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The Yeast Arf GTPase-activating Protein Age1 Is Regulated by Phospholipase D for Post-Golgi Vesicular Transport*

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Vesicular transport shuttles cargo among intracellular compartments. Several stages of vesicular transport are mediated by the small GTPase Arf, which is controlled in a cycle of GTP binding and hydrolysis by Arf guanine-nucleotide exchange factors and Arf GTPase-activating proteins (ArfGAPs), respectively. In budding yeast the Age2 + Gcs1 ArfGAP pair facilitates post-Golgi transport. We have found the *AGE1* gene, encoding another ArfGAP, can in high gene-copy number alleviate the temperature sensitivity of cells carrying mutations affecting the Age2 + Gcs1 ArfGAP pair. Moreover, increased *AGE1* gene dosage compensates for the complete absence of the otherwise essential Age2 + Gcs1 ArfGAP pair. Increased dosage of *SFH2*, encoding a phosphatidylinositol transfer protein, also allows cell growth in the absence of the Age2 + Gcs1 pair, but good growth in this situation requires Age1. The ability of Age1 to overcome the need for Age2 + Gcs1 depends on phospholipase D activity that regulates lipid composition. We show by direct assessment of Age1 ArfGAP activity that Age1 is regulated by lipid composition and can provide ArfGAP function for post-Golgi transport.

Eukaryotic cells move protein and membrane cargo between the plasma membrane and various organelles by a process termed vesicular transport. Each step in the vesicular-transport process, including vesicle generation, cargo packaging, vesicle targeting, and vesicle fusion, is controlled by a variety of proteins (for review, see Ref. 1). Regulated vesicular transport not only ensures the fidelity of cargo delivery but also maintains the structural and functional integrity of membrane organelles.

Each stage of vesicular transport relies on a specific set of proteins to form the transport vesicle and direct the vesicle to the target compartment. For example, vesicles involved in retrograde transport from the Golgi to the endoplasmic reticulum require coat protein complex I (COPI) and the small GTPase Arf1 (2, 3), whereas transport vesicles originating from the *trans*-Golgi network also require the Arf1 GTPase but use the clathrin coat complex (4, 5). Arf1 regulates recruitment of coat protein complex I and clathrin coat complexes to the donor membrane, where these coat complexes mediate the generation of transport vesicles by deforming the membrane. Arf regulatory activity depends on a cycle of GTP binding and hydrolysis, which is in turn regulated by two types of proteins, Arf guanine-nucleotide exchange factors and Arf GTPase-activating proteins (ArfGAPs).[6] Arf guanine-nucleotide exchange factors mediate the exchange of GDP for GTP on Arf to activate Arf and allow coat recruitment (6), whereas ArfGAPs stimulate hydrolysis of Arf-bound GTP to inactivate Arf and allow release of coat proteins from transport vesicles in preparation for fusion of the transport vesicle with a target membrane (7).

The budding yeast *Saccharomyces cerevisiae* contains ArfGAP proteins that are characterized by a zinc-binding motif ($CXXCX_{16}CXXC$, where C is cysteine and *X* is any amino acid) and a nearby invariant arginine residue, which together constitute a catalytic center known as the ArfGAP domain (4). Four yeast proteins, Gcs1, Glo3, Age2, and Age1, have been shown to exert ArfGAP activity *in vitro* (8–11), with the Age2 + Gcs1 ArfGAP pair providing essential overlapping function for post-Golgi transport (10).

Vesicular transport is also mediated by membrane lipid composition through the effects of lipid-protein interactions on protein function. The membrane lipid phosphatidylinositol is phosphorylated on the inositol ring to produce distinct phospholipid species that, like Arf, are involved in protein recruitment to membranes for vesicle formation. One family of yeast proteins that affects membrane lipid composition is defined by the Sec14 phosphatidylinositol transfer protein that is involved in vesicle formation for post-Golgi transport, presumably by creating a lipid composition permissive for vesicle formation. Indeed, we and others have shown that ArfGAP activity is modulated by lipid environment (12, 13) and that increased abundance of the Sec14 homologue Sfh2 enhances Gcs1 activity for post-Golgi transport (14).

We have investigated the function of the Age2 + Gcs1 pair for post-Golgi transport by identifying yeast genes that, in

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[6] The abbreviations used are: ArfGAP, Arf GTPase-activating protein; DAG, diacylglycerol; myr, myristoylated; RFP, red fluorescent protein.

TABLE 1
Yeast strains used in this study

Strain	Genotype[a]	Source
AAY10	*age2Δ::HIS3/AGE2 gcs1Δ::URA3/GCS1*	This study
AAY20	*age2Δ::HIS3/AGE2 gcs1Δ::LEU2/GCS1*	(14)
JBY10	*age2Δ::HIS3/AGE2 gcs1Δ::LEU2/GCS1 age1Δ::natMX4/AGE1*	This study
JBY12	*age2Δ::HIS3/AGE2 gcs1Δ::URA3/GCS1 sfh2Δ::kanMX4/SFH2*	This study
JBY29	*age2Δ::HIS3 gcs1Δ::LEU2* [pMG4-4]	This study
JBY65	*age2Δ::HIS3 gcs1Δ::LEU2* [pMG4-4]	This study
JBY64	*age2Δ::HIS3 gcs1Δ::LEU2 spo14Δ::kanMX4* [pMG4-4]	This study
PPY169	*age2Δ::HIS3 gcs1Δ::URA3* [pLAA314-3]	This study
JBY122	*age2Δ::HIS3 gcs1Δ::URA3* [pMG4-4]	This study
SL215	*age1Δ::natMX4*	This study
PPY203G-Chc1–11A	*ADE2 CHC1-mRFP::kanMX6*	This study
PPY203G-Snf7–4A	*ADE2 SNF7-mRFP::kanMX6*	This study
PPY203G-Erg6–7D	*ADE2 ERG6-mRFP::kanMX6*	This study
PPY203G-Chc1–28B	*age2Δ::HIS3 gcs1Δ::LEU2 ADE2 CHC1-mRFP::kanMX6* [pMG4-4]	This study
PPY203G-Snf7–5C	*age2Δ::HIS3 gcs1Δ::LEU2 ADE2 SNF7-mRFP::kanMX6* [pMG4-4]	This study
PPY203G-Erg6–1A	*age2Δ::HIS3 gcs1Δ::LEU2 ADE2 ERG6-mRFP::kanMX6* [pMG4-4]	This study
PPY205	*age2Δ::HIS3 gcs1Δ::natMX4 ADE2* [pMG4-4 and p416-GFP-Snc1]	This study

[a] All strains were derived from diploid W303 (*ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*) or its isogenic haploid derivatives W303-1A (*MAT***a**) and W303-1B (*MAT*α) (30); additional alterations to the W303 genotype are indicated.

increased dosage, alleviate the deleterious effects of impaired ArfGAP function. The *AGE1* gene is shown here to alleviate defects resulting from deficient ArfGAP function for post-Golgi transport. The *AGE1* gene was initially described as a suppressor of Arf1 temperature sensitivity (*SAT1*) (15) and was later renamed as encoding an ArfGAP with effector functions (*AGE1*) (11). Those studies implicated the Age1 protein in Arf1 function because increased *AGE1* gene dosage alleviates the temperature sensitivity caused by the *arf1-3* mutant allele (15). A more direct role for Age1 in Arf1 function was implied by the finding that the Age1 protein has ArfGAP activity *in vitro* (11). We show here that deletion of the *AGE1* gene compromises the ability of increased *SFH2* gene dosage to alleviate post-Golgi transport defects in cells lacking the Age2 + Gcs1 ArfGAP pair. Moreover, we find that the ability of *AGE1* to effectively alleviate post-Golgi transport defects depends upon phospholipase D, an effector of an Sfh2-mediated phosphoinositide metabolic pathway (16). In addition, we show that the *in vitro* ArfGAP activity of Age1 is stimulated by diacylglycerol (DAG), a downstream product of phospholipase D activity. Thus, our analysis highlights the importance of phospholipid metabolism for ArfGAP activity in post-Golgi vesicular transport.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids—Yeast strains used in this study are described in Table 1. Yeast cells were propagated and transformed using standard techniques. Genes encoding the organelle-specific red fluorescent protein (RFP) markers (17) were backcrossed three times into the W303 genetic background. Plasmids used in this study are described in Table 2. To generate the N-terminal deletion alleles of *AGE1* expressed from the endogenous *AGE1* promoter, a 600-bp sequence upstream of *AGE1* including the first two codons of *AGE1* was amplified by PCR and cloned into YEp351 to yield plasmid pSL485. The segments of Age1 encoding the various truncations plus ~300 bp of downstream sequence were amplified by PCR and cloned downstream of the *AGE1* promoter in pSL485 to yield the various high-copy plasmids expressing the *AGE1* truncations. The *AGE1* sequences from the high-copy plasmids were subcloned into pRS315 to create the low-copy plasmids expressing the *AGE1* truncations. For protein expression in bacteria, the entire *AGE1* open reading frame and *AGE1* codons 169–482 were separately subcloned into pET21b (Novagen) to create plasmids pSL363 and pSL396, respectively. To produce the Age1-GFP and Age1ΔN-GFP fusions, the *AGE1* open reading frame and *AGE1* sequence from codon 164 to 482 were amplified by PCR and inserted into vector pGREG600 by *in vivo* ligation in yeast (18), yielding plasmids pJB-Age1-GFP and pPPL149, respectively. A Yep213 yeast genomic library (a gift from D. Thomas) was used to identify dosage suppressors of the temperature sensitivity of *age2Δ gcs1-3* mutant cells.

Microscopy—Staining with the lipophilic dye FM 4-64 was performed as described (14). For subcellular localization of GFP and RFP protein fusions, cells were concentrated in growth medium by centrifugation just before analysis by fluorescence microscopy.

His_6-tagged Age1ΔN and Myristoylated-Arf1—For phospholipid binding and *in vitro* ArfGAP assays, bacterially expressed Age1ΔN-His_6 was purified and eluted from nickel-bead resin under native conditions according to the manufacturer's protocols (Qiagen Inc.).

To isolate myristoylated-Arf1-His_6 (myr-Arf1-His_6), bacterial cells carrying plasmids pET-Arf1 and pACYC/ET3d/yNMT were first grown at 37 °C to an A_{600} of 0.5. A 100-fold concentrated solution of 50 μM myristate, 6 μM bovine serum albumin was added to the culture and incubated for 20 min before inducing protein expression with 1 mM IPTG. The cells were then grown overnight at 25 °C, harvested, and frozen at −20 °C. Cell pellets were resuspended in 1 mg/ml lysozyme, 100 mM NaCl, 1 mM $MgCl_2$, 25 mM Tris-HCl, pH 8, and incubated at room temperature for 15 min, and the cells were lysed by adjusting the cell suspension to 0.2% Triton X-100. Lysates were incubated with 50 μg/ml DNase on ice for 1 h and centrifuged at 9000 × *g* for 30 min to pellet cell debris. Supernatants were adjusted to 300 mM NaCl, 10 mM imidazole, and treated with nickel-nitrilotriacetic acid beads (Qiagen Inc.) for 2 h at 4 °C. The nickel-nitrilotriacetic acid beads were collected in a disposable column and washed twice with

TABLE 2
Plasmids used in this study

Plasmid	Gene insert	Vector features	Source or reference
pRS314		*TRP1 CEN6*[a]	(31)
pLAA314–3	*gcs1-3*	*TRP1 CEN6*	(14)
pMG4-4	*gcs1-4*	*TRP1 CEN6*	(14)
pRS315		*LEU2 CEN6*	(31)
pSH4	*GCS1*	*LEU2 CEN6*	This study
pSL340	*AGE1*	*LEU2 CEN6*	This study
pSL494	*AGE1*Δ*N* (167–482)[b]	*LEU2 CEN6*	This study
pJB315-AGE1–61	*AGE1* (61–482)	*LEU2 CEN6*	This study
pJB315-AGE1–71	*AGE1* (71–482)	*LEU2 CEN6*	This study
pJB315-AGE1–81	*AGE1* (81–482)	*LEU2 CEN6*	This study
pJB315-AGE1–91	*AGE1* (91–482)	*LEU2 CEN6*	This study
pJB315-AGE1–101	*AGE1* (101–482)	*LEU2 CEN6*	This study
pJB315-AGE1–111	*AGE1* (111–482)	*LEU2 CEN6*	This study
pJB315-AGE1–121	*AGE1* (121–482)	*LEU2 CEN6*	This study
pJB315-AGE1–131	*AGE1* (131–482)	*LEU2 CEN6*	This study
pJB315-AGE1–141	*AGE1* (141–482)	*LEU2 CEN6*	This study
pJB315-AGE1–161	*AGE1* (161–482)	*LEU2 CEN6*	This study
pRS316		*URA3 CEN6*	(31)
pGCS1–316	*GCS1*	*URA3 CEN6*	This study
pJB1736	*AGE1*Δ*N* (167–482)	*URA3 CEN6*	This study
YEp351		*LEU2* 2μ[c]	(32)
pRS425		*LEU2* 2μ	(33)
pEP1	*GCS1*	*LEU2* 2μ	This study
pSL485	$AGE1_{prom}$	*LEU2* 2μ	This study
pLAA-GLL5	*AGE1*	*LEU2* 2μ	This study
pSL377	*AGE1*	*LEU2* 2μ	This study
pSL489	*AGE1*Δ*N* (167–482)	*LEU2* 2μ	This study
pJB351-AGE1–61	*AGE1* (61–482)	*LEU2* 2μ	This study
pJB351-AGE1–71	*AGE1* (71–482)	*LEU2* 2μ	This study
pJB351-AGE1–81	*AGE1* (81–482)	*LEU2* 2μ	This study
pJB351-AGE1–91	*AGE1* (91–482)	*LEU2* 2μ	This study
pJB351-AGE1–101	*AGE1* (101–482)	*LEU2* 2μ	This study
pJB351-AGE1–111	*AGE1* (111–482)	*LEU2* 2μ	This study
pJB351-AGE1–121	*AGE1* (121–482)	*LEU2* 2μ	This study
pJB351-AGE1–131	*AGE1* (131–482)	*LEU2* 2μ	This study
pJB351-AGE1–141	*AGE1* (141–482)	*LEU2* 2μ	This study
pJB351-AGE1–161	*AGE1* (161–482)	*LEU2* 2μ	This study
pSL344	*AGE2*	*LEU2* 2μ	This study
pRS426		*URA3* 2μ	(33)
pPP421	*GCS1*	*URA3* 2μ	This study
pSL473	*AGE1*	*URA3* 2μ	This study
pJB1737	*AGE1*Δ*N* (167–482)	*URA3* 2μ	This study
pCTY201	*SFH2*	*URA3* 2μ	(34)
pRS426-STT4	*STT4*	*URA3* 2μ	Scott Emr
pRS426-PIK1	*PIK1*	*URA3* 2μ	Scott Emr
pRS426-MSS4	*MSS4*	*URA3* 2μ	Scott Emr
pRS426-SPO14	*SPO14*	*URA3* 2μ	This study
pET21b		*Escherichia coli* expression	Novagen
pPPL21	*GCS1*	*E. coli* expression	(8)
pSL363	*AGE1*	*E. coli* expression	This study
pSL396	*AGE1*Δ*N*	*E. coli* expression	This study
pPPL149	$GAL1_{prom}$-*AGE1*Δ*N-GFP*	*URA3 CEN6*	This study
pJB-Age1-GFP	$GAL1_{prom}$ -*AGE1-GFP*	*URA3 CEN6*	This study
pGREG576	$GAL1_{prom}$ -*GFP*	*URA3 CEN6*	This study
pPPL165	$AGE1_{prom}$-*AGE1-GFP*	*LEU2 CEN6*	This study
p416-GFP-Snc1	*GFP-SNC1*	*URA3 CEN6*	(22)
pET-Arf1	$Arf1\text{-}His_6$	*E. coli* expression	(8)
pACYC/ET3d/yNMT	*NMT1*	*E. coli* expression	(35)

[a] Low-copy plasmid.
[b] Bracketed numbers indicate encoded amino acids; see "Experimental Procedures" for details.
[c] High-copy plasmid.

300 mM NaCl, 20 mM imidazole, 25 mM Tris-HCl, pH 8. Myr-Arf1-His_6 was eluted with 250 mM imidazole, 300 mM NaCl, 25 mM Tris-HCl, pH 8. Fractions enriched with myr-Arf1-His_6, identified by Bradford assay, were washed and concentrated with the use of Centricon filtering systems. Samples of myr-Arf1-His_6 were stored at −80 °C as protein preparations in 100 mM NaCl, 1 mM $MgCl_2$, 1 mM dithiothreitol, 20 mM Tris-HCl, pH 8, 10% glycerol.

Phospholipid Binding—Phospholipid strips (Echelon Inc.) were pretreated with 3% bovine serum albumin (BSA) in Tris-buffered saline + 0.1% Tween 20 (TBST) for 2 h. The strips were then incubated overnight at 4 °C in the dark with 0.5 μg/ml purified Age1ΔN-His_6 in TBST containing 1% BSA. The strips were washed with several changes of TBST over a 1-h period and then exposed to affinity-purified anti-Age1 antibodies, diluted in TBST containing 1% BSA, for 1.5 h at room temperature. The strips were washed with TBST, treated with horseradish peroxidase-conjugated goat anti-rabbit antibody for 1.5 h, and washed with TBST; strip-bound Age1ΔN-His_6 was detected by enhanced chemiluminescence as described by the manufacturer (Pierce). A GST-tagged C-terminal fragment of the *Legionella pneumophila* SidC protein (PI(4)P Grip, Echelon Inc.) was used as a control for binding specificity to the phospholipid strips. Bound protein was

detected with horseradish peroxidase-conjugated anti-GST antibody (GE Healthcare).

GTP Loading—Unilamellar lipid vesicles were prepared essentially as described (20) with minor modifications. Lipid mixtures were prepared in chloroform, evaporated as thin films, and then resuspended in 25 mM MOPS, pH 7.4, as a solution of either 10 mM dimyristoylphosphatidylcholine or 8.5 mM dimyristoylphosphatidylcholine, 1.5 mM dioleoylglycerol (mixture of 1,3 and 1,2 isomers) or 8.5 mM dimyristoylphosphatidylcholine, 1.5 mM phosphatidic acid. The lipid mixtures were then extruded by 20 passages through 100-nm pore filters to produce uniform-sized liposomes.

1-ml loading reactions prepared on ice consisted of 8 μM myr-Arf1-His_6, 1 mM ATP, 1 mM dithiothreitol, 1 μM [γ-^{32}P]GTP and unlabeled GTP (10,000–20,000 cpm/pmol), 2 mM EDTA, 1 mM $MgCl_2$, 50 mM NaCl, 25 mM MOPS, pH 7.4, and 100 μl of one of the lipid preparations, which were added after a brief warming of the reaction mixture. Reaction mixtures were incubated at 30 °C for 40 min and terminated by adjusting the $MgCl_2$ concentration to 2 mM and icing the reaction. Unincorporated GTP was removed by harvesting and washing the GTP-loaded myr-Arf1 with Amicon Ultra-2 centrifugal filter units with Ultracel-10 membranes.

GAP Assay—GTP hydrolysis was assayed essentially as previously described (20). 100-μl reactions consisted of varying amounts of Age1ΔN-His_6 in 5 mM $MgCl_2$, 1 mM ATP, 100 μg/ml BSA, 25 mM MOPS, pH 7.4, and 10 μl of [γ-^{32}P]GTP-loaded myr-Arf1-His_6, added last to initiate the reaction. Assays were incubated at 30 °C for 15 min and terminated with the addition of 500 μl of a cold charcoal suspension and incubation on ice for 30 min. The samples were centrifuged for 1 min, 300 μl of the supernatant was transferred to scintillation mixture, and the amount of radioactivity released was quantified by scintillation counting.

RESULTS

Increased Abundance of the AGE1 Gene Alleviates the Effects of ArfGAP Deficiencies Impairing Post-Golgi Transport—The *gcs1-3* or *gcs1-4* mutation in combination with the *age2*Δ gene deletion results in double-mutant cells that exhibit a temperature sensitivity for growth associated with defective post-Golgi transport (10, 14). To further characterize Age2 + Gcs1 ArfGAP function, we sought genes that, in increased gene dosage, alleviate this temperature sensitivity. We introduced a yeast genomic library into *age2*Δ *gcs1-3* double-mutant cells and selected for colony formation at 37 °C. Of the 8 colonies capable of growth at 37 °C, 6 carried genomic inserts containing *AGE2*, and 2 were found to carry the *AGE1* gene (Fig. 1*A*). *AGE1* encodes a protein with 40% identity and 62% similarity to the ArfGAP Gcs1 and that has ArfGAP activity *in vitro* (11). Thus, increasing the gene dosage for another ArfGAP, Age1, alleviates the growth defect caused by deficient ArfGAP function in post-Golgi transport.

Increased AGE1 Dosage Restores Vesicular Transport in Cells with Impaired ArfGAP Function—The ability of increased *AGE1* gene dosage to allow growth by *age2*Δ *gcs1-3* cells probably results from the restoration of effective transport that is compromised in the ArfGAP mutant cells. To address this possibility directly, we monitored the effects of increased *AGE1* dosage on the trafficking of two reporters that depend on vesicular transport for proper localization.

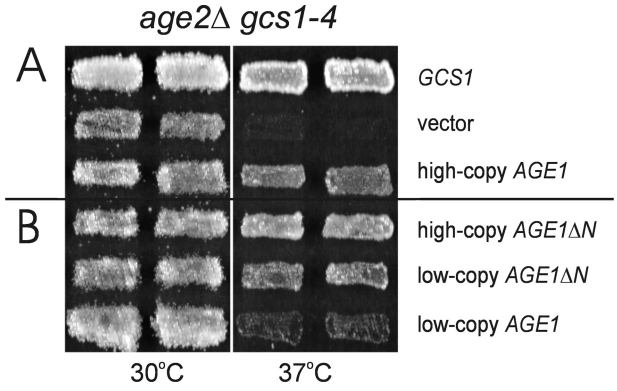

FIGURE 1. **Increased *AGE1* dosage alleviates *age2*Δ *gcs1-4* temperature sensitivity.** Patches of cells harboring plasmid-borne *GCS1* or *AGE1* (*A*) and *AGE1*Δ*N* or *AGE1* (*B*) were grown on selective medium at 23 °C, replica-plated to enriched medium, and incubated at 37 °C for 1 day or at 30 °C for 2 days. For each situation, two independent yeast transformants are displayed.

The lipophilic dye FM 4-64 provides a sensitive measure of post-Golgi function for the endocytic transport pathway (21). FM 4-64 is internalized from the cell surface via endocytosis, transported through endosomal compartments, and delivered to the vacuolar periphery. Activity of the Age2 + Gcs1 ArfGAP pair mediates this delivery of FM 4-64 to the vacuole (10). At the restrictive temperature, *age2*Δ *gcs1-3* cells carrying vector alone exhibited impaired endocytic transport such that FM 4-64 extensively stained the cytoplasm (Fig. 2*A*), indicating that the membrane-bound dye remained trapped in endosomal compartments. In marked contrast, *age2*Δ *gcs1-3* mutant cells carrying either a low-copy *GCS1* plasmid or a high-copy *AGE1* plasmid effectively transported FM 4-64 to the vacuole periphery, yielding the typical ring staining pattern seen in a cell with intact endocytic transport. Thus, the Age1 ArfGAP can provide function for post-Golgi transport.

Another measure of post-Golgi transport is the recycling of the v-SNARE Snc1 (22). Snc1 mediates the targeting of vesicles from the Golgi to the plasma membrane and is then retrieved from the plasma membrane to the endosomes and subsequently transported to the *trans*-Golgi network. This retrieval pathway recycles Snc1 for successive rounds of transport to the cell surface. In wild-type cells, green fluorescent protein (GFP) fused to Snc1 is seen at Golgi and endosomal compartments but is enriched at the plasma membrane of the growing bud (22). This localization of GFP-Snc1 was also observed at 37 °C in *age2*Δ *gcs1-4* cells carrying a *GCS1* plasmid (Fig. 2*B*). In contrast, *age2*Δ *gcs1-4* cells carrying empty vector exhibited punctate GFP-Snc1 staining without staining at the plasma membrane, indicative of a block in transport of GFP-Snc1 from internal compartments to the cell surface. Although the majority of *age2*Δ *gcs1-4* cells carrying an *AGE1* plasmid displayed GFP-Snc1 localized at internal structures, a portion of the population exhibited GFP-Snc1 at the plasma membrane, a situation not observed in mutant cells carrying vector alone. Thus, an increased dosage of

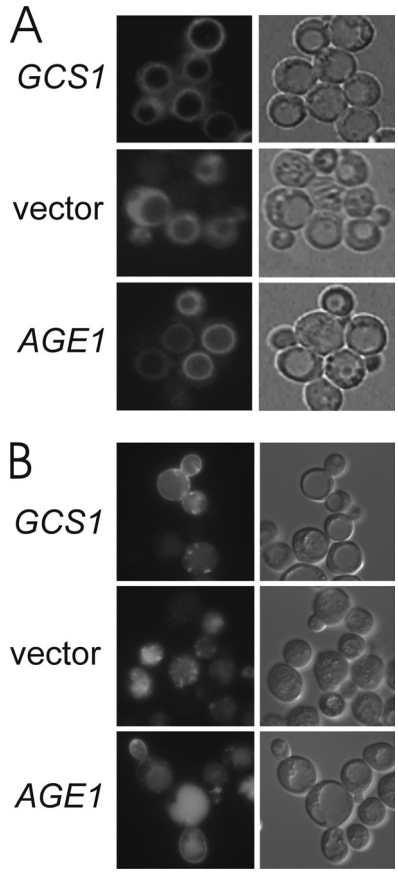

FIGURE 2. **Increased *AGE1* dosage restores post-Golgi function in *age2*Δ *gcs1* cells.** Cells were visualized by fluorescence (*left panels*) and differential interference contrast microscopy (*right panels*). *A*, mutant *age2*Δ *gcs1-3* cells growing at 30 °C and carrying plasmid-borne *GCS1* or *AGE1* genes were stained with FM 4-64 as described (14) and then incubated in fresh medium at 37 °C for 45 min before visualization. *B*, mutant *age2*Δ *gcs1-4* cells carrying p416-GFP-Snc1 and plasmid-borne *GCS1* or *AGE1* genes were grown at 23 °C and then incubated at 37 °C for 3 h before visualization.

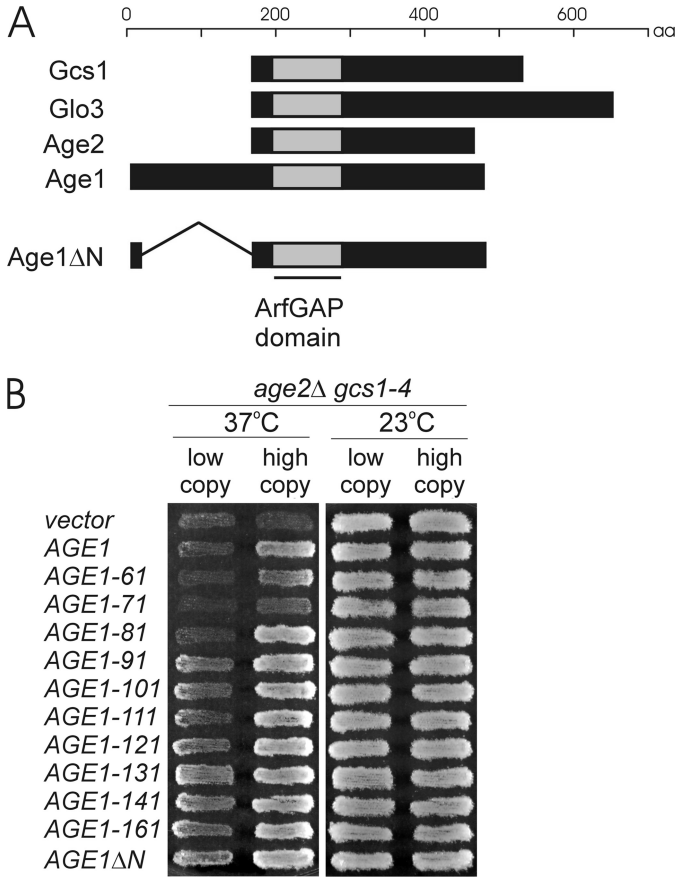

FIGURE 3. **The N terminus of Age1 inhibits the ability of Age1 to alleviate *age2*Δ *gcs1-4* temperature sensitivity.** *A*, schematic alignment of four yeast proteins with ArfGAP activity and the Age1ΔN protein is shown. *B*, patches of cells harboring plasmid-borne N-terminal truncations of Age1 were grown on selective medium at 23 °C, replica-plated to fresh medium, and incubated at 37 °C for 1 day or 23 °C for 2 days. The allele number refers to the codon that is fused downstream of the promoter and the first two codons of *AGE1*.

AGE1 can restore (albeit partially) the ability of *age2*Δ *gcs1-4* mutant cells to properly traffic GFP-Snc1.

N-terminal Sequences of the Age1 Protein Restrict Its Post-Golgi Activity—The Age1 protein differs from other members of the yeast ArfGAP family by possessing a long N-terminal extension (Fig. 3*A*). To assess the involvement of these N-terminal sequences in Age1 function, we created the *AGE1*Δ*N* allele, which is expressed from the endogenous *AGE1* promoter but lacks the sequences encoding codons 3–166 of the Age1 protein (Fig. 3*A*). A similarly truncated Age1 protein retains ArfGAP activity *in vitro* (11).

Temperature-sensitive *age2*Δ *gcs1-4* mutant cells carrying a high-copy *AGE1*Δ*N* plasmid grew at 37 °C and actually grew better than cells carrying the high-copy intact *AGE1* gene (Fig. 1*B*). When these *AGE1* alleles were on low-copy plasmids, the difference was even more pronounced; cells carrying a low-copy *AGE1*Δ*N* plasmid grew well at 37 °C, whereas cells harboring a low-copy *AGE1* plasmid did not (Fig. 1*B*). The absence of residues 3–166 from the N terminus of Age1, therefore, results in enhanced ability to alleviate the growth defects of mutant cells, suggesting that N-terminal sequences have a pronounced effect upon Age1 activity.

To localize potential regulatory regions within the Age1 N terminus, we assessed various *AGE1* truncation alleles for alleviation of growth defects of mutant cells. The truncation alleles were constructed in a manner similar to *AGE1*Δ*N*, with the deletion of sequences from codon 3 to downstream codons ranging from codon 60 to 160. When expressed from low-copy plasmids, truncated proteins lacking the first 80 residues or fewer failed, like intact Age1, to alleviate the temperature sensitivity of *age2*Δ *gcs1-4* cells (Fig. 3*B*). These truncations did, however, alleviate growth defects when expressed from high-copy plasmids. In contrast, truncations lacking the first 90 or more residues of Age1 were effective in alleviating the growth defects even at low plasmid copy number.

Increased Age1 Abundance Can "Bypass" the Need for an Otherwise Essential ArfGAP Pair—The alleviation by *AGE1* (or *AGE1* truncations) of temperature sensitivity in the situations described above took place in cells with mutant Gcs1 proteins that may provide residual Gcs1 activity. Therefore, we determined the ability of increased Age1 abundance to compensate for (bypass) the complete absence of the Age2 + Gcs1 essential protein pair. Diploid cells heterozygous for *age2*Δ and *gcs1*Δ deletion mutations were transformed with a high-copy *AGE1* or *AGE1*Δ*N* plasmid or empty vector and

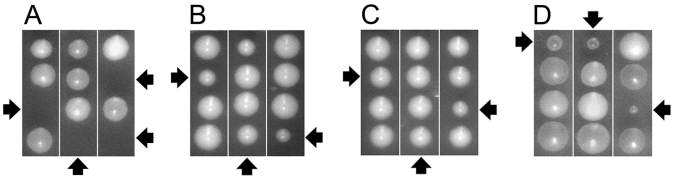

FIGURE 4. **Increased abundance of Age1, Age1ΔN, or Sfh2 bypasses the requirement for the essential Age2 + Gcs1 ArfGAP pair.** Diploid cells heterozygous for *age2*Δ and *gcs1*Δ deletion mutations and harboring high-copy plasmids carrying vector (*A*), *AGE1* (*B*), *AGE1*Δ*N* (*C*), or *SFH2* (*D*) were sporulated, and the resulting haploid segregants were incubated on solid selective medium at 30 °C for 5 days. *Arrows* indicate *age2*Δ *gcs1*Δ double-mutant segregants that are either nonviable (*A*) or kept alive by the plasmid-borne gene (*B–D*).

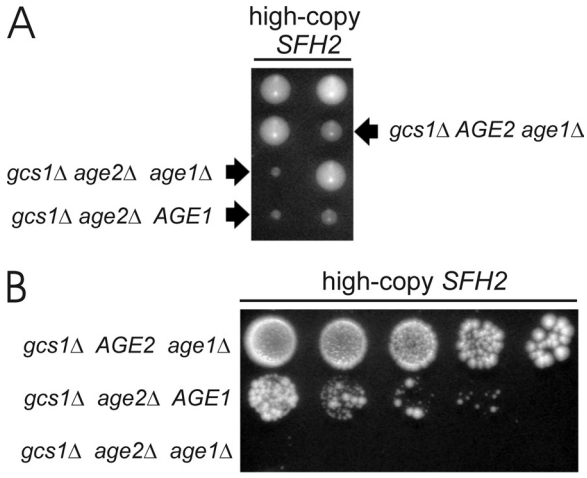

FIGURE 5. **Effective *SFH2* bypass depends on Age1.** *A*, diploid cells heterozygous for *gcs1*Δ, *age2*Δ, and *age1*Δ deletion mutations and carrying a high-copy *SFH2* plasmid were sporulated, and the resulting haploid segregants were incubated on solid synthetic complete medium for 5 days at 30 °C. *B*, equal numbers of cells from colonies identified in *panel A* were serially diluted (5-fold dilutions), spotted onto solid enriched medium, and incubated for 5 days at 30 °C.

then induced to undergo meiosis (sporulation). Resulting haploid segregants lacking both the Age2 and Gcs1 proteins were recovered when the *AGE1* or *AGE1*Δ*N* plasmid was present (Fig. 4, *B* and *C*), an effect not seen for empty vector (Fig. 4*A*). These results indicate that the lethality due to the absence of Age2 + Gcs1 can be circumvented by increased abundance of the Age1 ArfGAP or its N-terminal-truncated derivative.

Increased SFH2 Gene Dosage Also Bypasses the Need for the Age2 + Gcs1 Pair—Previously we showed that increased dosage of *SFH2*, encoding a member of the Sec14 family of phosphatidylinositol transfer proteins, alleviates the temperature sensitivity of *age2*Δ *gcs1-4* double-mutant cells (14). Here we found that haploid cells harboring a high-copy *SFH2* plasmid and both *age2*Δ and *gcs1*Δ deletion mutations were viable (Fig. 4*D*). Therefore, increased *SFH2* gene dosage, like increased *AGE1* dosage, also bypasses the need for the Age2 + Gcs1 pair.

Age1 Is Needed for Effective SFH2 Bypass of the Age2 + Gcs1 Pair—Sfh2 activity may influence ArfGAP activity through modification of the lipid environment (13, 14). The finding that increased dosage of the *SFH2* gene bypasses the need for the Age2 + Gcs1 ArfGAP pair may reflect an altered lipid environment that permits vesicular transport in the absence of ArfGAP activity or that allows another ArfGAP to substitute for the missing Age2 and Gcs1. We, therefore, assessed the involvement of Age1 in this *SFH2* effect. Diploid cells heterozygous for *gcs1*Δ, *age2*Δ, and *age1*Δ deletion mutations and harboring a high-copy *SFH2* plasmid were induced to undergo meiosis. Both *gcs1*Δ *age2*Δ *AGE1* (double-mutant) and *gcs1*Δ *age2*Δ *age1*Δ (triple-mutant) haploid segregants with high-copy *SFH2* grew to form small colonies on synthetic complete medium (Fig. 5*A*), but a significant difference in growth was evident when the cells were grown on enriched medium (Fig. 5*B*). In total we assessed 11 double-mutant segregants and 6 triple-mutant segregants, and all exhibited consistent behaviors; that is, double-mutant cells harboring the high-copy *SFH2* plasmid grew better than triple-mutant cells (lacking the *AGE1* gene) harboring the *SFH2* plasmid. Thus, Age1 is required for efficient bypass, by increased *SFH2* dosage, of the need for the Age2 + Gcs1 pair.

*Phospholipase D Mediates AGE1 Alleviation of age2*Δ *gcs1-4 Temperature Sensitivity*—Sfh2 activity is suggested to affect post-Golgi transport by indirectly influencing phospholipase D activity, an enzyme activated by phosphoinositides (16). Sfh2 is thought to mediate phosphoinositide synthesis by delivering substrate to lipid kinases, such as Stt4, that in turn provide stimulatory phosphoinositides for phospholipase D (16).

If increased *SFH2* gene dosage results in a lipid environment that permits Age1 to functionally replace Age2 + Gcs1, then the absence of Sfh2 or downstream effectors of Sfh2 may compromise the ability of Age1 to replace the essential Age2 + Gcs1 ArfGAP pair. Cells deleted for the lipid kinase genes are inviable, preventing assessment of genetic interactions in cells lacking these genes. Deletion of the *SFH2* gene did not impair the ability of *AGE1* to bypass the lethality of *age2*Δ *gcs1*Δ cells (data not shown). In contrast, deletion of the phospholipase D gene, *SPO14,* did compromise the ability of *AGE1* and *AGE1*Δ*N* to alleviate the temperature sensitivity of *age2*Δ *gcs1-4* cells (Fig. 6). These results indicate that phospholipase D, a downstream target of Sfh2 activity, is required for aspects of Age1 function.

Increased Gene Dosage for Phosphatidylinositol Kinases Fails to Compensate for a Post-Golgi ArfGAP Defect—To determine if potential downstream effectors of Sfh2 activity could mimic the *SFH2* relief of growth inhibition for mutant cells with inadequate post-Golgi ArfGAP activity, we tested high-copy plasmids encoding the lipid kinases Stt4, Mss4, and Pik1 for the ability to support 37 °C growth of *age2*Δ *gcs1-4* cells. In contrast to what was seen for *age2*Δ *gcs1-4* cells carrying the *SFH2, AGE1,* or *GCS1* plasmids, increased dosage of lipid kinase genes failed to restore high temperature growth (data not shown). Unlike increased *SFH2* gene dosage itself, increased dosage of genes whose functions may be affected by Sfh2 activity does not alleviate the temperature sensitivity of *age2*Δ *gcs1-4* cells.

*Age1-GFP and Age1*Δ*N-GFP Colocalize with Golgi and Endosomal Markers*—The observations that increased levels of Age1 can provide post-Golgi function suggest that Age1 might be localized to Golgi and/or endosomal compartments. Age1-GFP and Age1ΔN-GFP fusions, expressed under con-

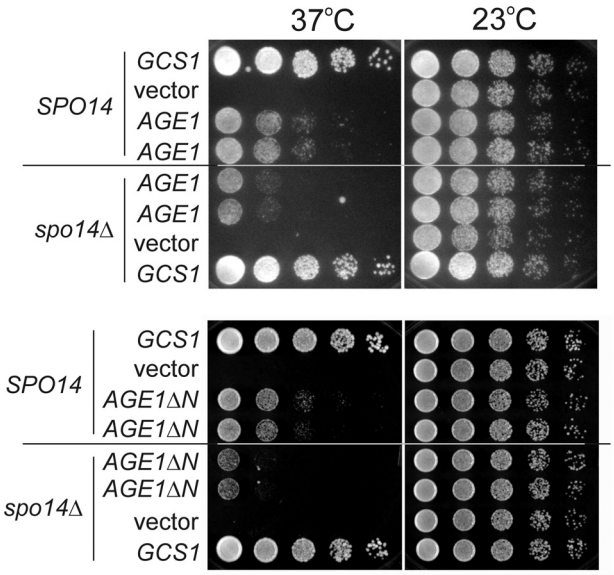

FIGURE 6. **Effective alleviation by *AGE1* and *AGE1*Δ*N* of the post-Golgi ArfGAP defect depends on *SPO14*.** *age2*Δ *gcs1-4* cells with the wild-type *SPO14* gene or a *spo14*Δ deletion mutation and carrying either *GCS1*, high-copy *AGE1*, or low-copy *AGE1*Δ*N* plasmids were grown in selective medium at 23 °C. Equal numbers of cells were then serially diluted (5-fold dilutions), spotted onto solid enriched medium, and incubated at 37 and 23 °C for 2 days. Two independent transformants are shown for cells carrying the *AGE1* or *AGE1*Δ*N* plasmid.

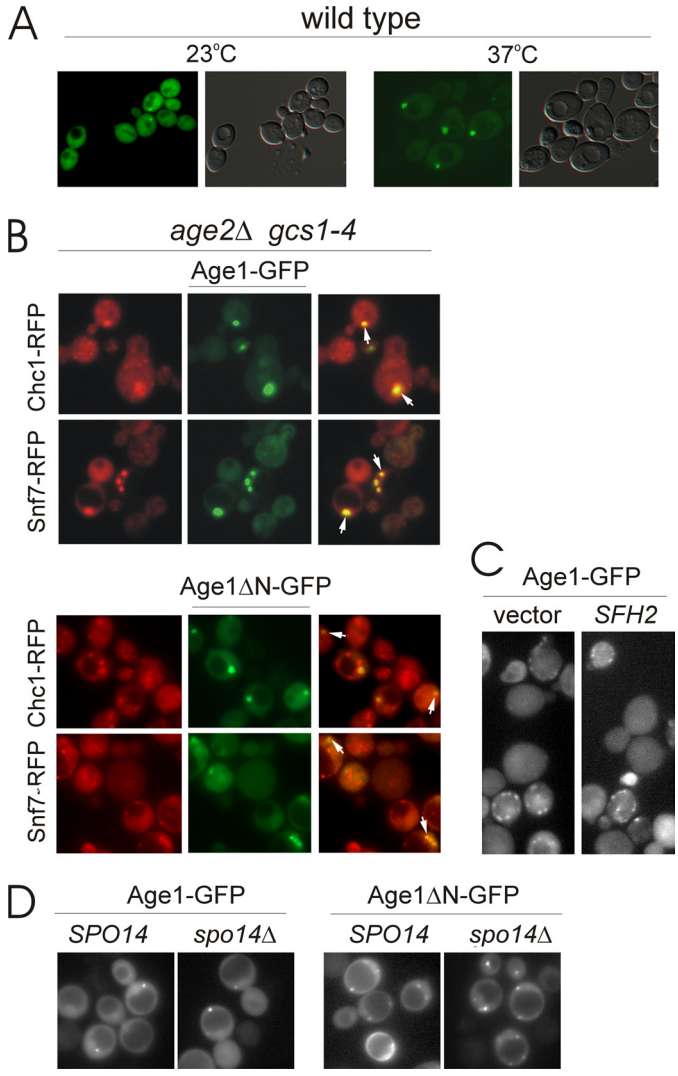

FIGURE 7. **Age1-GFP localization at the *trans*-Golgi and endosome.** *A*, with galactose as the sole carbon source, cells were grown at 23 °C, diluted in fresh medium, and grown for an additional 16 h at 23 or 37 °C before visualization. The localization of Age1ΔN-GFP, expressed under control of the *GAL1* promoter, was assessed in wild-type cells at 23 °C and 37 °C. Cells were visualized by fluorescence (*left panels*) and differential interference contrast microscopy (*right panels*). *B*, the colocalization of Age1ΔN-GFP and Age1-GFP, expressed under control of the *GAL1* promoter, with RFP organelle markers was assessed in *age2*Δ *gcs1-4* cells at 37 °C. *Arrows* indicate representative colocalization. *C*, the localization of Age1-GFP, expressed under control of the *AGE1* promoter, was monitored in proliferating *age2*Δ *gcs1-4* cells also carrying an empty vector or a high-copy *SFH2* plasmid and incubated for 2 h at 37 °C. *D*, the localization of Age1ΔN-GFP and Age1-GFP, expressed under control of the *GAL1* promoter, was analyzed at 37 °C in *age2*Δ *gcs1-4* cells with the wild-type *SPO14* gene or a *spo14*Δ deletion mutation.

trol of the inducible *GAL1* promoter, were used to investigate the localization of Age1 protein. The expression of each GFP fusion, like the increased expression of wild-type Age1 and Age1ΔN, alleviated the temperature sensitivity of *age2*Δ *gcs1-4* cells (data not shown), demonstrating that both Age1-GFP and Age1ΔN-GFP retain biological function.

In wild-type cells at 23 °C, Age1ΔN-GFP was found throughout the cytoplasm and in punctate dots (Fig. 7*A*). At 37 °C, Age1ΔN-GFP (Fig. 7*A*) and Age1-GFP (data not shown) were in large punctate dots with minimal cytoplasmic staining. These dots were typically present once or twice per cell and were often localized near the vacuole. Under the same growth conditions and temperatures, wild-type cells expressing GFP itself failed to exhibit any punctate staining (data not shown).

In *age2*Δ *gcs1-4* cells grown at the restrictive temperature of 37 °C, both Age1-GFP and Age1ΔN-GFP exhibited punctate staining similar to that seen in wild-type cells (Fig. 7*B*). To determine whether the punctate staining corresponded to Golgi or endosomal compartments, we assessed the colocalization of the GFP fusions with organelle-specific RFP fusion proteins (17). Both Age1-GFP and Age1ΔN-GFP localization coincided with the compartments visualized by Chc1-RFP and Snf7-RFP, markers localized at *trans*-Golgi and endosomal compartments, respectively. The localization of Age1-GFP and Age1ΔN-GFP to Golgi/endosomal compartments is consistent with the ability of Age1 to alleviate deleterious effects caused by the absence of post-Golgi ArfGAPs.

Altered Gene Dosage of SFH2 or SPO14 Does Not Alter Age1-GFP Distribution—The ability of Sfh2 to bypass the need for the essential Age2 + Gcs1 ArfGAP pair in an Age1-dependent manner raises the possibility that increased Sfh2 abundance leads to the recruitment or localization of Age1 to Golgi/endosomal compartments. To address this possibility, we monitored the localization of Age1-GFP, whose expression was controlled by the native *AGE1* promoter. A low-copy plasmid harboring *AGE1pr-AGE1-GFP*, like a low-copy *AGE1* plasmid, was unable to alleviate the temperature sensitivity of *age2*Δ *gcs1-4* cells unless a high-copy *SFH2* plasmid was also present (data not shown). Irrespective of the presence or absence of a high-copy *SFH2* plasmid, Age1-GFP exhibited a punctate staining pattern in *age2*Δ *gcs1-4* cells incubated for a short duration at 37 °C, consistent with Golgi/endosomal lo-

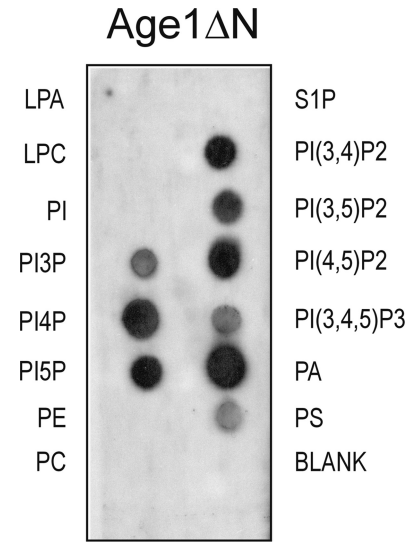

FIGURE 8. **Phospholipid binding by Age1ΔN.** Phospholipid membrane strips were incubated overnight at 4 °C with proteins (0.5 μg/ml), affinity-purified under native conditions from bacterial cells, and then washed and treated with Age1-specific affinity-purified antibodies; proteins bound to the phospholipid membrane strip were detected by enhanced chemiluminescence. No signal was detected when a membrane strip was treated with proteins isolated from bacteria carrying empty vector and probed with Age1-specific antibodies (data not shown). Incubation with 2.5 μg of C-terminal fragment of SidC, a phosphatidylinositol (*PI*) 4-phosphate (*PI(4)P*)-binding protein (29), demonstrated preferential binding of the protein to phosphatidylinositol 4-phosphate and to a much lesser extent to phosphatidylinositol 3,4-diphosphate (*PI(3,4)P2*) and phosphatidylinositol 4,5-diphosphate (*PI(4,5)P2*) (data not shown). The different pattern of binding for this phosphatidylinositol 4-phosphate (*PI(4)P*)-binding protein *versus* Age1ΔN-His_6 demonstrates that phospholipid binding is protein-specific. *PE*, phosphatidylethanolamine; *PC*, phosphatidylcholine; *PA*, phosphatidic acid; *PS*, phosphatidylserine; *PI*, phosphatidylinositol; *LPA*, lysophosphatidic acid; *LPC*, lysophosphocholine; *S1P*, sphingosine-1-phosphate.

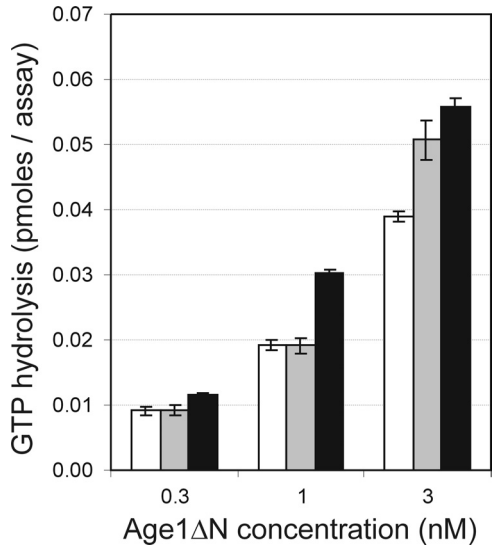

FIGURE 9. **DAG stimulates *in vitro* ArfGAP activity of Age1ΔN.** Activation of hydrolysis of GTP-loaded myristoylated-Arf1 by Age1ΔN-His_6 was assessed in assays in which the lipid content was either 100% dimyristoylphosphatidylcholine (*white bar*), 85% dimyristoylphosphatidylcholine, 15% phosphatidic acid (*gray bar*), or 85% dimyristoylphosphatidylcholine, 15% dioleoylglycerol (*black bar*). The low level of background radioactivity observed in the absence of Age1 protein was subtracted from all data values. S.D. were calculated from triplicate samples.

calization (Fig. 7*C*). Thus, an increased abundance of Sfh2 does not appear to alter the localization of Age1.

The ability of increased Age1 abundance to alleviate the temperature sensitivity of *age2*Δ *gcs1-4* cells is compromised in the absence of Spo14, suggesting the possibility that Age1 fails to localize properly in cells lacking Spo14. To test this, we assessed the localization of GFP fusions in *spo14*Δ cells. Age1-GFP and Age1ΔN-GFP were localized to punctate dots in both wild-type and *spo14*Δ cells (Fig. 7*D*), indicating that Spo14 is not required for the localization of Age1 to Golgi/endosomal compartments.

Age1ΔN Binds Phospholipids—Gcs1 binds to a subset of phospholipids *in vitro* (14). The finding that *SFH2*-mediated bypass may act through Age1 led us to ask whether Age1, like Gcs1, binds phospholipids. The Age1ΔN protein and intact Age1 were expressed in bacteria as His_6-tagged proteins; only the Age1ΔN protein could be isolated in soluble form under non-denaturing conditions, so intact Age1 protein was not tested. Using commercial phospholipid strips, affinity-purified soluble Age1ΔN-His_6 avidly bound phosphatidic acid (*PA*), phosphatidylinositol 4,5-diphosphate (*PI(4,5)P2*), and phosphatidylinositol 3,4-diphosphate (*PI(3,4)P2*)and moderately bound phosphatidylserine (*PS*) and phosphatidylinositol 3-phosphate (*PI3P*) (Fig. 8). This pattern of lipid binding suggests that Age1 may be regulated by the phospholipid content of membranes.

Age1 ArfGAP Activity in Vitro Is Enhanced by DAG—The *in vitro* activation of GTP-bound Arf1 hydrolysis by Gcs1 and by the rat ArfGAP1 is enhanced by long-chain forms of DAG (23). Phospholipase D produces phosphatidic acid with DAG as a secondary product. Our finding that phospholipase D may influence Age1 function to alleviate the defect in *age2*Δ *gcs1-4* cells suggests that the ArfGAP activity of Age1 may be stimulated by phosphatidic acid or DAG. To address this possibility directly, we employed an *in vitro* assay to assess the stimulation by Age1ΔN-His_6 of Arf1-bound GTP hydrolysis in the presence or absence of phosphatidic acid or DAG. In our assay, GTP-Arf1 is bound to unilamellar lipid vesicles of uniform size. Substituting 15% of the lipid content with DAG resulted in increased stimulation of GTP hydrolysis, which was greater than that observed when the lipid content was substituted with 15% phosphatidic acid (Fig. 9). At 1 nM Age1ΔN-His_6, the presence of DAG enhanced GTP hydrolysis by 50% compared with what was seen in the absence of DAG. DAG, a downstream product of phospholipase D activity, stimulates the *in vitro* ArfGAP activity of Age1.

DISCUSSION

We show here that an increased abundance of Age1 and various Age1 truncations alleviates the deleterious effects caused by deficient Age2 + Gcs1 activity for post-Golgi transport. These effects of increased Age1 ArfGAP abundance are consistent with previous observations that endocytic transport is delayed in both *age1*Δ *arf1*Δ and *age1*Δ *age2*Δ double-mutant cells (11), suggesting an Age1 involvement in post-Golgi function. Elimination of the N-terminal 90 residues of Age1, external to the ArfGAP domain, improved the ability of the Age1 protein to alleviate the temperature sensitivity of *age2*Δ *gcs1-4* double-mutant cells. Hence, the N-terminal re-

gion of Age1, specifically the first 90 residues, has a negative effect on Age1 function. Sequence analysis of the N-terminal region of Age1 did not reveal a motif that was conserved in the protein sequences of other eukaryotes.

The ability to compensate for the complete absence of Age2 + Gcs1 implies that Age1ΔN and Age1 provide essential functions that are normally supplied by the missing ArfGAP pair. Because Arf activity is essential in yeast (24), one function most likely supplied by Age1 and Age1ΔN is ArfGAP activity to regulate the GTPase cycle of Arf for post-Golgi transport. The Gcs1 ArfGAP also stimulates the interactions between v-SNAREs and Arf1/coatomer as determined by *in vitro* assays (25). These interactions are thought to promote vesicle "priming" to produce v-SNARE-equipped vesicles competent to deliver cargo to target compartments. In the absence of the proteins that normally stimulate incorporation of v-SNAREs into transport vesicles, Age1 and Age1ΔN may be capable of fulfilling this function. Thus, Age1 may support vesicular transport through ArfGAP activity and/or as a priming component for vesicle maturation.

A particularly informative finding here is that increased dosage of the *SFH2* gene, encoding a phosphatidylinositol transfer protein, restores growth to cells lacking the otherwise essential Age2 + Gcs1 ArfGAP pair. Routt *et al.* (16) characterized a phosphoinositide metabolic pathway fed by increased *SFH2* dosage that augments the delivery of substrates to lipid kinases, which in turn produce phosphoinositides stimulatory for phospholipase D activity. The alleviating effect of increased *SFH2* dosage on *age2*Δ *gcs1-4* temperature sensitivity depends on the phospholipase D gene *SPO14* (Ref. 14 and data not shown). Phospholipase D cleavage of phosphatidylcholine yields phosphatidic acid, which is then hydrolyzed to DAG, and we found that alleviation of *age2*Δ *gcs1-4* temperature sensitivity can also be achieved by supplying DAG exogenously (14). Therefore, the mechanism by which *SFH2* bypasses the need for the Age2 + Gcs1 ArfGAP pair is likely through Sfh2 enhancement of a phospholipid metabolic pathway leading to phospholipase D.

Surprisingly, increasing the dosage of genes encoding downstream effectors of Sfh2 did not alleviate the temperature sensitivity of *age2*Δ *gcs1-4* double mutants. In contrast to our earlier observation (14), a multicopy *SPO14* plasmid failed to relieve the temperature sensitivity of *age2*Δ *gcs1-4* cells. Further analysis indicated that our initial observation was due to a second-site spontaneous mutation independent of the *SPO14* plasmid. Our findings, therefore, suggest that alleviation of *age2*Δ *gcs1-4* temperature sensitivity is due to increased flux through the Sfh2-mediated phosphoinositide metabolic pathway rather than increased activity of pathway proteins downstream of Sfh2.

Effective *SFH2*-mediated relief of the lethality of the *age2*Δ *gcs1*Δ combination depends on the chromosomal copy of *AGE1,* implying that *SFH2* bypass acts through Age1. Furthermore, this bypass raises the possibility that the alleviation of *age2*Δ *gcs1-4* growth defect by a multicopy *SFH2* plasmid is independent of the *gcs1-4* protein. Instead, the alleviation by *SFH2* may operate mainly, if not exclusively, through Age1. We found that purified Age1ΔN protein binds to several phospholipids and Age1-GFP, expressed at high or low levels, localized to Golgi and endosomal compartments. Therefore, Age1 may be regulated by phospholipid composition at the membranes of post-Golgi compartments.

A multicopy *AGE1* plasmid was found to overcome the lethality of the *age2*Δ *gcs1*Δ combination even in cells lacking the *SFH2* gene. This finding suggests that the remaining Sfh proteins, known to have overlapping function with Sfh2 (16), may provide compensatory activity. Consistent with this idea, increasing *SFH3, SFH4,* and *SFH5* dosage also alleviates the temperature sensitivity of *age2*Δ *gcs1-4* cells, albeit poorly in comparison to *SFH2* (14).

We show here that the phospholipase D enzyme Spo14, which is required for the alleviation of *age2*Δ *gcs1-4* temperature sensitivity by increased *SFH2* gene dosage (14), is also required for the alleviation of that temperature sensitivity by *AGE1* dosage, reinforcing the idea that phosphatidic acid and/or DAG, the immediate and downstream products of phospholipase D activity, enhance Age1 activity. Interactions between ArfGAPs, specifically Gcs1, and phospholipase D have previously been described for sporulating cells, where Gcs1 is required for phospholipase D function during formation of the prospore membrane (26). The *in vitro* GAP activities of Gcs1 and its rat ArfGAP1 ortholog are stimulated by phosphatidylinositol 4,5-diphosphate, phosphatidic acid, and most effectively, DAG (12, 23). In similar fashion, we show here that long-chain DAG also stimulates the *in vitro* ArfGAP activity of Age1. The genetic interactions identified here among *SFH2*, *SPO14*, and *AGE1* and the stimulation of Age1 *in vitro* ArfGAP activity by DAG suggest a mechanism for Age1-mediated effects on post-Golgi vesicular transport; an Sfh2-mediated phosphoinositide pathway increases the abundance of phospholipids to activate phospholipase D activity, which in turn augments the pool of DAG to stimulate the ArfGAP activity of Age1 at post-Golgi membranes.

Our data suggest that Age1, like Gcs1, may be recruited to membranes through recognition of localized lipid packing and the resulting membrane topology. Gcs1 protein has an ArfGAP1 lipid packing sensor motif that regulates recruitment to membranes of high curvature (13). Although Age1 lacks an obvious ArfGAP1 lipid packing sensor motif, the involvement of phospholipase D and DAG in Age1 activity suggests that Age1 might also be recruited to highly curved membranes; the products of phospholipase D activity contribute to high membrane curvature (27). Because the overexpression of *SFH2* or the deletion of *SPO14* did not alter the Golgi/endosomal punctate staining of Age1-GFP, changes in membrane lipid content may affect the putative recruitment of Age1 at a local level that cannot be discerned by fluorescence microscopy.

The effect of depleting the cellular pool of DAG in mammalians cells has been reported (28). A diminished DAG pool results in the inhibition of Golgi-to-endoplasmic reticulum retrograde transport as a consequence of decreased abundance of ArfGAP1 at Golgi membranes. This decrease in Golgi-localized ArfGAP1 is associated with an accumulation of coat protein complex I-coated buds, implying that ArfGAP1 is required to complete membrane fission as part of

the process of transport-vesicle production. The consequences of depleted DAG pools in mammalian cells raises the possibility that DAG pools in yeast regulate the recruitment of Age1 ArfGAP to participate in the production of transport vesicles at post-Golgi membranes.

In summary, increasing the activity of a phosphoinositide pathway can restore viability to *age2*Δ *gcs1*Δ mutant cells lacking the essential Age2 + Gcs1 post-Golgi ArfGAP pair. The phospholipids generated lead to the activation of phospholipase D, and the lipid products then stimulate Age1 activity to provide essential post-Golgi ArfGAP function.

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