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The Gcs1 Arf-GAP Mediates Snc1,2 v-SNARE Retrieval to the Golgi in Yeast

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Gcs1 is an Arf GTPase-activating protein (Arf-GAP) that mediates Golgi–ER and post-Golgi vesicle transport in yeast. Here we show that the Snc1,2 v-SNAREs, which mediate endocytosis and exocytosis, interact physically and genetically with Gcs1. Moreover, Gcs1 and the Snc v-SNAREs colocalize to subcellular structures that correspond to the *trans*-Golgi and endosomal compartments. Studies performed in vitro demonstrate that the Snc-Gcs1 interaction results in the efficient binding of recombinant Arf1Δ17N-Q71L to the v-SNARE and the recruitment of purified coatomer. In contrast, the presence of Snc had no effect on Gcs1 Arf-GAP activity in vitro, suggesting that v-SNARE binding does not attenuate Arf1 function. Disruption of both the *SNC* and *GCS1* genes results in synthetic lethality, whereas overexpression of either *SNC* gene inhibits the growth of a distinct subset of COPI mutants. We show that GFP-Snc1 recycling to the *trans*-Golgi is impaired in *gcs1*Δ cells and these COPI mutants. Together, these results suggest that Gcs1 facilitates the incorporation of the Snc v-SNAREs into COPI recycling vesicles and subsequent endosome–Golgi sorting in yeast.

INTRODUCTION

Protein and lipid transport between intracellular compartments is required for the functional and structural integrity of organelles in eukaryotic cells. This transport is mediated by carrier vesicles generated by protein-based coat complexes. The COPI coat consists of the Arf1 small GTPase and coatomer and confers intra-Golgi and Golgi-to-ER retrograde transport (Kreis *et al.*, 1995; Kirchhausen, 2000; Spang, 2002; Nie *et al.*, 2003). Coatomer consists of seven subunits: αCOP (Sec33, 160 kDa); βCOP (Sec26, 110 kDa); β′COP (Sec27, 102 kDa); γCOP (Sec21, 98 kDa); δCOP (60 kDa); ϵCOP (Sec28, 35 kDa); and ζCOP (20 kDa), which are conserved from yeast to mammals. Coatomer subunits can be divided into two subcomplexes: the B subcomplex (COPI B) composed of the α, β′, and ϵ subunits; and the F subcomplex (COPI F), consisting of the β, δ, γ, and ζ subunits (Eugster *et al.*, 2000; McMahon and Mills, 2004). Interestingly, the γ subunit of COPI F shows structural similarity to components of the clathrin adaptor, AP2 (Hoffman *et al.*, 2003), whereas COPI B has been suggested to be clathrinlike (McMahon and Mills, 2004).

Arf GTPases undergo a cycle of GTP binding and hydrolysis to regulate vesicle formation from a variety of intracellular compartments, such as the Golgi and endosomes (Kreis *et al.*, 1995; Spang, 2002; Nie *et al.*, 2003). In the GTP-bound and myristoylated form, Arf1 becomes membrane-bound and drives COPI vesicle formation in vitro from Golgi membranes and defined liposomes (Spang *et al.*, 1998). Membrane-localized GTP-bound Arf1 recruits coatomer to create a molecular platform capable of deforming the lipid bilayer and leading to vesiculation. Thus, the regulation of Arf function is critical for the initial steps that lead to vesicle biogenesis.

GTP binding and hydrolysis on Arf is mediated by guanine nucleotide exchange factors and GTPase-activating proteins, known as Arf-GEFs and Arf-GAPs, respectively. Because vesicle uncoating is a prerequisite for fusion, Arf-GAP activity is thought to fulfill a role in the removal of coatomer, although roles in cargo packaging and vesicle formation have also been shown (Nickel *et al.*, 1998; Pepperkok *et al.*, 2000; Lanoix *et al.*, 2001; Rein *et al.*, 2002). Although in vitro studies suggest that myristoylated Arf1 and coatomer are sufficient to drive vesicle formation (Spang *et al.*, 1998; Springer *et al.*, 1999), these studies utilized amounts of protein that are unlikely to be physiological. Thus, other factors may be needed for Arf recruitment to membranes. These factors include p23 family members and SNAREs (Gommel *et al.*, 2001; Rein *et al.*, 2002). Recent work demonstrated that yeast Arf1 interacts directly with the SNAREs involved in ER–Golgi transport in a manner requiring Arf-GAP catalytic activity (Rein *et al.*, 2002; Randazzo and Hirsch, 2004). Moreover, Arf-GAP function was shown to be sufficient for COPI coat recruitment even in the absence of activated Arf (Reinhard *et al.*, 1999; Rein *et al.*, 2002; Yang *et al.*, 2002; Lee *et al.*, 2004). Thus, it has been suggested that Arf-GAP is a component of coatomer (Yang *et al.*, 2002; Lewis *et al.*, 2004) and that its catalytic activity is necessary for coat formation and vesicle production (Lee *et al.*, 2004).

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Table 1. Yeast strains used in this study

Strain	Genotype	Source
AH109	*MAT***a** *trp1-901 leu2-3, 112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1UAS-GAL1TATA-HIS3 GAL2UAS-GAL2TATA-ADE2 URA3::MEL1UAS-MEL1TATA-lacZ*	Clontech
Y153	*MAT***a** *gal4 gal80 his3 trp-902 ade2-101 ura3-52 leu2-3,-112 URA3::GAL-lacZ LYS2::Gal-HIS3*	S. Elledge
RH268-1C	*MAT***a** *can1 his4 leu2 trp1 bar1-1 end4-1*	H. Riezman
GWK8A	*MAT***a** *can1 his3 leu2 trp1 ade2 gcs1::URA3*	P. Poon
GWK8A pRS1-1	*MAT***a** *can1 leu2 trp1 ade2 gcs1::URA3* pRS1-1	P. Poon
RSY1309	*MAT***a** *his3-Δ leu2-3,112 lys2-801 suc2-9 sec21-2*	A. Spang
RSY1312	*MAT***a** *leu2-3,112 trp1 ura3-52 sec27-1*	A. Spang
RDY241	*MATα leu2 ura3 trp1 ade2 his3 lys2 sec28Δ::HIS3*	R. Duden
RDY260	*MATα leu2 ura3 sec33-1*	R. Duden
JG8 T15:85 (*sncΔ*)	*MAT***a** *can1 his3 leu2 snc1::URA3 snc2::ADE8* pTGAL-SNC1	J. Gerst
SP1	*MAT***a** *can1 his3 leu2 trp1 ura3 ade8*	J. Gerst
SP1-SEC7RFP	*MAT***a** *can1 his3 leu2 ura3 ade8 trp1::TRP1::TPI1-SEC7RFP*	J. Gerst
MRY1	*MAT***a** *can1 his3 leu2 trp1 ura3 ade8 gcs1::LEU2*	This study
MRY2	*MAT***a** *can1 his3 leu2 trp1 ura3 ade8 gcs1::URA3*	This study
MRY3	*MAT***a** *can1 his3 leu2 lys2 trp1 ura3 ade2 gcs1::LEU2*	This study
MRY4	*MAT***a** *can1 his3 leu2 lys2 trp1 ura3 ade2 gcs1::URA3*	This study
MRY5 (*sncΔ gcs1Δ*)	*MAT***a** *can1 his3 snc1::URA3 snc2::ADE8 gcs1::LEU2* pTGAL-SNC1	This study
PPY169-4	*MATα leu2Δ-0 lys2Δ-0 his3Δ1 ura3Δ-0 gcs1Δ::Nat-R mfa1ΔMFApr-HIS*	This study
W303-1a	*MAT***a** *can1 his3 leu2 lys2 trp1 ura3 ade2*	J. Hirsch
rcy1Δ	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rcy1Δ::kanMX*	Euroscarf

Rein *et al.* (2002) found that yeast COPI (e.g., Arf1 and coatomer) bound to the Bet1, Bos1, and Sec22 ER–Golgi SNAREs in vitro (Rein *et al.*, 2002). This binding occurred only after preincubation of the SNAREs with either of the two Arf-GAPs known to facilitate ER–Golgi transport (e.g., Gcs1 and Glo3; Poon *et al.*, 1999). As SNAREs are central components of the vesicle docking and fusion machinery (reviewed in Chen and Scheller, 2001), Arf1-SNARE-coat interactions may be required to generate SNARE-equipped fusion-competent vesicles in vivo.

Because Arf1-GTP and coatomer binding to the SNAREs is Arf-GAP–dependent, it implies that Gcs1 and Glo3 may have two distinct functions. The first is to initiate Arf1-GTP binding, presumably by inducing a conformational change in SNARE structure and allowing for coat association. The second function is to uncoat the vesicle after budding has occurred by catalyzing GTP hydrolysis on Arf1. This additional role presumably allows the uncoated vesicles to undergo docking and fusion at the appropriate acceptor compartment.

We have been studying the Snc1 and Snc2 v-SNAREs that participate in both exocytosis and endocytosis in yeast (Protopopov *et al.*, 1993; Gurunathan *et al.*, 2000). These v-SNAREs partner with the Sso1,2 and Sec9 t-SNAREs to mediate exocytic functions (Brennwald *et al.*, 1994; Couve and Gerst, 1994) and with the Tlg1,2 and Vti1 t-SNAREs to mediate endocytic functions (Bryant and James, 2003). Thus, the Snc v-SNAREs, which are members of the synaptobrevin/VAMP family, engage in multiple transport steps and recycle continually between the plasma membrane and *trans*-Golgi via early endosomes (Lewis *et al.*, 2000; Hettema *et al.*, 2003). Efficient Snc1 recycling to the early endosome requires the sorting nexin, Snx4, which is involved in protein retrieval from endosomes to the Golgi (Hettema *et al.*, 2003). Here we show that the Snc1,2 v-SNAREs and Gcs1 Arf-GAP interact physically and genetically, leading to v-SNARE recycling to the *trans*-Golgi. This recycling process appears to involve coatomer, as well as Snx4, and thus, may represent a novel trafficking pathway from sorting endosomes back to the Golgi.

MATERIALS AND METHODS

Media, DNA, and Genetic Manipulations

Yeast were grown in standard growth media containing either 2% glucose or 3.5% galactose. Synthetic complete (SC) and drop-out media were prepared similar to that described (Rose *et al.*, 1990). Standard methods were used for the introduction of DNA into yeast and the preparation of genomic DNA (Rose *et al.*, 1990).

Growth Tests

Yeast were grown on synthetic and rich growth media (Rose *et al.*, 1990). For cold-sensitive growth tests on plates, yeast were grown to stationary phase, normalized for optical density, diluted serially, and plated by drops onto solid medium preincubated at different temperatures. For growth tests involving *sncΔ* or *sncΔ gcs1Δ* cells, which carry a galactose-inducible form of *SNC1*, cells were first grown to stationary phase on galactose-containing synthetic medium. Next, a portion of the cells was shifted to glucose-containing medium for 24 h to induce the *sncΔ* phenotype. Cultures were then normalized for optical density, diluted serially, and plated by drops onto solid medium preincubated at different temperatures. For temperature-sensitive growth of COPI mutants, cells were grown to midlog phase on synthetic medium before normalization, serial dilution, and plating onto solid medium preincubated at different temperatures. Calcofluor resistance was measured by adding between 50 and 150 μg/ml fluorescent brightener 28 (Sigma, St. Louis, MO) to plates and plating serial dilutions of the different strains by drops.

Yeast Strains and Plasmids

Yeast strains are listed in Table 1. Vectors included: pRS313 (*CEN, TRP1*); pRS315 (*CEN, LEU2*); pRS316 (*CEN, URA3*); YCp50 (*CEN, URA3*); pAD11 (*CEN, HIS3*); pRS426 (2μ, *URA3*); pAD4Δ (2μ, *LEU2, ADH1* promoter); and pAD54 and pAD6 (same as pAD4Δ, but containing sequences encoding the HA or Myc epitopes, respectively). Previously described *SNC* expression plasmids included: pADH-SNC1 (Gerst *et al.*, 1992); pADH-SNC2, pADH-HASNC1, and pTGAL-SNC1 (Protopopov *et al.*, 1993); and pADH-mycSNC2 and pADH-HASNC2 (Lustgarten and Gerst, 1999). Previously described plasmids for the bacterial expression of Gcs1 and Arf1 included pPPL21 and pET-Arf1H, respectively (Poon *et al.*, 1996). A plasmid expressing recombinant *N*-myristoyltransferase in bacteria, pACY177/ET3d/yNMT (Haun *et al.*, 1993), was used to create myristoylated Arf1, as described (Poon *et al.*, 1996). Plasmid YIplac204-T/C-SEC7-dsRED.T4 was generously provided by B. Glick (University of Chicago, IL).

Plasmids created for this study are listed in Table 2. Sequences of the oligonucleotides used will be provided on request. Disruption constructs for *GCS1* that do not interfere with adjacent open reading frames were created by amplifying a region corresponding to 1999 bp upstream of the start codon and 1455 bp downstream of the stop codon of *GCS1* from genomic DNA. This PCR product was cloned into pGEM-T-Easy to give pGEM-GCS1. Next, a fragment containing either *URA3* or *LEU2* was cloned into the PmeI-XbaI sites of *GCS1* in pGEM-GCS1. Digestion with PmeI and XbaI resulted in the removal of

Table 2. Expression plasmids used in this study

Plasmid name	Gene	Backbone	Sites	Type	Selectable marker	Created by
pAD54-cSNC1	*cSNC1* (*SNC1* cDNA)	pAD54	SalI-SacI	2μ	*LEU2*	M. Robinson
pAD54-GFP-cSNC1	*GFP* (w/o ATG and STOP)	pAD54-cSNC1	SalI-SacI	2μ	*LEU2*	M. Robinson
pAD54-GFP-SNC2	*GFP* (w/o ATG and STOP)	pADH-HASNC2	SalI-SacI	2μ	*LEU2*	M. Robinson
pGADT7-SNC1	*SNC1*[a]	pGADT7	EcoRI-SacI	2μ	*LEU2*	M. Robinson
pGADT7-SNC2	*SNC2*[a]	pGADT7	EcoRI-SacI	2μ	*LEU2*	M. Robinson
pHADH-mycSNC1	*SNC1*	pAD11	BamHI	*CEN*	*HIS3*	Gerst Lab
pHADH-mycSNC2	*SNC2*	pAD11	BamHI	*CEN*	*HIS3*	Gerst Lab
pRS426-HA-cSNC1	*HA-cSNC1*	pRS426	BamHI	2μ	*URA3*	M. Robinson
pRS426-HA-SNC2	*HA-SNC2*	pRS426	BamHI	2μ	*URA3*	M. Robinson
pRS315-HA-GFP-cSNC1	*HA-GFP-cSNC1*	pRS315	BamHI	*CEN*	*LEU2*	M. Robinson
pRS315-HA-GFP-SNC2	*HA-GFP-SNC2*	pRS315	BamHI	*CEN*	*LEU2*	M. Robinson
pRS316-HA-mRFP-cSNC1	*HA-mRFP-cSNC1*	pRS316	BamHI	*CEN*	*URA3*	R. Kama
pPP381-39	$SNC2^{21\text{-}348}$[a]	pGAD424	BamHI-Sau3A	2μ	*LEU2*	P. Poon
pPPL92	$SNC2^{1\text{-}92}$	pET32mlic				P. Poon
pAD54-GCS1	*GCS1*	pAD54	SalI-SacI	2μ	*LEU2*	M. Robinson
pAD54-DsRedT4-GCS1	*DsRedT4*	pAD54-GCS1	SalI-SalI	2μ	*LEU2*	M. Robinson
pAD54-GFP-GCS1	*GFP* (w/o ATG & STOP)	pAD54-GCS1	SalI-SalI	2μ	*LEU2*	M. Robinson
YCp50-GCS1-DsRedT4	*GCS1-DsRedT4*	YCp50	BamHI	*CEN*	*URA3*	M. Robinson
pRS315-GFP-GCS1	HA-GFP-GCS1	pRS315	BamHI	*CEN*	*LEU2*	M. Robinson
pRS426-HA-GCS1	HA-GCS1	pRS426	BamHI	2μ	*URA3*	M. Robinson
pSH4	*GCS1*	pRS315		*CEN*	*LEU2*	P. Poon
pGBKT7-GCS1	*GCS1*[b]	pGBKT7	NcoI-SalI	2μ	*TRP1*	M. Robinson
pLM60	*GCS1*[b]	pGBD-C2	EcoRI-ClaI	2μ	*TRP1*	L. Murray
pLM61	$GCS1^{1\text{-}6782}$	pGBD-C2	EcoRI-ClaI	2μ	*TRP1*	L. Murray
pLM62	$GCS1^{145\text{-}1059}$[b]	pGBD-C2	EcoRI-ClaI	2μ	*TRP1*	L. Murray
pLM63	$GCS1^{349\text{-}1059}$[b]	pGBD-C2	EcoRI-ClaI	2μ	*TRP1*	L. Murray
pLM64	$GCS1^{409\text{-}1059}$[b]	pGBD-C2	EcoRI-ClaI	2μ	*TRP1*	L. Murray
pLM65	$GCS1^{1\text{-}417}$[b]	pGBD-C2	EcoRI-ClaI	2μ	*TRP1*	L. Murray
pSP10C	$SNC2^{153\text{-}348}$[a]	pGAD-C3	ClaI	2μ	*LEU2*	L. Murray
pGEM-GCS1-LEU2	*gcs1::LEU2*	pGEM-T-Easy	PmeI-XbaI		*LEU2*	M. Robinson
pGEM-GCS1-URA3	*gcs1::URA3*	pGEM-T-Easy	PmeI-XbaI		*URA3*	M. Robinson
pPP269	*GCS1*[b]			2μ	*TRP1*	P. Poon
pPP329	*GCS1*	YEp352		2μ	*URA3*	P. Poon
pRS315-GFP-TLG2	*GFP-TLG2*	pRS315	BamHI	*CEN*	*LEU2*	M. Robinson
YCp50-DsRedT4-AGE2	*DsRedT4-AGE2*	YCp50	BamHI	*CEN*	*URA3*	M. Robinson
pRS313-GFP-YIF1	*HA-GFP-YIF1*	pRS313	BamHI	*CEN*	*TRP1*	R. Kama
pSE1112	*SNF1*[b]			2μ	*TRP1*	P. Poon
pCL1	*GAL4*			2μ	*LEU2*	Clontech

[a] Fused with transactivating domain of Gal4.
[b] Fused with DNA-binding domain of Gal4.

nucleotides 213–592 from the coding region of *GCS1*. Subsequent insertion of either the *URA3* or *LEU2* selectable marker gave plasmids pGCS1::URA3 and pGCS1::LEU2, respectively. A ~6-kb disruption fragment was excised from pGCS1::URA3 or pGCS1::LEU2 by digestion with NotI and was used to transform both wild-type and *snc*Δ null cells.

Synthetic Genetic Analysis

For synthetic genetic analysis (SGA), a query strain (PPY169-4) was constructed by replacing the *GCS1* gene with a nourseothricin-resistance cassette (Goldstein and McCusker, 1999) via homologous recombination. The cassette was created by PCR amplification using oligonucleotides bearing sequences flanking the *GCS1* coding region and plasmid p4339 as a template (Tong *et al.*, 2001). The replacement of *GCS1* in PPY169-4 was verified by PCR analysis. An automated approach was then used to cross strain PPY169-4 with the yeast gene-deletion collection, and the resulting haploid double segregants were screened for synthetic-lethal combinations as described previously (Tong *et al.*, 2001).

Microscopy

GFP and RFP fluorescence in strains expressing the appropriate GFP- and DsRedT4/mRFP-tagged fusion proteins was visualized by confocal microscopy (Bio-Rad, Hercules, CA).

Immunoprecipitation

Protein–protein interactions were monitored by the immunoprecipitation (IP) from cell extracts, as described in (Couve and Gerst, 1994) except that a 10 mM Tris (pH 7.5), 1 mM EDTA buffer was substituted for phosphate-buffered saline. Monoclonal antisera included anti-myc antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-HA antibodies (gift of Michael Wigler, Cold Spring Harbor Laboratory). Anti-myc antibodies were used for IP (4 μl per reaction) and detection (1:1000). Anti-HA antibodies were also used for IP (0.8 μl per reaction) and detection (1:7000). Polyclonal antibodies included anti-Gcs1 antibodies (1:2500). Samples of TCLs and immunoprecipitates were resolved by electrophoresis and detected by Western blotting. Detection was performed using enhanced chemiluminescence (Amersham).

Two-Hybrid Assay

Assessment of the Snc v-SNARE and Gcs1 Arf-GAP interaction in β-galactosidase assays (Figure 1, A and D) was performed using Y153 cells in the yeast two-hybrid assay (Durfee *et al.*, 1993). Transformants were patched onto selective synthetic medium, before being subjected to lifts onto nitrocellulose filters and lysis in liquid nitrogen. β-Galactosidase assays were performed using standard procedures. Assessment of the Snc v-SNARE and Gcs1 Arf-GAP interaction in drop tests (Figure 1B) was performed using AH109 cells in the yeast two-hybrid assay, as described by Durfee *et al.* (1993). Transformants were grown in liquid selective medium, diluted serially, and plated by drops onto solid medium lacking histidine and containing 0–2 mM 3-aminotriazole.

In Vitro Arf-GAP Activity Assays

To assess the effects of Snc2 on Gcs1 Arf-GAP activity in vitro, recombinant His6-tagged Gcs1 (plasmid pPPL21) and His6-tagged Snc2 (plasmid pPPL92), which lacks the transmembrane domain, were expressed in *Escherichia coli*

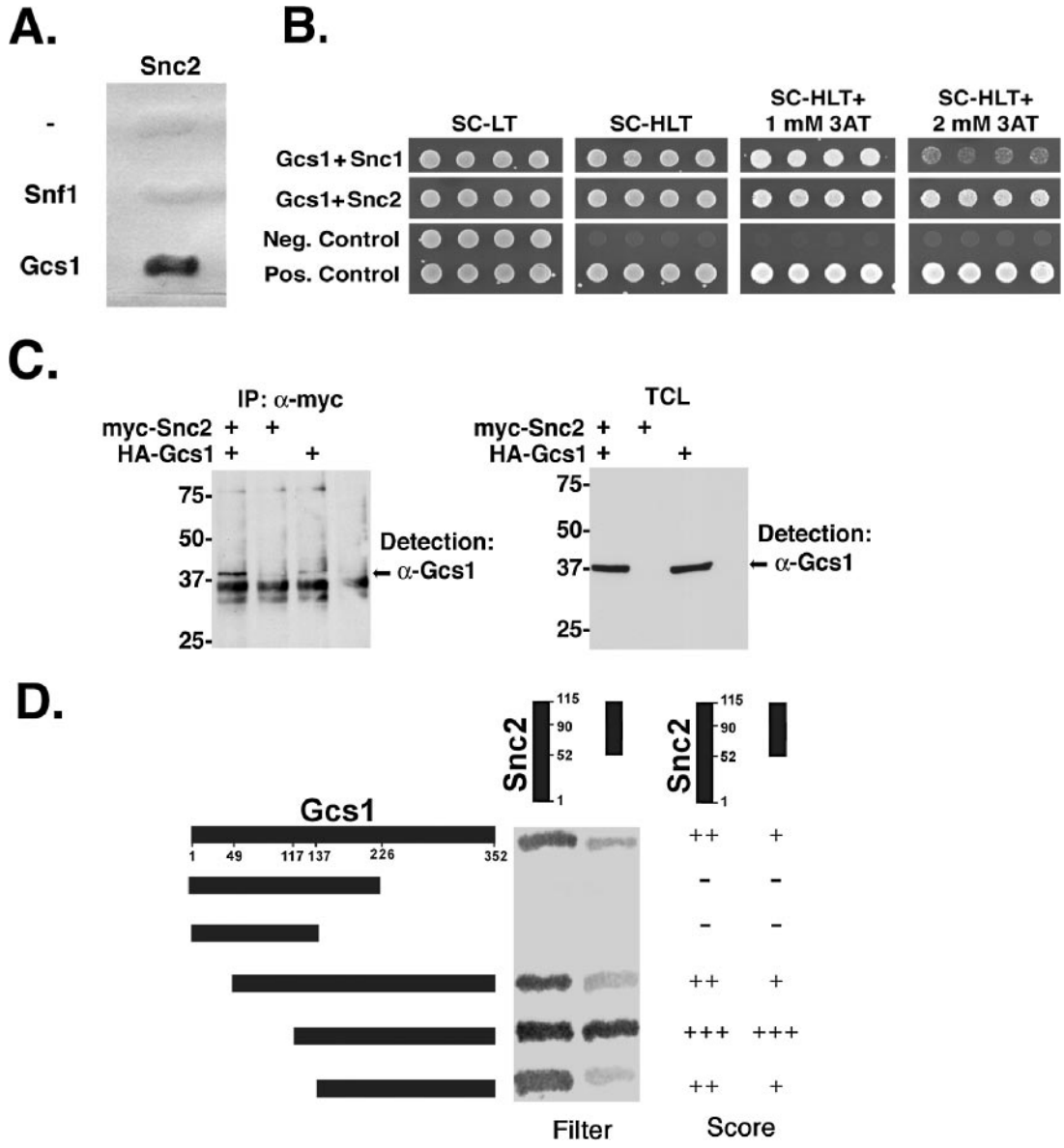

Figure 1. The Snc v-SNAREs interact physically with Gcs1. (A) Snc2 interacts with Gcs1, as assayed using the two-hybrid *lac*Z detection assay. Yeast (Y153) were transformed with a prey plasmid expressing Snc2 fused to the transactivating domain of Gal4 ($Snc2^{8-115}$; plasmid pPP381-39) and bait plasmids, including vectors expressing the DNA-binding domain of Gal4 alone (−; plasmid pGBT9), or fused with Snf1 (Snf1; plasmid pSE1112) or Gcs1 (Gcs1; plasmid pPP269). Cells were grown in patches on selective medium, replica plated onto nitrocellulose filters, lysed in liquid nitrogen, and measured for β-galactosidase activity using standard techniques. (B) Snc1 and 2 interact with Gcs1, as assayed using the two-hybrid 3-AT growth assay. Bait plasmids expressing the Gal4-transactivating domain fused to either Snc1 or Snc2 (Snc1 or Snc2; plasmids pGADT7-SNC1 and pGADT7-SNC2, respectively), along with a plasmid expressing the Gal4 DNA-binding domain fused to Gcs1 (Gcs1; pGBKT7-GCS1), were transformed into AH109 cells and examined for their ability to grow on medium lacking histidine and containing 3-aminotriazole (3-AT). Cells were plated by serial dilution on selective medium (SC-LT), selective medium lacking histidine (SC-HLT), and the same medium with or without the addition of either 1 mM or 2 mM 3-AT. Negative control (Neg. control) consisted of empty vectors expressing the DNA-binding and TA domains alone. A positive control consisted of an empty vector plus a plasmid (pCL1) expressing full-length Gal4. Cells were grown for 48-72 h at 30°C. (C) HA-Gcs1 coimmunoprecipitates with myc-Snc2. *gcs1*Δ (MRY4) cells bearing: (1) a multicopy plasmid expressing HA-tagged Gcs1 (pAD54-GCS1) and a single-copy plasmid expressing myc-tagged Snc2 (pHADH-mycSNC2); (2) either expression plasmid (e.g., pAD54-GCS1 or pHADH-mycSNC2) alone along with the appropriate control vector (e.g., pAD54 or pAD11); or (3) both control vectors (pAD54 and pAD11) were grown to log phase, lysed, and processed for coIP with anti-myc antibodies. The immunoprecipitation lanes (IP) and total cell lysate (TCL) shown were detected with anti-Gcs1 antibody (1:2500). (D) The Arf-GAP domain of Gcs1 and amino terminus of Snc2 are dispensable for the Gcs1-Snc2 two-hybrid interaction. Yeast (Y153) was transformed with prey plasmids expressing either Snc2 or a truncated form of Snc2, $Snc2^{52-115}$, fused to the TA domain of Gal4 (plasmids pPP381-39 and pSP10C, respectively) and bait plasmids expressing Gcs1 ($Gcs1^{1-352}$) or truncated forms of Gcs1 (e.g., $Gcs1^{1-139}$; $Gcs1^{1-226}$, $Gcs1^{49-352}$; $Gcs1^{117-352}$; and $Gcs1^{137-352}$; plasmids pLM65, pLM61, pLM62, pLM63, and pLM64, respectively) fused to the DNA-binding domain of Gal4. Cells were grown in patches on selective medium, replica plated onto nitrocellulose filters, lysed in liquid nitrogen, and measured for β-galactosidase activity using standard techniques. Shown are a representative filter after β-galactosidase detection (Filter) and a qualitative assessment of β-galactosidase activity (Score).

BL21 and purified in native form using standard protocols (Poon *et al.*, 2001). First, 50 ng of purified Gcs1 was mixed with varying amounts of His6-Snc2, ranging from 50 ng to 25 μg, and incubated on ice in 80 μl of 12.5% glycerol, 0.125% bovine serum albumin (fraction V), 1.25 mM DTT, 1.25 mM ATP, 1.25 mM MgCl, 187 mM KOAc, and 31.3 mM MOPS buffer at pH 7.5. After 3 h of incubation, GAP activity was assayed by the addition of 20 μl γ-^{32}P-GTP-bound myristoylated Arf1, incubation at 30°C for 15 min, and subsequent assessment of GTP-hydrolysis, as previously described (Poon *et al.*, 2001).

In Vitro Arf- and Coatomer-binding Assays

Truncated genes encoding SNAREs lacking their transmembrane domains were cloned into vector pETGEXCT (Sharrocks, 1994). N- and C-terminal GST-tagged SNARE fusion proteins were expressed in *E. coli* and purified using standard procedures. Gcs1 and Arf1ΔN17-Q71L expression in *E. coli* and purification were performed as described (Rein *et al.*, 2002). Coatomer was purified from yeast as described (Hosobuchi *et al.*, 1992). Pulldown assays employing immobilized SNARE-GST fusion proteins were performed essentially as described (Rein *et al.*, 2002). In brief, 5 μg SNARE-GST fusion proteins were immobilized onto GSH-agarose (Sigma) and subsequently incubated for 1 h at 4°C with 20 nM recombinant Gcs1 in a total reaction volume of 100 μl in BBP (25 mM HEPES, pH 6.8, 300 mM KOAc, 1 mM DTT, 0.5 mM $MgCl_2$, and 0.2% Triton X-100). Gcs1 was removed from the reaction by three washes with BBP. The beads were incubated with 40 nM coatomer and 7.3 μM recombinant Arf1ΔN17-Q71L for 1 h at 4°C, washed three times with BBP and once with 20 mM HEPES, pH 6.8. The proteins bound to the beads were separated by SDS-PAGE, visualized by Fairbanks staining, and visualized using the Odyssey system (Li-Cor).

RESULTS

The Snc2 v-SNARE Interacts with the Gcs1 Arf-GAP in the Two-Hybrid Assay

The yeast two-hybrid screen was used to identify proteins that interact with the Gcs1 Arf-GAP. Full-length Gcs1 fused to the DNA binding domain of Gal4 was used as bait and screened with a yeast genomic library fused to the transactivating domain of Gal4. One of the candidate prey genes identified in this assay encoded the yeast synaptobrevin/VAMP ortholog, Snc2, as shown in Figure 1A. Interestingly, a systematic genome-wide two-hybrid screen also identified Snc2 as interacting with Gcs1 (Ito *et al.*, 2001). Thus, independent two-hybrid screens suggest that this Arf-GAP and a post-Golgi v-SNARE interact physically. This interaction could be of consequence for post-Golgi vesicular transport as the Snc v-SNAREs mediate both endo- and exocytosis (Protopopov *et al.*, 1993; Gurunathan *et al.*, 2000) and cycle between the plasma membrane and the Golgi (Lewis *et al.*, 2000). Indeed, a role for Gcs1 in post-Golgi vesicle biogenesis has already been proposed (Poon *et al.*, 2001).

To further extend our observations using two-hybrid analysis, we assessed protein–protein interactions with either full-length Snc1 or Snc2 (e.g., Snc1$^{2\text{-}116}$ and Snc2$^{2\text{-}115}$) fused to the transactivating domain of Gal4 and Gcs1 fused to the DNA-binding domain (Figure 1B). When tested for the ability to confer growth in the absence of histidine and in the presence of a metabolic inhibitor of His3 (3-aminotriazole [3-AT]), we found that either v-SNARE could do so in the presence of Gcs1. Thus, both Snc1 and Snc2 interact with this Arf-GAP. However, the Snc1-Gcs1 interaction was more sensitive to higher concentrations of 3-AT (Figure 1B), indicating that it may be weaker than that of Snc2-Gcs1. Thus, both lines of experimentation verify an interaction between the Gcs1 Arf-GAP and the Snc1,2 v-SNAREs.

Snc v-SNAREs Coimmunoprecipitate with Gcs1

Because the Snc v-SNAREs interact physically with Gcs1 in the two-hybrid assay (Figure 1, A and B), we next examined whether these proteins coimmunoprecipitate. In the absence of a functional copy of *GCS1*, yeast cells are unable to reenter the cell cycle at 14°C and are rendered cold-sensitive (Ireland *et al.*, 1994). For the coimmunoprecipitation experiments, we used an HA-tagged version of Gcs1 that was deemed functional by virtue of its ability to confer cold-resistant growth to *gcs1-1* and *gcs1*Δ mutant cells (our unpublished observations). We found that HA-tagged Gcs1 coprecipitated with myc-tagged Snc2 from lysates prepared from wild-type cells expressing these proteins (Figure 1C). A single band corresponding to a molecular mass of about 40 kDa was observed in precipitates from cells expressing both proteins. This signal was specific, but weak, being eliminated by relatively low concentrations of salt (i.e., 130 mM NaCl; our unpublished observations). Thus, these proteins interact physically, but perhaps not tightly, in vivo. Similar results were obtained with myc-Snc1 and HA-Gcs1 (our unpublished observations).

The Arf-GAP Domain of Gcs1 Is Not Required for the Interaction with Snc2

To determine which regions of Gcs1 and Snc2 are required for their physical association, truncated forms of the proteins were tested by two-hybrid analysis (Figure 1D). Deletions in the amino terminus of Gcs1 (Gcs1$^{49\text{-}352}$, Gcs1$^{117\text{-}352}$, and Gcs1$^{137\text{-}352}$), which effectively remove the Arf-GAP domain (amino acid residues 8-129) or portions thereof, did not abolish and even enhanced the interaction with Snc2 isolated in the initial screen (e.g., Snc2$^{8\text{-}115}$). In contrast, the amino terminus of Gcs1 alone (Gcs1$^{1\text{-}139}$ and Gcs1$^{1\text{-}226}$) conferred no β-galactosidase activity when coexpressed with Snc2. This lack of interaction was not due to insufficient protein expression because Western blot analysis indicated that the amino terminal Gcs1 fusion protein was as abundant as the full-length Gcs1 fusion (our unpublished observations). These results suggest that the Arf-GAP domain of Gcs1 is dispensable for the interaction with Snc2.

Deletion of the first 51 amino acids of Snc2 did not abolish the interaction with either full-length Gcs1 or its amino terminus–truncated forms. Thus, the Gcs1-interacting domain resides in the carboxy terminus of the v-SNARE, which encompasses the SNARE domain (Fasshauer *et al.*, 1998). As access to the transmembrane region of Snc2 is unlikely in vivo, this result implies that Gcs1 binds to the t-SNARE–interacting domain of the protein.

A Genetic Interaction between the SNC and GCS1 Genes

We next assessed the significance of the Gcs1-Snc interaction in vivo using a genetic approach. We examined whether the combined disruption of the *SNC* and *GCS1* genes leads to synthetic defects, which would indicate a related function in post-Golgi transport. We first disrupted *GCS1* in the same genetic background (SP1 wild-type cells) as that used to generate the *snc*Δ strain (Protopopov *et al.*, 1993). The disruption of *GCS1* led to cold sensitivity on synthetic and rich medium (YPD) at 15°C in the SP1 background (Figure 2A and our unpublished results), as shown previously in the W303 background (Ireland *et al.*, 1994). The disruption of *GCS1* in SP1 cells also resulted in an inhibition of growth at 37°C on rich medium (our unpublished results).

We examined whether overexpression of the *SNC* genes or reexpression of *GCS1* could confer cold-resistant growth to *gcs1*Δ cells. We found that *HA-GCS1* expression from a multicopy plasmid or *GCS1-RFP* expression from a single copy plasmid could confer growth at 15°C to *gcs1*Δ cells. In contrast, overexpression of the *SNC* genes had no effect and could not confer growth at 15°C (Figure 2A). This verified that the *GCS1* expression constructs are functional and could restore cold-resistant growth to *gcs1*Δ cells. We note that overexpression of either *HA-GCS1* (Figure 2A) or native *GCS1* (our unpublished observations) from multicopy plasmids has a mild inhibitory

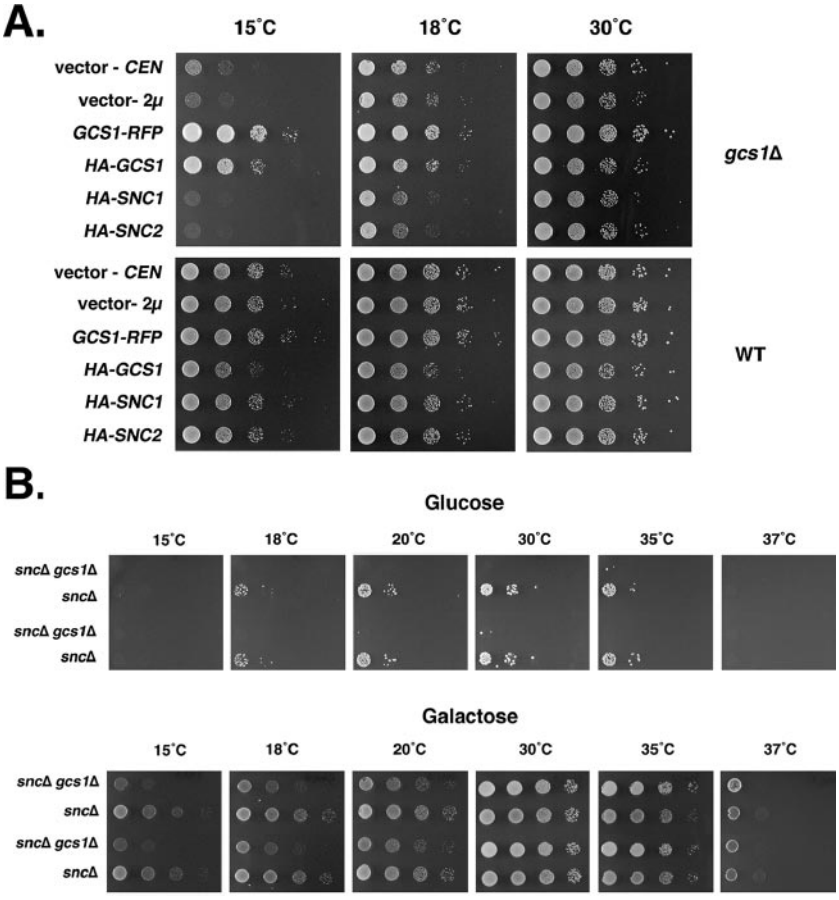

Figure 2. Deletion of both the *SNC1,2* and *GCS1* genes results in lethality. (A) *gcs1*Δ cells are cold-sensitive and are rescued by plasmids expressing *GCS1*. SP1 wild-type cells and a *gcs1*Δ disruption strain (MRY1) were transformed with multicopy plasmids expressing *SNC1* (pRS426-HA-cSNC1), *SNC2* (pRS426-HA-SNC2), *GCS1* (pRS426-HA-GCS1), or a single copy plasmid expressing *GCS1* fused to *RFP* (YCp50-GCS1-DsRedT4) were grown to stationary phase (72 h), plated serially on selective medium at various temperatures, and incubated for 2-11 d. Cells bearing empty multicopy (pRS426; vector: 2μ) or single copy (YCp50; vector: *CEN*) plasmids were used as controls. *gcs1*Δ cells were grown at 15°C for 11 d; at 18°C for 6 d; and at 30°C for 2 d. Wild-type cells were grown at 15°C for 8 d; at 18°C for 5 d; and at 30°C for 2 d. (B) Combined *snc*Δ and *gcs1*Δ null mutations are synthetically lethal. *snc*Δ (JG8 T15:85) or *snc*Δ *gcs1*Δ (MRY5) cells, which both bear plasmid pTGAL-SNC1, were grown to stationary phase (48 h) on galactose-containing medium (which induces expression from the *GAL*-inducible *SNC1* gene). Cells were diluted to 1 OD_{600}/ml either in galactose-containing medium or glucose-containing medium (to deplete Snc1) for 24 h, before being serially diluted and plated onto either galactose-containing (Galactose) or glucose-containing (Glucose) solid medium. Cells were grown for 4-11 d on glucose at various temperatures: 15°C, 11 d; 18°C, 8 d; 20°C, 7 d; 30°C, 4 d; 35°C, 4 d; and 37°C, 6 d. Cells were grown for 3-9 d on galactose at various temperatures: 15°C, 9 d; 18°C, 6 d; 20°C, 3 d; 30°C, 3 d; 35°C, 3 d; and 37°C, 4 d.

effect on wild-type cells at lower temperatures (15 and 18°C; Figure 2A). This is likely to result from a general inhibitory effect the Arf-GAP has on Arf function.

Next, we disrupted *GCS1* in *snc*Δ null cells, which are temperature-sensitive on synthetic medium and unable to grow on amino acid–rich medium (Protopopov *et al.*, 1993). To maintain viability, *SNC1* was expressed in the *snc*Δ *gcs1*Δ strain under the control of an inducible *GAL* promoter and the cells grown on galactose-containing medium. On shifting the cells to glucose-containing medium the *snc*Δ phenotype becomes apparent after 12 h (Protopopov *et al.*, 1993). We grew *snc*Δ and *snc*Δ *gcs1*Δ cells to stationary phase and examined their growth upon plating onto solid medium at different temperatures. Cells lacking the *SNC* genes alone were both cold- and temperature-sensitive for growth on glucose-containing medium, as previously shown (Protopopov *et al.*, 1993 and our unpublished results), but could grow slowly at 18–35°C. In contrast, cells disrupted in both the *SNC* v-SNARE genes and the *GCS1* Arf-GAP gene were unable to grow on glucose-containing medium at any temperature (Figure 2B). However, both *snc*Δ and *snc*Δ *gcs1*Δ cells were fully viable when maintained on galactose-containing medium (Figure 2B), whereon Snc1 is expressed. Thus, synthetic lethality is observed between the *gcs1*Δ and *snc*Δ mutations and suggests that these gene products provide related functions that allow for an essential transport activity.

Gcs1 Colocalizes with the Snc v-SNAREs

As the Snc v-SNAREs and Gcs1 interact both physically and genetically, we examined whether these proteins colocalize in yeast (Figure 3). We used functional (Figure 2A and our unpublished observations) green and red fluorescent protein (GFP and RFP, respectively) derivatives expressed from low copy plasmids. Both GFP-Snc1 and GFP-Snc2 strongly labeled the yeast plasma membrane and some cytoplasmic structures and weakly labeled vacuolar membranes (Figure 3A). This corresponds well with the pattern of labeling described for GFP-Snc1 (Gurunathan *et al.*, 2000; Lewis *et al.*, 2000). The cytoplasmic structures observed earlier with GFP-Snc1 included the *trans*-Golgi and endosomes (Lewis *et al.*, 2000; Galan *et al.*, 2001). Importantly, these v-SNAREs have been demonstrated to recycle back to the *trans*-Golgi via early endosomes in a manner dependent on the involvement of Rcy1, Ric1, and Ypt6, which mediate endosome–Golgi transport (Lewis *et al.*, 2000; Galan *et al.*, 2001; Lafourcade *et al.*, 2004). Rcy1 is an F-box protein involved in recycling at endosomes (Wiederkehr *et al.*, 2000), whereas Ric1 is part of the GEF complex for the rab-GTPase Ypt6 at the TGN (Siniossoglou *et al.*, 2000). In our colabeling experiments, we found that both GFP-Snc1 and GFP-Snc2 localized with Gcs1-RFP at a subset of large punctate structures present in the cytoplasm, but not at the plasma membrane (Figure 3A). The intracellular localization of Gcs1 was previously unknown, though its role in post-Golgi vesicle biogenesis (Poon *et al.*, 2001) suggested a possible Golgi or endosomal localization. Thus, the internal compartments colabeled by Gcs1-RFP and GFP-Snc1 (or GFP-Snc2) are likely to be late Golgi or endosomal in nature.

To better address the intracellular localization of Gcs1, we examined the location of Gcs1 tagged either at the carboxy terminus with RFP or at the amino terminus with GFP along with markers of other endomembrane compartments or

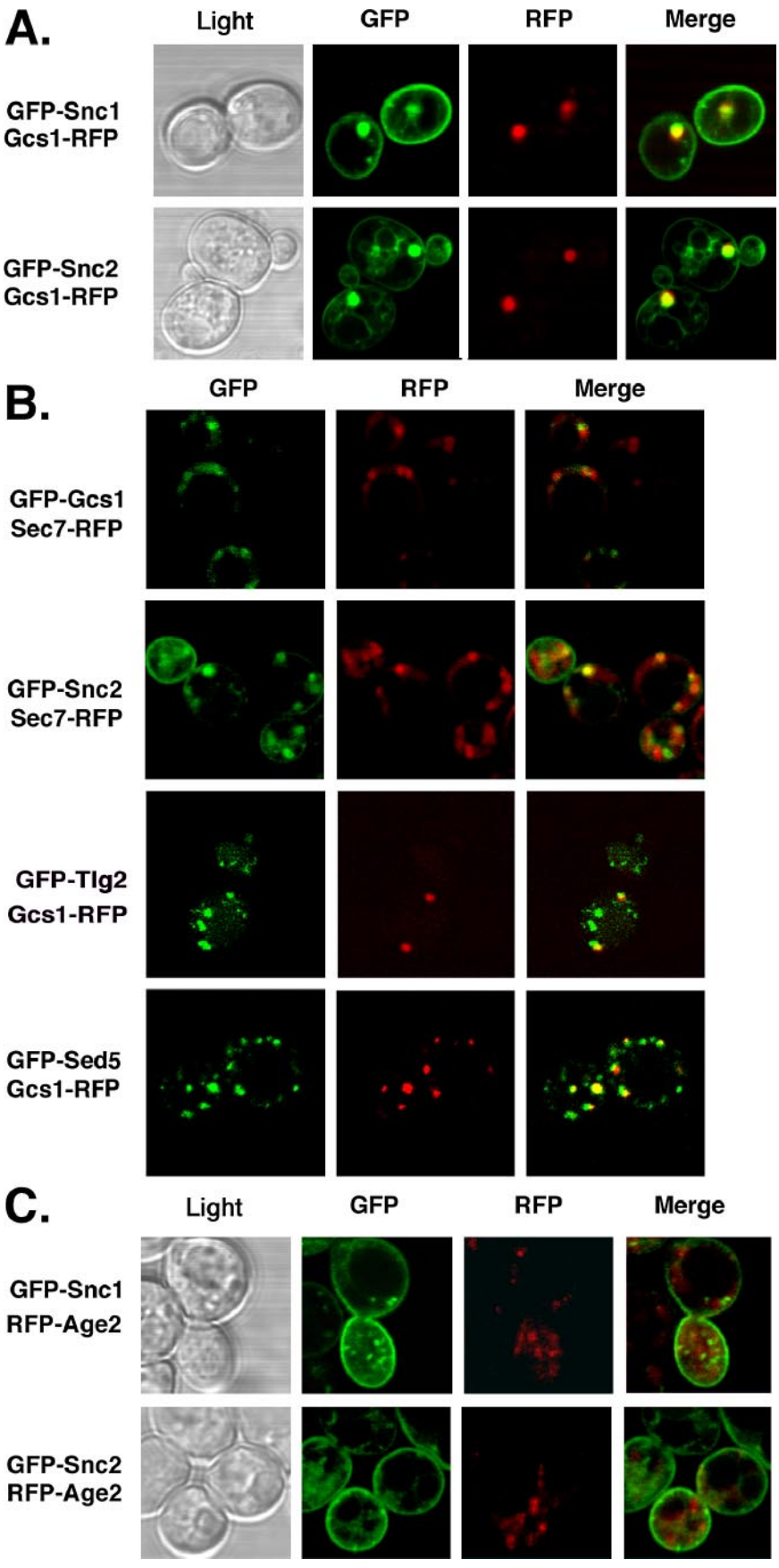

Figure 3. Snc v-SNAREs and Gcs1 colocalize to late Golgi and endosomal compartments. (A) A subset of internalized Snc1,2 v-SNAREs colocalizes with Gcs1. Single-copy plasmids producing Snc1 or Snc2 tagged with GFP at their amino termini (GFP-Snc1; pRS315-GFP-cSNC1 and GFP-Snc2; pRS315-GFP-SNC2) were transformed into wild-type yeast having a single-copy plasmid expressing Gcs1 tagged at its carboxy terminus with RFP (Gcs1-RFP; YCp50-GCS1-DsRedT4). Cells were visualized by confocal microscopy to show excitation at the appropriate wavelengths ("GFP" and "RFP" windows, respectively). "Merge" represents the merger of the windows. (B) Gcs1 colocalizes with Golgi and early endosomal markers. Wild-type cells bearing an integrated copy of Sec7-RFP (SP1-SEC7RFP), a late Golgi marker, were transformed with single-copy plasmids expressing Gcs1 or Snc2 tagged at the amino terminus with GFP (GFP-Gcs1; pRS315-GFP-GCS1 and GFP-Snc2; pRS315-GFP-SNC2, respectively) and examined for fluorescent marker colocalization. Similarly, cells bearing a single-copy plasmid expressing Gcs1-RFP (YCp50-GCS1DsRedT4) were transformed with plasmids expressing GFP-tagged Tlg2 (GFP-Tlg2; pRS315-GFP-TLG2) or Sed5 (GFP-Sed5; pRS315-GFP-SED5) and examined for colocalization. (C) The Snc1,2 v-SNAREs do not colocalize with Age2. Single-copy plasmids expressing GFP-Snc1 (pRS315-GFP-cSNC1) or GFP-Snc2 (pRS315-GFP-SNC2) and Age2 tagged at the amino terminus with RFP (RFP-Age2; YCp50-DsRedT4-AGE2) were transformed into wild-type cells and visualized by confocal microscopy.

structures (Figure 3B). We found that Gcs1 colocalized in part with both Sed5 and Sec7, which are early and late Golgi markers, respectively (Franzusoff *et al.*, 1991; Hardwick and Pelham, 1992). This colocalization was limited to a subset of punctate structures and correlated well with the known functional overlap between Gcs1 and the Glo3 and Age2 Arf-GAPs, which facilitate Golgi–ER and post-Golgi transport, respectively (Poon *et al.*, 1999, 2001). In addition, Gcs1 also colocalized to a subset of compartments labeled by Tlg2 (Figure 3B), a t-SNARE involved in endocytosis and the delivery of proteins to endosomes and the vacuole (Abeliovich *et al.*, 1998; Holthuis *et al.*, 1998; Seron *et al.*, 1998). In contrast, Gcs1 did not colocalize with a preautophagosomal marker, Aut7/Apg8 (Kim *et al.*, 2001; our unpublished observations). Together, these results imply that Gcs1 resides in both Golgi and endosomal compartments.

We also found that Snc2 colocalized well with Sec7 (Figure 3B), as predicted (Lewis *et al.*, 2000; Galan *et al.*, 2001). This colocalization was observed at numerous cytoplasmic structures that are thought to correspond to the *trans*-Golgi (Lewis *et al.*, 2000; Galan *et al.*, 2001), but which may include early endosomes. In contrast, there was little to no colocalization between GFP-Snc1 (or GFP-Snc2) and the Age2 Arf-GAP (Figure 3C). This implies that the site of interaction between the Snc v-SNAREs and Gcs1 may be distinct from the intracellular locale governed by Age2. Although we cannot exclude the possibility that fluorescent protein-tagged Gcs1, as well as the tagged organellar markers, do not induce changes in the morphology and distribution of intracellular trafficking compartments, our results are consistent with Gcs1 and Snc v-SNAREs colocalizing at late or post-Golgi (endosomal) structures.

Snc-Gcs1 Interactions Do Not Alter Arf-GAP Activity In Vitro

Because the Snc v-SNAREs and Gcs1 interact and colocalize at an endosomal compartment, we examined the functional consequences of this interaction. First we examined whether Snc2 v-SNARE binding to Gcs1 alters its ability to activate GTP hydrolysis by Arf1. Recombinant His_6-Snc2, lacking the transmembrane domain, was mixed with His_6-tagged Gcs1 in the presence of recombinant myristoylated Arf1 prebound to GTP and subsequent GTP hydrolysis was measured in vitro. It was found that the presence of Snc2 had no effect on GTP hydrolysis by Arf1 (Figure 4A). This suggests that the Snc v-SNAREs do not alter Gcs1-mediated GTP-hydrolysis.

Snc-Gcs1 Interactions Promote Arf1 Binding and Coatomer Recruitment In Vitro

Because the Snc v-SNAREs physically interact with Gcs1 (Figure 1), but do not regulate Gcs1 GAP activity in vitro (Figure 4A), we examined whether Gcs1 modulates the binding of Arf1 to the v-SNARE. Previous work suggested that Arf-GAP-SNARE interactions prime the v-SNARE to bind Arf1 and allow for subsequent coat recruitment (Rein *et al.*, 2002). Specifically, the catalytic interaction of either Glo3 or Gcs1 with the ER–Golgi v-SNAREs (e.g., Bet1, Bos1, and Sec22) resulted in the binding of Arf1ΔN17-Q71L and the acquisition of coat in a nucleotide- and GAP activity–independent manner in vitro. Thus, Arf-GAP-SNARE interactions have been proposed to recruit both SNAREs and coat proteins to the sites of vesicle formation (Rein *et al.*, 2002).

To determine whether Gcs1 fulfills a similar role with the Snc v-SNAREs, we substituted Snc1 or Snc2 for the ER–Golgi v-SNAREs in this in vitro binding assay. First, we examined whether GST-tagged Snc1,2 v-SNAREs could precipitate Gcs1 in vitro (Figure 4B). We found that Snc1-GST and Snc2-GST could precipitate recombinant Gcs1 as well as Sec22-GST (Figure 4B). Next, when used in the in vitro binding assay either GST-Snc1 or Snc1-GST was readily able to recruit purified coatomer in a Gcs1-dependent manner (Figure 4C; lanes 4, 6, 10, and 12). This reaction does not appear to require Arf1, as COPI was recruited in its absence (see lanes 4 and 10, Figure 4C). This property was observed previously with the ER–Golgi SNAREs (Rein *et al.*, 2002). Arf1ΔN17-Q71L binding to Snc1 or to Snc2 (our unpublished observations), however, was dependent on the addition of Gcs1 to the in vitro assay (Figure 4C; lanes 2, 6, 8, and 12). In contrast, GST alone was unable to recruit either Arf1ΔN17-Q71L or coatomer (Figure 4D). Thus, the Snc v-SNAREs interact with both Arf and coatomer, which is suggestive of a post-Golgi role for this complex in vivo.

Synthetic Lethal Interactions with gcs1Δ

To identify factors that facilitate Gcs1-mediated post-Golgi transport, we used a nonbiased screen to select for gene deletions that are synthetically lethal in combination with *gcs1Δ*. By exhaustive screening against a yeast deletion library of nonessential genes, we found that a number of deletions are synthetically lethal in combination with the *gcs1Δ* mutation. These included deletions in genes encoding the other known Arf-GAPs, *GLO3* and *AGE2* (Poon *et al.*, 1999, 2001). In addition, the screen identified numerous genes encoding factors involved in Golgi–endosome transport, including: *TLG2*, *VPS1*, *VPS51*, and *YPT31* (see Table 3). These findings are consistent with a role for Gcs1 in post-Golgi transport.

Snc v-SNARE Overexpression Inhibits the Growth of Certain Coatomer Mutants

Because the Snc v-SNAREs recruit COPI coat components in a Gcs1-dependent manner in vitro (Figure 4C), we examined the significance of this in vivo. Overexpression of the *SNC* genes is known to rescue mutations affecting partner t-SNAREs from the plasma membrane (i.e., *sec9-4* and *sso2-1*) as well as a mutation in a SNARE regulator (*sec1-1*; Couve and Gerst, 1994; Gerst, 1997). We decided to examine the effect of *SNC1* and *SNC2* overexpression in mutants of coatomer (i.e., *sec21-2*, *sec27-1*, and *sec33-1*). Interestingly, the growth of *sec27-1* and *sec33-1* cells, which express mutated components of the clathrinlike B subcomplex of COPI (McMahon and Mills, 2004), was significantly inhibited by the overexpression of either *SNC1* or *SNC2* (Figure 4E). In contrast, the overexpression of either *SNC1* or *SNC2* in *sec21-2* cells, which expresses a mutated component of the adaptor-like F subcomplex of COPI (McMahon and Mills, 2004), had no deleterious effect (Figure 4F). Thus, we could identify genetic interactions between the Snc v-SNAREs and components of the COPI B subcomplex. Because Gcs1 interacts physically with the Snc proteins, we addressed the possibility that overproduction of the v-SNAREs could cause a decrease in the Arf-GAP available for membrane transport. However, an increase in *GCS1* expression did not alleviate the effects of *SNC* overexpression in the COPI B mutants (Figure 4, D and E).

The Deletion of GCS1 Alters GFP-Snc1 Recycling

Because of a functional overlap with Age2, Gcs1 was proposed to play a role in post-Golgi protein sorting (Poon *et al.*, 2001). In addition, the present study shows the colocalization of Gcs1 with Golgi and endosomal markers, as well as physical and genetic interactions with v-SNAREs that facilitate post-Golgi transport. Because GFP-Snc1 recycles through early

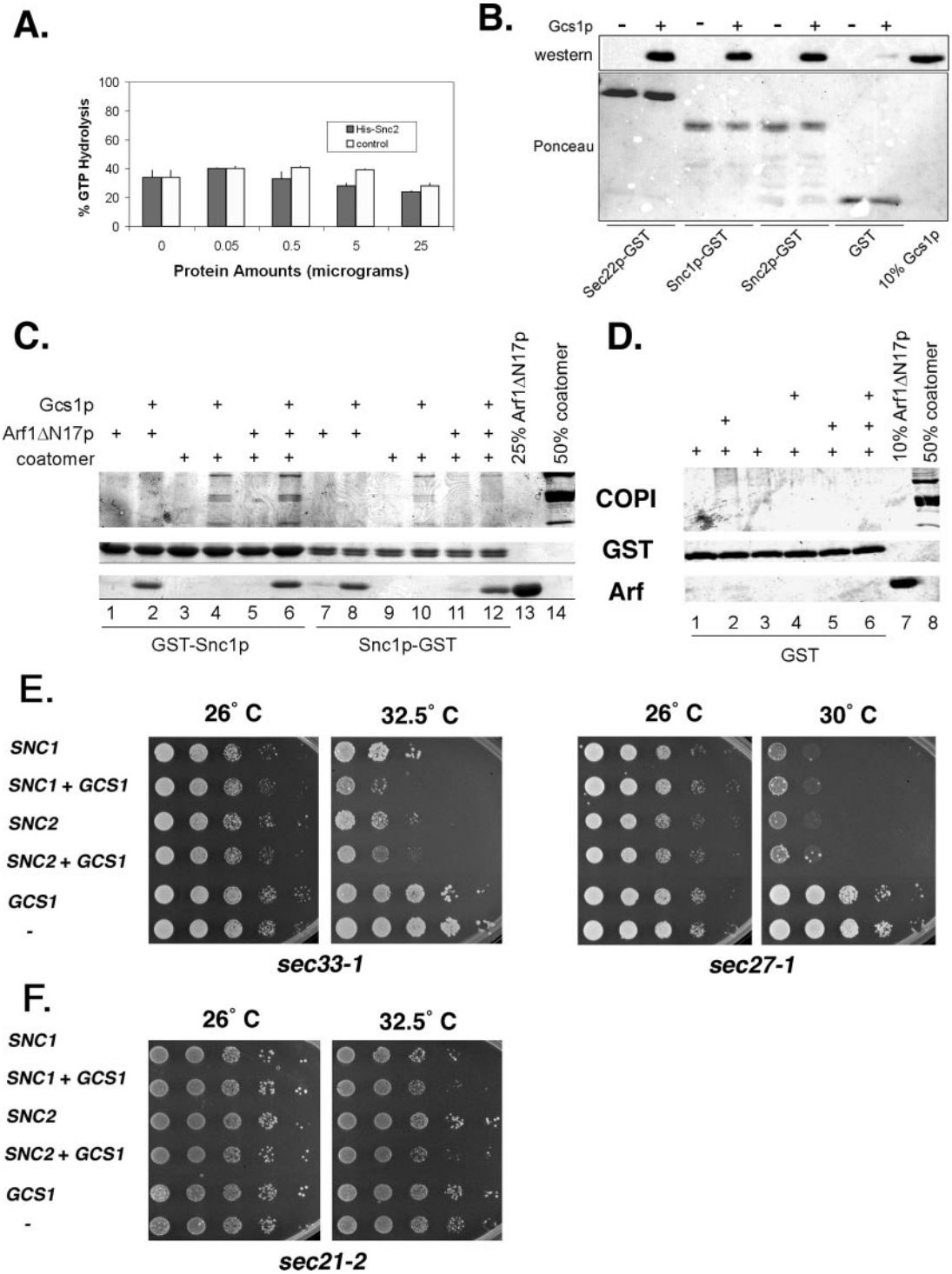

Figure 4. Snc v-SNAREs bind to Arf1ΔN17-Q71L and coatomer in a Gcs1-dependent manner in vitro and interact genetically with specific COPI subunits. (A) Recombinant Snc2 does not alter Gcs1 Arf-GAP activity in vitro. Recombinant His_6-tagged Gcs1 and His_6-tagged Snc2 (His-Snc2) or His_6-tagged Gcs1 alone (control) were mixed with GTP-bound myristoylated Arf1, and Arf-GAP activity was measured in vitro as described (see *Materials and Methods*). (B) Recombinant Gcs1 binds to the Snc v-SNAREs in vitro. Purified recombinant Snc1-GST, Snc2-GST, Sec22-GST, or GST alone (5 μg) were incubated with or without recombinant Gcs1 (20 nM) and a GST-pull down assay was performed. Samples subjected to SDS-PAGE and analyzed in immunoblots with anti-Gcs1 antibodies. Ponceau staining of the nitrocellulose filter after gel transfer is shown as a control for the amounts of GST fusion proteins added. Ten percent of added Gcs1 is shown as a control for loading. (C) Recombinant Snc1 binds to Arf1ΔN17-Q71L and purified coatomer in a Gcs1-dependent manner in vitro. Purified recombinant GST-Snc1 or Snc1-GST (5 μg) were incubated with recombinant Gcs1 (20 nM), recombinant Arf1ΔN17-Q71L (7.3 nM), and purified coatomer (40 nM), alone or in combination (see *Materials and Methods*). Binding was carried out for 1 h at 4°C. Samples were resolved on SDS-PAGE gels and visualized by Fairbanks (Coomassie R) staining. Loadings corresponding to 25% of added Arf1ΔN17-Q71L and 50% of added coatomer are shown in lanes 13 and 14, respectively. (D) GST alone does not bind recombinant Arf1ΔN17-Q71L or purified coatomer. Purified GST was incubated with recombinant Gcs1,

Table 3. Genes that are synthetic lethal with *gcs1*Δ

ORF	Gene name	Role
YIL044c	*AGE2*	Membrane trafficking
YDL192w	*ARF1*	Membrane trafficking
YAL026c	*DRS2*	Membrane trafficking
YKL204w	*EAP1*	Translation inhibition
YER122c	*GLO3*	Membrane trafficking
YOR070c	*GYP1*	Membrane trafficking
YMR224c	*MRE11*	DNA nuclease
YJL117w	*PHO86*	Phosphate transport
YGL167c	*PMR1*	Ion transport
YIL067w	*SEC28*	Membrane trafficking
YDR320c	*SWA2*	Membrane trafficking
YOL018c	*TLG2*	Membrane trafficking
YOR115c	*TRS33*	Membrane trafficking
YER151c	*UBP3*	Ubiquitin protease
YKL080w	*VMA5*	Membrane trafficking
YLR447c	*VMA6*	Membrane trafficking
YKR001c	*VPS1*	Membrane trafficking
YKR020w	*VPS51*	Membrane trafficking
YER031c	*YPT31*	Membrane trafficking
YBR111c	*YSA1*	Nuc. diphos. sugar hydrolase
YEL048c		Unknown
YGL081w		Unknown

endosomes back to the Golgi (Lewis *et al.*, 2000), we examined the trafficking of this v-SNARE in cells lacking the *GCS1* gene (Figure 5A). In addition, we also examined GFP-Snc1 localization in *rcy1*Δ cells, which are defective in early endosome–Golgi sorting (Galan *et al.*, 2001), and *end4-1* cells, which are defective in the endocytosis of endocytic markers such as GFP-Snc1 (Lewis *et al.*, 2000). Unlike in wild-type cells, we found that GFP-Snc1 accumulated in intracellular compartments in both *rcy1*Δ and *gcs1*Δ cells, while being restricted to the plasma membrane in *end4-1* cells (Figure 5A). Thus, proper GFP-Snc1 recycling is largely inhibited in the absence of Gcs1. This result is identical to that shown earlier for *rcy1*Δ and other mutations in proteins that facilitate early endosome–Golgi transport (Galan *et al.*, 2001).

To examine whether GFP-Snc1 reaches the late Golgi in *gcs1*Δ cells we performed a colocalization study with GFP-Snc1 and Sec7-RFP in both wild-type and *gcs1*Δ cells (Figure 5B). We found that Snc1 could not colocalize effectively with Sec7 in the absence of *GCS1*. In contrast, these proteins readily colocalize in wild-type cells (Figures 3B and 5B). Similar results were obtained using another Golgi marker, Yif1 (Matern *et al.*, 2000), that colocalizes in part with Gcs1 (our unpublished observations). We found that GFP-Yif1 could not colocalize with mRFP-Snc1 in the absence of *GCS1* (Figure 5C). In contrast, partial colocalization is observed between mRFP-Snc1 and GFP-Yif1 in wild-type cells (Figure 5C). Thus, the ability of Snc1 to recycle to the *trans*-Golgi (e.g., Sec7 compartment) is dependent on the Gcs1 Arf-GAP. We note that both Golgi markers (Sec7, Yif1) appeared to be more widely distributed and less punctate in *gcs1*Δ cells, indicating a possible alteration in Golgi morphology in the absence of Gcs1 function.

Figure 4 (cont). recombinant Arf1ΔN17-Q71L, and purified coatomer, alone or in combination, as described above. Binding and detection were performed as under *Materials and Methods*. (E) *SNC1* and *SNC2* overexpression inhibits the growth of COPI B subcomplex mutants. *sec27-1* and *sec33-1* cells were transformed with multicopy plasmids expressing *SNC1* (pAD54-cSNC1) or *SNC2* (pAD54-SNC2) and either a control vector (pAD54 or pRS426) or a multicopy plasmid expressing *GCS1* (pPP329). Cells were grown to midlog phase on selective medium, diluted serially, and plated on solid medium at different temperatures. (F) *SNC1* or *SNC2* overexpression do not inhibit the growth of a mutant in the COPI F subcomplex. *sec21-2* cells were transformed with plasmids expressing *SNC1* (pAD54-cSNC1) or *SNC2* (pAD54-SNC2) and either a control vector (pAD54) or a multicopy plasmid expressing *GCS1* (pPP329). Cells were grown to midlog phase on selective medium, diluted serially, plated on solid medium and incubated at different temperatures for 48 h.

GFP-Snc1 Localization Is Altered in COPI B and ESCRT Mutants

As GFP-Snc1 retrieval to the Golgi is blocked in *gcs1*Δ cells (Figure 5) and the *SNC* genes interact genetically with mutations in COPI B (Figure 4E), we tested whether mutants in the COPI B subcomplex play a role in Snc1 recycling. We followed the localization of GFP-Snc1 in COPI B mutants and a variety of other cell types (Figure 6). In wild-type cells, we found that GFP-Snc1 gave typical plasma membrane staining that was slightly bud-enriched (Lewis *et al.*, 2000), a process requiring endocytosis (Valdez-Taubas and Pelham, 2003). Similar results were observed in COPI B mutants (e.g., *sec27-1*, *sec28*Δ) and an ESCRT-I mutant (*vps23*Δ at 26°C; however, the extent of plasma membrane labeling seen on the buds of these mutants was considerably stronger than that observed on the buds of wild-type cells. This bud-enriched pattern of labeling differed greatly from GFP-Snc1 labeling of the entire plasma membrane in endocytosis-deficient *end4-1* cells. In addition, it differed from the extensive pattern of internal GFP-Snc1 labeling seen in a COPI F mutant (*sec21-2*), as well as *sec33-1* cells. This is probably because both *sec21-2* and *sec33-1* mutants are impaired in transport through the early secretory pathway at 26°C (Wuestehube *et al.*, 1996), unlike *sec27-1* and *sec28*Δ cells. The internal pattern of GFP-Snc1 labeling was reminiscent of that seen in *rcy1*Δ mutants, which are defective in Snc1 recycling from early endosomes to the Golgi (Galan *et al.*, 2001). Our results imply that Snc1 v-SNARE retrieval and recycling through early endosomes to the Golgi is also impaired in *sec27-1* and *sec28*Δ cells, resulting in their retargeting to the bud plasma membrane.

*gcs1*Δ *Cells Are Calcofluor-sensitive*

Mutations in both the *GCS1* and *AGE2* Arf-GAP genes lead to defects in post-Golgi transport. In particular, combined *gcs1* and *age2* mutations led to the impaired delivery of internalized Ste3 mating-factor receptor and the vital dye, FM4-64, to the vacuole (Poon *et al.*, 2001). This result suggests that Gcs1 might facilitate protein retrieval from endosomes to other organelles.

Here we show that the deletion of *GCS1* strongly affects the recycling of GFP-Snc1 from early endosomes to the Golgi (Figure 5). Yet, cells lacking *GCS1* tend to grow normally on synthetic medium at temperatures above 15°C (Figure 2A) and have only minor defects in protein trafficking to the cell surface (Poon *et al.*, 1996). This suggests that protein export pathways are not markedly affected by loss of the Gcs1 Arf-GAP, as long as the Age2 Arf-GAP is present. To verify that export from the Snc1 recycling compartment is not abolished, we examined whether *gcs1*Δ cells are resistant to calcofluor, a molecule that binds to chitin and inhibits cell growth. Cells that are resistant to calcofluor have either a mutation in chitin synthase III (Chs3) or are defective in Chs3 export from the chitosome, which is analogous to the early endosome (Valdivia *et al.*, 2002). However, we found that *gcs1*Δ cells are generally sensitive to calcofluor, unlike control *chs6*Δ cells (our unpublished observations). This im-

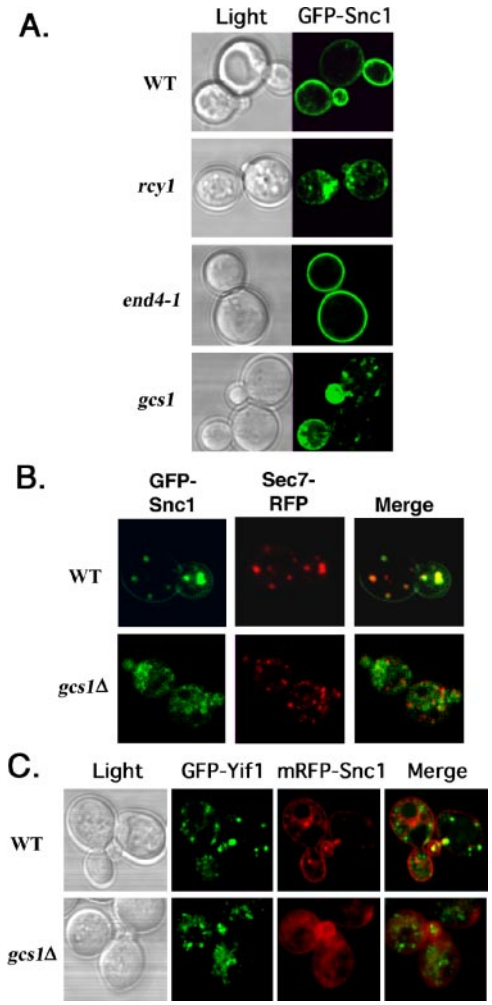

Figure 5. Snc v-SNARE retrieval to the *trans*-Golgi is defective in *gcs1*Δ cells. (A) GFP-Snc1 recycling is defective in *gcs1*Δ cells. Wild-type (SP1), *rcy1*Δ, *gcs1*Δ (MRY2), and *end4-1* cells expressing GFP-Snc1 from a single-copy plasmid (pRS315-GFP-cSNC1) were grown to midlog phase and processed for confocal fluorescence microscopy. (B) GFP-Snc1 is unable to access the Sec7 compartment (e.g., *trans*-Golgi) in the absence of Gcs1. Wild-type (SP1) and *gcs1*Δ (MRY4) cells were transformed with a linearized *SEC7-RFP* integrating plasmid (YIplac204-T/C-SEC7-dsRED.T4) and correctly integrated *SEC7-RFP*–expressing cells were transformed with a single copy plasmid expressing GFP-Snc1 (pRS315-GFP-cSNC1), and examined for fluorescence using confocal microscopy. (C) GFP-Snc1 is unable to access the Yif1 compartment (e.g., Golgi) in the absence of Gcs1. Wild-type (SP1) and *gcs1*Δ (MRY3) cells were transformed with single copy plasmids expressing mRFP-Snc1 (pRS316-mRFP-cSNC1) and GFP-Yif1 (pRS313-GFP-YIF1), and examined for fluorescence using confocal microscopy.

plies that the loss of Gcs1 function does not alter export of Chs3 to the cell surface.

Gcs1 Coimmunoprecipitates with Snx4

Normal cycling of the Snc v-SNAREs requires additional proteins, including the sorting nexins that play a role in retrieval, often as part of multiprotein complexes (Carlton *et al.*, 2005). In particular, the Snx4 sorting nexin is required for the retrieval of GFP-Snc1 from post-Golgi endosomes (Hettema *et al.*, 2003). Because Gcs1 and Snx4 both mediate

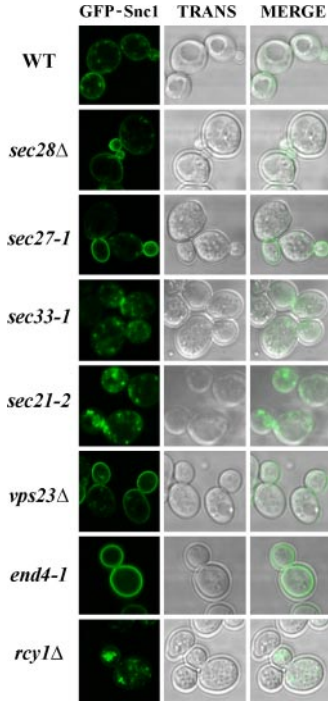

Figure 6. GFP-Snc1 is enriched on the bud plasma membrane in certain COPI mutants and in an ESCRT mutant. Wild-type yeast (W303-1a; WT) and mutants in COPI F (*sec21-2*), COPI B (*sec27-1*, *sec28*Δ, and *sec33-1*), ESCRT (*vps23*Δ), *RCY1* (*rcy1*Δ), and *END4* (*end4-1*) were transformed with a single-copy plasmid expressing GFP-Snc1 (pRS315-GFP-cSNC1) and examined by confocal microscopy.

GFP-Snc1 recycling and physically interact with Snc1 (Hettema *et al.*, 2003 and this study), we determined whether Gcs1 and Snx4 form a complex. We expressed both myc-tagged Snx4 and HA-tagged Gcs1 in wild-type cells and examined whether they can coimmunoprecipitate from cell lysates (Figure 7). We found that Gcs1 coprecipitates with Snx4 in a specific manner. No band corresponding to Gcs1 was detected in precipitates formed in the absence of either myc-Snx4 or HA-Gcs1. In contrast, a control reaction employing the anti-myc antibody to bring down myc-Snc2 demonstrated that it specifically precipitated an HA-tagged protein, in this case the Sso1 t-SNARE (Figure 7). Thus, Gcs1 and Snx4 interact in a specific manner.

DISCUSSION

Coatomer recruitment to Golgi membranes is necessary for formation of the COPI vesicles involved in intra-Golgi and Golgi–ER retrograde transport in yeast and mammals (Kirchhausen, 2000; Spang, 2002; McMahon and Mills, 2004). Despite the established role for coatomer, studies in mammalian cells also describe a post-Golgi role for coatomer and COPI in the transport of proteins to endosomes and multivesicular bodies (Whitney *et al.*, 1995; Aniento *et al.*, 1996; Gu and Gruenberg, 2000; Faure *et al.*, 2004). Coatomer binding in all systems is Arf-dependent and, thus, a mechanism for the recruitment of Arf and Arf-like proteins to different membranes also must depend on specific recruiting factors.

Here we demonstrate that the yeast exo- and endocytic v-SNAREs, Snc1 and Snc2, interact genetically and physically with the Gcs1 Arf-GAP (Figures 1 and 2) and colocalize with Gcs1 to late Golgi and endosomal compartments (Fig-

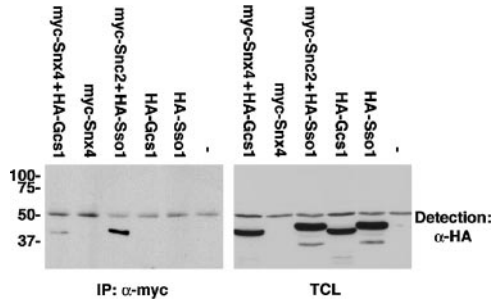

Figure 7. Gcs1 coimmunoprecipitates with Snx4. Wild-type (W303) cells were transformed with plasmids expressing both myc-Snx4 and HA-Gcs1 (pRS426-HA-GCS1 and pAD6-SNX4, respectively), or either myc-Snx4 or HA-Gcs1 alone. Empty vectors (pRS426 or pAD6) were also used as controls. In addition, cells used for a control immunoprecipitation were transformed with plasmids expressing myc-Snc2 and HA-Sso1 (pADH-myc-SNC2 and pRS426-HA-SSO1, respectively) or HA-Sso1 alone. Cells were grown to midlog phase and were processed for immunoprecipitation (see *Materials and Methods*). Immunoprecipitation (IP) was performed with anti-myc antibodies, whereas Western blotting was performed with both anti-myc and anti-HA antibodies. Blots for both the IP reactions and samples (50 μg) of the total cell lysates (TCL) were probed in parallel.

ure 3, A and B). This Arf-GAP has been previously shown to facilitate ER–Golgi and post-Golgi transport (Poon *et al.*, 1999, 2001). Thus, we hypothesize that the Snc v-SNAREs are actively involved in recruiting Gcs1 and, subsequently, Arf1 to these membranes. This idea is supported by in vitro binding data demonstrating the recruitment of coatomer to the Arf-GAP-v-SNARE complex (Figure 4C). One functional consequence of this interaction is retrieval of the Snc v-SNAREs and, perhaps, other cargo proteins to the *trans*-Golgi. Indeed, Snc1 does not reach the *trans*-Golgi, as visualized by Sec7-RFP or GFP-Yif1, in cells lacking *GCS1* (Figure 5, B and C). This role for Gcs1 is similar to that described for Rcy1 and Snx4, which also interact with Snc1 and mediate its retrieval to the *trans*-Golgi (Galan *et al.*, 2001; Hettema *et al.*, 2003; Chen *et al.*, 2005). Consistent with a role for Gcs1 in Snc1,2 recycling, we demonstrate that Gcs1 binds to the Snx4 sorting nexin (Figure 7). This suggests that the v-SNARE recruits a complex involving a sorting nexin, an Arf-GAP, Arf, and a coat for retrieval to the Golgi from endosomal compartments (see model, Figure 8). Thus, in the absence of any of these factors (i.e., Gcs1, Snx4, COPI B, etc.), Snc v-SNARE retrieval to the Golgi is altered.

Other components are also involved in GFP-Snc1 recycling to the Golgi (Lafourcade *et al.*, 2004), including the Ypt31,32 GTPases that facilitate Golgi export (Jedd *et al.*, 1997) and act upstream of Rcy1 (Chen *et al.*, 2005). In earlier work, a correlation between GFP-Snc1 phosphorylation and its presence at the cell surface was demonstrated (Galan *et al.*, 2001). Recently Chen *et al.* (2005) suggested that Ypt31,32 regulate the phosphorylation state of the Snc v-SNAREs, implying that phosphorylation targets these v-SNAREs for recycling (Chen *et al.*, 2005). This method of targeting would seem to be an important mechanism for controlling SNARE recycling and a potential means for facilitating interactions with either Gcs1 or Snx4, for example. However, we have been unable to demonstrate the phosphorylation of either endogenous or epitope-tagged Snc proteins expressed in yeast, either by in vivo labeling or by mobility shift analysis employing alkaline phosphatase treatment using wild-type, *rcy1*Δ, or *end4-1* cells (Couve *et al.*, 1995 and our unpublished

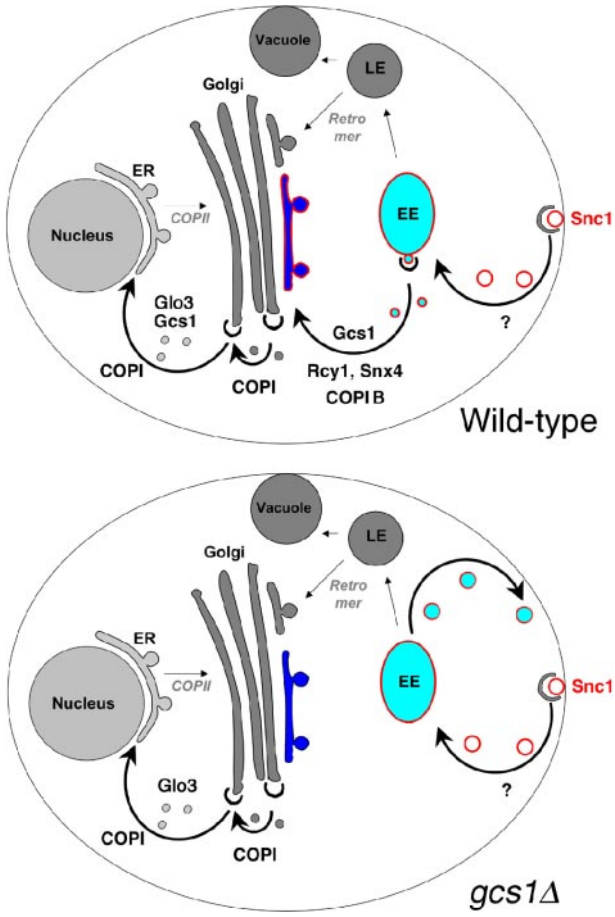

Figure 8. A model for Snc v-SNARE recycling involving Gcs1, Snx4, Rcy1, and COPI. After exocytosis, the Snc1 v-SNARE (designated with red line) undergoes retrieval from the cell surface to the early endosome (EE; light blue fill) and then to the *trans*-Golgi (blue fill). We propose that in wild-type cells, Gcs1 acts at the level of early endosomes to retrieve Snc1 back to the Golgi. This requires other proteins known to mediate Snc1 recycling to the Golgi, including Snx4 and Rcy1. Thus, Snc1 accumulates at the level of early endosomes in the absence of Gcs1 (*gcs1*Δ cells). We also propose that a subset of COPI subunits is involved in this step, either directly or indirectly. COPI is known to mediate retrograde Golgi–ER and intra-Golgi transport in yeast and mammals, as well as late endosome (LE)–multivesicular body transport in mammals. Thus COPI, like clathrin, acts as a coat for multiple trafficking pathways. In the absence of certain COPI B subunits (e.g., sec27-1 cells after temperature-shifting or *sec28*Δ cells), Snc1 is recycled back to the plasma membrane presumably by secretory vesicles derived from endosomal compartments.

observations). In contrast, GFP-Snc1 can clearly be modified into a form whose mobility is altered by phosphatase treatment, as demonstrated by several studies (Galan *et al.*, 2001; Hettema *et al.*, 2003; Chen *et al.*, 2005) as well as by us (our unpublished observations). This finding is consistent with the idea that GFP, and not the v-SNARE, is the phosphorylated substrate observed under these conditions. Thus, the role of phosphorylation as a recycling signal for v-SNAREs warrants further study.

In addition to its well-described role in Golgi–ER transport, the involvement of coatomer has also been demonstrated at the level of protein sorting to endosomes and multivesicular bodies in mammals (Whitney *et al.*, 1995; Aniento *et al.*, 1996; Gu and Gruenberg, 2000; Faure *et al.*,

2004). We show here that certain COPI subunits in yeast may assume this additional role by mediating protein retrieval from endosomes to the Golgi. For example, recombinant Snc v-SNAREs recruit purified coatomer in an in vitro binding assay (Figure 4C). Also, the *SNC* genes interact genetically with those encoding COPI B subunits, but not a COPI F subunit (Figure 4, E and F). Finally, GFP-Snc1 labeling of the plasma membrane of the growing bud is noticeably heightened in certain COPI B mutants, but not in a COPI F mutant (Figure 6). As a similar result was obtained in the ESCRT I mutant, *vps23*Δ (Figure 6), which is likely to be defective in its ability to target GFP-Snc1 for vacuolar degradation, it suggests that Snc1 recycles to the plasma membrane under conditions where trafficking to endosomal compartments is affected (see model, Figure 8). Unlike the *gcs1*Δ mutant, wherein recycling GFP-Snc1 accumulates in early endosomes (Figure 5), the enhanced bud localization observed with *sec27-1* and *sec28*Δ cells suggests an additional role for COPI (but not Gcs1) in sorting to multivesicular bodies (G. Gabriely and J. E. Gerst, our unpublished observations). These results suggest a broad role for COPI in endosome–Golgi transport and warrant further investigation.

Our findings support earlier studies that demonstrate a connection between Arf1 GTPases and v-SNAREs in conferring coat recruitment (Gommel *et al.*, 2001; Rein *et al.*, 2002; Lee *et al.*, 2004). This connection may guarantee incorporation of a v-SNARE into nascent vesicles in order to make them fusion-competent at the appropriate acceptor compartment. The classical view for Arf-GAP function has been that GAP-mediated hydrolysis of GTP on Arf1 leads to vesicle uncoating to allow for subsequent fusion (Tanigawa *et al.*, 1993; Bigay *et al.*, 2003; Reinhard *et al.*, 2003). Yet, other studies support the idea that Arf-GAP activity is required for the packaging of cargo into COPI vesicles (Nickel *et al.*, 1998; Pepperkok *et al.*, 2000; Lanoix *et al.*, 2001; Rein *et al.*, 2002; Lee *et al.*, 2004) and for COPI vesicle biogenesis (Yang *et al.*, 2002; Lee *et al.*, 2004). Our findings neither contradict nor reconcile these differing views, but may suggest additional functions for Gcs1 that are independent of its Arf-GAP activity. This is based on the fact that Snc binding does not involve the Arf-GAP domain of Gcs1 (Figure 1D) nor alters Gcs1 Arf-GAP activity (Figure 4A).

Finally, it has been suggested that Arf-GAPs themselves are coat components (Yang *et al.*, 2002; Lewis *et al.*, 2004). For example, interactions between Arf-GAPs and coat proteins that function in post-Golgi transport, including the GGA and AP3 clathrin adaptor proteins, have been demonstrated (Randazzo and Hirsch, 2004). Arf-GAPs have been shown to interact physically with coat proteins by two-hybrid analysis and coimmunoprecipitation. In particular, Glo3, a yeast Arf-GAP that acts with Gcs1 upon Golgi–ER transport (Poon *et al.*, 1999) binds to coatomer in both in vitro and two-hybrid assays (Eugster *et al.*, 2000; Lewis *et al.*, 2004). Although Gcs1 is not readily detected in COPI vesicles (Lewis *et al.*, 2004), this Arf-GAP may play a role in post-Golgi transport along with members of the COPI coat, as suggested here for the COPI B subcomplex. The possibility exists that multiple subpopulations of COPI occur in yeast, as was recently shown for mammalian cells (Malsam *et al.*, 2005). These different coats may define distinct sorting routes (i.e., Golgi–ER, intra-Golgi, early endosome–Golgi, and late endosome-MVB).

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