrying a plasmid with the *GCS1* gene (*glo3Δ*) or an "empty" vector (*gcs1-28 glo3Δ*) were spotted as serial dilutions onto rich solid growth medium (YEPD), rich solid growth medium with 100mM $CaCl_2$, rich solid growth medium with 100mM $MgCl_2$, or rich solid growth medium with 100mM NaCl. Cells were incubated at 37°C for 2 days.

possible that increased abundance of Sly41 may suppress *gcs1-28 glo3Δ* temperature sensitivity through a similar cation-mediated mechanism.

To test whether increased cation concentration in the growth medium can suppress *gcs1-28 glo3Δ* temperature sensitivity, *gcs1-28 glo3Δ* cells were tested for growth at 37°C on rich medium supplemented with 100 mM $CaCl_2$, 100 mM $MgCl_2$, or 100 mM NaCl. As expected, *gcs1-28 glo3Δ* cells incubated on rich medium failed to grow at 37°C (Figure 13), whereas *gcs1-28 glo3Δ* cells incubated on rich medium supplemented with 100 mM $CaCl_2$, 100 mM $MgCl_2$, or 100 mM NaCl were able to grow at 37°C (Figure 13). The order for robustness of suppression is $CaCl_2 > MgCl_2 >$ NaCl. These data indicate that the temperature-sensitive phenotype of *gcs1-28 glo3Δ* cells can be suppressed by increased cation concentration in the growth medium. Since increasing the cation concentration in the growth medium, or increased *SLY41* gene dosage, suppress both a *gcs1-28* temperature-sensitive allele and a *ypt1* temperature-sensitive allele, *gcs1-28 glo3Δ* temperature sensitivity may be suppressed through a cation-mediated mechanism, as has been suggested for suppression of *ypt1* temperature sensitivity.

13. The temperature-sensitive phenotype of *gcs1-28 glo3Δ* cells is alleviated by increasing the osmotic strength of the growth medium

I have found that increasing the concentration of cations in the growth medium suppresses the temperature sensitivity of *gcs1-28 glo3Δ* cells. However, the addition of

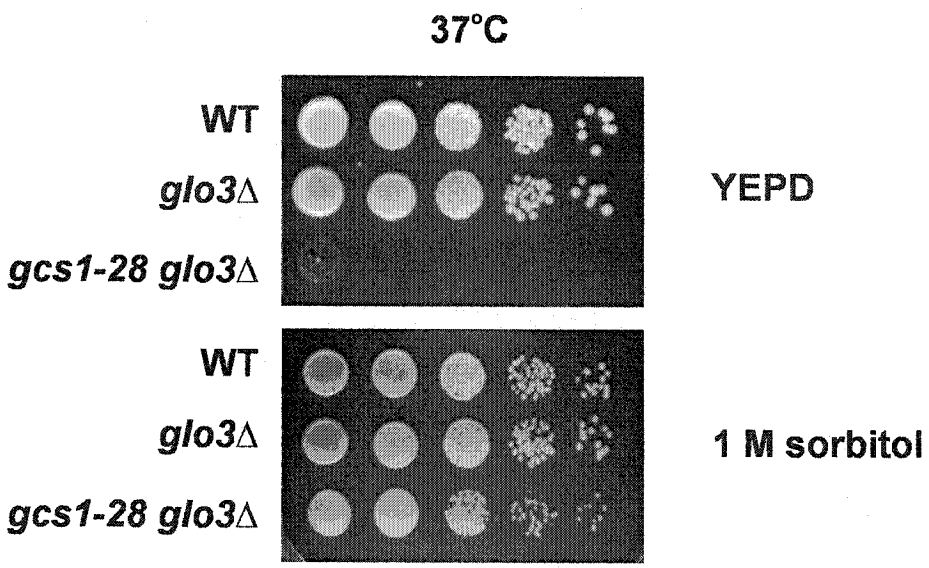

Figure 14. The *gcs1-28 glo3Δ* temperature-sensitive phenotype is osmo-remediable. Wild-type cells (WT), *gcs1-28 glo3Δ* cells carrying a plasmid with the *GCS1* gene (*glo3Δ*), or *gcs1-28 glo3Δ* cells carrying an "empty" vector (*gcs1-28 glo3Δ*) were spotted as serial dilutions onto rich solid growth medium (YEPD) or rich solid growth medium with 1 M sorbitol. Cells were incubated at 37°C for 2 days.

cations to the growth medium also increases the osmotic strength of the growth medium, and therefore may suppress *gcs1-28 glo3Δ* temperature sensitivity by osmotic stabilization of these cells. To test this possibility, *gcs1-28 glo3Δ* cells were incubated at 37°C on growth medium containing sorbitol, an osmotic stabilizer. Whereas *gcs1-28 glo3Δ* cells incubated on rich medium failed to grow at 37°C, *gcs1-28 glo3Δ* cells incubated on rich medium supplemented with sorbitol to a 1 M final concentration grew as well as wild-type cells did at 37°C (Figure 14). These results show that *gcs1-28 glo3Δ* temperature sensitivity is alleviated by increasing the osmotic strength of the growth medium, and therefore *gcs1-28 glo3Δ* temperature sensitivity is not specifically remediated by increased cation concentration in the growth medium. Thus, the temperature-sensitive phenotype of *gcs1-28 glo3Δ* cells is responsive to the osmotic strength of the growth medium.

14. *gcs1Δ* single-mutant cells, *glo3Δ* single-mutant cells, and *gcs1-28 glo3Δ* double-mutant cells have defects in cell-wall structure

Mutant cells that are rescued by increasing the osmotic strength of the growth medium (also known as osmotic remediation or osmotic stabilization) often have an aberrant cell wall structure (Hampsey, 1997). Since many components required for cell-wall biosynthesis are trafficked through the secretory pathway (especially through the Golgi apparatus), a breakdown in vesicular transport caused by inadequate ArfGAP function required for retrograde vesicular transport could ultimately lead to aberrant cell-

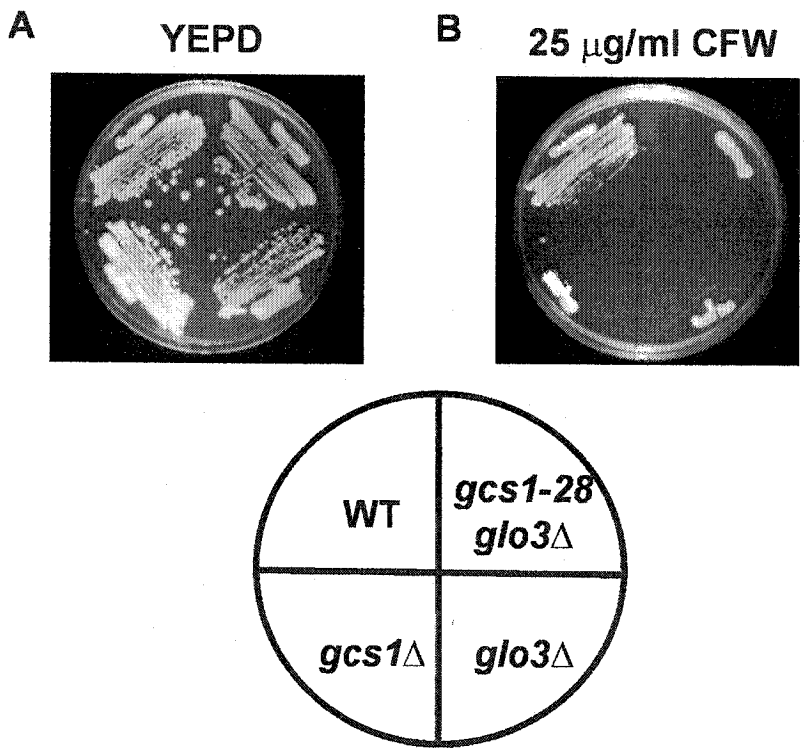

Figure 15. ***gcs1Δ*****,** ***glo3Δ*****, and** ***gcs1-28 glo3Δ*** **cells are sensitive to calcofluor white.** Wild-type (WT), *gcs1Δ*, *glo3Δ*, and *gcs1-28 glo3Δ* cells were streaked for single colonies on rich solid growth medium (YEPD) or rich solid growth medium with 25 μg/ml calcofluor white (CFW). Cells were incubated at 30°C for 3 days.

wall structure. Often cells that have an aberrant cell-wall structure are sensitive to the presence of calcofluor white (CFW) in the growth medium (Hampsey, 1997). Therefore, to test whether *gcs1Δ* single-mutant cells, *glo3Δ* single-mutant cells, or *gcs1-28 glo3Δ* double-mutant cells have an aberrant cell-wall structure, these cells were tested for sensitivity to the presence of calcofluor white in the growth medium.

Wild-type, *gcs1Δ*, *glo3Δ*, and *gcs1-28 glo3Δ* cells grew equally well on growth medium lacking calcofluor white (Figure 15A), whereas only wild-type cells were able to grow on growth medium containing calcofluor white (Figure 15B). These results demonstrate that individual deletion of the *GCS1* and *GLO3* genes, or the *gcs1-28 glo3Δ* double-mutant situation, causes sensitivity to calcofluor white. Therefore, ArfGAP function may be required for cell-wall integrity, since any perturbation of ArfGAP function leads to an aberrant cell-wall structure.

15. Synthetic enhancement of *gcs1-28 glo3Δ* temperature sensitivity by the presence of the *ssd1-d* allele

Many proteins have been implicated in cell-wall structure. A recent report has indicated that the Ssd1 protein is involved with the integrity of cell-wall structure, raising the possibility that yeast genetic backgrounds carrying an *ssd1-d* allele may pre-dispose cells to a weakened cell wall (Kaeberlein and Guarente, 2002). *SSD1* is a polymorphic gene, and several versions of the *SSD1* gene are found in various yeast genetic backgrounds (Jorgensen et al., 2002). These *SSD1* alleles have been classified as *SSD1-V* (viable) and *ssd1-d* (defective) based on the ability to confer Ssd1 function (Sutton et al., 1991). All of

my experiments have been performed in strain W303, which has been shown to carry a recessive mutant allele of *SSD1*, known as *ssd1-d* (Jorgensen et al., 2002). Therefore, it is possible that the osmotic remediation of *gcs1-28 glo3Δ* temperature sensitivity is due to the osmotic suppression of the effects of the *ssd1-d* allele rather than the *gcs1-28* allele, suggesting that the presence of the *ssd1-d* allele synthetically enhances *gcs1-28 glo3Δ* temperature sensitivity. If there is indeed a synthetic enhancement of *gcs1-28 glo3Δ* temperature sensitivity by the presence of the *ssd1-d* allele in the genetic backgorund, the wild-type *SSD1* gene should alleviate *gcs1-28 glo3Δ* temperature sensitivity by complementation of the *ssd1-d* mutation.

To determine whether the wild-type *SSD1* gene can alleviate the temperature sensitivity of *gcs1-28 glo3Δ* cells, *gcs1-28 glo3Δ* cells were transformed with a low-copy plasmid carrying the wild-type *GCS1* gene, a low-copy plasmid carrying the wild-type *SSD1* gene, and an "empty" vector. As shown in Figure 16, *gcs1-28 glo3Δ* cells carrying either the low-copy *GCS1* plasmid or the low-copy *SSD1* plasmid were able to grow at 37°C, whereas *gcs1-28 glo3Δ* cells carrying the "empty" vector failed to grow. Therefore, complementation of the *ssd1-d* allele by the wild-type *SSD1* gene alleviates *gcs1-28 glo3Δ* temperature sensitivity, demonstrating a genetic interaction between the *gcs1-28 glo3Δ* double-mutant situation and the *ssd1-d* allele.

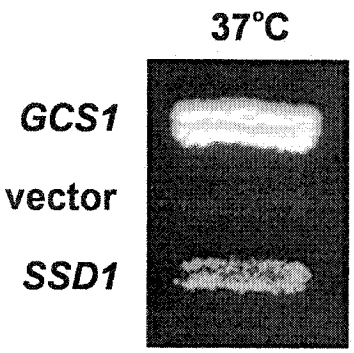

Figure 16. *SSD1* alleviates *gcs1-28 glo3Δ* temperature sensitivity. *gcs1-28 glo3Δ* cells were transformed with a *GCS1* plasmid (*GCS1*), a low-copy "empty" vector (vector), or a low-copy *SSD1* plasmid (*SSD1*). Transformed cells were spread as patches on selective solid growth medium, incubated at 30°C for 2 days, and then replica-plated to rich solid growth medium and incubated at 37°C for 3 days.

16. The presence of the wild-type *SSD1* gene does not restore vesicular transport in *gcs1-28 glo3Δ* cells at 37°C

Other groups have reported that the wild-type *SSD1* gene can suppress a variety of temperature-sensitive mutations in components of the vesicular transport machinery, and in some cases *SSD1*-mediated suppression results in restoration of vesicular transport in these mutants (Li and Warner, 1996; Li and Warner, 1998; Rosenwald et al., 2002). I have identified a genetic interaction between the *gcs1-28 glo3Δ* double-mutant situation and the *ssd1-d* allele, raising the possibility that Ssd1 may have a role to play in Gcs1- and Glo3-mediated vesicular transport. Therefore, the alleviation of *gcs1-28 glo3Δ* temperature sensitivity by the wild-type *SSD1* gene may result from the restoration of vesicular transport at 37°C.

To determine whether the wild-type *SSD1* gene can restore vesicular transport in *gcs1-28 glo3Δ* cells at 37°C, the processing of CPY was assessed. *gcs1-28 glo3Δ* cells carrying a low-copy *GCS1* plasmid, a low-copy *SSD1* plasmid, or an "empty" vector were grown to mid-log phase at 30°C , then transferred to 37°C and subjected to pulse-chase labeling and immunoprecipitation of CPY. *gcs1-28 glo3Δ* cells carrying the low-copy *SSD1* plasmid or the "empty" vector failed to process p1CPY to mCPY after 60 minutes of chase with unlabeled methionine and cysteine (Figure 17, lanes 4 and 6), whereas *gcs1-28 glo3Δ* cells carrying the low-copy *GCS1* plasmid were able to process p1CPY to mCPY during the same time period (Figure 17, lane 2). These data indicate that the presence of the wild-type *SSD1* gene in *gcs1-28 glo3Δ* cells at 37°C does not permit transport of p1CPY and p2CPY through the secretory pathway to the vacuole.

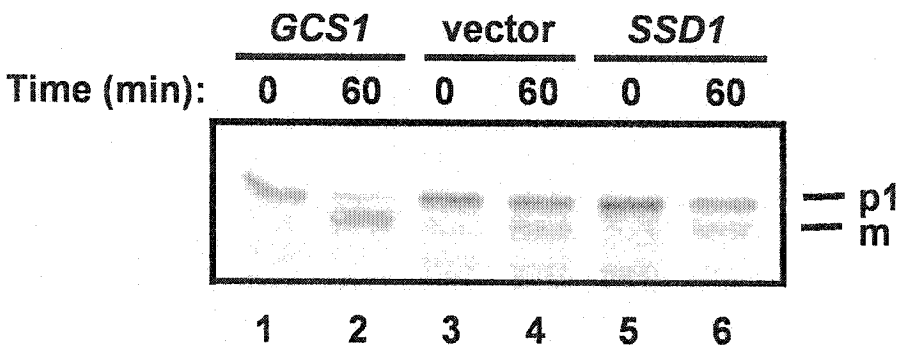

Figure 17. *SSD1* does not restore CPY processing in *gcs1-28 glo3Δ* cells at 37°C. *gcs1-28 glo3Δ* cells carrying a *GCS1* plasmid (*GCS1*), a low-copy "empty" vector (vector), or a low-copy *SSD1* plasmid (*SSD1*) were inoculated into growth medium lacking methionine and cysteine and grown at 37°C for 30 minutes, pulsed with [^{35}S]-methionine, [^{35}S]-cysteine for 7 minutes, and then chased for 60 minutes with excess unlabeled methionine and cysteine. At 0 and 60 minutes samples were taken, cells were lysed, and CPY was immunoprecipitated from the extract. Immunoprecipitants were resolved by SDS-PAGE, and CPY was detected by autoradiography. The p1 (ER-form) and m (mature form) species of CPY are indicated.

Therefore, the wild-type *SSD1* gene does not restore vesicular transport in *gcs1-28 glo3Δ* cells at the non-permissive temperature.

17. The *SLY41* gene is a dosage suppressor of *glo3Δ* cold sensitivity

I have established that increased dosage of the *SLY41* gene suppresses *gcs1-28 glo3Δ* temperature sensitivity. The *SLY41* gene encodes a putative small-ion transporter, and has previously been suggested to dosage-suppress other defects in vesicular transport through a cation-dependent mechanism that may bypass the need for the mutant protein in vesicular transport (Daschler et al., 1991). Although increased cation concentration in the growth medium suppresses *gcs1-28 glo3Δ* temperature sensitivity, the addition of compounds (such as sorbitol) that increase the osmolarity of the growth medium also suppresses the *gcs1-28 glo3Δ* temperature-sensitive phenotype. Therefore, it is unlikely that increased abundance of Sly41 suppresses *gcs1-28 glo3Δ* temperature sensitivity through a cation-dependent mechanism. These results raise the possibility that increased abundance of the Sly41 protein suppresses *gcs1-28 glo3Δ* temperature sensitivity *via* a novel mechanism, likely by facilitating enfeebled Gcs1-28 ArfGAP function at the non-permissive temperature. To better characterize the mechanism of *SLY41* dosage suppression, the effect of increased *SLY41* gene dosage on other situations in which ArfGAP function for retrograde transport is enfeebled was assessed.

Single-mutant *glo3Δ* cells exhibit a cold-sensitive phenotype at 15°C (Poon et al., 1999). Since the only ArfGAP normally able to provide function for retrograde transport

in the absence of Glo3 is Gcs1, this finding indicates that Gcs1 function for retrograde vesicular transport is inherently cold-sensitive (Poon et al., 1999). That is, Gcs1 function for retrograde vesicular transport is enfeebled at 15°C. If increased abundance of the Sly41 protein can indeed facilitate Gcs1 ArfGAP function for retrograde vesicular transport, then the *SLY41* gene may also dosage-suppress the inherent cold sensitivity of the Gcs1 ArfGAP for retrograde vesicular transport in *glo3Δ* mutant cells.

To test this hypothesis, *glo3Δ* mutant cells were transformed with a low-copy *GLO3* plasmid, a high-copy *SLY41* plasmid, or an "empty" vector. *glo3Δ* cells carrying the "empty" vector failed to grow at 15°C, whereas *glo3Δ* cells carrying either the low-copy *GLO3* plasmid or the high-copy *SLY41* plasmid grew equally well at 15°C (Figure 18). Therefore, increased abundance of the Sly41 protein suppresses the cold sensitivity of Gcs1 ArfGAP function for retrograde vesicular transport. Together with the observation that increased dosage of the *SLY41* gene suppresses *gcs1-28 glo3Δ* temperature sensitivity, these results suggest that increased abundance of the Sly41 protein may generally facilitate Gcs1 function under conditions where the Gcs1 protein is enfeebled.

18. Increased abundance of the Sly41 homologue Yjl193w does not suppress *gcs1-28 glo3Δ* temperature sensitivity or *glo3Δ* cold sensitivity

Saccharomyces cerevisiae contains two genes that could provide Sly41 function, *SLY41* itself and the uncharacterized open reading frame *YJL193W*. The polypeptide encoded by *YJL193W* is 51 % similar and 32 % identical to Sly41 (Figure 19A). This situation raises

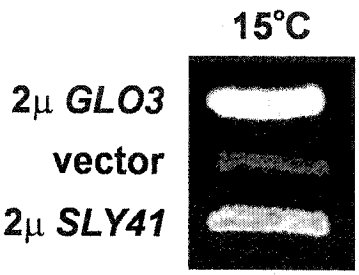

Figure 18. Increased dosage of *SLY41* suppresses the cold sensitivity of *glo3Δ* cells. *glo3Δ* cells were transformed with a high-copy plasmid carrying the *GLO3* gene (2μ *GLO3*), an "empty" high-copy vector (vector), or high-copy plasmid carrying the *SLY41* gene (2μ *SLY41*). Cells were spread as patches on a selective solid growth medium, incubated at 30°C for 2 days, and then replica-plated to rich solid growth medium and incubated at 15°C for 7 days.

the possibility that Sly41 and Yjl193w constitute a protein family, and therefore may share similar functions. Therefore, increased abundance of the Yjl193w protein may be able to suppress *gcs1-28 glo3Δ* temperature sensitivity and *glo3Δ* cold sensitivity, as is the case for increased abundance of the Sly41 protein.

The *YJL193W* gene was amplified by PCR, using yeast genomic DNA as a template, with oligonucleotides that amplify the *YJL193W* ORF flanked by 500 bp of upstream and downstream DNA sequence, and the resulting PCR product was subcloned into a high-copy vector. To determine whether the *YJL193W* gene is a dosage suppressor of *gcs1-28 glo3Δ* temperature sensitivity, *gcs1-28 glo3Δ* cells were transformed with a low-copy *GCS1* plasmid, the high-copy *YJL193W* plasmid, or an "empty" vector. *gcs1-28 glo3Δ* cells carrying the low-copy *GCS1* plasmid were able to grow at 37°C, whereas *gcs1-28 glo3Δ* cells carrying either the high-copy *YJL193W* plasmid or the "empty" vector failed to grow at 37°C (Figure 19B). These data suggest that *YJL193W* is not a dosage suppressor of *gcs1-28 glo3Δ* temperature sensitivity.

To test whether *YJL193W* is a dosage suppressor of *glo3Δ* cold sensitivity, *glo3Δ* mutant cells were transformed with a low-copy *GLO3* plasmid, the high-copy *YJL193W* plasmid, or an "empty" vector. *glo3Δ* mutant cells carrying either the high-copy *YJL193W* plasmid or the "empty" vector plasmid failed to grow at 15°C, whereas *glo3Δ* cells carrying the low-copy *GLO3* plasmid were able to grow (data not shown). This result suggests that *YJL193W* is not a dosage suppressor of *glo3Δ* cold sensitivity. Taken together, the above results suggest that although Yjl193w and Sly41 are homologous proteins, the functions of these two proteins in relation to ArfGAP activity are different –

increased abundance of Sly41 suppresses the effects of enfeebled ArfGAP function, whereas increased abundance of Yjl193w does not.

Figure 19. The Sly41 homologue Yjl193w does not suppress *gcs1-28 glo3Δ* temperature sensitivity. (A) Pairwise sequence alignment showing similarity between the Sly41 and Yjl193w polypeptides. Amino acid residues shading in black are absolutely conserved, whereas residues shaded in gray indicate a conserved property of the residue; similarly, in the 'consensus' line asterisks indicate absolute conservation and dots indicate property conservation. (B) *gcs1-28 glo3Δ* cells were transformed with a *GCS1* plasmid (*GCS1*), a high-copy "empty" vector (vector), or a high-copy *YJL193W* plasmid (*YJL193W*). Transformed cells were spread as patches on selective solid growth medium, incubated at 30°C for 2 days, and then replica-plated to rich solid growth medium and incubated at 37°C for 3 days.

A

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Sly41          1 KRFTQLFPSNTQGYLPEVDLRTTIICSIWYVTSSISSNLSKAILRTFNHPIALTELQFLV
Yjl193w        1 -MFQQLSAS-IR-----HNAHIIFLCISWYFISSLASQVTKQVLTVCPLPLFLGEFQFIY
consensus      1 . * **  *.* .....   .*  .*  **  **. *...* .*      *. * * **.

Sly41         61 SAVLCVGFASIVNLFRLPRLKHTKFSKALNSFPDGILPEY-LDG--NFRS----SILHKF
Yjl193w       54 TAVL-AWFTCYIAYS-FP-----GF---YRIFPNGTFPEYYIDDREETSRAARKESKLSSL
consensus     61 .***.  *   .  . *..... *...   ** *  ***..* ..  * ....* *

Sly41        114 LVP-SKLVLMTTFPMGIFQFIGHITSHKAVSMIPVSLVHSVKALSPIITVGYYKFFEHR-
Yjl193w      104 IIPPSKPILQTVLPLGLFQFVGKYFGHTATSLVPVSTVASIKTLSPMFILLLQKILKIST
consensus    121 ..*.** .* *  *.*.***.*.   * * *..*** * *.* ***.   .     *

Sly41        172 YYNSMTY-YTLLLLIFGVMTTCWSTHGSKRASDNKSGSSLIGLLFAFISMIIFVAQNIFA
Yjl193w      164 LKITLTLIFSLCTLVLGVWIIVQEDNRSPASSNELREFSKYGVICAMISMFIFVLQNIYG
consensus    181    ..* ...*  *. **          *   *       *  *.. * *** *** ***..

Sly41        231 KNILTIRRKVGILPSSSTDDVTSKEG-QE---SLDK---TRFSPLQVDKITILFYCSCIG
Yjl193w      224 KTVFTYRSQT--DESQSNSGFSRQESSILPLYEKLLDEKLVAKKKPKSYDKLTLMIYISLVG
consensus    241 * . * *   ..  * *     . * . *... ** ... .  *   **.*.. * * .*

Sly41        284 FSLTLLPFLTGEL---MHGGSVIN--DLTLET--VALVAIHGIAHFFQAMLAFQLIGLLS
Yjl193w      282 FCLSFGWEITLEFPVLFRYFFQINSSSTVIKAFPVSLFLLNGTFHFIQAMITFHLLGEVS
consensus    301 * *.   *.* * ... .    **..   .  ..* *  . *  ** ***. * *.* .*

Sly41        337 SINYSVANIMKRIVVISVA-LFWETKLNFFQVFGVILTIAGLYGYDKWG-LS--KKDGRQ
Yjl193w      342 TLTYSIANLMKRFAIIAVSWVFIGRRITWLQVFGLVLNTLGLFLYERCTSQSKIKAKIRP
consensus    361 .. **.**.***  .* * ..*   .. . ****..*   **. *..  . *..*   *
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B

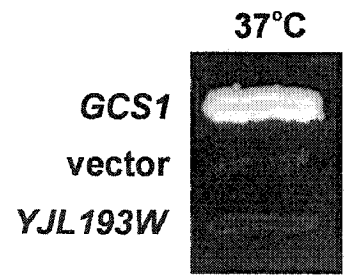

Figure 19

Part C: Analysis of Gcs1 and Glo3 ArfGAP function at the molecular level for retrograde vesicular transport

1. Non-equivalent contributions of the Gcs1 and Glo3 ArfGAPs to retrograde vesicular transport

During the course of my studies on the temperature-sensitive *gcs1-28* allele, evidence began to mount suggesting that the Gcs1 and Glo3 ArfGAPs may not have equivalent functions for retrograde vesicular transport (Dogic et al., 1999; Eugster et al., 2000). Dogic et al. (1999) isolated a mutant allele of *GLO3*, known as *ret4-1*, in a screen for novel mutations affecting the retrieval of cargo proteins from the Golgi to the ER. In follow-up experiments, Dogic et al. (1999) determined the effects of individual deletion of each of the other five genes encoding known or putative ArfGAP proteins (*GCS1*, *AGE1*, *AGE2*, *SPS18*, and *GTS1*). Interestingly, only mutation or deletion of the *GLO3* gene affected retrograde vesicular transport; deletion of the *GCS1* gene appeared to have no effect on the retrograde vesicular-transport process (Dogic et al., 1999). A report by Eugster et al. (2000) examined potential protein-protein interactions between the Gcs1 ArfGAP, the Glo3 ArfGAP, and components of the COPI vesicle coat complex (also known as coatomer), using the yeast two-hybrid system. The Glo3 ArfGAP was found to make protein-protein interactions with the Sec21 (γ-COP) and Sec27 (β'-COP) components of coatomer, but the Gcs1 ArfGAP did not display an interaction with the coatomer proteins (Eugster et al., 2000). These observations raise the possibility that the

Gcs1 ArfGAP may have an indirect role in retrograde vesicular transport, suggesting that the Glo3 ArfGAP is the predominant ArfGAP mediating retrograde vesicular transport.

While inadequacy of both Gcs1 and Glo3 ArfGAP function leads to a severe defect on vesicular transport and cell growth, even single deletion of the *GLO3* gene leads to a slowed growth rate and causes measurable defects in the secretory pathway even though the Gcs1 ArfGAP is still present (Poon et al., 1999). These observations raise the possibility that Gcs1 may facilitate vesicular transport in the absence of Glo3 by substituting (albeit somewhat poorly) for Glo3 function in the retrograde-transport process. Indeed, mutation of the Gcs1 ArfGAP (Gcs1-28) leads to severely decreased Gcs1-mediated vesicular transport in the absence of Glo3 under non-permissive conditions.

I have shown that the temperature-sensitive phenotype of *gcs1-28 glo3Δ* cells is suppressed by increased abundance of the Age1 ArfGAP. A mutation that destroys the ArfGAP activity of Age1 also removes the ability of increased Age1 abundance to supress the *gcs1-28 glo3Δ* temperature-sensitive phenotype. Furthermore, a mutation that removes the first 164 residues of the Age1 protein (Age1-Δ164) augments the ability of Age1 to suppress the effects of defective Gcs1-28 function for retrograde vesicular transport. Therefore, the inadequate ability of the Gcs1-28 ArfGAP to provide Glo3 function for retrograde vesicular transport can be improved by manipulation (either increased abundance or mutation) of another ArfGAP, namely Age1. Such findings suggest that a loss of Glo3 ArfGAP function for retrograde vesicular transport can be

partially compensated by other ArfGAP proteins – either the wild-type Gcs1 protein under normal conditions, or a combination of Gcs1-28 and Age1 function under conditions where Gcs1 mediation of Glo3 function is compromised. These observations suggest that the Gcs1 ArfGAP may not normally participate in retrograde vesicular transport, or may play only a minor role in the retrograde-transport process. Nonetheless, Gcs1 can supply Glo3-like ArfGAP function in the absence of Glo3. Based on these observations, I have determined the contributions of the Gcs1 and Glo3 ArfGAPs to retrograde vesicular transport at the molecular level.

2. The Glo3 ArfGAP interacts with Sec21, the γ-COP component of the coatomer complex, *in vivo*

Using the yeast two-hybrid system, a previous study has demonstrated direct protein-protein interactions between the Glo3 ArfGAP and the coatomer proteins Sec21 and Sec27 (Eugster et al., 2000). Interestingly, this study failed to identify any protein-protein interactions between the Gcs1 ArfGAP and coatomer proteins, even though both the Gcs1 and Glo3 ArfGAPs can function for retrograde vesicular transport (Poon et al., 1999). Such results suggest that the Gcs1 and Glo3 ArfGAPs may not have equivalent function for retrograde vesicular transport. Thus, I first sought to determine whether these ArfGAP-coatomer interactions seen by yeast two-hybrid analyses reflect biological reality; that is, whether these protein-protein interactions take place *in vivo*.

To determine whether the Gcs1 and Glo3 ArfGAPs can interact with coatomer proteins *in vivo*, protein extracts from wild-type cells were subjected to

immunoprecipitation using anti-Sec21 antibodies, and co-precipitation of the Gcs1 and Glo3 ArfGAPs was assessed. As shown in Figure 20A, the Glo3 ArfGAP was co-immunoprecipitated along with the Sec21 component of the coatomer complex; however, the Gcs1 ArfGAP failed to co-precipitate with the Sec21 component of the coatomer complex. These results indicate that the Glo3 ArfGAP physically associates with the Sec21 component of the coatomer complex *in vivo*. Since no interaction was found between the Sec21 component of the coatomer complex and the Gcs1 ArfGAP under the conditions used in these experiments, it is possible that the Gcs1 ArfGAP does not interact with coatomer *in vivo*.

The Gcs1 ArfGAP can facilitate retrograde vesicular transport in the absence of Glo3 (Poon et al., 1999), raising the possibility that Gcs1 may physically associate with the coatomer complex only in the absence of any Glo3 protein. To determine whether Gcs1 can physically associate with coatomer in the absence of the Glo3 ArfGAP, a protein extract from *glo3Δ* mutant cells was subjected to immunoprecipitation using anti-Sec21 antibodies, and the co-precipitation of the Gcs1 ArfGAP was assessed. Once again, the Gcs1 ArfGAP failed to co-precipitate with the Sec21 component of the coatomer complex (Figure 20B), suggesting that Gcs1 may not physically associate with coatomer *in vivo*.

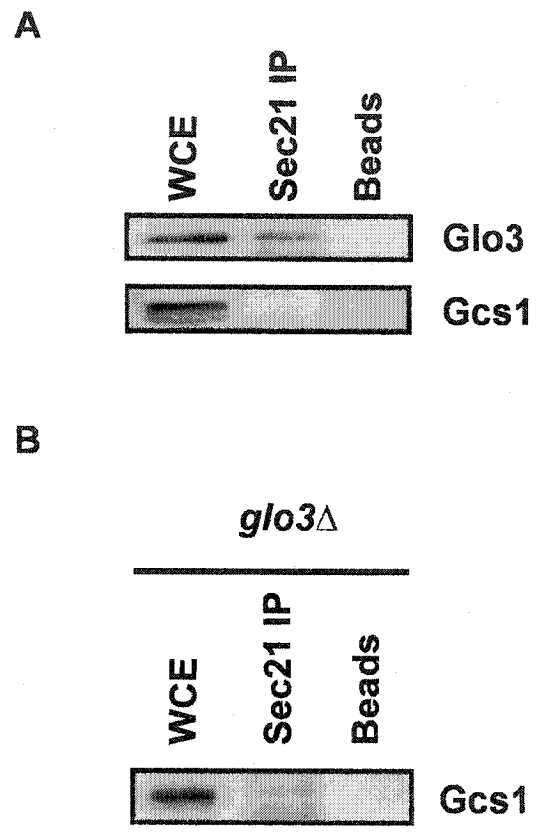

Figure 20. Glo3, but not Gcs1, co-immunoprecipitates with the coatomer complex. (A) Protein extracts from a wild-type yeast strain were incubated with anti-Sec21 antibody, followed by incubation with Protein-A-agarose beads. Beads were pelleted by centrifugation, washed, and resuspended in 1× Laemmli buffer. Samples were resolved on a 10% SDS-PAGE, transferred to PVDF, and analyzed with antibodies against Gcs1 and Glo3. (B) The co-immunoprecipitation experiment was repeated using *glo3Δ* cells. The samples were analyzed as in (A) with antibodies against Gcs1. WCE: whole cell extract.

3. The Glo3 ArfGAP is a component of COPI vesicles

The identification of a Glo3-coatomer interaction *in vivo* raised the possibility that interaction may take place on Golgi membranes and/or on the COPI vesicles themselves. To determine whether Glo3 is incorporated into the coat of COPI vesicles through association with coatomer, Golgi membranes were isolated from wild-type yeast cells and used for an *in vitro* vesicle-budding reaction (Spang and Schekman, 1998). These experiments were performed in collaboration with Dr. Anne Spang (Friedrich Miescher Laboratorium in Tubingen, Germany), and the data that appear in Figure 21 are courtesy of Dr. Spang. Enriched Golgi membranes were incubated with purified coatomer, Arf1 and guanine nucleotide to stimulate COPI-vesicle formation. The vesicles were then separated from the Golgi membrane by a velocity-sedimentation gradient, collected, and further purified by buoyant density centrifugation. The vesicle fraction was then subjected to Western blot analysis, revealing that the Glo3 protein is indeed found on COPI vesicles generated *in vitro* from Golgi membranes (Figure 21). Thus, the Glo3 protein is a component of COPI vesicles.

4. Expression of a mutant Glo3 protein, Glo3-R59K, impairs growth

I have found that the Glo3 ArfGAP interacts with the Sec21 component of the COPI vesicle coat *in vivo*, and that the Glo3 protein itself is a component of the COPI vesicle coat. Such an interaction is likely to have consequences for COPI-vesicle dynamics; therefore, I next assessed the specific requirement for Glo3 ArfGAP function in COPI-mediated vesicular transport. Members of the ArfGAP protein family have an invariant

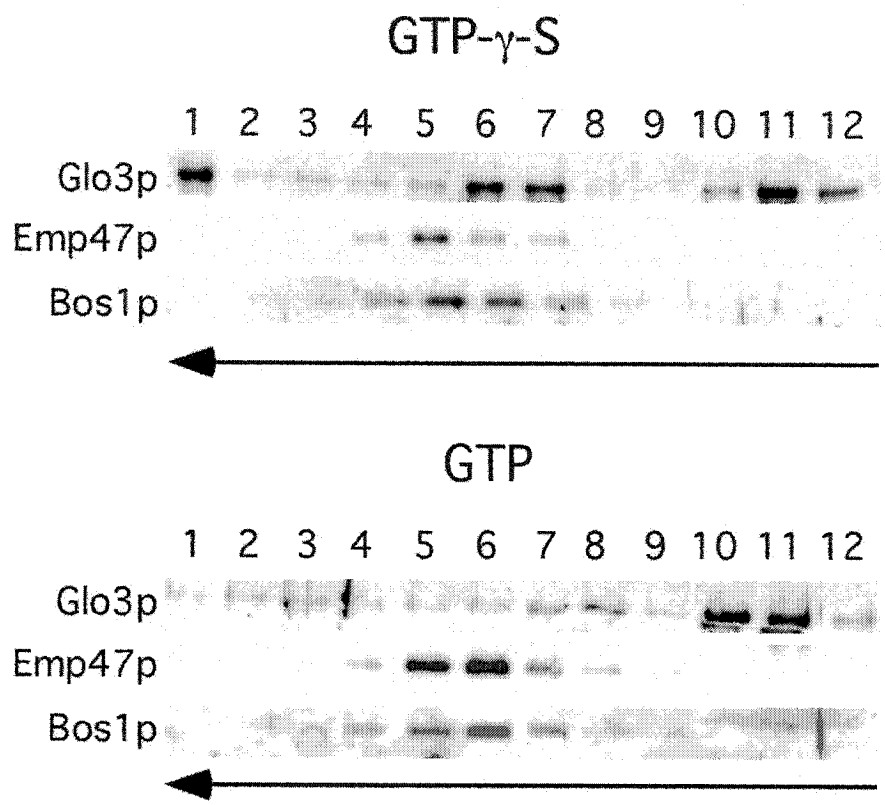

Figure 21. The Glo3 ArfGAP is found on the coat of vesicles budded from Golgi membranes *in vitro*. Golgi membranes from wild-type cells were incubated with Arf1, coatomer and GTPγS or GTP to generate COPI vesicles. The vesicles were separated from the Golgi membranes by velocity sedimentation centrifugation. The vesicle peak was collected and floated on a buoyant-density gradient. Fractions were collected, precipitated and analyzed by immunoblot. Emp47 and Bos1 are vesicle proteins that indicate the location of the vesicle fraction. The arrows indicate the movement of the lipid particles in the gradient (Figure courtesy of Dr. Anne Spang).

arginine residue located just downstream of the zinc-binding domain (Figure 22A), and mutation of this invariant arginine in Gcs1 destroys Gcs1 ArfGAP activity (Yanagisawa et al., 2001). Therefore, to generate a mutant version of Glo3 that lacks ArfGAP activity a lysine residue was substituted for the invariant arginine residue at position 59 (Glo3-R59K). This mutant form of Glo3 is referred to as an "ArfGAP-dead" protein, since it lacks measurable ArfGAP activity as determined by an *in vitro* ArfGAP assay (Figure 22B; ArfGAP assay and figure courtesy of Dr. Pak Phi Poon, Dalhousie University). Since this mutant Glo3 may exert negative effects on cell growth, I placed the *glo3-R59K* mutant allele under control of the inducible *MET3* promotor (Mao et al., 2002; *MET3pr-glo3-R59K*, as shown in Figure 25C). In the presence of methionine, expression of the *glo3-R59K* gene is repressed, whereas in the absence of methionine expression of the *glo3-R59K* gene is induced. As assessed by Western blot analysis, the relative abundance of Glo3-R59K protein expressed from the *MET3* promotor was similar to the level of Glo3 protein expressed from the endogenous *GLO3* promotor (data not shown). Therefore, the Glo3-R59K protein is expressed at a level that reflects the situation for intact Glo3 protein.

As shown in Figure 23A, the presence of a plasmid without any *GLO3* sequences (*MET3pr*-empty) or with the wild-type *GLO3* gene under control of the *MET3* promotor (*MET3pr-GLO3*) did not impair the growth of mutant cells lacking a chromosomal copy of the *GLO3* gene. In contrast, expression of *glo3-R59K* was markedly detrimental to the growth of mutant cells lacking a chromosomal copy of the *GLO3* gene. However, expression of *glo3-R59K* in cells *with* a chromosomal copy of the wild-type *GLO3* gene

Figure 22. Mutation of the arginine at position 59 in Glo3 destroys ArfGAP activity *in vitro*. (A) Schematic representation of the arginine-to-lysine mutation in *glo3-R59K* allele. The shaded box indicates the coding region for the ArfGAP domain, and the black bar indicates the position of the R59K mutation. (B) Glo3-R59K-His6× was expressed in *E. coli*, purified using NiNTA-agarose, and used for an *in vitro* ArfGAP assay as previously described (Poon et al., 1999). ■ = Glo3, ● = Glo3-R59K (Figure courtesy of Dr. Pak Phi Poon).

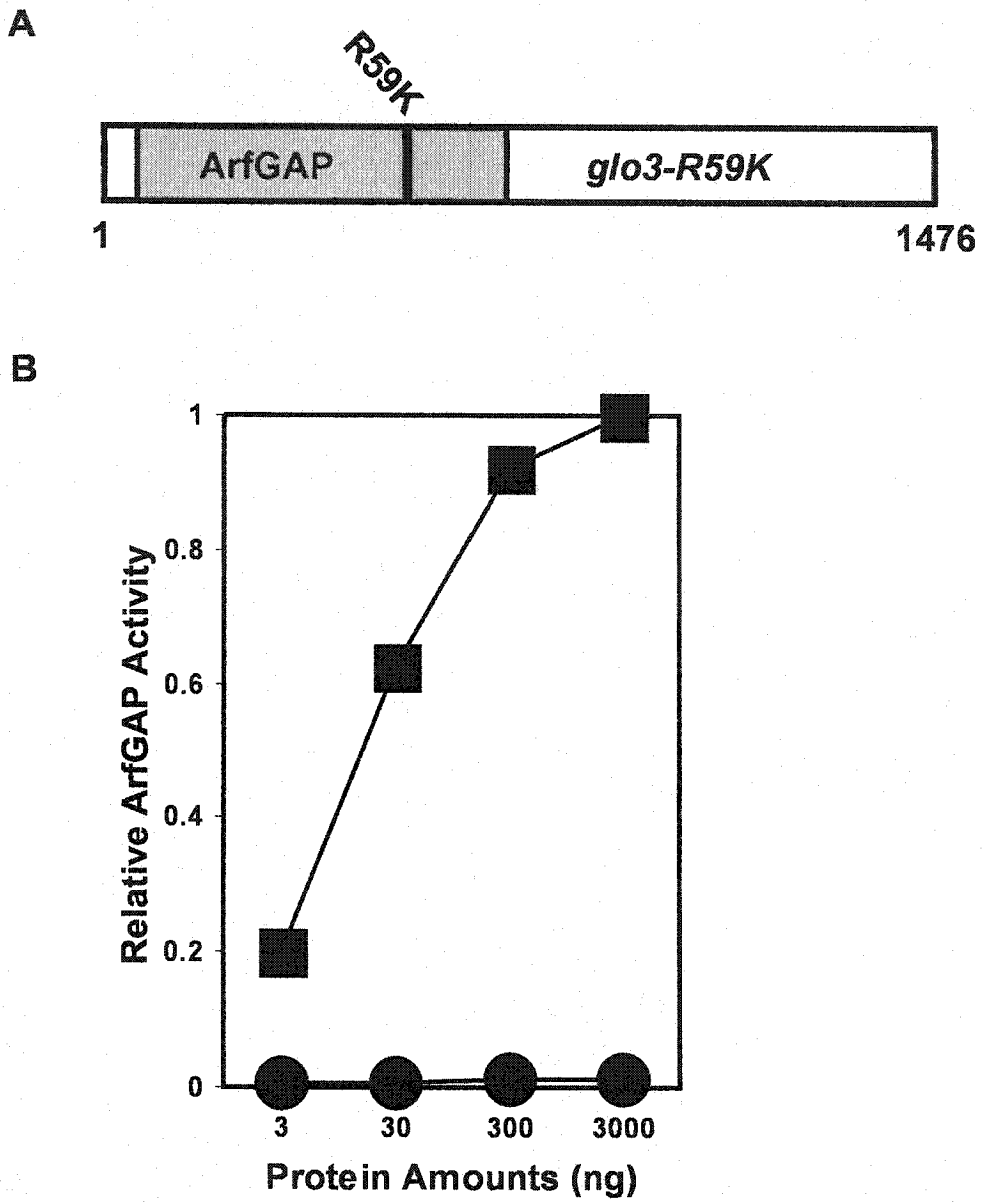
A
R59K
ArfGAP
glo3-R59K
1
1476
B
Relative ArfGAP Activity
1
0.8
0.6
0.4
0.2
0
3
30
300
3000
Protein Amounts (ng)

Figure 22

had no effect on cell viability, even when *glo3-R59K* is expressed from a high-copy plasmid (Figure 23B and 23C). These results show that the Glo3-R59K protein inhibits growth when it is the only version of the Glo3 protein in the cell. Furthermore, since the Gcs1 ArfGAP can facilitate retrograde vesicular transport in the absence of Glo3, these data indicate that Glo3-R59K interferes with the ability of the Gcs1 ArfGAP to facilitate retrograde vesicular transport.

5. Glo3-R59K interacts with Sec21 *in vivo*

I have determined that the Glo3 ArfGAP interacts with the Sec21 component of the COPI coat, and that this interaction leads to the incorporation of Glo3 into the COPI vesicle coat. Furthermore, I have also found that a mutant version of the Glo3 protein exerts a negative effect on cell growth when it is the only version of Glo3 in the cell. These observations raise the possibility that the lethality imposed by the Glo3-R59K mutant protein may be due to a loss of association with Sec21, and thus a failure to incorporate Glo3-R59K into the COPI vesicle. To test this hypothesis, a protein extract from *glo3Δ* mutant cells expressing *glo3-R59K* was subjected to immunoprecipitation with anti-Sec21 antibodies and the co-precipitation of Glo3-R59K was assessed. Glo3-R59K does indeed co-precipitate along with Sec21 (Figure 24), indicating that the mutation present in Glo3-R59K does not disrupt the association of Glo3-R59K with coatomer. Therefore, the inhibition of growth imposed by Glo3-R59K is not due to a loss of interaction between Glo3-R59K and Sec21.

Figure 23. Expression of the mutant Glo3-R59K protein causes lethality. (A) Expression of the *glo3-R59K* allele is controlled by the *MET3* promoter (*MET3pr-glo3-R59K*). *MET3* promoter sequences are indicated by the box at the left of the diagram, whereas *glo3-R59K* sequences are indicated by the boxed arrow at the right of the diagram. Transcription of the *glo3-R59K* allele proceeds from the *MET3* promoter, as indicated by the small arrow. (B) *glo3Δ* yeast cells harbouring a low-copy plasmid with the *MET3pr-glo3-R59K* gene were streaked for single colonies on medium containing methionine (Met+) to repress *glo3-R59K* expression or medium lacking methionine (Met-) to induce *glo3-R59K* expression. Plates were incubated at 30°C for two days. (C) Wild-type yeast cells harbouring a low-copy plasmid with the *MET3pr-glo3-R59K* construct were streaked for single colonies on medium containing methionine (Met+) to repress *glo3-R59K* expression or media lacking methionine (Met-) to induce *glo3-R59K* expression. Plates were incubated at 30°C for two days.

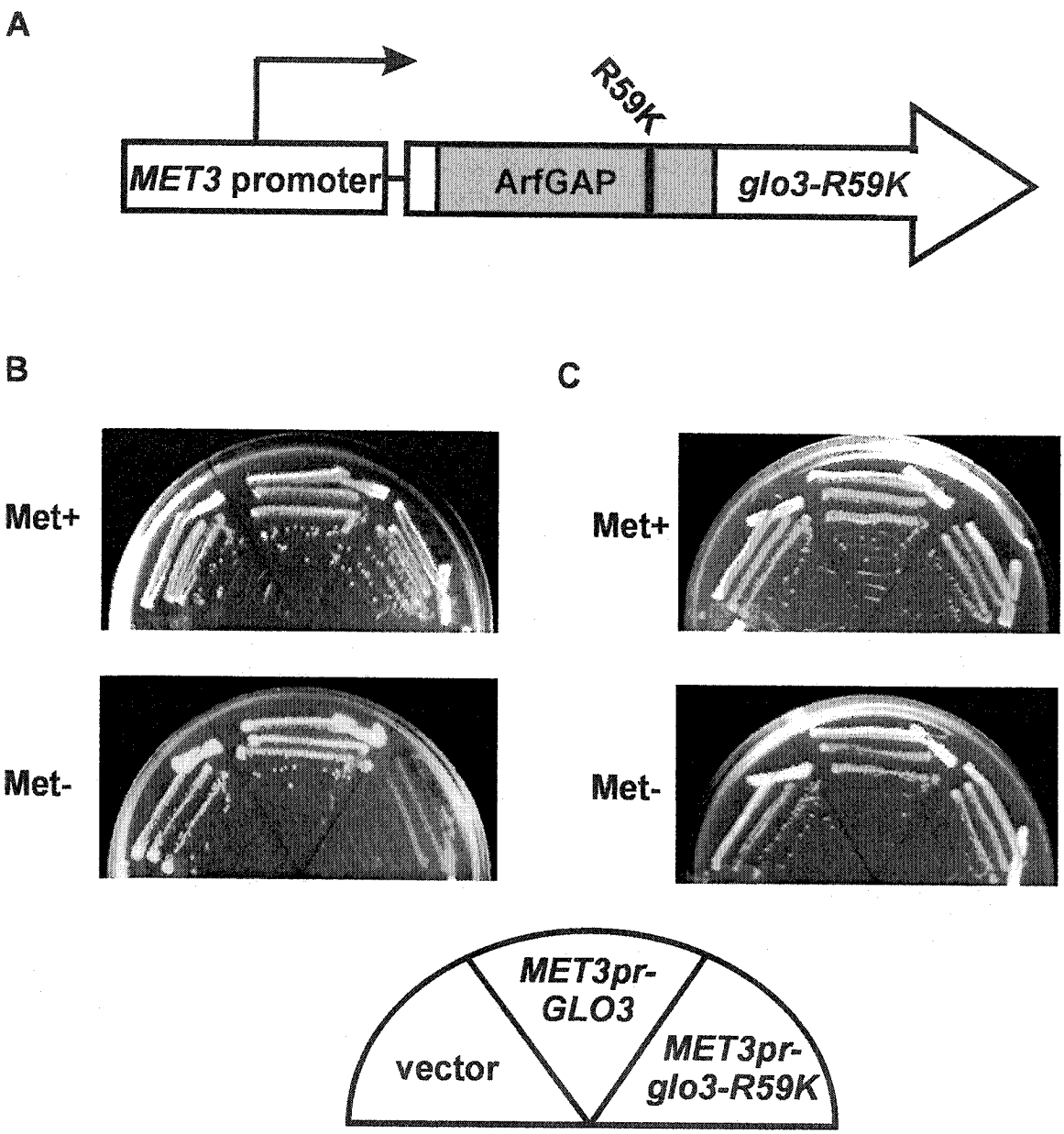
A
R59K
MET3 promoter
ArfGAP
glo3-R59K
B
C
Met+
Met-
Met+
Met-
MET3pr-
GLO3
vector
MET3pr-
glo3-R59K

Figure 23

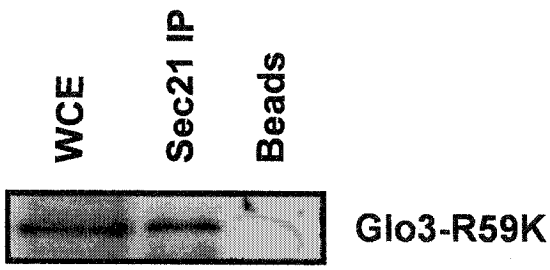

Figure 24. Glo3-R59K co-immunoprecipitates with the coatomer complex. Protein extracts from *glo3Δ* mutant cells expressing *glo3-R59K* were incubated with anti-Sec21 antibody, followed by incubation with Protein-A-agarose beads. Beads were pelleted by centrifugation, washed, and resuspended in 1× Laemmli buffer. Samples were resolved by 10% SDS-PAGE, transferred to PVDF, and probed with Glo3 antibodies.

6. Glo3-R59K impairs vesicular transport

I have found that Glo3-R59K impairs growth when it is the only version of Glo3 in the cell. This toxicity could be due to a block of vesicular transport, or Glo3-R59K may possibly affect some other process. To investigate the effect of ArfGAP-dead Glo3-R59K on vesicular transport in mutant cells lacking a chromosomal copy of the *GLO3* gene, cells were examined by electron microscopy. It has previously been reported that ER membranes accumulate in cells in which both Gcs1 and Glo3 ArfGAP function is impaired (Poon et al., 1999). Such ER accumulation is a hallmark of defects in vesicular transport, and is indicative of the indirect effect of perturbations in retrograde vesicular transport on anterograde vesicle traffic. *glo3Δ* mutant cells harboring either the *glo3-R59K* allele or the wild-type *GLO3* gene expressed from the *MET3* promoter were transferred to medium lacking methionine to induce expression from the *MET3* promoter. After incubation for 6 hours, cells were collected and prepared for electron microscopy as described (Eitzen et al., 1997). As shown in Figure 25A, cells expressing Glo3-R59K accumulated a significant amount of ER membrane. In contrast, cells expressing wild-type Glo3 protein did not show any accumulation of ER membrane (Figure 25B). These results show that expression of the Glo3-R59K mutant protein causes cells to accumulate ER membranes, suggesting a breakdown in the vesicular transport process.

To further examine the effect of Glo3-R59K on vesicular transport in mutant cells lacking a chromosomal copy of the *GLO3* gene, the processing state of carboxypeptidase Y (CPY) was assessed. Mutant cells carrying the *MET3pr*-"empty" plasmid or the

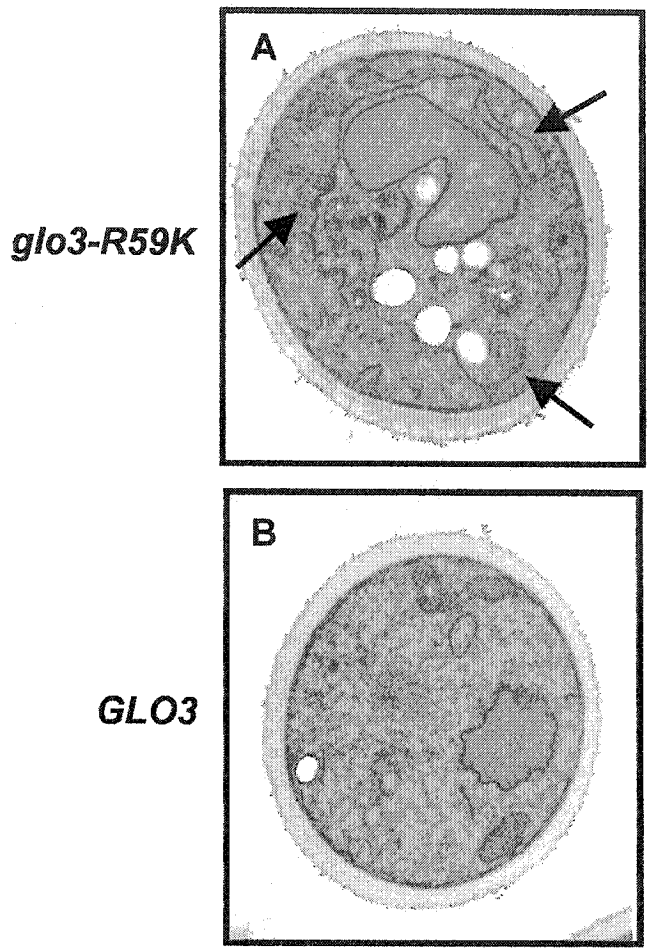

Figure 25. Glo3-R59K causes accumulation of ER membrane. *glo3Δ* cells carrying (A) the *MET3pr-glo3-R59K* plasmid or (B) the *MET3pr-GLO3* plasmid were transferred to medium lacking methionine and incubated for 6 hours at 30°C. Cells were then collected and fixed for examination by electron microscopy. Arrows indicate accumulated ER membranes.

MET3pr-GLO3 plasmid processed p1CPY to mCPY within 30 minutes of exposure to radiolabel (Figures 26, lanes 2 and 5). In contrast, mutant cells expressing Glo3-R59K from the *MET3pr-glo3-R59K* plasmid were ineffective in converting the precursor p1 form of CPY to mCPY even after 60 minutes (Figure 26, lane 9). Therefore, transport of CPY is slowed in mutant cells expressing Glo3-R59K, indicating that vesicular transport is disrupted in these mutant cells. Taken together with the observation of elaborated ER membranes in mutant cells expressing *glo3-R59K*, these data indicate that Glo3-R59K inhibits growth by perturbation of vesicular transport. Furthermore, since the Gcs1 ArfGAP is still present in cells expressing *glo3-R59K*, Glo3-R59K interferes with the ability of Gcs1 to facilitate retrograde vesicular transport.

7. Increased gene dosage of *GCS1* suppresses the lethality imposed by Glo3-R59K

Glo3-R59K interferes with the ability of the Gcs1 ArfGAP to mediate retrograde vesicular transport, perhaps by preventing Gcs1 from making the interactions required to facilitate retrograde vesicular transport. Such interactions may be restored by increasing the abundance of the Gcs1 ArfGAP, thereby allowing Gcs1 to "out-compete" the mutant version of Glo3 for interacting partners. Thus, increased gene dosage of *GCS1* may suppress the lethality imposed by *glo3-R59K* expression.

To test this hypothesis, cells lacking a chromosomal copy of the wild-type *GLO3* gene and harbouring the *MET3pr-glo3-R59K* plasmid were transformed with a high-copy *GLO3* plasmid, a high-copy *GCS1* plasmid, or an "empty" vector. Cells expressing the mutant version of Glo3 were able to grow on medium lacking methionine if these mutant

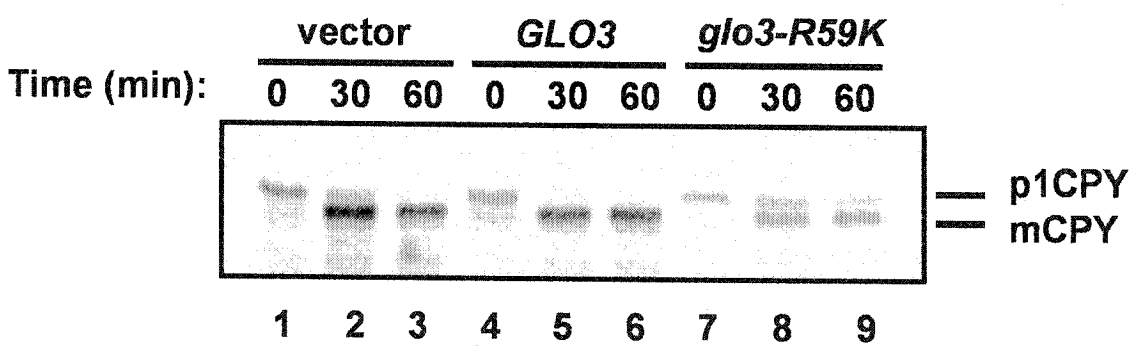

Figure 26. Glo3-R59K causes defects in CPY maturation. Proliferating cells were exposed to [^{35}S] methionine and cysteine for 7 minutes and sampled without a "chase" with unlabeled amino acids at 0, 30, and 60 minutes. CPY was immunoprecipitated, resolved by SDS-PAGE, and detected by autoradiography.

cells also had increased abundance of either the Glo3 or Gcs1 ArfGAP, whereas mutant cells carrying the "empty" vector failed to grow on medium lacking methionine (Figure 27). These results demonstrate that increasing the abundance of the Gcs1 ArfGAP abrogates the lethality caused by *glo3-R59K* expression and allows retrograde transport to proceed in the presence of Glo3-R59K.

8. Intact Glo3 function is required for the generation of COPI vesicles

Mutant Glo3-R59K protein may impair retrograde vesicular transport either by affecting COPI vesicle uncoating, or by affecting the generation of COPI-coated vesicles. To determine if Glo3-R59K inhibits COPI-coated vesicle generation, I assessed the ability of Glo3-R59K to mediate the production of COPI vesicles using an *in vitro* vesicle-budding reaction (Spang and Schekman, 1998). Once again, these vesicle-budding experiments were performed in collaboration with Dr. Anne Spang, and Figure 28 is courtesy of Dr. Spang. Actively proliferating cells lacking a chromosomal copy of the wild-type *GLO3* gene and harbouring the *MET3pr-glo3-R59K* plasmid were transferred to medium lacking methionine to induce expression of the mutant Glo3 protein. After a 6-hour incubation, cells were harvested and Golgi membranes were isolated. Vesicles were generated from the isolated Golgi membranes, and COPI vesicles were separated from the Golgi by velocity sedimentation and further purified by buoyant density flotation. The vesicle fraction was then subjected to Western blot analysis. As expected, Golgi membranes isolated from cells carrying an "empty" vector or a *MET3pr-GLO3* plasmid were able to form COPI coated vesicles *in vitro* in the presence of GTP (Figure 28). However, Golgi

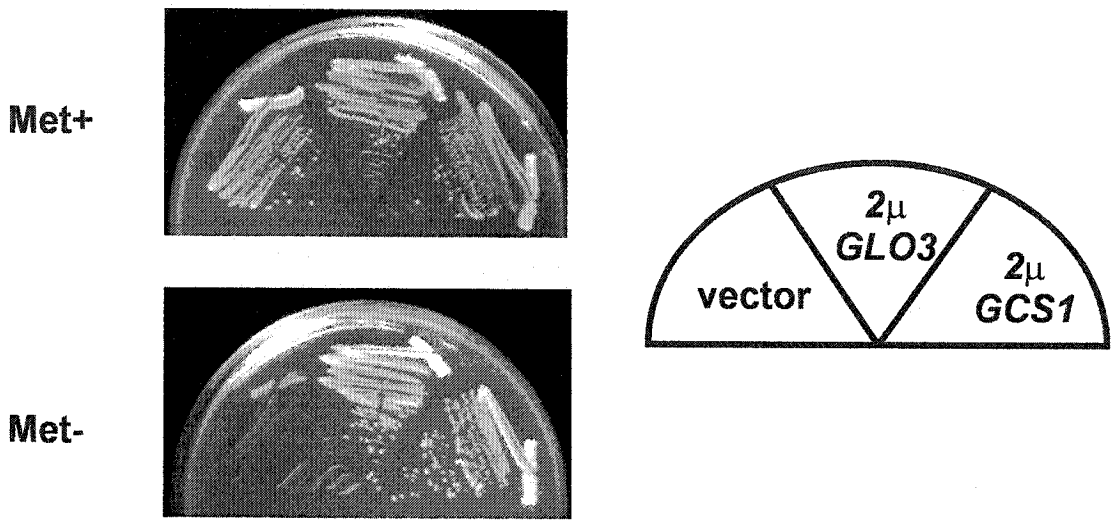

Figure 27. Increased abundance of the Gcs1 ArfGAP suppresses Glo3-R59K lethality. *glo3Δ* yeast cells harbouring a low-copy plasmid with the *MET3pr-glo3-R59K* gene were transformed with either an "empty" high-copy plasmid, a high-copy plasmid carrying the *GLO3* gene, or a high-copy plasmid carrying the *GCS1* gene. Cells were streaked for single colonies on either Met+ or Met- medium and incubated at 30°C for two days.

membranes isolated from cells carrying the *MET3pr-glo3-R59K* plasmid failed to produce COPI vesicles in the presence of GTP (Figure 28). These data demonstrate that intact Glo3 function is required for the generation of COPI vesicles *in vitro*.

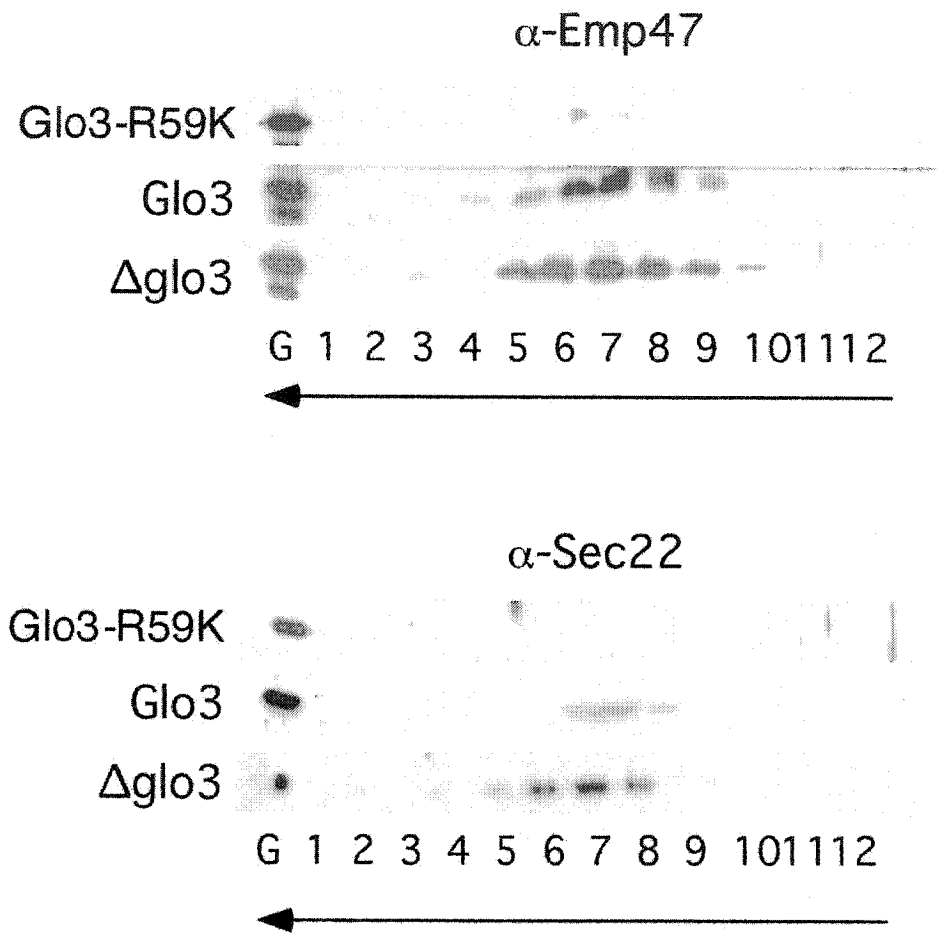

Figure 28. Glo3-R59K prevents the generation of COPI vesicles *in vitro*. COPI vesicles were generated from Golgi membranes isolated from *glo3Δ* yeast cells carrying the *MET3pr-glo3-R59K*, *MET3pr-GLO3*, and *MET3pr*-empty plasmids, which were incubated in the presence of GTP and COPI components. The vesicles were purified over a velocity gradient, and subsequently floated on a Nycodenz gradient. Fractions were collected from the top, resolved by SDS-PAGE, and analyzed by immunoblot. The arrows indicate the direction of movement of lipid particles within the gradient.

IV. DISCUSSION

1. General remarks

In the budding yeast *Saccharomyces cerevisiae*, the Gcs1 + Glo3 ArfGAP pair provides essential overlapping function for retrograde vesicular transport from the Golgi apparatus to the ER (Poon et al., 1999), whereas the Gcs1 + Age2 ArfGAP pair provides essential overlapping function for transport from the *trans*-Golgi network (Poon et al., 2001). I have investigated the role of ArfGAPs in retrograde vesicular transport using a temperature-sensitive version of the Gcs1 protein that is only defective for retrograde transport at the non-permissive temperature. Mutant cells that lack chromosomal copies of the *GCS1* and *GLO3* genes and carry the temperature-sensitive *gcs1-28* allele on a plasmid (i.e. *gcs1-28 glo3Δ* double-mutant cells) are able to grow at permissive temperatures of 23°C or 30°C, but fail to grow at the non-permissive temperature of 37°C. This growth defect reflects an impairment of the essential retrograde-transport process.

Using a dosage-suppressor approach, I have identified several yeast genes that, when present in increased copy number, permit *gcs1-28 glo3Δ* double-mutant cells to grow at the non-permissive temperature. These dosage-suppressor genes encode proteins that are likely to be involved in the same process as, or processes related to, the function of the Gcs1 ArfGAP for retrograde transport (Prelich, 1999). Thus, the identification of dosage suppressors of defective Gcs1 ArfGAP function for retrograde vesicular transport has permitted the further characterization of the role of Gcs1 in this process.

At the same time, I have also characterized the individual contributions of the Gcs1 and Glo3 ArfGAPs to retrograde vesicular transport at the molecular level. Recent evidence has suggested that Gcs1 and Glo3 may not contribute equally to retrograde transport (Dogic et al., 1999; Eugster et al., 2000). Indeed, deletion of the *GLO3* gene was found to impart a more severe defect on cell growth and retrograde vesicular transport than does deletion of the *GCS1* gene (Poon et al., 1999; Dogic et al., 1999). Furthermore, yeast two-hybrid analysis has shown that the Glo3 ArfGAP physically interacts with the Sec21 (γ-COP) and Sec27 (β'-COP) components of the COPI vesicle coat complex (also known as coatomer), whereas the Gcs1 ArfGAP was not found to interact with any components of the COPI complex by the same two-hybrid approach (Eugster et al., 2000). Using these observations as a starting point, I have investigated ArfGAP–coatomer interactions *in vivo* and have characterized the effect of a non-functional Glo3 protein on the ability of the Gcs1 ArfGAP to mediate retrograde vesicular transport.

I have found that the Glo3 ArfGAP physically associates with coatomer *in vivo*, whereas I was unable to detect any similar interactions between the Gcs1 ArfGAP and coatomer. In collaboration with Dr. Anne Spang at the Friedrich Miescher Laboratorium in Tuebingen, Germany, I have found that the association of Glo3 with coatomer allows the Glo3 ArfGAP to become a component of the COPI vesicle coat. Moreover, expression of a non-functional Glo3 protein that lacks ArfGAP activity impairs cell growth and vesicular transport, even in the presence of the Gcs1 ArfGAP. This finding supports the idea that Gcs1 may replace Glo3 activities for retrograde vesicular transport

only in the absence of the Glo3 protein. Furthermore, my collaboration with Dr. Spang has revealed an unexpected role for ArfGAP function in the generation of COPI vesicles.

2. Suppression of the effects of deficient Gcs1 and Glo3 ArfGAP function for retrograde vesicular transport by the Age1 ArfGAP

To characterize the role of the Gcs1 ArfGAP in retrograde vesicular transport from the Golgi apparatus to the ER, I isolated dosage suppressors of the temperature-sensitive phenotype of *gcs1-28 glo3Δ* double-mutant cells, which results from deficient ArfGAP function for retrograde vesicular transport at the non-permissive temperature (37°C). Through this analysis, I have identified the *AGE1* gene as a dosage suppressor of defective Gcs1-28 ArfGAP function for retrograde vesicular transport.

The *AGE1* gene was originally isolated and characterized as a suppressor of Arf1 temperature sensitivity (*SAT1*; Zhang et al., 1998), but was later renamed as an ArfGAP with effector functions (*AGE1*; Zhang et al., 2003). The Age1 protein was previously implicated in vesicular-transport processes mediated by Arf1 because of the ability of increased Age1 abundance to suppress the temperature-sensitive defect of the *arf1-3* mutant allele (Zhang et al., 1998). A dosage-suppressor analysis for suppressors of another situation involving Gcs1, the impairment of transport from the *trans*-Golgi network caused by deficient Gcs1 and Age2 activities, also identified *AGE1* as a dosage suppressor (Auger, 2000). Recently, a direct role for Age1 in Arf1 function was suggested by the finding that the Age1 protein has ArfGAP activity for Arf1 *in vitro*

(Zhang et al., 2003). All of these data imply that the Age1 protein may function for the vesicular transport process.

The discovery that increased abundance of the Age1 ArfGAP suppresses the effects of deficient Gcs1 and Glo3 ArfGAP function for retrograde vesicular transport, and of deficient Gcs1 and Age2 ArfGAP function for transport from the *trans*-Golgi network (Auger, 2000), provides the first evidence that Age1 can act as an ArfGAP *in vivo*. Indeed, increased abundance of Age1 partially suppresses the disruption of vesicular transport associated with *gcs1-28 glo3Δ* double-mutant cells. Moreover, the ability of increased Age1 abundance to facilitate retrograde transport is dependent on Age1 ArfGAP activity, since a substitution in the Age1 protein that destroys this ArfGAP activity also abrogates Age1 suppression of *gcs1-28 glo3Δ* temperature sensitivity. Thus, Age1 is able to perform ArfGAP function for vesicular transport *in vivo*, at least when present in increased abundance.

Although increased Age1 abundance can overcome the effects of defective Gcs1 and Glo3 function, increased Age1 abundance cannot substitute for Gcs1 and Glo3 ArfGAP function. Increased Age1 abundance does not replace (bypass) Gcs1 and Glo3 ArfGAP function for retrograde vesicular transport, since *gcs1Δ glo3Δ* double-mutant cells must retain the plasmid borne *gcs1-28* allele even in the presence of increased *AGE1* gene dosage. Furthermore, even though the increased dosage of the *age1-Δ164* mutant allele allows even better growth of *gcs1-28 glo3Δ* double-mutant cells at the non-permissive temperature than does increased dosage of the wild-type *AGE1* gene, increased Age1-Δ164 abundance still cannot replace Gcs1 and Glo3 function for

retrograde vesicular transport (data not shown). Since some Gcs1-28 function is required for the viability of *gcs1-28 glo3Δ* double-mutant cells even in the presence of increased Age1 ArfGAP abundance, Age1 must be deficient for some Gcs1 and Glo3 ArfGAP activities.

The Gcs1 ArfGAP has been implicated in cellular activities other than vesicular transport, namely actin cytoskeletal organization through actin remodeling (Blader et al., 1999). Perhaps mutant versions of Gcs1, such as the mutant version encoded by the *gcs1-28* allele, retain some necessary capacity for actin remodelling. As mentioned previously, Gcs1 shares essential overlapping function with the Glo3 ArfGAP for retrograde transport, and with the Age2 ArfGAP for transport from the *trans*-Golgi network (Poon et al., 1999; Poon et al., 2001), such that the Glo3 ArfGAP facilitates retrograde transport, and the Age2 ArfGAP facilitates transport from the *trans*-Golgi network, in the absence of Gcs1. Nevertheless, *gcs1Δ* single-mutant cells are viable, raising the possibility that either Glo3 or Age2 (or a combination of these two ArfGAPs) carries out some Gcs1-mediated actin remodeling activities in *gcs1Δ* mutant cells. Indeed, *gcs1Δ* mutant cells were shown to have actin cytoskeleton defects under various stress conditions (such as 0.9 M NaCl, 40 mM NaF, or the presence in the growth medium of Lat-B, a drug that interferes with actin polymerization; Blader et al., 1999), implying that Glo3 and Age2 may substitute poorly for Gcs1-mediated actin-remodeling activities. When cells lack either the Glo3 or Age2 proteins, even mutant versions of the Gcs1 ArfGAP (defective for vesicular transport) may be necessary for actin remodelling. Both *gcs1-28 glo3Δ* and *gcs1-3 age2Δ* double-mutant cells have demonstrable defects in vesicular transport, but

may not display any defect in actin cytoskeleton structure at the non-permissive temperature, a finding that would suggest the mutant Gcs1-28 and Gcs1-3 ArfGAPs retain actin remodeling activities even under non-permissive conditions. Therefore, although increased abundance of the Age1 ArfGAP restores vesicular transport in *gcs1-28 glo3Δ* cells at the non-permissive temperature, it is entirely possible that the Age1 protein cannot perform Gcs1-mediated actin remodeling activities, even when Age1 is present in increased abundance. An inability of Age1 to supply Gcs1-mediated actin activities provides an explanation for the inability of the Age1 ArfGAP to replace Gcs1 and Glo3 function for retrograde transport.

3. Localization and regulation of ArfGAP function

Mounting evidence indicates that ArfGAPs may be able to function at multiple vesicular-transport stages. Gcs1 has essential overlapping function with the Glo3 ArfGAP for retrograde vesicular transport from the ER to the Golgi (Poon et al., 1999), and also shares an essential overlapping function with the Age2 ArfGAP for transport from the *trans*-Golgi network (Poon et al., 2001). Therefore, the Gcs1 ArfGAP is able to function at two distinct vesicular-transport stages. Exactly how Gcs1 activities for each of these two transport stages are regulated remains to be seen. However, it has been hypothesized the sequences external to the ArfGAP domain are involved in the localization and regulation of ArfGAP function (Aoe et al., 1999).

The *in vivo* activity of the mammalian ARF1 GAP requires a non-catalytic domain present within the carboxy terminus (Aoe et al., 1999). Similarly, the *in vitro*

ArfGAP activity of a carboxy-terminal truncation of the Gcs1 protein (Gcs1-7) is significantly reduced (Poon et al., 1996), indicating that sequences external to the ArfGAP domain of yeast ArfGAP family members are also required for effective ArfGAP function. Of particular interest in this study is the finding that removal of the amino-terminal sequences external to the ArfGAP domain of the Age1 protein (Age1-Δ164) actually improves the ability of Age1 to suppress the effects of deficient Gcs1 and Glo3 ArfGAP function in *gcs1-28 glo3Δ* double-mutant cells. Moreover, the *age1-Δ164* mutant allele is able to suppress the temperature-sensitive phenotype of *gcs1-28 glo3Δ* cells even when present in low copy number. Such a finding suggests that Age1 ArfGAP function may be regulated by sequences within the amino-terminal extension, providing additional evidence to support the hypothesis that ArfGAP activities are regulated by sequences external to the ArfGAP domain itself.

Why does the Age1 amino-terminal extension exert a negative effect on the ability of Age1 to suppress the effects of deficient Gcs1 and Glo3 ArfGAP function? A recent study exploring Age1 ArfGAP activity found that a mutant Age1 protein lacking the amino-terminal region (Age1Δ5) retains ArfGAP activity in an *in vitro* assay (Zhang et al., 2003). My *in vivo* results support this finding, as I have found that Age1 ArfGAP activity is required for suppression of *gcs1-28 glo3Δ* temperature sensitivity, and that the *age1-Δ164* mutant allele lacking the coding sequences for the Age1 amino-terminal region in fact displays increased suppressor function. The Age1Δ5 and Age1-Δ164 mutant proteins lack the same 164 amino acids of the amino-terminal region of Age1, and thus represent the same polypeptide sequence. Therefore, the amino-terminal extension of

Age1 is not required for Age1 ArfGAP activity. Zhang et al. (2003) suggest that the amino-terminal extension of Age1 may regulate ArfGAP function by acting as a negative regulator through specific protein contacts. Indeed, the amino-terminal extension of Age1 may bind as-yet-unknown negative regulators of ArfGAP activity and/or ArfGAP function. A yeast two-hybrid analysis of the Age1 amino-terminal extension may reveal novel negative regulators of ArfGAP function.

An alternative hypothesis concerns a role for the amino-terminal extension of Age1 in regulating Age1 ArfGAP activity through intra-peptide interactions, perhaps by masking sites of interaction with the Arf GTPase. However, the Age1Δ5 mutant protein was not found to possess increased ArfGAP activity compared with wild-type Age1 in an *in vitro* assay (Zhang et al., 2003), and therefore it is unlikely that the amino-terminal extension of Age1 negatively regulates ArfGAP activity through these intra-peptide interactions. Yet another possible mechanism for Age1 regulation by the amino-terminal extension concerns the localization of the Age1 protein within the cell. The amino acid sequence of this region contains a putative transmembrane domain (data not shown), which may anchor Age1 at a specific organelle or membrane location. Similarly, specific proteins may bind to the amino-terminal extension to facilitate the retention of Age1 at a particular location. A proteome-wide analysis of protein localization (Huh et al., 2003) has shown that Age1 tagged with green fluorescent protein (GFP) has a disperse cytoplasmic distribution within the cell, and therefore may not be localized to any specific organelle or location. Nonetheless, further analysis of the subcellular location of

both Age1 and the amino-terminally truncated Age1-Δ164 protein will provide evidence as to whether the amino-terminal extension is specifically required for Age1 localization.

The ability of increased Age1 abundance to suppress the effects of deficient ArfGAP activities for two distinct stages of vesicular transport speaks to the promiscuity of ArfGAP function. The Gcs1 ArfGAP is known to function for retrograde vesicular transport *and* for transport from the *trans*-Golgi network (Poon et al., 1999, 2001), implying that some ArfGAPs can facilitate more then one distinct vesicular-transport processes. Indeed, increased abundance of the Age1 ArfGAP facilitates both retrograde transport and transport from the *trans*-Golgi (this study; Auger, 2000) when these transport processes are compromised. In two independent studies, increased abundance of Glo3 was found to suppress the effects of deficient Gcs1 and Age2 ArfGAP function for transport from the *trans*-Golgi network (Auger, 2000; Zhang et al., 2003), providing additional evidence for the promiscuity of ArfGAP function. However, since I have found that increased abundance of the Age2 ArfGAP does not suppress the effects of deficient Gcs1 and Glo3 ArfGAP function for retrograde transport, not all ArfGAPs share such promiscuity. Therefore, only certain ArfGAPs (namely Glo3 and Age1) display promiscuous function when present in increased abundance.

But why is the ArfGAP function of Age1 and Glo3 promiscuous only when these proteins are present in increased abundance? One possibility is an alteration in the ratio of a regulator protein to the ArfGAPs themselves. When ArfGAPs are in excess, specific factors that localize and/or regulate ArfGAP function at a particular vesicular-transport stage may be limiting, and therefore some ArfGAP protein could be freed from this

regulation to mediate other vesicular-transport stages. As mentioned above, such regulation may require the binding of the regulatory protein(s) to sequences external to the ArfGAP domain. The generation of chimeric ArfGAP proteins containing various combinations of these regions would allow the determination of which ArfGAP regions are necessary for regulatory control. For example, a chimeric ArfGAP containing the Glo3 ArfGAP domain and the Age2 carboxy-terminal region may not provide function for retrograde vesicular transport, if this chimera is subject to Age2-specific regulation and/or localization. Conversely, substitution of the carboxy-terminal region of the Age2 protein with the Age1 carboxy-terminal region may now allow Age2 to suppress the effects of deficient Gcs1 and Glo3 ArfGAP function for retrograde vesicular transport. The identification of the regions of ArfGAPs that regulate their function, and the regulatory proteins that may be involved, await further investigation. Yeast two-hybrid analysis may prove useful in identifying the regulatory proteins that bind regions external to the ArfGAP domain to regulate ArfGAP function, likely in a transport-stage specific manner.

4. A normal function for the Age1 ArfGAP

The finding that the Age1 ArfGAP provides function for vesicular transport when present in increased abundance begs the question of whether Age1 normally functions for vesicular transport, and if so, at what particular transport stage. Zhang et al. (2003) have inferred a subtle role for Age1 in the endocytic pathway, based on the observation that deletion of other ArfGAP genes in combination with an *age1Δ* mutation causes a subtle

endocytic defect. Nonetheless, an exact cellular role for the Age1 ArfGAP remains elusive. *age1Δ* single-mutant cells do not display any obvious phenotype; thus other proteins likely replace lost Age1 function in *age1Δ* mutant cells. Such proteins must share an overlapping function with Age1, and are likely to function for the same or a similar process as the Age1 ArfGAP itself. Therefore, the identification of the proteins that share an overlapping function with Age1 will allow a determination of normal Age1 function.

The combination of individual mutations in two genes that encode proteins having a related function, or that are involved in the same process, often leads to a loss of viability or impaired function, a phenomenon known as synthetic lethality. That is, individual mutations of each gene do not cause a growth defect on their own; however, the combination of the two mutations leads to a synthetic-lethal phenotype. Therefore, synthetic lethality is a useful tool for discovering relationships between a gene of unknown function and genes that have known functions. To identify those genes that share an overlapping function with *AGE1* (and therefore mutation of these genes resulting in synthetic lethality when combined with an *age1Δ* mutation), an initial analysis of genetic interactions between an *age1Δ* mutation and mutations of genes of the secretory pathway (*sec* mutants) was undertaken. Unfortunately, none of the *sec* genes tested displayed any detectable synthetic lethality with the *age1Δ* mutation (data not shown). Furthermore, an analysis of *age1Δ* genetic interactions with mutations in other ArfGAP genes showed no genetic interactions (Zhang et al., 2003).

A broader approach to identify genes that share overlapping function with *AGE1* relies on a global analysis of *age1Δ* genetic interactions. Systematic genetic array (SGA) analysis makes use of a collection of ~4700 viable single-gene disruption yeast strains and robotic manipulations to systematically generate double-mutant cells containing the mutation of interest (in this case *age1Δ*) and each of the gene disruptions from the arrayed collection (Tong et al., 2001). The double-mutant cells generated are then scored for growth defects; double-mutant derivatives that fail to grow, or that are impaired, are scored as displaying a synthetic-lethal interaction. Through this process, the genetic interactions of an *age1Δ* mutant can be identified on a global scale. Although SGA analysis has yielded some potential genetic interactions for *age1Δ* mutants, my further analyses of these interactions has thus far not identified any gene that has a true genetic interaction with *age1Δ* (data not shown). Since the yeast ArfGAP family consists of six members, the redundancy of ArfGAP function may be a confounding factor in any SGA analyses. For example, some combination of the remaining five ArfGAP proteins may be capable of providing Age1 function in *age1Δ* cells, so that multiple (more than two) ArfGAP mutations may be necessary to detect synthetic lethality. Because the Gcs1 ArfGAP is known to mediate at least two distinct vesicular-transport stages, it is possible that a combination of Gcs1 and another ArfGAP may be able to mediate Age1 activities in the absence of Age1 itself. This genetic situation effectively masks any genetic interactions that an *age1Δ* mutant may otherwise display. Future SGA analyses using *age1Δ gcs1Δ* double-mutant cells as a starting point may overcome this problem.

Additionally, approaches using *age1Δ age2Δ* or *age1Δ glo3Δ* double-mutant cells as a starting point for SGA analyses may also be informative.

5. Suppression of the effects of deficient Gcs1 and Glo3 ArfGAP function for retrograde vesicular transport by Sly41

My search for proteins involved in the function of the Gcs1 ArfGAP for retrograde vesicular transport also identified the *SLY41* gene as a dosage-suppressor of *gcs1-28 glo3Δ* temperature sensitivity. In addition to alleviating the growth defect of *gcs1-28 glo3Δ* double-mutant cells at the non-permissive temperature, increased abundance of Sly41 also restores vesicular transport in these cells. Furthermore, increased *SLY41* gene dosage was found to suppress the cold-sensitive phenotype of *glo3Δ* mutant cells, suggesting that increased abundance of Sly41 may allow the Gcs1 ArfGAP to function better under conditions where Gcs1 is enfeebled.

The *SLY41* gene was originally identified as a suppressor of the loss of *YPT1* (*SLY* gene) in a selection for genes that could alleviate the growth defect of *ypt1Δ* mutant cells (Dascher et al., 1991). *YPT1* is an essential gene that encodes a member of the Rab GTPase family, which have been shown to mediate numerous aspects of vesicular transport (reviewed in Segev, 2001). The yeast Rabs were initially thought only to be involved in a tethering step of vesicle docking and fusion, but later were recognized to perhaps have a role in vesicle generation. The Ypt1 Rab protein acts at the *cis* Golgi, where it mediates vesicular transport from the ER to the *cis* Golgi and vesicular transport from the *cis* Golgi to intermediate Golgi compartments (Jedd et al., 1995).

As mentioned above, a hunt for genes that can bypass the need for Ypt1 function in vesicular transport identified the *SLY* genes: *SLY1*, *SLY2*, *SLY12*, and *SLY41* (Dascher et al., 1991). A mutant allele of *SLY1*, *sly1-20*, was found to suppress the effects of loss of *YPT1*, whereas increased gene dosage of *SLY2*, *SLY12*, and *SLY41* could suppress the *ypt1Δ* defect. *SLY2* was later found to be identical to *SEC22*, a gene encoding a SNARE protein involved in the early secretory pathway (Newman et al., 1992a); similarly, *SLY12* was found to be identical to another gene encoding a SNARE of the early secretory pathway, *BET1* (Newman et al., 1992b). Unfortunately, *sly41Δ* mutant cells do not display any obvious phenotype (Dascher et al., 1991), so that characterization of the normal function of Sly41 did not occur. The recent advent of SGA analysis (see above) may permit the assignment of a normal function for the Sly41 protein. Nonetheless, the finding that the *SLY* gene products suppress the effects of loss of Ypt1 function implicates the Sly proteins in Ypt1-related function.

A particularly striking finding is that the three *SLY* genes identified as dosage suppressors of *ypt1Δ* lethality (*BET1*, *SEC22*, and *SLY41*) can also act as dosage suppressors of the cold-sensitive phenotype of *glo3Δ* mutant cells (Poon et al., 1999; this study). *glo3Δ* single-mutant cells exhibit a cold-sensitive phenotype that is likely due to an inherent cold sensitivity of some aspect of Gcs1 ArfGAP function for retrograde vesicular transport, since Gcs1 is the only ArfGAP present that can normally mediate retrograde transport in the absence of Glo3 (Poon et al., 1999). Thus, some Gcs1-mediated function is enfeebled for retrograde transport in the cold. The ability of increased *SLY* gene dosage to alleviate *glo3Δ* cold sensitivity may reflect either an

improvement of Gcs1-related function or a replacement (bypass) of Gcs1-related function for retrograde transport by increased Sly protein abundance. The latter hypothesis is unlikely, since it is probable that some level of ArfGAP activity is required to facilitate transport (see section 2). However, the finding that increased abundance of either Sly41 or Bet1 (data not shown) suppresses the effects of deficient Gcs1-28 ArfGAP function in *gcs1-28 glo3Δ* cells at 37°C supports the hypothesis that the *SLY* genes may improve enfeebled Gcs1 ArfGAP function for retrograde transport.

6. A link between ArfGAPs and Ypt Rabs?

The finding that members of the *SLY* gene family (*BET1*, *SEC22*, and *SLY41*) suppress defects in both Rab and ArfGAP function identifies a genetic link between ArfGAP function for vesicular transport and the function of the Rab proteins in this process. This hypothesis is not unprecedented, since a previous study has identified a genetic link between ArfGEF function and Ypt Rab function in yeast (Jones et al., 1999). Jones et al. (1999) found that increased abundance of ArfGEF proteins could suppress the effects of deficient Ypt function, and conversely that increased Ypt protein abundance could suppress ArfGEF defects. These data led those authors to speculate on the existence of a Ypt-Arf GTPase cascade for vesicular-transport processes. In other words, vesicular-transport steps regulated by the Arf GTPases (such as vesicle generation and uncoating) may be linked to vesicular-transport steps regulated by the Rab GTPases (such as vesicle tethering and fusion) *via* the proteins that regulate the function of each class of GTPase. Together the Arf and Rab GTPases may regulate the secretory pathway.

I have some evidence of a genetic link between the ArfGAPs and the Ypt Rabs. Although ArfGAPs have not yet been found to directly suppress the effects of deficient Ypt function, the same cohort of genes (*BET1*, *SEC22*, and *SLY41*) suppresses the effects of defects in both ArfGAP and Ypt Rab function. These data alone are sufficient to indicate that ArfGAPs and Ypt Rabs may interact with similar substrates, and through such interactions may either regulate the activity of each other or regulate a common process. Recently, I have found evidence suggesting that ArfGAPs and Ypt Rabs may act in the same process to facilitate vesicular transport. As mentioned above, the Ypt Rab proteins are involved in tethering transport vesicles to a target compartment prior to vesicle fusion (Cao et al., 1998). I performed an SGA screen for gene deletions that cause synthetic lethality when combined with a *glo3Δ* mutation, and identified the *COD4*, *COD5*, and *DOR1* genes (data not shown). These genes encode members of the conserved oligomeric Golgi (COG) complex (and have been subsequently renamed *COG5*, *COG7*, and *COG8* respectively by Whyte and Munro [2002]) that is involved in vesicle tethering (Whyte and Munro, 2001, 2002). Therefore, these data suggest a possible role for ArfGAPs in vesicle tethering.

The COG complex has been implicated in the tethering of transport vesicles to the *cis* Golgi prior to vesicle fusion with this compartment (Whyte and Munro, 2002), and is comprised of eight protein subunits (previous names are indicated in parentheses): Cog1 (Sec36, Tfi1), Cog2 (Sec35), Cog3 (Sec34, Grd20), Cog4 (Cod1, Sgf1, Sec38, Tfi3), Cog5 (Cod4), Cog6 (Cod2, Sec37, Tfi2), Cog7 (Cod5), and Cog8 (Dor1). Interestingly, members of the COG complex have been shown to interact genetically and physically

with Ypt1, as well as with components of the COPI vesicle coat (Suvorova et al., 2002). These findings suggest that a link between the Ypt1 Rab and components of the retrograde-transport machinery may exist through the COG complex. My identification of genetic interactions between ArfGAPs and components of the COG complex, as well as the recognition of a cohort of genes that suppress the effects of both Ypt1 and ArfGAP defects, suggest that the yeast ArfGAPs are also genetically linked to COG function and Ypt1 function for vesicle tethering (see section 8 below for additional comments). Nonetheless, exactly how the ArfGAPs are involved in COG function and Ypt1 function remains unclear. Further characterization of links between the ArfGAPs and the Ypt Rabs, and perhaps their combined roles in vesicle tethering, awaits investigation.

7. Perturbation of vesicular transport causes sensitivity to decreased cell-wall integrity

Early studies of *ypt1* mutant cells found that the temperature-sensitive phenotype of a *ypt1-ts* mutation could be alleviated by increasing the concentration of cations (such as Ca^{2+} and Mg^{2+}) in the growth medium (Schmitt et al., 1988). *SLY41* was originally isolated as a dosage suppressor of the effects of lost Ypt1 function, and the Sly41 protein was shown to have homology to a small-ion transporter from spinach chloroplasts (Dascher et al., 1991). The seven predicted transmembrane domains of Sly41 are thought to be arranged to form a channel through which ions or small molecules may traverse a membrane (Dascher et al., 1991). Based on these observations, Dascher et al. (1991) hypothesized that increased Sly41 abundance suppresses the effects of lost Ypt1 function

by mediating the redistribution of ions within the cell, and therefore that suppression of the effects of the *ypt1Δ* defect by increased *SLY41* gene dosage acts *via* a mechanism similar to the cation-mediated suppression of the effects of the *ypt1-ts* mutation.

gcs1Δ single-mutant cells exhibit a cold-sensitive phenotype at 15°C for re-entry into the mitotic cell cycle from quiescence (known as stationary phase in yeast; Drebot et al., 1987), and this phenotype is thought to be due to defective vesicular transport, since several studies have implicated Gcs1 in the vesicular-transport process (Poon et al., 1996; Wang, 1996; Poon et al., 1999, 2001). Interestingly, much like the suppression of the *ypt1-ts* phenotype by increased cation concentration, the addition of cations (such as Ca^{2+}) to the growth medium allows *gcs1Δ* cells to re-enter the cell cycle at the non-permissive temperature (Ireland, 1994). Furthermore, I have found that an increased concentration of cations in the growth medium alleviates the temperature sensitivity of *gcs1-28 glo3Δ* double-mutant cells. Since the effects of ArfGAP defects and *ypt1* defects are both alleviated either by increasing the abundance of Sly41 or by increasing the concentration of cations in the growth medium, these findings raise the possibility that ArfGAPs and Ypt1 are involved in a similar aspect of vesicular transport (see Section 6). Moreover, both the *ypt1* and ArfGAP phenotypes may be suppressed through a similar cation-mediated mechanism.

Increasing the concentration of cations in the growth medium also increases the osmolarity of the medium. Growth defects that are alleviated by an increase in osmolarity are referred to as osmo-remediated growth defects (Hampsey, 1997). Osmo-remediation of growth defects likely reflects problems with cell-wall structure in the mutant cells,

which lead to a weakening of the cell wall and thus a fragile cell. Under conditions where cells are stressed (such as elevated temperature), a growth defect may be observed due to the weakened cell wall of the mutant cells. Indeed, *gcs1-28 glo3Δ* double-mutant cells display a growth defect at 37°C that is alleviated by increasing the osmolarity of the growth medium by the addition of cations such as Ca^{2+} and Mg^{2+}. Furthermore, the addition of 1 M sorbitol to the growth medium was also found to suppress the temperature-sensitive phenotype of *gcs1-28 glo3Δ* double-mutant cells. These data indicate that alleviation of the *gcs1-28 glo3Δ* growth defect at 37°C is dependent only on the osmolarity of the growth medium, rather than the cation-specific mechanism that has been suggested for suppression of the *ypt1-ts* growth defect by the addition of cations to the growth medium. Surprisingly, the original study showing the rescue of *ypt1-ts* temperature sensitivity by increased cation concentration did not assess whether the addition of sorbitol to the growth medium has the same effect (Schmitt et al., 1988). Therefore, it is possible that suppression of the effects of the *ypt1-ts* mutation is also dependent only on the osmolarity of the growth medium.

Why is the *gcs1-28 glo3Δ* double-mutant situation osmo-remediable? *gcs1Δ* single-mutant cells, *glo3Δ* single-mutant cells, and *gcs1-28 glo3Δ* double-mutant cells appear to have a weakened cell wall (when compared to wild-type cells) because these mutant cells are sensitive to the presence of calcofluor white, a compound that binds to the cell wall and disrupts cell-wall structure (Hampsey, 1997). A recent report has implicated the Ssd1 protein in the integrity of cell-wall structure, and suggests that Ssd1 is required for an intact cell wall (Kaeberlein and Guarente, 2002). *SSD1* was originally

identified as a suppressor of a *sit4Δ* mutation, which eliminates a gene that encodes a serine/threonine phosphatase (Sutton et al., 1991). The *SSD1* locus is naturally polymorphic, and therefore various yeast genetic backgrounds have different versions of the *SSD1* gene. Cells that carry the *SSD1-v* allele (or wild-type *SSD1*) display wild-type Ssd1 function, whereas cells that carry an *ssd1-d* allele (of which there are various versions, either point-mutations or truncations) do not have wild-type Ssd1 function. That is, cells with the *SSD1-v* allele are viable in the presence of a *sit4Δ* mutation, whereas cells with an *ssd1-d* allele are not viable in the presence of a *sit4Δ* mutation (Sutton et al., 1991). I have observed a genetic interaction between the *gcs1-28 glo3Δ* double-mutant situation and *SSD1*: addition of wild-type *SSD1* to *gcs1-28 glo3Δ* double-mutant cells was found to suppress the temperature-sensitive phenotype of these mutant cells. Furthermore, addition of wild-type *SSD1* to *gcs1Δ* single-mutant cells suppresses calcofluor white sensitivity (data not shown). All of my experiments have been performed with the yeast strain W303, which is known to contain an *ssd1-d* allele (Jorgensen et al., 2002). Construction of a *gcs1-28 glo3Δ* double-mutant situation in the yeast strain S288C, which has a chromosomal copy of wild-type *SSD1*, failed to generate a temperature-sensitive yeast strain (data not shown). Therefore, a genetic interaction between the *gcs1-28 glo3Δ* double-mutant situation and an *ssd1-d* allele is necessary for the temperature-sensitive phenotype of *gcs1-28 glo3Δ* mutant cells.

Although the addition of wild-type *SSD1* to *gcs1-28 glo3Δ ssd1-d* mutant cells was found to suppress the temperature-sensitive phenotype, wild-type *SSD1* was not able to restore vesicular transport in *gcs1-28 glo3Δ ssd1-d* mutant cells. This finding indicates

that the Ssd1 protein is probably not directly involved in ArfGAP-mediated vesicular transport. More likely, the *ssd1-d* mutant allele causes a cell-wall defect that exacerbates the vesicular-transport defect caused by the *gcs1-28 glo3Δ* double-mutant situation, leading to the thermosensitive phenotype of *gcs1-28 glo3Δ* mutant cells. In other words, a weakened cell-wall structure caused by the *ssd1-d* allele unmasks the temperature-sensitive phenotype of *gcs1-28 glo3Δ* double-mutant cells. Therefore, any situation that alleviates the effects of the cell-wall defect caused by the *ssd1-d* allele (such as increasing the osmolarity of the growth medium or addition of wild-type *SSD1*) will allow *gcs1-28 glo3Δ* mutant cells to grow at 37°C.

Why does a defect in cell-wall integrity exacerbate the phenotype of a mutant situation that affects vesicular transport? Enzymes that are responsible for the generation of cell-wall components must be trafficked to the cell surface, where these proteins perform their functions. Since vesicular transport is required for the movement of protein cargo throughout the cell, it is not unreasonable to speculate that vesicular transport is required for the localization of cell-wall enzymes and components to the cell surface. This is indeed what has been found for Chs3, a protein that has chitin synthase activity at the cell surface (Shaw et al., 1991; Ziman et al., 1998). Chs3 is stored in an intracellular pool (in an endosome-like compartment), from which Chs3 is transported to the plasma membrane *via* vesicular transport (Ziman et al., 1998). In fact, the transport of Chs3 to the cell surface appears to be regulated (Valdivia and Schekman, 2003), so that under conditions of cell-wall stress Chs3 is redistributed from intracellular stores to the plasma membrane *via* vesicular transport. This vesicular-transport activity is regulated by the

signalling MAP kinases Rho1 and Pkc1 (Valdivia and Schekman, 2003). These findings clearly link functional vesicular transport to cell-wall integrity. Since the Gcs1 and Glo3 ArfGAPs mediate vesicular transport early in the secretory pathway, any perturbation of Gcs1 and/or Glo3 ArfGAP function will have consequences downstream, ultimately enfeebling the transport of particular cell-wall enzymes, such as Chs3, to the plasma membrane. The result of defective transport is increased sensitivity to a weakened cell-wall structure (such as that caused by an *ssd1-d* mutation) in *gcs1* and *glo3* mutant cells.

8. Glo3 is the primary ArfGAP that facilitates retrograde vesicular transport

Both the Gcs1 and Glo3 ArfGAPs were previously shown to mediate retrograde vesicular transport (Poon et al., 1999). However, recent reports have brought into question whether Gcs1 and Glo3 contribute equally to the retrograde-transport process (Dogic et al., 1999; Eugster et al., 2000). I have investigated the contribution of the Gcs1 and Glo3 ArfGAPs to retrograde vesicular transport by generating and using a mutant version of Glo3 (Glo3-R59K) that lacks measurable ArfGAP activity as determined by an *in vitro* assay. If both Gcs1 and Glo3 contribute equally to retrograde transport, or if Gcs1 provides the majority of ArfGAP function, a decrease in transport efficiency would not be expected when the mutant Glo3-R59K protein is expressed, since the Gcs1 ArfGAP would still be able to provide adequate retrograde-transport function in the presence of this mutant Glo3. However, if Glo3 provides the majority of ArfGAP function that is required for retrograde transport, then expression of Glo3-R59K would be expected to interfere with

transport because Gcs1 does not contribute sufficiently to the retrograde-transport process to overcome any defect imposed by Glo3-R59K. I have found the latter case be true, since mutant Glo3-R59K protein exerts a negative effect on retrograde vesicular transport, even in the presence of the Gcs1 ArfGAP.

The finding that Glo3-R59K exerts a negative effect on the ability of Gcs1 to mediate retrograde vesicular transport suggests that the Glo3 and Gcs1 proteins perform distinct roles in vesicular transport. Indeed, it is reasonable to speculate that Gcs1 is only capable of providing a minor contribution to the retrograde-transport process. Cells with only Gcs1 for retrograde transport (and lacking any Glo3 protein) do display some impairment for growth, whereas cells lacking Gcs1 protein (but with intact Glo3) are proficient for retrograde functions (Poon et al., 1999). Therefore, the Gcs1 ArfGAP may only be capable of compensating for Glo3 function in the complete absence of Glo3. This suggestion is supported by a study by Dogic et al. (1999), which has shown that *glo3Δ* mutant cells display a defect in cargo retrieval from the Golgi to the ER, whereas deletion of each of the other five genes encoding ArfGAP proteins (including Gcs1) does not result in any defect in Golgi-to-ER cargo retrieval. Moreover, I have found that increased abundance of Gcs1 is able to overcome the lethality imposed by the mutant Glo3-R59K protein, implying that normally the Gcs1 ArfGAP performs Glo3-related activities poorly. It is only when the Gcs1 protein is present in increased abundance that Gcs1 can compete effectively with the mutant Glo3-R59K protein for Glo3-specific interactions. These data indicate that the Glo3 protein is predominantly involved in retrograde

transport, whereas the Gcs1 ArfGAP may not normally function in this process or perhaps plays only a minor role.

What role might the Gcs1 ArfGAP play in the retrograde vesicular-transport process? Glo3 appears to be involved in the generation of COPI vesicles (see section 9), whereas Gcs1 may play a role downstream of vesicle generation. Preliminary data identifying genetic interactions between a *glo3Δ* mutant (where Gcs1 is the only ArfGAP that can facilitate retrograde transport) and mutation of the COG vesicle-tethering complex (see section 6) suggest that the Gcs1 ArfGAP may have a role to play in COG-related activities, such as vesicle tethering. It is reasonable to speculate that a compromise in the "normal" activity of Gcs1 for retrograde transport (perhaps vesicle tethering) may cause a severe defect when Gcs1 is required to fulfill Glo3 activities. That is, a *glo3Δ* mutation combined with an enfeebled Gcs1 pathway (caused by mutation of the COG complex) leads to a synthetic lethal phenotype.

I have shown that the Glo3 ArfGAP interacts with the Sec21 coatomer component, and that Glo3 is a component of COPI vesicles, whereas Gcs1 is not. Therefore, Glo3 appears to act in a pathway with the Sec21 component of the coatomer complex to mediate retrograde transport. Genetic interactions between *gcs1Δ* and *glo3Δ*, *gcs1Δ* and *sec21-1* (Poon et al., 1999), COG complex components (*cog5Δ*, *cog7Δ*, and *cog8Δ*) and *glo3Δ* (this study), and COG complex components (*cog2* and *cog3*) and a *sec21* mutation (Suvorova et al., 2002) suggest that the COG complex and the Gcs1 ArfGAP may act in a pathway separate from the Glo3 pathway. Therefore, it is possible that the Glo3 pathway mediates early events in vesicular transport and the Gcs1 pathway

mediates later events. Any perturbation of either the Gcs1 or Glo3 pathway can be overcome due to the overlapping function shared by Gcs1 and Glo3. However, perturbation of both pathways simultaneously, such as occurs in *gcs1Δ glo3Δ*, *gcs1Δ sec21-1*, *glo3Δ cog5Δ*, *glo3Δ cog7Δ*, and *glo3Δ cog8Δ* double-mutants cells, causes a block of retrograde transport that affects cell growth and viability. Additional experiments to determine the exact relationship between the Gcs1 ArfGAP and the COG complex may provide support for this theory.

9. Intact ArfGAP function is required for the generation of COPI vesicles

The classical model for COPI-mediated vesicular transport proposed by Tanigawa et al. (1993) suggests that hydrolysis of Arf-bound GTP causes the dissociation of Arf and coatomer from the vesicle membrane, and therefore that GTP hydrolysis may only be required for the uncoating of COPI vesicles (Tanigawa et al., 1993). Thus, ArfGAPs have been thought to function primarily to induce shedding of the vesicle coat by stimulating GTP hydrolysis by Arf. Nevertheless, several studies have implied that ArfGAP proteins may provide additional functions for vesicular transport. Mounting evidence indicates that ArfGAPs may be required for the proper selection and packaging of cargo proteins and SNAREs into nascent COPI vesicles (Nickel et al., 1998; Pepperkok et al., 2000; Goldberg, 2000; Lanoix et al., 2001; Rein et al., 2002). Indeed, ArfGAP proteins have been hypothesized to facilitate cargo packaging through the actions of a "discard pathway" in which vesicle generation is aborted by ArfGAP function if improper cargo is packaged (Goldberg, 2000).

Two recent reports have addressed a role for ArfGAP proteins in the actual generation of COPI vesicles (Yang et al., 2002; Reinhard et al., 2003). Yang et al. (2002) suggested that the mammalian ARFGAP1 is a constituent of COPI vesicles, and that GTP hydrolysis (and thus ArfGAP activity) is necessary for COPI-vesicle generation. Reinhard et al. (2003) found no such requirement for ArfGAP activity during the generation of COPI vesicles in an *in vitro* reconstitution system, but instead found that ArfGAP activity is necessary and sufficient for the uncoating of COPI vesicles. Therefore, there is conflicting evidence concerning a role for ArfGAP function in COPI-mediated vesicular transport.

I have investigated the requirement for ArfGAP function for COPI-mediated vesicular transport using a mutant version of Glo3 that lacks measurable ArfGAP activity. I found that the Glo3 protein is a component of the COPI vesicle coat, that a mutant Glo3 protein (Glo3-R59K) has a negative effect on retrograde vesicular transport, and that Glo3-R59K mutant protein interferes with the generation of COPI vesicles *in vitro*. Based on these observations, I propose that functional Glo3 protein is indeed required for the generation of COPI-coated vesicles. Springer et al. (1999) hypothesized that Arf-mediated GTP hydrolysis early in vesicle biogenesis releases the Arf GTPase for subsequent rounds of coatomer recruitment. This hypothesis is known as the "priming complex" model for COPI-mediated vesicular transport. This model states that Arf is released by GTP hydrolysis after coat and cargo recruitment so that Arf proteins are made available for subsequent rounds of coat recruitment during vesicle generation. In support of this hypothesis, recent experiments have shown that coatomer remains on membranes

long after Arf-bound GTP hydrolysis has taken place (Presley et al., 2002). These findings indicate that, *in vivo*, ArfGAP activity may be necessary to facilitate release of the Arf GTPase to initiate subsequent rounds of Arf-mediated coatomer recruitment, and therefore support a role for ArfGAP activity in COPI vesicle generation. The finding that a mutant Glo3-R59K protein without ArfGAP function prevents the generation of COPI-coated vesicles *in vitro* lends further weight to the "priming complex" model.

The *in vitro* vesicle-budding assay performed by my collaborator, Dr. Anne Spang, demonstrates that vesicles can form even in the presence of GTPγS, a non-hydrolysable analogue of GTP. This finding indicates that in the *in vitro* assay, necessary components for vesicle generation, including Arf-GTP, are in excess so that, unlike the *in vivo* situation, there is no need to provide ArfGAP activity to release Arf proteins through GTP hydrolysis. Nevertheless, I have found that the presence of an impaired Glo3 ArfGAP protein severely inhibits vesicle production *in vitro*. Thus, other functions of the Glo3 ArfGAP protein in addition to the stimulation of GTPase activity may be required to generate transport vesicles.

Unlike Glo3, the Gcs1 ArfGAP protein does not appear to be part of a COPI coat complex. This finding suggests that Gcs1 and Glo3 proteins normally play distinct roles for vesicular transport, and it is only in the absence of one of these ArfGAP proteins that the other member of this protein pair is able to provide the missing ArfGAP function to maintain viability and vesicular-transport activity. The inhibitory effect of the Glo3-R59K mutant protein for cell growth and retrograde transport likely reflects interactions between Glo3-R59K and components of the vesicular-transport machinery, which in turn

lead to the formation of inactive (dead-end) complexes that effectively exclude Gcs1 and prevent the provision of ArfGAP function by Gcs1. Increased abundance of the Gcs1 protein in the presence of mutant Glo3-R59K may allow Gcs1 to compete effectively for these common components of the vesicular-transport machinery.

10. Concluding remarks

In this study, I have found that increased abundance of the Age1 ArfGAP facilitates retrograde vesicular transport in cells with inadequate Gcs1 and Glo3 ArfGAP function. This finding provides evidence that Age1 can perform ArfGAP activities for vesicular transport *in vivo*. I have also identified the *SLY41* gene as a dosage suppressor of the effects of deficient Gcs1 and Glo3 ArfGAP activity for retrograde vesicular transport. Based on previous studies of Sly41, this finding suggests a link between the Ypt1 Rab and ArfGAP functions for retrograde transport. Identification of *SLY41* as a dosage suppressor has also led to the identification of a cell-wall defect caused by perturbed retrograde transport, and this cell-wall defect is exacerbated by the presence of the *ssd1-d* allele. Finally, I have evaluated the contribution of the Gcs1 and Glo3 ArfGAPs to retrograde transport and find that Glo3 is the primary ArfGAP required for retrograde transport function. Furthermore, in collaboration with Dr. Anne Spang I have shown that Glo3 is a component of COPI vesicles and that intact Glo3 function is required for vesicle generation.

These studies provide a basis for further investigation of the roles of the Gcs1 and Glo3 ArfGAPs in retrograde vesicular transport. Future experimentation will provide a

clearer picture of the genetic relationships and biochemical mechanisms identified in this study, and thus will add to our knowledge of ArfGAP function for vesicular transport.

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