Dysregulated Arl1, a regulator of post-Golgi vesicle tethering, can inhibit endosomal transport and cell proliferation in yeast

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ABSTRACT **Small monomeric G proteins regulated in part by GTPase-activating proteins (GAPs) are molecular switches for several aspects of vesicular transport. The yeast Gcs1 protein is a dual-specificity GAP for ADP-ribosylation factor (Arf) and Arf-like (Arl)1 G proteins, and also has GAP-independent activities. The absence of Gcs1 imposes cold sensitivity for growth and endosomal transport; here we present evidence that dysregulated Arl1 may cause these impairments. We show that gene deletions affecting the Arl1 or Ypt6 vesicle-tethering pathways prevent Arl1 activation and membrane localization, and restore growth and trafficking in the absence of Gcs1. A mutant version of Gcs1 deficient for both ArfGAP and Arl1GAP activity in vitro still allows growth and endosomal transport, suggesting that the function of Gcs1 that is required for these processes is independent of GAP activity. We propose that, in the absence of this GAP-independent regulation by Gcs1, the resulting dysregulated Arl1 prevents growth and impairs endosomal transport at low temperatures. In cells with dysregulated Arl1, an increased abundance of the Arl1 effector Imh1 restores growth and trafficking, and does so through Arl1 binding. Protein sequestration at the *trans*-Golgi membrane by dysregulated, active Arl1 may therefore be the mechanism of inhibition.**

Monitoring Editor
Francis A. Barr
University of Liverpool

Received: Sep 14, 2010
Revised: Mar 31, 2011
Accepted: May 4, 2011

INTRODUCTION

Molecular switches control many central processes in the cell. Among these molecular switches are the small monomeric G proteins. These proteins are GTPases: They are converted to an activated configuration by binding GTP and are then deactivated by hydrolysis of the bound GTP to GDP. To perform its functions, a G protein typically cycles between the activated, GTP-bound state and the deactivated, GDP-bound state. The activation of GDP-bound G proteins is a controlled process carried out by proteins termed guanine-nucleotide exchange factors (GEFs), which interact with GDP-bound G proteins to stimulate exchange of the bound GDP for GTP. The deactivation of G proteins by GTP hydrolysis is also a controlled process, mediated by GTPase-activating proteins (GAPs). In general, G proteins have little intrinsic GTPase activity, so the actions of GAPs are critical for proper G-protein function.

One of the intracellular processes regulated by G proteins is vesicular transport, a remarkably sophisticated process characteristic of eukaryotic cells that ensures that cellular constituents, including proteins and lipids, are properly localized through transport among cellular compartments such as the endoplasmic reticulum, Golgi apparatus, endosomes, lysosomes/vacuoles, and the plasma membrane. This transport relies on transport vesicles that are formed from one membrane compartment and fuse with another to release associated protein and lipid "cargo" molecules. Regulation is exerted on several aspects of vesicular transport, including transport-vesicle

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E10-09-0765) on May 11, 2011.

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Author contributions: J.J.R.B., P.P.P., R.A.S., and G.C.J. conceived and designed the experiments. J.J.R.B., P.P.P., J.D.D., and X.W. performed the experiments. J.J.R.B., P.P.P., J.D.D., X.W., R.A.S., and G.C.J. analyzed the data. J.J.R.B., R.A.S., and G.C.J. drafted the article. J.J.R.B. prepared the digital images.

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Abbreviations used: Arf, ADP-ribosylation factor; Arl, Arf-like; BSA, bovine serum albumin; FM 4–64, *N*-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl) hexatrienyl) pyridinium dibromide; GAP, GTPase-activating protein; GARP, Golgi-associated retrograde protein; GEF, guanine-nucleotide exchange factor; GFP, green fluorescent protein; GRIP, golgin-97, RanBP2α, Imh1p, and p230/golgin-245; NatC, N-terminal acetyltransferase; ORF, open reading frame; SNARE, SNAP (Soluble NSF Attachment Protein) REceptor.

Supplemental Material can be found at:
http://www.molbiolcell.org/content/suppl/2011/05/09/mbc.E10-09-0765.DC1.html

production, cargo packaging within newly formed vesicles, and docking of the vesicle with the appropriate target membrane (Derby and Gleeson, 2007; Spang, 2008; Beck *et al.*, 2009). Small monomeric G proteins have been implicated in the regulation of each of these aspects of the vesicular-transport process.

Most features of vesicular transport are conserved from humans to yeast (Bonifacino and Glick, 2004); the budding yeast *Saccharomyces cerevisiae* has been a widely used experimental system in the characterization of this conserved process. One of the GAPs involved in the regulation of vesicular transport in yeast is a protein named Gcs1. We showed that Gcs1 is a GAP for the Arf (ADP-ribosylation factor) type of G protein belonging to the Ras superfamily of monomeric G proteins, and is a functional ortholog of the rat protein ArfGAP1 (Poon *et al.*, 1996, 1999). The GTPase cycle of Arf is important for formation of transport vesicles (Lewis *et al.*, 2004) and for release of associated coat proteins in preparation for vesicle fusion with a target membrane (Antonny *et al.*, 1997). By regulating Arf G proteins through its ArfGAP activity, the Gcs1 protein affects various vesicular-transport stages, including post-Golgi transport (reviewed in Donaldson and Klausner, 1994; Boman and Kahn, 1995; Lemmon and Traub, 2000). In addition, we and others have shown that Gcs1 works in vitro to "prime" various SNARE (SNAP [Soluble NSF Attachment Protein] REceptor) proteins allowing interactions with Arf1 and coatomer on the membrane (Rein *et al.*, 2002; Robinson *et al.*, 2006). These interactions form a priming complex that is required for vesicle biogenesis (Springer *et al.*, 1999). This priming activity of Gcs1 is independent of its GAP activity.

Others have shown that Gcs1 can also act as a GAP for the Arl1 (Arf-like) G protein (Liu *et al.*, 2005). Arl1 is a member of the Arf subfamily of small G proteins; yeast Arl1 is 55% identical to yeast Arf1 and 52% identical to yeast Arf2 (Lee *et al.*, 1997). Like the Arf G proteins, Arl1 also functions in vesicle transport, specifically in the docking of transport vesicles at target membranes, a process facilitated by vesicle-tethering factors that are effectors of Arl1. Vesicle tethering is an important late step in vesicular transport to ensure proper cargo delivery. There are two general classes of vesicle-tethering factors: long coiled-coil proteins, and multi-subunit tethering complexes. Vesicle-tethering factors are found on the surfaces of target membranes and/or transport vesicles to allow correct target compartment recognition for vesicle docking and fusion (Whyte and Munro, 2002; Bonifacino and Glick, 2004; Cai *et al.*, 2007; Sztul and Lupashin, 2009). Thus Gcs1 is a dual-specificity GAP regulating two small G proteins, Arf and Arl1, that are involved in vesicular transport; Arf is involved in early steps of the vesicular-transport process such as vesicle biogenesis, whereas Arl1 is involved in later steps such as tethering, which leads to vesicle docking and fusion.

A common feature of most tethering factors is direct interaction with small Ras-related G proteins, specifically those of the Arl and Rab (Ypt in yeast) families (Sztul and Lupashin, 2009). Therefore, the pathways that regulate the activation and membrane localization of these small G proteins are implicated in vesicle-tethering events. One such pathway is the "Arl1 pathway" (Munro, 2005), which involves multiple proteins that function to recruit and activate Arl1 on the *trans*-Golgi membrane. In this pathway, N-terminal acetylation of the Arl-family G protein Arl3, by the N-terminal acetyltransferase complex (NatC) composed of Mak3, Mak10, and Mak31, allows Arl3 recruitment to the *trans*-Golgi membrane through interaction with the membrane-spanning Sys1 protein (Behnia *et al.*, 2004; Setty *et al.*, 2004). Activation of Arl3 to the GTP-bound form then allows activation and relocalization of the Arl1 G protein from the cytoplasm to the *trans*-Golgi membrane (Panic *et al.*, 2003b; Setty *et al.*, 2003). On activation, Arl1 recruits vesicle-tethering factors to the *trans*-Golgi membrane, including the long coiled-coil Imh1 protein and the multi-subunit GARP (Golgi-associated retrograde protein) tethering complex consisting of four subunits (Vps51, Vps52, Vps53, and Vps54; Panic *et al.*, 2003b; Setty *et al.*, 2003). Another small G protein involved in vesicle tethering at the *trans*-Golgi membrane is Ypt6, the yeast homologue of mammalian Rab6. The Ypt6 pathway involves the activation of Ypt6 by its heterodimeric GEF composed of Ric1 and Rgp1 (Siniossoglou *et al.*, 2000). Activated Ypt6 then recruits the GARP tethering complex. Thus the Arl1 and Ypt6 pathways regulate vesicle-tethering events at the *trans*-Golgi membrane. Genetic interactions between components of the Arl1 and Ypt6 pathways are consistent with them being parallel pathways with overlapping functions, and some level of cross-talk between the two pathways has been suggested by the fact that Arl1 and Ypt6 share the ability to bind GARP (Panic *et al.*, 2003b).

The Gcs1 protein, despite its dual-specificity GAP activities for both Arf and Arl1 proteins, is dispensable for life: Yeast cells lacking Gcs1 due to deletion of its structural gene are able to grow and form colonies. These *gcs1*Δ mutant cells, however, are cold sensitive, unable to begin cell proliferation and colony formation at the low growth temperature of 14°C (Drebot *et al.*, 1987; Ireland *et al.*, 1994). This cold sensitivity for growth in the absence of Gcs1 protein is accompanied by defective endosomal transport (Wang *et al.*, 1996). The underlying cause of the cold sensitivity and transport defects in *gcs1*Δ cells is unknown. We have used genetic procedures to gain a better appreciation of the mechanism responsible for this cold sensitivity and for the endosomal transport defect. Genetic screens for situations that alleviate the cold sensitivity of *gcs1*Δ cells identified multiple genes involved in vesicle-tethering pathways at the *trans*-Golgi membrane. We then used genetic, cell biological, and biochemical experiments to elucidate the roles that these vesicle-tethering pathways play in imposing the defects that result from the absence of Gcs1. Our data suggest that a Gcs1 activity independent of its GAP activity regulates active Arl1, allowing growth and transport processes to function properly in the cold. We propose that dysregulated active Arl1 resulting from the absence of this GAP-independent Gcs1 function is responsible for the cold sensitivity and transport defects of *gcs1*Δ cells.

RESULTS

Elimination of the Arl1 vesicle-tethering pathway at the *trans*-Golgi membrane alleviates cold sensitivity caused by the absence of Gcs1

Inadequate Gcs1 function impairs cell proliferation at low growth temperatures (Drebot *et al.*, 1987; Ireland *et al.*, 1994). To identify proteins that impose this impairment, we assessed whether *gcs1*Δ cold sensitivity can be alleviated by the deletion of protein-coding genes. A query strain lacking the *GCS1* gene was crossed to each member of the collection of yeast deletion strains (~4700 different gene deletions) (Giaever *et al.*, 2002), and double-mutant haploid derivatives were generated, each deleted for the *GCS1* gene plus one of these yeast genes (Tong *et al.*, 2001). Deletion mutations that alleviate the *gcs1*Δ cold sensitivity were identified by the ability of double-mutant cells to form colonies at 14°C. These procedures identified 92 genes that, when deleted, allowed *gcs1*Δ mutant cells to form colonies at low growth temperatures (Supplemental Table 1). Strikingly, 5 of these 92 genes encode members of the Arl1 pathway, a well-conserved, vesicular-transport pathway involved in transport-vesicle tethering at the *trans*-Golgi membrane (Munro, 2005). Tetrad analysis

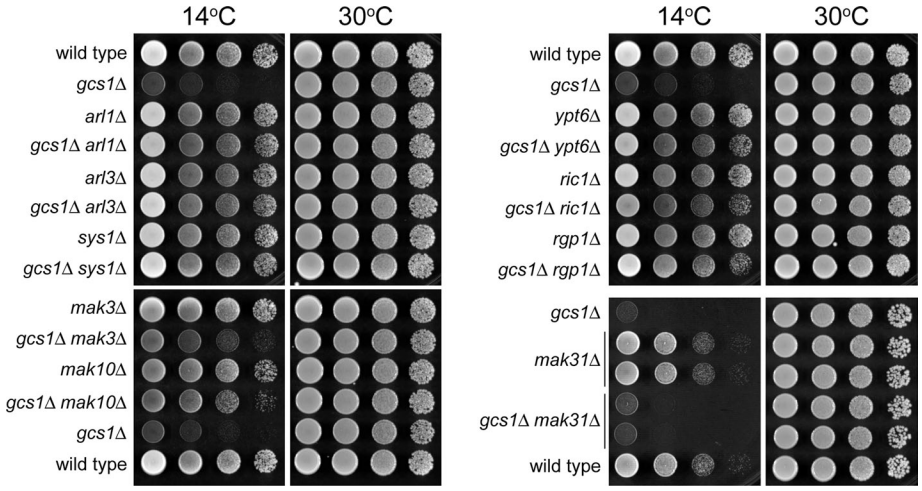

FIGURE 1: Deletion of genes in the Arl1 and Ypt6 pathways alleviates *gcs1*Δ cold sensitivity. Isogenic strains deleted for the indicated genes were grown to stationary phase; 10-fold serial dilutions were spotted onto solid enriched medium and incubated at 14°C and 30°C.

confirmed that these five gene deletions (*arl1*Δ, *arl3*Δ, *sys1*Δ, *mak3*Δ, and *mak10*Δ) alleviate *gcs1*Δ cold sensitivity; shown in Figure 1 is the growth of representative double-mutant segregants in a serial dilution assay.

To confirm that the *arl1*Δ deletion mutation alleviates *gcs1*Δ cold sensitivity, the deletion was reconstructed in the widely used W303 strain background. Confirming the findings of our initial screen, the *arl1*Δ deletion mutation also alleviated *gcs1*Δ cold sensitivity in this context (e.g., see Figure 6 later in the paper). Thus impairment of the Arl1 pathway relieves the inhibition of cell proliferation seen in the absence of the Gcs1 protein. The Arl1 pathway functions to recruit Arl1 to the *trans*-Golgi membrane, where it becomes activated and membrane bound. Deletion of any component of the Arl1 pathway blocks Arl1 activation and membrane localization (Panic *et al.*, 2003b; Setty *et al.*, 2003, 2004; Behnia *et al.*, 2004). These results suggest that, in the absence of Gcs1, active Arl1 inhibits growth in the cold.

The NatC subunit Mak31 is not involved in the inhibition imposed by the Arl1 pathway

N-terminal acetylation of the Arl3 protein by the NatC complex is required for Golgi targeting of Arl3, which is essential for proper Arl1 pathway function (Behnia *et al.*, 2004; Setty *et al.*, 2004). The NatC complex is composed of a catalytic subunit, Mak3, and two auxiliary or regulatory subunits, Mak10 and Mak31 (Polevoda and Sherman, 2001). All three subunits of NatC are required for the N-terminal acetylation of several NatC substrates tested in vitro (Polevoda and Sherman, 2001); however, Mak31 is not required for the N-terminal acetylation of Arl3 (Setty *et al.*, 2004). Our screen for deletion mutations that alleviate *gcs1*Δ cold sensitivity identified multiple members of the Arl1 pathway, including Arl3 and the NatC subunits Mak3 and Mak10, but did not identify the third subunit, Mak31. We directly tested whether deletion of the *MAK31* gene alleviates *gcs1*Δ cold sensitivity. Consistent with Mak31 being dispensable for the acetylation of Arl3, we found that deletion of *MAK31* did not alleviate *gcs1*Δ cold sensitivity: *gcs1*Δ *mak31*Δ double-mutant cells remained cold sensitive (Figure 1). This observation is in agreement with results showing that Arl3 is properly localized to the Golgi in *mak31*Δ cells but not in *mak3*Δ or *mak10*Δ cells (Setty *et al.*, 2004), and that the Arl1 effector Imh1 is still normally targeted to the Golgi in *mak31*Δ cells while being completely mislocalized in *mak3*Δ and *mak10*Δ cells (Behnia *et al.*, 2004).

Elimination of Arl1 alleviates an endocytic transport defect caused by the absence of Gcs1

Endocytosis is impaired in *gcs1*Δ cells attempting to resume cell proliferation from stationary phase in the cold, and conditions identified previously that alleviate the cold-sensitive growth defect of *gcs1*Δ cells also relieve this endocytosis defect (Wang *et al.*, 1996). To assess whether the absence of Arl1 relieves the endocytosis impairment caused by the *gcs1*Δ mutation, we used the lipophilic dye FM 4–64 (*N*-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl) pyridinium dibromide) to monitor endocytic transport. After FM 4–64 staining of the plasma membrane, cells were incubated in fresh medium, and transport of the dye was monitored over time by fluorescence microscopy. If endocytic transport is functional, membrane-bound FM 4–64 is internalized from the plasma membrane, transported through intermediate endocytic compartments, and accumulated at the vacuolar membrane (Vida and Emr, 1995). Wild-type cells resuming cell proliferation from stationary phase at 14°C transported FM 4–64 to the vacuolar membrane resulting in a characteristic ring-staining pattern (Figure 2). As expected, the use of both stationary-phase cells and a low incubation temperature led to kinetics of dye transport that were considerably

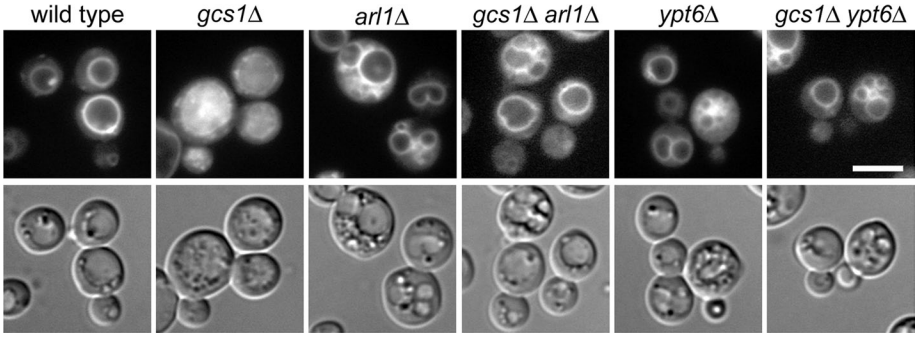

FIGURE 2: Deletion of the *ARL1* or *YPT6* gene restores effective endocytic transport in *gcs1*Δ cells. Stationary-phase cells deleted for the indicated genes were loaded with the lipophilic dye FM 4–64 on ice before being diluted in fresh enriched medium and incubated at 14°C. Membrane transport was assessed by fluorescence microscopy after 0, 5, 10, and 24 h. The FM 4–64 stain began to accumulate at the vacuole in all strains except *gcs1*Δ by 10 h, and clear vacuolar ring staining was apparent in the majority of cells by 24 h. In the *gcs1*Δ control strain, the FM 4–64 stain remained trapped in endocytic compartments within the cytoplasm and had not been transported to the vacuolar membrane by 24 h. Data from the 24-h time point are shown. Dye-bound membranes were visualized by fluorescence microscopy (top), and cells were visualized by differential interference contrast microscopy (bottom). Under these assay conditions, all strains remained greater than 95% viable for up to 96 h; cell viability was assessed by methylene blue dye exclusion. Scale bar, 5 μm.

slower than those of actively proliferating cells at 30°C (Vida and Emr, 1995; Wang *et al.*, 1996). In contrast to wild-type cells, *gcs1*Δ cells treated in the same way, although capable of internalizing the dye from the plasma membrane, were unable to deliver dye to the vacuole, and the dye remained trapped in endocytic compartments (Wang *et al.*, 1996; Figure 2). Deletion of *ARL1*, which alleviated the cold sensitivity of *gcs1*Δ cells, also relieved the endocytic transport defect in *gcs1*Δ cells: FM 4–64 was efficiently transported to the vacuole in *gcs1*Δ *arl1*Δ double-mutant cells (Figure 2). As previously reported (Bonangelino *et al.*, 2002), the *arl1*Δ mutation resulted in moderately fragmented vacuoles. The additional deletion of the *GCS1* gene from *arl1*Δ mutant cells did not appear to affect this fragmentation. In any case, deletion of the *ARL1* gene, which alleviates *gcs1*Δ cold sensitivity, also restores effective endosomal transport. This result suggests that, in addition to inhibiting growth, active Arl1 also inhibits endosomal transport in the cold when Gcs1 is absent.

Gcs1 GAP activity is dispensable for low-temperature growth and effective endocytosis

The Gcs1 protein has been well characterized as a GAP for the small G proteins Arf1 and Arf2 (Poon *et al.*, 1996), and also has GAP activity for the related GTPase Arl1 (Liu *et al.*, 2005). This observation, coupled with the findings that active Arl1 may have deleterious consequences in the absence of Gcs1, raised the possibility that the Arl1GAP activity of Gcs1 is critical for cell proliferation and endosomal transport at 14°C.

To assess the involvement of the Arl1GAP activity of Gcs1 in the Gcs1-dependent processes at issue here, we wished to test a mutant version of Gcs1 lacking Arl1GAP activity. ArfGAP-deficient mutant versions of Gcs1 exist in which Arg-54 is substituted with alanine (R54A), lysine (R54K), or glutamine (R54Q). These changes dramatically impair the in vitro ArfGAP activity of each mutant protein (Yanagisawa *et al.*, 2002). To assess whether these substitutions also impair the Arl1GAP activity of Gcs1, we used an in vitro assay to test the most conservative of the Gcs1-R54 substitutions (R54K) for Arl1GAP activity. The stimulation of GTP hydrolysis on GTP-bound Arl1, and Arf1, was measured in the presence of Gcs1-R54K over a range of protein concentrations; wild-type Gcs1 protein and bovine serum albumin (BSA) protein were used as positive and negative controls, respectively (Figure 3A). Increasing amounts of BSA had no stimulatory effect on Arl1- or Arf1-bound GTP hydrolysis, indicating that any stimulation of GTP hydrolysis in the presence of wild-type or mutant Gcs1 is specific. Increasing amounts of wild-type Gcs1 protein resulted in a dose-dependent increase in Arl1- and Arf1-bound GTP hydrolysis, consistent with Gcs1 acting as a GAP for these two GTPases (Poon *et al.*, 1996; Liu *et al.*, 2005). The mutant Gcs1-R54K protein, however, displayed only weak GAP activity in vitro not only against Arf1, but also against Arl1 (Figure 3A), indicating that the R54K substitution also disrupts the Arl1GAP activity of Gcs1. It is likely that the R54A and R54Q substitutions have the same effect.

To assess whether the Arl1GAP (and ArfGAP) activity of Gcs1 is required for growth in the cold, we tested the ability of the GAP-deficient R54 mutant versions of Gcs1 to provide function for growth at 14°C. Each of these GAP-deficient Gcs1 mutant proteins, expressed from genes present at only one or two copies per cell, relieved the cold sensitivity of *gcs1*Δ cells (Figure 3B), indicating that these mutant forms of Gcs1 provide function for low-temperature growth. We also assessed the requirement of Gcs1 GAP activity for effective endocytosis. Fluorescence microscopy revealed that *gcs1*Δ cells expressing GAP-deficient Gcs1-R54K were able to efficiently transport the lipophilic dye FM 4–64 to the vacuole, whereas in

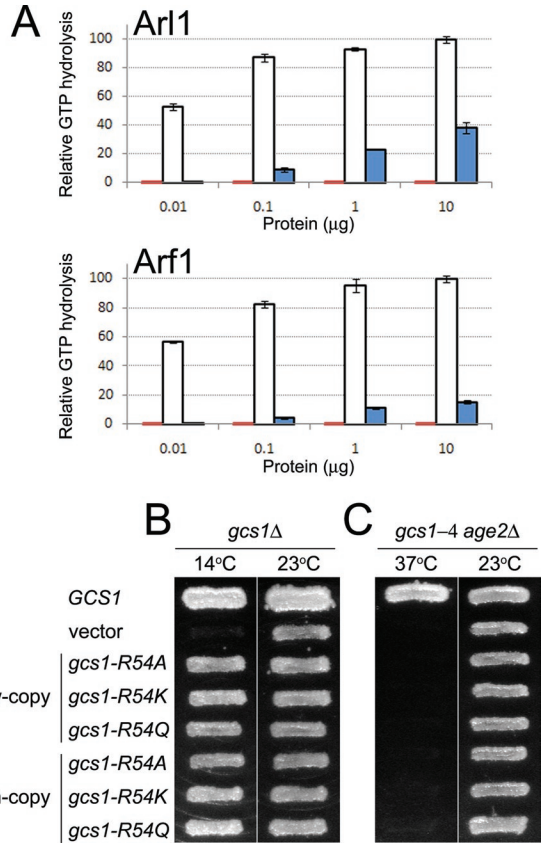

FIGURE 3: GAP-deficient Gcs1-R54K provides a GAP-independent Gcs1 activity that alleviates *gcs1*Δ cold sensitivity. (A) Activation of GTP hydrolysis by GTP-loaded myristoylated Arl1 (top) and myristoylated Arf1 (bottom) was assessed in the presence of BSA (red bar), wild-type Gcs1 (white bar), and mutant Gcs1-R54K (blue bar), over a range of protein concentrations. The assays were carried out in triplicate (1 SD is displayed), and the data presented are from one of two repeated experiments. (B) Cold-sensitive *gcs1*Δ cells harboring plasmids expressing the indicated genes were grown to stationary phase on solid selective medium before being replica-plated to solid enriched medium and incubated at 14°C and 23°C. Four individual transformants were assessed for each plasmid and in each case showed consistent behavior. (C) Temperature-sensitive *gcs1–4 age2*Δ cells harboring the same plasmids as in (B) were grown on solid selective medium and then replica-plated to solid enriched medium for further incubation at 37°C and 23°C. Four individual transformants were assessed for each plasmid and in each case showed consistent behavior.

*gcs1*Δ control cells carrying an empty vector, the dye remained trapped in endocytic compartments (Figure 4). Thus, like *gcs1*Δ cold sensitivity, defective endocytic transport in *gcs1*Δ mutant cells is remedied by a GAP-deficient version of Gcs1.

In light of the low levels of GAP activity measured in vitro, we wanted to see if the Gcs1-R54 mutants provide Gcs1 GAP activity in vivo. We therefore determined whether expression of each R54 mutant protein could provide Gcs1 ArfGAP activity known to be required in the absence of another ArfGAP, Age2 (Poon *et al.*, 2001). The GAP-deficient Gcs1 mutants failed to provide ArfGAP activity in two temperature-sensitive mutational situations, *gcs1–4 age2*Δ (Wong *et al.*, 2005) and *gcs1–3 age2*Δ (Poon *et al.*, 2001), even when overexpressed from high-copy plasmids (Figure 3C and unpublished data). Thus the R54A, R54K, and R54Q mutant forms of Gcs1 fail to provide Gcs1 ArfGAP activity in vivo but do provide the

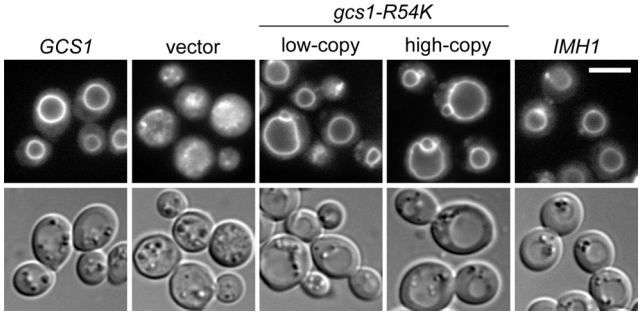

FIGURE 4: Defective endocytic transport in *gcs1*Δ cells is restored by ArfGAP-deficient Gcs1-R54K and by increased abundance of Imh1. Stationary-phase *gcs1*Δ cells harboring plasmids expressing the indicated genes were loaded with the lipophilic dye FM 4–64 on ice before being diluted in fresh enriched medium and incubated at 14°C. Membrane transport was assessed by fluorescence microscopy after 0, 5, 13, 23, 29, 36, 46, and 53 h. Cells expressing plasmid-borne *GCS1* displayed clear vacuolar ring staining by 23 h. The FM 4–64 stain began to accumulate at the vacuolar membrane in cells expressing plasmid borne *gcs1-R54K* and *IMH1* by 29 h, and clear vacuolar ring staining was apparent in these strains by 46 h. In the *gcs1*Δ vector control strain, the FM 4–64 stain remained trapped in endocytic compartments within the cytoplasm and had not been transported to the vacuolar membrane by 53 h. Data from the 53-h time point are shown. Dye-bound membranes were visualized by fluorescence microscopy (top), and cells were visualized by differential interference contrast microscopy (bottom). Under these assay conditions, all strains remained greater than 95% viable for up to 96 h; cell viability was assessed by methylene blue dye exclusion. Scale bar, 5 μm.

Gcs1 activity needed to alleviate *gcs1*Δ growth and endocytic transport defects in the cold.

These data provide little support for the notion that the Arl1-related activity of Gcs1 that is critical for growth and endocytic transport in the cold is its Arl1GAP (or ArfGAP) activity, and suggest that the GAP activity of Gcs1 is dispensable for these processes. We therefore propose that another Arl1-related activity of Gcs1, independent of GAP activity, is important for preventing the deleterious effects of activated Arl1.

Elimination of the Ypt6 vesicle-tethering pathway at the *trans*-Golgi membrane also alleviates the cold sensitivity and endocytic transport defect caused by the absence of Gcs1

Inspection of the list of gene deletions that alleviated the cold sensitivity of *gcs1*Δ cells (Supplemental Table 1) indicated that, as described earlier in this article for the Arl1 pathway, the inactivation of another vesicle-tethering pathway at the *trans*-Golgi membrane also has beneficial effects for *gcs1*Δ cells. Three of the gene deletions (*ypt6*Δ, *ric1*Δ, and *rgp1*Δ) eliminate members of the Ypt6 pathway (Siniossoglou *et al.*, 2000). Tetrad analysis confirmed that each of these gene deletions alleviates *gcs1*Δ cold sensitivity; shown in Figure 1 is the growth of representative double-mutant segregants in a serial dilution assay. The *ypt6*Δ deletion also had this effect in the W303 genetic background (unpublished data), confirming the generality of this effect. Like *arl1*Δ, the *ypt6*Δ deletion also relieved the endocytic transport defect caused by the *gcs1*Δ mutation (Figure 2). As previously reported (Tsukada *et al.*, 1999), the *ypt6*Δ mutation resulted in moderately fragmented vacuoles. The additional deletion of the *GCS1* gene from *ypt6*Δ mutant cells did not appear to affect this fragmentation. The Arl1 pathway and the Ypt6 pathway operate similarly, by the activation and recruitment, through GTP binding, of small G proteins: in one case Arl1, and in the other, Ypt6. In this GTP-bound state, each of these G proteins is membrane bound and active. Therefore, like active Arl1, active Ypt6 is also implicated in the growth and transport defects exhibited by *gcs1*Δ cells.

The Ypt6 pathway is required for normal localization of the Arl1 effector Imh1

As an effector of activated Arl1, the Imh1 protein is thought to be involved in tethering of transport vesicles at the *trans*-Golgi membrane, facilitating target-membrane recognition and vesicle fusion. Proper functioning of the Arl1 pathway results in recruitment of Imh1 to the *trans*-Golgi membrane through direct interaction of Imh1 with activated Arl1 (Munro and Nichols, 1999; Panic *et al.*, 2003a; Munro, 2005). Deletion of any of the known members of the Arl1 pathway (Arl1, Arl3, Sys1, Mak3, Mak10) blocks the activation of Arl1, and results in the mislocalization of Imh1. Expression in wild-type cells of a version of Imh1 fused to green fluorescent protein (GFP-Imh1) reveals punctate structures characteristic of proper Golgi localization, but only diffuse cytoplasmic staining when the Arl1 pathway and Arl1 activation are defective (Panic *et al.*, 2003b; Setty et al., 2003, 2004; Behnia *et al.*, 2004).

We used localization of a GFP-Imh1 fusion protein as a readout to indicate whether any of the other gene deletions that alleviated *gcs1*Δ cold sensitivity prevent the activation of Arl1. A GFP-Imh1 fusion protein was expressed in the 92 deletion strains identified here (Supplemental Table 1), and in a blinded experiment the staining pattern for GFP-Imh1 was assessed for each. GFP-Imh1 was mislocalized and exhibited a diffuse cytoplasmic pattern in several of these deletion strains. As expected, cells of the five strains with Arl1-pathway deletions (*arl1*Δ, *arl3*Δ, *sys1*Δ, *mak3*Δ, and *mak10*Δ) were found to mislocalize GFP-Imh1. Surprisingly, cells of the three strains with Ypt6-pathway deletions (*ypt6*Δ, *ric1*Δ, and *rgp1*Δ) also mislocalized GFP-Imh1 (Figure 5, top row). Three other deletions that caused mislocalization of GFP-Imh1 were *ypr050c*Δ, *ylr261c*Δ, and *ydr136c*Δ; each of these deletions eliminates a dubious open reading frame (ORF) that overlaps a gene in the Arl1 or Ypt6 pathway (*MAK3, YPT6,* and *RGP1,* respectively). The most likely interpretation is that each of these deletions impairs the bona fide gene with which it overlaps.

These results indicate that our blinded analysis of the alleviating deletion strains was robust in the ability to identify known components required for the proper localization of GFP-Imh1 and revealed that the Ypt6 pathway is also required for the proper localization of GFP-Imh1. This finding was unexpected; the Ypt6 and Arl1 pathways have been considered to be parallel pathways (Graham, 2004), although with some "cross-talk" between them (Panic *et al.*, 2003b). The fact that deletion of the genes encoding the GEF for Ypt6 (Ric1–Rgp1) affects GFP-Imh1 localization in the same way as deletion of *YPT6* itself leads us to conclude that it is the activated, GTP-bound form of Ypt6 that is required for proper GFP-Imh1 localization.

The Ypt6 pathway mediates the normal localization of proteins in the Arl1 pathway upstream of Imh1

Each protein in the Arl1 pathway is needed for the recruitment of activated Arl1 to the *trans*-Golgi membrane; activated Arl1 then recruits Imh1. Inactivation of any protein in the Arl1 pathway results in the mislocalization of both Arl1-GFP and GFP-Imh1 fusion proteins, so that the normal punctate staining patterns of these proteins become diffuse and cytoplasmic (Panic *et al.*, 2003b; Setty *et al.*, 2003, 2004; Behnia *et al.*, 2004). The simplest explanation for the mislocalization of GFP-Imh1 in the absence of a functional Ypt6 pathway,

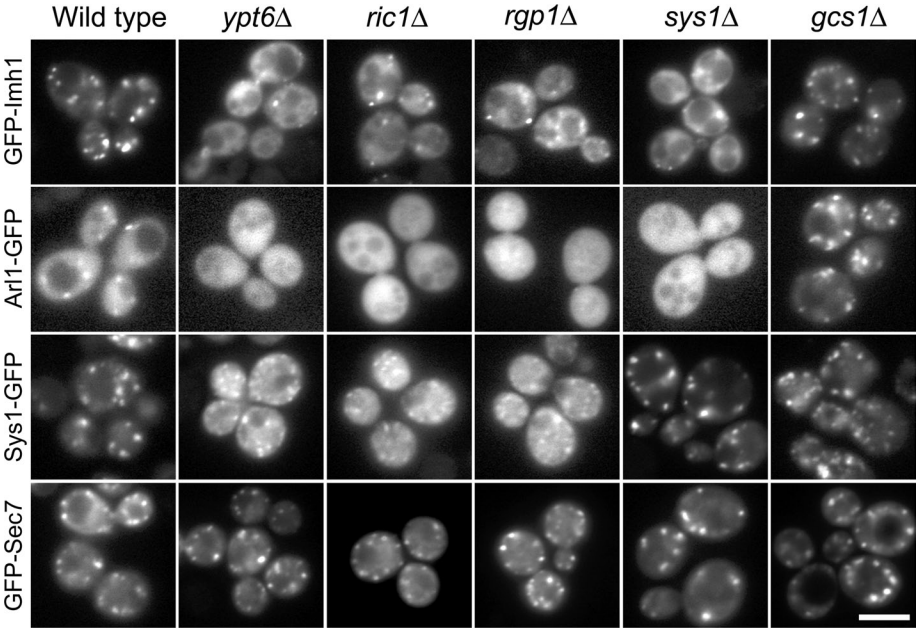

FIGURE 5: Golgi localization of proteins in the Arl1 pathway is disrupted by the deletion of genes in the Ypt6 pathway. Fluorescence micrographs of live yeast cells with the indicated gene deletions and harboring plasmids expressing GFP-Imh1, Arl1-GFP, Sys1-GFP, or GFP-Sec7 are displayed. Scale bar, 5 μm.

as shown earlier in the text, is that activated Ypt6 is needed for the recruitment of activated Arl1 to the *trans*-Golgi membrane. To address this possibility, we assessed the localization of Arl1-GFP and Sys1-GFP fusion proteins in cells deleted for genes of the Ypt6 pathway. Similar to what was seen for the mislocalization of GFP-Imh1, the punctate staining pattern of Arl1-GFP and Sys1-GFP was lost when components of the Ypt6 pathway were absent (Figure 5). In these cells the distribution of Arl1-GFP was diffuse and cytoplasmic, leading to homogeneous staining of the cytoplasm. Unlike Arl1-GFP, Sys1-GFP was not homogeneously distributed within the cytoplasm, but rather appeared somewhat granular, with many small dots throughout the cytoplasm. Because Sys1 is an integral membrane protein predicted to have four *trans*-membrane segments (Tsukada and Gallwitz, 1996), the Sys1-GFP fusion protein most likely remains associated with some membranous structures, resulting in the small dots and granular staining pattern. The mislocalization of GFP-Imh1, Arl1-GFP, and Sys1-GFP is apparently due to defects in targeting these proteins rather than a general disruption of the Golgi apparatus, because the Golgi marker GFP-Sec7 retained its normal punctate distribution in *ypt6*Δ, *ric1*Δ, and *rgp1*Δ mutant cells (Figure 5).

These data show that the Ypt6 pathway is required for the normal localization of proteins in the Arl1 pathway. The similar effects seen by deleting genes for the Ypt6 GEF (Ric1–Rgp1) and for Ypt6 itself indicate that the GTP-bound, activated form of Ypt6 mediates the Golgi localization of Sys1. The loss of Golgi-localized Sys1 in the absence of activated Ypt6 results in the mislocalization of proteins downstream of Sys1 in the Arl1 pathway, including Arl1 and Imh1.

Cytoplasmic Imh1 does not itself alleviate *gcs1*Δ cold sensitivity

The finding that the Ypt6 pathway is required for effective Arl1-pathway function reveals two features shared by the three alleviating deletion mutations affecting the Ypt6 pathway and the five alleviating deletion mutations affecting the Arl1 pathway: All result in the depletion of activated Arl1 at the *trans*-Golgi membrane and, as a result, all cause an abnormal cytoplasmic localization of Imh1. We therefore considered the possibility that increased levels of cytoplasmic Imh1 might be the common condition that allows Ypt6-pathway and Arl1-pathway impairment to alleviate *gcs1*Δ cold sensitivity.

If alleviation of *gcs1*Δ cold sensitivity by the *arl1*Δ mutation does indeed function through increased abundance of cytoplasmic Imh1, then deletion of the *IMH1* gene should abolish the beneficial effects of the *arl1*Δ deletion. To determine whether Imh1 is required for *arl1*Δ relief of *gcs1*Δ cold sensitivity, we compared the 14°C growth of *gcs1*Δ *arl1*Δ double-mutant segregants with that of *gcs1*Δ *arl1*Δ *imh1*Δ triple-mutant segregants. Deletion of the *IMH1* gene did not affect the ability of the *arl1*Δ mutation to alleviate *gcs1*Δ cold sensitivity: Triple-mutant (*gcs1*Δ *arl1*Δ *imh1*Δ) segregants grew as well as double-mutant (*gcs1*Δ *arl1*Δ) segregants (Figure 6). Imh1 is not required for the effects of the *arl1*Δ mutation; therefore, cytoplasmic Imh1 is not the feature of the *arl1*Δ cells that produces these effects. We are precluded from conducting a similar analysis to assess if Imh1 is required for *ypt6*Δ alleviation of *gcs1*Δ cold sensitivity because combining the *ypt6*Δ and *imh1*Δ deletions causes lethality (Tsukada *et al.*, 1999; Setty *et al.*, 2003; Tong *et al.*, 2004; our unpublished results).

Increased abundance of Imh1 alleviates *gcs1*Δ cold sensitivity and endocytic transport defects

In addition to the screen described earlier in the text for gene deletions that alleviate *gcs1*Δ cold sensitivity, we also undertook a complementary approach to identify genes that when overexpressed have the same effect. Mutant cells lacking the *GCS1* gene were transformed with a high-copy yeast genomic library to identify genes that, in increased copy number, alleviate *gcs1*Δ cold sensitivity (Wang *et al.*, 1996). This screen identified four genes (*YCK2, YCK3, YPT31,* and *YPT32*) the beneficial effects of which have already been reported (Wang *et al.*, 1996; Zhang *et al.*, 2002); a fifth

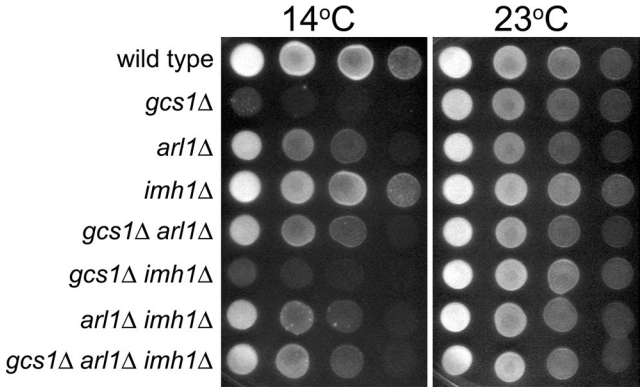

FIGURE 6: Imh1 is not required for *arl1*Δ alleviation of *gcs1*Δ cold sensitivity. Isogenic strains deleted for the indicated genes were grown to stationary phase; 10-fold serial dilutions were spotted onto solid enriched medium and incubated at 14°C and 23°C.

gene is described here. One library plasmid that alleviated *gcs1*Δ cold sensitivity harbored a 4.1-kb yeast genomic DNA insert containing the entire 2.7-kb *IMH1* ORF. On direct testing, *gcs1*Δ cells carrying a plasmid providing increased expression of Imh1 or GFP-Imh1 were no longer cold sensitive (unpublished data and Figure 7B). These findings suggest that increased abundance of the Arl1 effector Imh1 overcomes the growth inhibition of *gcs1*Δ cells.

We also assessed whether overexpression of *IMH1* could restore effective endocytosis in *gcs1*Δ cells. Fluorescence microscopy revealed that *gcs1*Δ cells carrying a high-copy plasmid expressing Imh1 efficiently transported the lipophilic dye FM 4–64 to the vacuole, whereas the dye remained trapped in endocytic compartments in *gcs1*Δ control cells carrying an empty vector (Figure 4). Thus similar to *gcs1*Δ cold sensitivity, defective endocytic transport in *gcs1*Δ mutant cells is remedied by increased abundance of the Arl1 effector Imh1.

Arl1 binding by the GRIP domain of Imh1 is necessary and sufficient for Imh1 effects

The Arl1 effector Imh1 is a long coiled-coil protein with a conserved GRIP (golgin-97, RanBP2α, Imh1p, and p230/golgin-245) domain. This GRIP domain, comprising the C-terminal 50 residues of the 911-residue Imh1 protein, mediates the interaction between Imh1 and activated Arl1 (Barr, 1999; Kjer-Nielsen *et al.*, 1999; Munro and Nichols, 1999; Panic *et al.*, 2003b; Setty *et al.*, 2003). As described earlier in the text, Imh1 is recruited to the Golgi membrane by binding-activated Arl1, and is cytoplasmically localized when the Arl1 or Ypt6 pathway is defective. Cytoplasmic Imh1 does not contribute to the alleviation of *gcs1*Δ cold sensitivity in the *arl1*Δ situation; therefore, we considered the possibility that increased abundance of Imh1 alleviates *gcs1*Δ cold sensitivity through Arl1 binding. To assess involvement of Arl1 binding by Imh1, we used the Imh1-Y870A mutant form of Imh1 in which Tyr-870 in the GRIP domain is substituted with alanine, a change that abolishes Arl1 binding (Panic *et al.*, 2003b). Fluorescence microscopy confirmed that GFP-Imh1-Y870A in *gcs1*Δ cells had the same diffuse cytoplasmic localization that has been reported in wild-type cells (Panic *et al.*, 2003b), consistent with the failure of Imh1-Y870A to bind activated Arl1 and become localized to the *trans*-Golgi membrane (Figure 7A). Although the abundances of Imh1 and Imh1-Y870A GFP-fusion proteins are similar (Panic *et al.*, 2003b), we found that increased expression of the wild-type GFP-Imh1 fusion protein alleviated *gcs1*Δ cold sensitivity, whereas increased expression of the mutant GFP-Imh1-Y870A fusion protein failed to do so (Figure 7B). Thus Arl1 binding by Imh1 is necessary for the alleviation of *gcs1*Δ cold sensitivity by increased Imh1 levels.

The GRIP domain of Imh1 is sufficient to bind Arl1 and target a GFP-tagged GRIP domain to the Golgi, resulting in punctate staining (Kjer-Nielsen *et al.*, 1999; Munro and Nichols, 1999; Setty *et al.*, 2003). We therefore constructed and tested a plasmid expressing GFP fused to the C-terminal 177 residues of Imh1 containing the GRIP domain (Setty *et al.*, 2003). Fluorescence microscopy confirmed that, like GFP-Imh1, this GFP-GRIP fusion protein was targeted to the Golgi in wild-type and *gcs1*Δ cells, as revealed by punctate staining in cells of both genotypes (Figure 7A). As expected, Golgi targeting of the GFP-GRIP fusion protein was absent in cells lacking Arl1 (Panic *et al.*, 2003b; Setty *et al.*, 2003; Figure 7A). As seen for the wild-type GFP-Imh1 fusion protein, increased expression of the GFP-GRIP fusion protein alleviated *gcs1*Δ cold sensitivity (Figure 7B). Thus the GRIP domain of Imh1, which is sufficient for Arl1 binding and Golgi localization, is also sufficient for alleviation of *gcs1*Δ cold sensitivity. The long coiled-coil N-terminal domain of

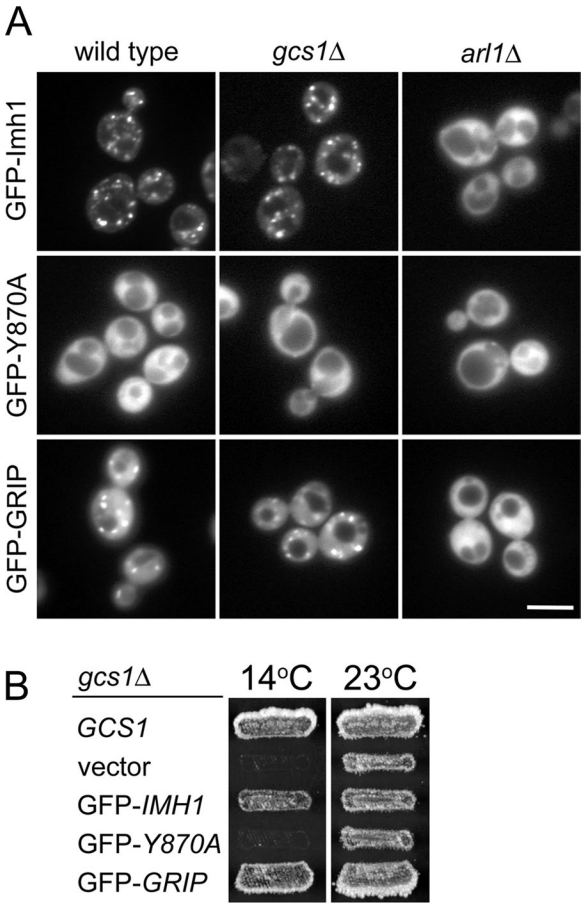

FIGURE 7: Binding of Arl1 by the GRIP domain of Imh1 is necessary and sufficient for Imh1-mediated alleviation of *gcs1*Δ cold sensitivity. (A) Fluorescence micrographs of live yeast cells with the indicated gene deletions each harboring low-copy plasmids expressing N-terminally GFP-tagged Imh1, Imh1-Y870A, or Imh1 GRIP domain (C-terminal 177 residues of Imh1) from the *TPI1* promoter. Scale bar, 5 μm. (B) Growth of the same *gcs1*Δ cells as in panel A, plus *gcs1*Δ cells harboring vector or a *GCS1* plasmid as controls for cold sensitivity. Transformants were patched on solid selective medium and grown to stationary phase before being replica-plated to the same medium for incubation at 14°C and 23°C. Four individual transformants were assessed for each plasmid and in each case showed consistent behavior.

Imh1, comprising 80% of the protein and involved in normal Imh1 function, is dispensable for Imh1-mediated alleviation of *gcs1*Δ cold sensitivity. This finding suggests that the effect of increased Imh1 abundance may be brought about by perturbing other Arl1 interactions through competition for Arl1 binding sites, rather than by increased Imh1 function itself. Other participants in this proposed competition remain to be identified.

DISCUSSION

Previous investigations from our laboratory demonstrated that the yeast Gcs1 protein is required for cell proliferation and endocytic transport in the cold (Drebot *et al.*, 1987; Ireland *et al.*, 1994; Wang *et al.*, 1996). Here we have expanded our understanding of the conditions that impose these *gcs1*Δ defects. We propose that the defects exhibited in the cold by cells lacking Gcs1 are consequences of altered Arl1 protein activity caused by the absence of Arl1 regulation by Gcs1. We imagine that the cold-induced nature of the *gcs1*Δ defects is a combination of dysregulated active Arl1 and decreased

membrane fluidity that results in the cold; in contrast, dysregulated Arl1 is tolerated at normal growth temperatures where membrane fluidity does not impose additional stress. Although Gcs1 is a dual-specificity GAP for Arl1 and Arf1 (Poon *et al.*, 1996; Liu *et al.*, 2005; Figure 3A), and as such regulates Arl1 deactivation through stimulation of GTP hydrolysis, our results using GAP-deficient mutants of Gcs1 suggest that the Gcs1 activity that is important for cell proliferation and endocytic transport in the cold is independent of Gcs1 GAP activity.

The ability of a yeast ArfGAP protein to provide a conditionally essential function independent of its GAP activity is not without precedent. One example involves the Glo3 protein, a member of the ArfGAP family in yeast that regulates retrograde transport from the *cis*-Golgi to the endoplasmic reticulum (Poon *et al.*, 1999). Glo3 is nonessential at permissive growth temperatures of 23°C and 30°C but, like Gcs1, is essential for growth in the cold; *glo3*Δ cells are cold sensitive (Poon *et al.*, 1999). We have shown that the GAP activity of Glo3 is not required for growth in the cold; a central portion of Glo3 lacking the GAP domain is sufficient to alleviate *glo3*Δ cold sensitivity (Schindler *et al.*, 2009). Another GAP-independent function, which is shared by Gcs1 and Glo3, is involved in the priming of SNARE proteins. Independent of their GAP activities, Gcs1 and Glo3 proteins induce a conformational change in SNARE proteins that allows subsequent interaction of the SNAREs with Arf1 and coatomer in vitro (Rein *et al.*, 2002; Robinson *et al.*, 2006). These interactions allow the formation of priming complexes that are, in turn, required for vesicle biogenesis (Springer *et al.*, 1999). GAPs like Gcs1 clearly can exert important non-GAP activities.

Our analysis implicates multiple components of the Arl1 and Ypt6 pathways in the conditional growth impairment caused by the absence of Gcs1. Gene deletions eliminating components of the Arl1 and Ypt6 pathways relieved the low-temperature growth inhibition and endocytic transport defect caused by the absence of Gcs1. Each component of the Arl1 pathway is required for the activation and Golgi localization of Arl1, and we found that the Ypt6 pathway is also required for the activation and Golgi localization of Arl1. These results suggest that both pathways impose the low-temperature growth inhibition and endocytic transport defect through a common activated-Arl1 mechanism.

Two vesicle-tethering factors, Imh1 and the multi-subunit GARP complex, are known effectors of the Arl1 and Ypt6 pathways, respectively (Panic *et al.*, 2003b; Setty *et al.*, 2003). Although not known to be an effector of Arl1, GARP binds Arl1 through a direct interaction with the Vps53 GARP subunit (Panic *et al.*, 2003b). Despite this potential for cross-talk between the two pathways, the finding that Ypt6 influences the Arl1 pathway was unexpected. We show that a lack of activated Ypt6, due to a lack of Ypt6 GEF activity or of Ypt6 itself, dramatically impairs the Golgi localization of Sys1, which in turn results in the impaired localization of proteins downstream of Sys1 in the Arl1 pathway, namely Arl1 and Imh1. The converse, however, is not true: The Arl1 pathway is not required for proper Ypt6 pathway function and GARP localization. In contrast to what is seen for cells lacking Ypt6, the absence of Arl1 or Sys1 does not affect localization of a Vps54-GFP GARP subunit (Panic *et al.*, 2003b; our unpublished results), suggesting that Ypt6 is sufficient for the proper localization of GARP. Vesicle tethering at the *trans*-Golgi membrane is an essential function, as suggested by the observation that deletion of both the *ARL1* and *YPT6* genes is lethal (Setty *et al.*, 2003; Tong *et al.*, 2004; our unpublished results). Thus the impairment of the Arl1 pathway due to the absence of Ypt6 must not completely eliminate Arl1-pathway function. It is therefore likely that, in the absence of Ypt6, at least some Arl1 is properly localized. Although we did not observe any punctate localization of Arl1-GFP in the absence of Ypt6, we noted that cells lacking Ypt6 display some localization of Sys1-GFP and GFP-Imh1 to punctate structures that may correspond to proper Golgi localization.

The mechanism by which Ypt6 contributes to Sys1 localization remains to be determined. To maintain residence at the *trans*-Golgi membrane, Sys1 may be excluded from transport vesicles formed at the *trans*-Golgi membrane and/or recycled from endosomal compartments back to the *trans*-Golgi membrane. The Ypt6 pathway is required for the localization of *trans*-Golgi membrane proteins through the latter mechanism, so that Golgi-resident proteins are retrieved from endosomes through retrograde vesicular transport (Tsukada and Gallwitz, 1996; Siniossoglou *et al.*, 2000; Bensen *et al.*, 2001). It is therefore likely that, without normal levels of activated Ypt6, Sys1 is not properly retrieved from endosomes, resulting in its mislocalization. The fact that cells are still viable in the absence of Ypt6 suggests that sufficient Sys1 function may be provided by newly made Sys1 delivered by the secretory pathway to the *trans*-Golgi membrane. Indeed, increased abundance of members of the Arl1 pathway, including Sys1, alleviates the inherent temperature sensitivity caused by the absence of Ypt6 (Tsukada and Gallwitz, 1996; Bensen *et al.*, 2001). The beneficial effects in these cells of increased abundance of members of the Arl1 pathway suggest that the decreased Arl1-pathway function in the absence of Ypt6 becomes inadequate at high growth temperatures, but that increased Sys1 abundance and/or increased rate of Sys1 production improves Arl1-pathway function to a level that supports growth at these temperatures.

We found that increased expression of the Arl1-effector protein Imh1 alleviates the cold sensitivity of *gcs1*Δ mutant cells. Moreover, increased expression of only the GRIP domain of Imh1 was able to alleviate this cold sensitivity, suggesting that normal Imh1 function may not be required to overcome the impairment imposed by the absence of Gcs1. Conversely, the lack of Imh1 did not affect the impairment of *gcs1*Δ cells (*gcs1*Δ *imh1*Δ double-mutant cells remain cold sensitive; Figure 6) or the beneficial effects of eliminating Arl1 (*gcs1*Δ *arl1*Δ *imh1*Δ triple-mutant cells grew and formed colonies at low growth temperature; Figure 6). We therefore propose that an increased abundance of the GRIP domain of Imh1 alleviates the cold sensitivity imposed by dysregulated active Arl1 by saturating Arl1 binding sites and displacing some cellular component. That is, the sequestration by active Arl1 of some factor imposes the low-temperature growth impairment, and this sequestration is minimized by the presence of the Gcs1 protein or by an increased abundance of Imh1 or the Arl1-binding GRIP domain of Imh1. Deletion mutations affecting the Arl1 and Ypt6 pathways could also decrease Arl1 sequestration of this factor either by the straightforward elimination of Arl1 in the case of *arl1*Δ or by impaired activation and membrane localization of Arl1 in the case of the other Arl1- and Ypt6-pathway deletions. In this model, Arl1 binding of the factor is normally regulated (either directly or indirectly) by a GAP-independent function of Gcs1, and only becomes problematic in the absence of this regulation. Release of this hypothesized sequestered factor from Arl1 may allow this factor to provide function for growth and transport; alternatively, the binding of this factor to active Arl1 may create a toxic complex that inhibits growth and transport in *gcs1*Δ cells. Identification of the proposed Arl1-binding protein may be necessary to distinguish between these two possibilities.

Several Arl1-binding proteins have been identified in both yeast and mammalian systems. Mammalian cells have four GRIP-domain proteins (golgin-245/p230, golgin-97, GCC88, GCC185) that have all been reported to bind Arl1 (Lu and Hong, 2003; Panic *et al.*,

2003a), although Arl1 binding by GCC88 and GCC185 is controversial (Derby *et al.*, 2004; Burguete *et al.*, 2008; Houghton *et al.*, 2009). In yeast, Imh1 is the only known GRIP-domain protein; therefore the factor that is displaced by Imh1 binding of Arl1 is not likely a GRIP-domain protein. Proteins that bind Arl1 but do not have a GRIP domain have been identified in mammalian cells (Munro, 2005); the only one with a homologue in yeast is SCOCO (short coiled-coil protein of unknown function), but the yeast homologue, Slo1 (SCOCO-like ORF), binds Arl3 and not Arl1 (Panic *et al.*, 2003b). In yeast, the GARP tethering complex binds activated Arl1 through an interaction with the Vps53 GARP subunit (Panic *et al.*, 2003b), raising the possibility that GARP might be the factor sequestered by dysregulated active Arl1. To address this possibility, we assessed the requirement for GARP in *arl1*Δ-mediated alleviation of *gcs1*Δ cold sensitivity. We found that neither of the GARP subunits tested (Vps53 or Vps51) are required for *arl1*Δ-mediated alleviation, because *gcs1*Δ *arl1*Δ *vps53*Δ and *gcs1*Δ *arl1*Δ *vps51*Δ triple-mutant cells grew and formed colonies at 14°C (our unpublished results). Thus GARP is not required for growth, ruling out the simple model in which GARP is the factor needed for growth in the cold but sequestered by dysregulated Arl1. In the alternative model, in which GARP sequestration by dysregulated Arl1 creates a toxic complex, the deletion of GARP should alleviate the *gcs1*Δ defects, just as deletion of *ARL1* does. Deletion of any of the genes encoding GARP subunits, however, is lethal in *gcs1*Δ cells with dysregulated Arl1 (our unpublished results), arguing against the existence of a toxic GARP–Arl1 complex. These observations reveal complex functional interactions that remain to be fully characterized. Further investigation is required to identify the proposed factor sequestered by dysregulated Arl1 that inhibits growth and endocytic transport in the cold.

MATERIALS AND METHODS

Yeast strains, plasmids, and growth conditions

Yeast strains used in this study are described in Supplemental Table 2. Standard techniques were used for the propagation, transformation, and genetic manipulation of yeast cells. To assess cold sensitivity, yeast cells were first grown to stationary phase by incubation at 30°C for 5 to 7 d in liquid culture for serial dilution assays or as patches of cells on solid medium. If the cells of interest contained a plasmid, they were grown under selective conditions for that plasmid; otherwise, enriched medium was used. For serial dilution assays, stationary-phase cells were concentrated to 1×10^8 or 1×10^9 cells/ml, and 5 μl of 10-fold serial dilutions was spotted onto solid medium and incubated at 14°C. A Coulter (Hialeah, FL) electronic particle counter model ZM was used to determine cell concentrations.

Plasmids used in this study are described in Supplemental Table 3. To construct pGRIP-177, an *Eco*R1 digest was used to remove all *IMH1* coding sequences from pLK. A ~700-base-pair region of *IMH1* including 534 base pairs of the *IMH1* ORF (encoding the C-terminal 177 residues of Imh1) and ~180 base pairs of downstream flanking sequence was amplified from pLK and cloned back into the *Eco*RI-digested pLK backbone. Sequencing confirmed that this plasmid encodes an N-terminally tagged GFP-Imh1 GRIP-domain fusion protein, encompassing the C-terminal 177 amino acids of Imh1, that is expressed from the *TPI1* promoter. The plasmids encoding GAP-deficient Gcs1-R54 mutants were constructed by subcloning ~1.8-kb fragments containing the *gcs1-R54A*, *gcs1-R54K,* and *gcs1-R54Q* alleles plus ~300 base pair upstream and ~500 base pair downstream flanking sequences from pCTY922, pCTY924, and pCTY925 (Yanagisawa *et al.*, 2002) into pRS315, pRS425, pRS316, and pRS426 (Sikorski and Hieter, 1989; Christianson *et al.*, 1992). To express His6-tagged Arl1 and Gcs1-R54K in bacteria, the coding information for each gene was amplified by PCR and subcloned into pET21b and pET166 to produce plasmids pRA8 and pPP14:142, respectively.

Genetic screens

To screen for gene deletions that alleviate *gcs1*Δ cold sensitivity, the query strain PPY169–4 was crossed to the yeast deletion collection (Giaever *et al.*, 2002), and double-mutant cells were obtained as described (Tong *et al.*, 2001). Cell manipulation was carried out using a Bio-Rad (Hercules, CA) VersArray colony arrayer housed in a Bio-Rad VersArray environmental control chamber. Double-mutant cells (each deleted for *GCS1* and a nonessential gene) were incubated at 30°C for 7 d to allow the cells to enter stationary phase and were then pinned to fresh medium and incubated at 14°C for 7 d. Double-mutant cells that formed a colony were considered to have a deletion that putatively alleviates *gcs1*Δ cold sensitivity. This screen was repeated four times and identified 170 gene deletions in total, 34 of which were identified in two or more screens. Multiple double-mutant clones from each of the 170 candidate strains were further assessed for growth at 14°C by patching the cells on solid medium, growing to stationary phase, and replica-plating to fresh solid medium for incubation at 14°C. These tests showed that 92 double-mutant strains consistently grew at 14°C (Supplemental Table 1). Remarkably, 11 of the deletions eliminate neighboring genes on chromosome XIII; we believe that they represent a linkage group. Such a linkage group would be identified in our analysis if the parental strain used to create these particular deletion mutations contained an unmarked mutation that alleviates *gcs1*Δ cold sensitivity. The position of the linkage group indicates where this mutation exists. It is therefore likely that one of the genes in the middle of this linkage group is a bona fide alleviator of *gcs1*Δ cold sensitivity when deleted or mutated; its identification requires further analysis.

The screen for yeast genes that alleviate *gcs1*Δ cold sensitivity when expressed at increased dosage was as reported (Wang *et al.*, 1996).

Localization of GFP fusion proteins

Cells expressing GFP-tagged proteins were grown overnight in medium that maintained selection for any plasmids. Log-phase cells were concentrated by centrifugation immediately before mounting for visualization using a Zeiss Axiovert 200 inverted microscope (Thornwood, NY). Images were captured with a Hamamatsu (Solna, Sweden) ORCA-R2 digital monochromatic camera, and contrast was enhanced using Adobe Photoshop software. For each situation, at least three individual transformants were assessed and found to display the same GFP staining pattern.

FM 4–64 endocytosis assays

Cells were treated essentially as described (Wang *et al.*, 1996). Before transfer to fresh medium, stationary-phase cells were stained with 40 μM FM 4–64 (Invitrogen, Carlsbad, CA) in YEPD-rich medium for 1 h on ice. Before staining, cells were grown to stationary phase in YEPD medium unless they contained a plasmid, in which case selection for the plasmid was maintained. After staining, cells were grown in YEPD medium. Cells were transferred to synthetic complete medium for visualization. Images were captured and processed as described for GFP fluorescence.

Methylene blue viability assay

Viability assays were carried out essentially as described (Gurr, 1965). Samples were concentrated by centrifugation and mixed on a

coverslip with an equal volume of a 0.02% methylene blue solution in phosphate-buffered saline, pH 7.4. Prepared cells were visualized by light microscopy; dead cells stained blue, and living cells remained unstained. For each sample at least 500 cells were counted. The numbers of blue (dead) and white (viable) cells were used to determine percent viability of the culture.

GTPase-activating protein assay

Arl1-His6 and Arf1-His6 were each coexpressed with *N*-myristoyltransferase in BL21(DE3) bacterial cells and purified following the procedure to isolate Arf1-His6 as described (Benjamin *et al.*, 2011). Myristoylated GTPase was loaded with radioactive GTP, and GAP activity was assessed as described (Huber *et al.*, 2001; Poon *et al.*, 2001). His6-Gcs1 and His6-Gcs1-R54K proteins were prepared in parallel as previously described (Huber *et al.*, 2001). The isolated GAP proteins were of equivalent purity as assessed by protein separation using SDS–PAGE followed with Coomasie blue staining.

ACKNOWLEDGMENTS

We thank Sean Munro, Christopher Burd, and Joel Moss for plasmids; Steve Whitefield and the Dalhousie University Cellular Microscopy and Digital Imaging facility for technical assistance with microscopy; and David Carruthers, Kendra Walker, and Rakhna Araslanova for technical assistance. This work was supported by funding (to G.C.J. and R.A.S.) from the Canadian Institutes of Health Research (CIHR). J.J.R.B. was supported by a trainee award from The Beatrice Hunter Cancer Research Institute with funds provided by The Terry Fox Foundation Strategic Health Research Training Program in Cancer Research at CIHR.

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